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CIRCULATING BIOMARKERS AND CLINICAL FACTORS ASSOCIATED WITH PROGNOSIS IN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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CONTENTS

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and, despite recent advances in its management, it is still burdened with high mortality. Prognostic prediction in HCC is very complex and several variables (among which tumor burden, residual liver function and clinical conditions) must be considered. With the aim of accurately stratify patient prognosis, reliable circulating biomarkers are urgently needed. Indeed, alpha-fetoprotein (AFP), despite being commonly used in HCC, is not completely satisfactory in prognostic prediction.

The primary aim of this thesis was to evaluate as potential circulating prognostic biomarkers some molecules involved in HCC development and progression. In particular, serpins (squamous cell carcinoma antigen [SCCA]-IgM), angiogenesis molecules (hypoxia-inducible factor [HIF]-1α and vascular endothelial growth factor [VEGF]), microRNAs (miR-21 and miR-122), prostaglandins (prostaglandin E_2) and inflammatory-based scores (platelets-to-lymphocytes ratio [PLR] and neutrophils-to-lymphocytes ratio [NLR]) were investigated. In the second part of the thesis, surveillance, cancer stage and treatment, which are essential clinical aspects to be considered for improving patient survival, were investigated.

The results obtained demonstrate that the biomarkers evaluated are potentially useful in stratifying patient prognosis and deserve a validation in large prospective studies. Moreover, the results of this thesis confirm the central importance of surveillance, provide the rationale for appropriate staging and treatment of large monofocal tumors, show the changes over time of transarterial chemoembolization application and effectiveness, and demonstrate the efficacy and safety of capecitabine in the treatment of HCC. In this personalized and precision medicine era, the development of prognostic and predictive biomarkers, possibly useful also in guiding treatment, and a careful clinical management are fundamental to improve patient survival.

CHAPTER 1

Introduction

Filippo Pelizzaro

INTRODUCTION

Despite the advancements in its management, hepatocellular carcinoma (HCC) has still a very high mortality (5-year survival rate of 18% in the United States and 20% in Italy (1,2)) and in most geographical areas the annual HCC mortality is almost similar to its incidence. Prognostic assessment in patients with HCC is complex, because several variables (among which tumor burden, residual liver function and clinical conditions) are involved in affecting the survival. Although potentially helpful in improving survival stratification, prognostic and predictive biomarkers in HCC are currently lacking and their identification is an urgent unmet need (3). Indeed, the most widely used and accepted serum marker, alpha-fetoprotein (AFP), has a suboptimal prognostic accuracy (4). In the last years, a lot of efforts have been made by researchers worldwide in identifying reliable non-invasive biomarkers. Despite the vast amount of evidence produced in this field, the perfect biomarker has not been identified yet and, unfortunately, I will not be able to do that at the end of the book. Nevertheless, I am reporting here the results of several studies aimed at evaluating the potential usefulness of several circulating molecules (serpins, mediators of angiogenesis, microRNAs, inflammatory mediators, inflammatory-based scores), which are promising in the prediction of HCC patient survival.

After evaluating biomarkers potentially useful in the prognostic prediction, some clinical aspects associated with patient survival were investigated. In particular, three main themes were addressed: the surveillance of patients at risk, the correct staging allocation of large monofocal tumors and the treatment with locoregional and systemic therapies.

The thesis is composed by sixteen chapters, including this chapter. Chapter 2 and 3 provide the background, with a brief overview on HCC in Chapter 2 and an updated review on circulating biomarkers in Chapter 3. Aims of the thesis are elucidated in Chapter 4. Chapter from 5 to 10 cover the studies on biomarkers, showing that some of these molecules are promising as prognostic

stratification tools. After discussing about microRNA-21 and microRNA-122 as prognostic biomarkers in HCC and their correlation with HIF-1α in Chapter 7, Chapter 8 contains data about microRNA-21 and HIF-1α circulating levels in different phases of chronic liver diseases and their correlation with liver fibrosis and liver function laboratory tests. In Chapter 11 and 12 the main topic is surveillance. The former includes a study investigating the association of surveillance with long-term survival, while the latter reports on the comparison between 3-months vs. 6-months surveillance interval in terms of survival benefit. The correct staging and treatment of large monofocal tumors is evaluated in Chapter 13. In Chapter 14, I assessed whether and how treatment with transarterial chemoembolization, one of the most widely used therapies worldwide, and its related survival changed over the last thirty decades in Italy. Finally, Chapter 15 is dedicated to the evaluation of capecitabine safety and efficacy in advanced stage patients. Chapter 16 reports general conclusions of the research.

Studies on biomarkers were conducted enrolling patients with HCC managed at the Gastroenterology Unit of the Padova University Hospital, and data were analyzed at the Gastroenterology Unit laboratory. For the studies on inflammatory-based biomarkers and for the majority of clinical studies reported in the second part of the thesis, data were retrieved from the Italian Liver Cancer (ITA.LI.CA) database. This is a multicenter registry collecting data of patients with HCC managed in 24 participating Institutions from 1988, and nowadays it is one of the largest European databases. The study reporting about capecitabine treatment in advanced HCC patients is the result of a multicenter collaboration between three Institutions of the Veneto Oncology Network.

REFERENCES

1. Jemal A, Ward EM, Johnson CJ, Cronin KA, Ma J, Ryerson B, et al. Annual Report to the Nation on the Status of Cancer, 1975-2014, Featuring Survival. J. Natl. Cancer Inst. 2017;109.
2. Italian Association of Cancer Registries (AIRTUM) - available at https://www.registri-tumori.it/cms/pubblicazioni/i-numeri-del-cancro-italia-2020.
3. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.
4. Galle PR, Foerster F, Kudo M, Chan SL, Llovet JM, Qin S, et al. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. Liver Int. 2019;39:2214–2229.

CHAPTER 2

Hepatocellular carcinoma: a general overview

Filippo Pelizzaro

EPIDEMIOLOGY

Primary liver cancer was the sixth most common cause of cancer and the second leading cause of cancer related death worldwide, with 906,000 incident cases and 830,000 deaths globally in 2020 (1). These figures are projected to increase in the near future, and more than 1 million people are estimated to die from liver cancer in 2030 (2). Hepatocellular carcinoma (HCC) accounts for the vast majority of primary liver cancers (85%) and constitutes a major global health problem.

Recent epidemiological studies show that both incidence and mortality rates of liver cancer have decreased in many high-risk countries in Eastern and South-Eastern Asia (including China) since the late 1970s and in Japan since the 1990s (3,4). Liver cancer incidence and mortality declined also in Italy since 1995 (3,4). On the contrary, these rates have progressively increased in North America (United States and Canada) and in North-West Europe (3,4). In most regions, incidence and mortality are 2 to 3 times higher among men than women (1).

The decrease in the prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, as well as the decrease in exposure to aflatoxin, have likely contributed to the declining incidence of liver cancer in high-risk regions (5). By contrast, the increased incidence in low-risk areas is likely related to the rising prevalence of obesity and metabolic disorders such as diabetes, thus offsetting the gain obtained through reduction of HBV and HCV prevalence (6,7).

RISK FACTORS

HCC is rare among patients without liver disease and in approximately 90% of cases the underlying etiology is known (8). In the majority of cases, HCC develops in patients with liver cirrhosis. All etiologic forms of cirrhosis may be complicated by HCC and, overall, one-third of cirrhotic patients will develop HCC during their lifetime (9). Long-term follow-up studies demonstrated that approximately 1-8% of patients with cirrhosis develop HCC per year, with a higher risk in patients

with chronic viral hepatitis (2%/year in patients with HBV-related cirrhosis and 3-8% in HCV-related cirrhosis) (10).

HBV infection is the main cause of HCC worldwide. Although HBV vaccination reduces the incidence of tumor development (11,12), many unvaccinated persons infected with HBV are still present, mostly in Asia and sub-Saharan Africa (13). Dietary exposure to aflatoxin B1 amplifies the risk of HCC in patients with chronic HBV infection, through a specific mutation in *TP53* at position 249 (R $\rightarrow$ S) (14). In Western countries and in Japan, the main cause of HCC is HCV infection. As opposed to HBV, that has a direct oncogenic effect regardless of the degree of the underlying liver fibrosis (15), HCC rarely occurs in HCV-infected patients without advanced fibrosis. For patients with alcoholic liver cirrhosis, an increased risk of HCC has been reported from most part of the world (16,17). Regarding other risk factors, an established association with HCC is reported for hemochromatosis and alpha-1-antitrypsin deficiency (18). Patients with hemochromatosis develop HCC in up to 45% of cases, almost exclusively in the cirrhotic phases of the disease (19,20).

Non-alcoholic fatty liver disease (NAFLD) associated with metabolic syndrome, diabetes and obesity is becoming increasingly important as a risk factor for HCC from an epidemiological point of view (21–23). The incidence of HCC caused by NAFLD is increasing worldwide, and particularly in developed countries (24–26). Between 500,000 and 900,000 new cases of HCC are estimated in the United States as a result of the high prevalence of metabolic syndrome and NAFLD (27) and the incidence of NAFLD-associated HCC is expected to increase by 122% between 2016 and 2030 (28). In NAFLD, the reported HCC incidence is very heterogeneous, ranging from 0.25% to 7.6% (29). Moreover, HCC is known to develop in the absence of cirrhosis in a not negligible proportion of patients with NAFLD (30,31).

Although the link between cigarette smoking and HCC occurrence was historically conflicting, recent evidence support the existence of an association (32,33). Infection with human immunodeficiency

virus (HIV) appears to be a co-factor in the development of HCC, increasing the risk in patients with chronic viral hepatitis (34). As far as protective factors are concerned, several epidemiological studies demonstrate an inverse association between coffee consumption and liver cancer risk (35). Available antiviral therapies decrease but do not eliminate the risk of HCC. In patients with chronic hepatitis B, interferon, nucleotide and nucleoside analogues favorably reduce HCC incidence (36–38), with a further decline after the first 5 years of entecavir and tenofovir disoproxil fumarate therapy (39,40). In patients with HCV infection who achieve a sustained virological response (SVR) with interferon-based treatment regimens compared to those not having response, the risk of HCC is reduced from 6.2% to 1.5% (41). The protective effect of SVR has also been reported among patients treated with Direct-acting Antiviral Agents (DAAs) (42–45). Nevertheless, patients receiving DAAs typically have more advanced fibrosis than those who were treated with interferon-based regimens (46) and, as far as we know, cirrhotic patients at baseline should continue to be surveilled for HCC occurrence after viral eradication (47).

SURVEILLANCE

Surveillance efficacy

Cancer surveillance aims at reducing disease-specific mortality through a diagnosis at early stages that, in turn, increases the opportunity to deliver curative treatments. Benefits of surveillance have been demonstrated in two randomized controlled trials (RCT) conducted in China among HBV infected patients (48,49). Surveillance with liver ultrasound (US) and AFP every 6 months, despite the suboptimal adherence to the surveillance program (55%), was able to reduce HCC-related mortality compared to no surveillance (49). However, these results cannot be generalized to patients undergoing surveillance in Western countries, who mostly have cirrhosis from HCV, alcohol and/or NAFLD. Cirrhotic patients are older, have more comorbidities and a higher competing risk of

liver-related mortality compared to patients with chronic HBV. Moreover, abdominal US may have lower sensitivity in patients with a cirrhotic liver and in obese patients (the prevalent population at risk of HCC in the West). Finally, cirrhotics have fewer curative options than patients with chronic hepatitis (the presence of portal hypertension often precludes liver resection). No RCT evaluated the effect of HCC surveillance in patients with cirrhosis. Nevertheless, several population and non-population based cohorts and cost-effectiveness analyses reinforce the benefits of regular surveillance (50–57). A metanalysis of 47 studies (including 15,158 patients with cirrhosis) demonstrated that surveillance was associated with early tumor detection (odds ratio [OR] 2.08, 95% CI 1.80-2.37), curative treatment receipt (OR 2.24, 95% CI 1.99-2.52) and improved survival (OR 1.90, 95% CI 1.67-2.17; 3-year survival rate of 50.8% in surveillance group vs. 27.9% in patients diagnosed symptomatically or incidentally) (58). These studies are heterogeneous as far as etiology of underlying liver disease, stage ad surveillance protocols. Moreover, almost all suffer from methodological biases, such as lead-time bias (apparent improvement of survival due to anticipated diagnosis) and length time bias (over-representation of slow-growing tumors in surveillance groups). However, despite all these limitations, the overall consistency in the results from the available cohort studies suggests that surveillance is likely beneficial in cirrhotic patients. The definitive proof of the surveillance benefit could be obtained from an RCT, but randomized data for surveillance vs. no surveillance are not likely to be forthcoming, because patients and their clinicians strongly prefers surveillance. One study underscores these difficulties, showing that 204/205 patients (99.5%) declined to assume the risk of being randomly assigned to no surveillance group (59). Therefore, international societies continue to rely on observational data to develop recommendation for HCC surveillance.

Target populations

Surveillance is strongly recommended in patients with cirrhosis, irrespective of its etiology (Table 1). In fact, cost-effectiveness studies indicate that an HCC incidence of 1.5%/year or greater warrants surveillance in cirrhotics (60–62). However, surveillance is not cost-effective in patients with advanced liver impairment (Child-Pugh class C) or decompensation in Child-Pugh class B (large ascites, hepato-renal syndrome, jaundice), because no effective HCC therapies are available when liver transplantation is not an option (50). By contrast, surveillance should be offered to cirrhotic patients awaiting liver transplantation because the detection of HCC could modify both priority on the waiting list and transplantability.

Table 1. Recommendation for HCC surveillance (modified from EASL Clinical Practice Guidelines: management of hepatocellular carcinoma (63))

Cirrhotic patients, Child-Pugh stage A and B
Cirrhotic patients, Child-Pugh C awaiting liver transplantation
Non-cirrhotic HBV patients at intermediate or high risk of HCC* (according to PAGE-B[†] classes for Caucasian subjects, respectively 10–17 and ≥18 score points)
Non-cirrhotic F3 patients, regardless of etiology may be considered for surveillance based on an individual risk assessment

* Patients at low HCC risk left untreated for HBV and without regular six months surveillance must be reassessed at least yearly to verify progression of HCC risk.

[†] PAGE-B (Platelet, Age, Gender, hepatitis B) score is based on decade of age (16–29 = 0, 30–39=2, 40–49=4, 50–59=6, 60–69=8, ≥70=10), gender (M=6, F=0) and platelet count (≥200,000/ μL = 0, 100,000–199,999/μL = 1, <100,000/μL = 2): a total sum of ≤9 is considered at low risk of HCC (almost 0% HCC at five years) a score of 10–17 at intermediate risk (3% incidence HCC at five years) and ≥18 is at high risk (17% HCC at five years) (64)

Some patients who have liver disease without cirrhosis should also be enrolled in surveillance programs, such as patients with chronic HBV infection. These patients have an HCC risk higher than the general population but lower compared to HBV-related cirrhotics. Caucasian HBV patients could be stratified into three different at-risk groups (low, intermediate and high) according to the PAGE-B classification (64). Patients in the low HCC risk class (PAGE-B score < 9) seldom develop HCC up to 10 years after starting NUC (39,64), and therefore do not require surveillance. Various other scoring systems are available to quantify HCC risk in HBV patients, but none are universally accepted because of suboptimal validation across geographical regions (37). The European Association for

the Study of the Liver (EASL) endorses the PAGE-B score for risk prediction and consequent surveillance recommendations in patients with chronic HBV infection (63).

Patients with chronic HCV infection and advanced fibrosis, defined by the Metavir system as a score of F3 or higher (on a scale from F0 to F4, whit higher scores indicating more severe fibrosis), are also at significant risk of developing HCC (65). Indeed, patients with advanced fibrosis are at risk of being understaged and the transition from advanced fibrosis to cirrhosis cannot be accurately defined in most instances. This leads international societies to recommend surveillance in these patients (63). In patients with advanced fibrosis, SVR achieved with DAAs decreases but do not eliminate the risk of HCC (41,43,44). A recent study including more than 8700 HCV-cirrhotic patients followed after a successful treatment with DAAs demonstrated that the annual risk of HCC was 1.8% (significantly lower than the 2-8% reported for previous cohorts, but still higher than the 1.5%/year threshold that should warrant surveillance) (42). Although there is consensus that these patients deserve surveillance, it is uncertain whether HCC risk will sufficiently decrease over time, so that HCC surveillance could be discontinued. A recent study with a median follow-up of 3.7 years suggests that the risk may remain high enough in the intermediate term to require ongoing HCC surveillance (47).

Although cirrhosis appears to be the main risk factor for HCC in NAFLD, carcinogenesis may occur in these patients even in the absence of advanced fibrosis/cirrhosis (66,67). Recently, it has been claimed that 20% of NAFLD patients with HCC had no evidence of cirrhosis based on a detailed medical record review (68). Other studies have reported an even higher proportion of NAFLD-related HCC cases (10-75%) that developed in the absence of cirrhosis (69). However, the actual risk of HCC in NAFLD non-cirrhotic patients is unknown and probably very low. Therefore, in the absence of cirrhosis surveillance is not recommended, until tools to identify high-risk patients will be developed in future studies.

Surveillance tests

Tests that can be used in HCC surveillance include imaging and serological examinations. Liver US has been long regarded as the standard surveillance test for HCC and it is one of the most frequently used. Advantages of US relies on the absence of risks, non-invasiveness, good acceptance by patients, moderate cost and ability to early detect the onset of other complications of cirrhosis (e.g., portal vein thrombosis or subclinical ascites). Nonetheless, detection of HCC in a context of a nodular cirrhotic liver is particularly challenging, because the coarse pattern at US may mask the presence of small tumors. In one of the randomized trials evaluating surveillance in patients with HBV infection, the sensitivity of US was 84% for any stage HCC and 63% for early-stage HCC (70). As expected, the diagnostic performance of US decreases in patients with cirrhosis. A metanalysis of cohort studies in cirrhotics reported a pooled sensitivity and specificity for the detection of any stage HCC of 84% (95% CI 76-92%) and 91% (95% CI 86-94%), respectively. The pooled sensitivity of US dropped to 47% (95% CI 33-61%) for the detection of early-stage HCC (71).

Based on data available for HCC diagnosis, cross-sectional imaging modalities, such as computed tomography (CT) and magnetic resonance imaging (MRI), may have high accuracy. However, very few studies investigated CT and MRI-based surveillance (72,73). Despite having satisfactory sensitivities, CT and MRI appear to be not cost-effective, also because of the considerable rates of false positive results that trigger further investigations (72). Moreover, radiation risk due to repeated exposure to CT scan, the high cost of MRI and the need for contrast injection make their use in long term surveillance debatable. In the setting of the waiting list for liver transplantation these circumstances are overcome, and CT/MRI may be alternative to US for surveillance.

The most widely serum biomarker used in surveillance is AFP, with a level of 20 ng/mL being the most commonly used cut-off to trigger further examinations in clinical practice. Notably, AFP has been mainly tested in diagnostic rather than surveillance setting, and this is relevant because its

usefulness in surveillance cannot be extrapolated form its performance as diagnostic test. A metanalysis of 5 studies evaluating AFP at the cut-off of 20 ng/mL in cirrhotic patients demonstrated sensitivities ranging from 41% to 65% and specificities from 80% to 94% for HCC at any stage (74). Sensitivity of AFP for early-stage HCC were even lower (32-49%). Reasons for the suboptimal performance of AFP as a serological surveillance test are twofold. Firstly, fluctuating levels of AFP in patients with cirrhosis may reflect HCC development, but also flares of HBV/HCV infections or exacerbations of the underlying liver disease (75). Secondly, only a small proportion of tumors (10-20%) have abnormal AFP serum levels in early-stage (76–78).

Although AFP is insufficiently accurate when used alone, its addition to US has been proposed as a means of increasing the sensitivity of surveillance, particularly with regard to early tumor detection. When combined with US, AFP is able to provide additional detection of early HCC only in 6-8% of cases not previously identified by imaging (79). However, a very recent metanalysis found that US with AFP had a significantly higher sensitivity than US alone (relative risk 1.23, 95% CI 1.08-1.41) (71). The pooled sensitivities with and without AFP for early HCC detection were 63% (95% CI 48-75%) and 45% (95% CI 30-62%), respectively (p=0.002).

Although the hope is that better imaging and serologic tests will be available in the future, these new strategies will require extensive evaluation before routine adoption in clinical practice. In the meantime, we will continue to depend on liver US-based surveillance, with or without AFP, and efforts should be made in maximizing US quality and surveillance utilization.

Surveillance interval

The ideal interval of surveillance should be dictated by the rate of tumor growth (tumor volume doubling time) and tumor incidence in the target population. Based on the available knowledge of tumor volume doubling time (80), a 6 months interval appears to be a reasonable choice. This is

confirmed also by studies showing that longer intervals were associated with worse survival (56), while a tighter schedule did not translate in any clinical benefit (55).

Although the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Disease (AASLD) and the Asian Pacific Association for the Study of the Liver (APASL) recommend the adoption of a 6-months interval in all patients (63,81,82), Japanese guidelines suggest to shorten the surveillance interval to 3-months in patients at "extremely-high risk" of developing HCC (i.e., HBV and HCV-related cirrhotics) (83). However, this proposal does not rely on experimental results.

DIAGNOSIS

In patients with cirrhosis, HCC could be diagnosed with the use of contrast-enhanced imaging techniques (Figure 1). Imaging-based diagnosis relies on the peculiar vascular derangement occurring during hepatic carcinogenesis (84) and the high pre-test probability of HCC in the setting of cirrhosis (85–88). During the malignant transformation of hepatocytes, a shift occurs in vascularization: benign lesions (e.g., regenerative and dysplastic nodules) are supplied by branches of the portal system, whereas malignant nodules receive blood from the hepatic artery (84). The typical hallmark of HCC is the combination of hypervascularity in the late arterial phase (defined as arterial phase hyperenhancement [APHE]) and wash-out in portal venous and/or delayed phases. In cirrhotic patients with nodules larger than 1 cm in diameter, this pattern has a sensitivity of 66-82% and a specificity higher than 90% for HCC diagnosis (89).

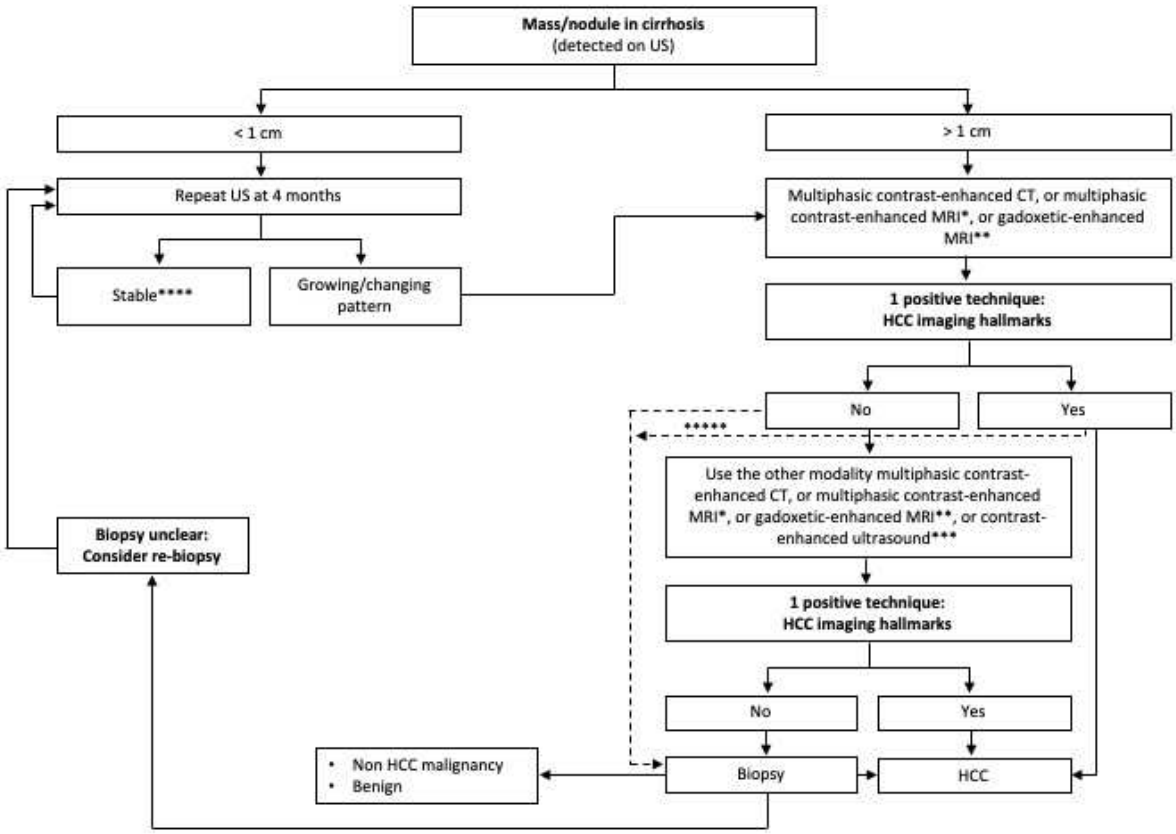

Figure 1. Diagnostic algorithm for a liver nodule in a patient with cirrhosis (adapted from the EASL guidelines (63)). *Using extracellular MR contrast agents or gadobenate dimeglumine. **Using the following diagnostic criteria: arterial phase hyperenhancement (APHE) and washout on the portal venous phase. ***Using the following diagnostic criteria: arterial phase hyperenhancement (APHE) and mild washout after 60 s. ****Lesion <1 cm stable for 12 months (three controls after four months) can be shifted back to regular six months surveillance. *****Optional for center-based programs.

The Liver Imaging Reporting and Data System (LI-RADS) uses APHE together with non-peripheral washout, presence of enhancing capsule and threshold growth to classify hepatic nodules on the basis of the likelihood of being HCC (90). Although this system allows the standardization of the reporting and data collection of imaging techniques, it is not universally accepted in diagnosis of HCC (63). In fact, some imaging features not related to tumor enhancement, such as the presence of tumor capsule or tumor growth over time, have not been prospectively validated. Moreover, L2, L3 and L4 LI-RADS classes might be helpful to stratify the risk of HCC in individual nodules (corresponding respectively to low, intermediate or high probability of HCC), but none of these classes rule out the presence of HCC (91).

In most studies, there was a trend toward higher sensitivity of MRI compared to CT, with specificity ranging between 85% and 100% (92). Results vary according to HCC size, with MRI performing better than CT particularly in small lesions (93). A metanalysis of forty studies demonstrated that in tumors smaller than 20 mm sensitivity is 48% and 62% for CT and MRI respectively vs. 92% and 95% respectively in HCC equal or larger than 20 mm (93). However, a very recent prospective multicenter study demonstrated that CT and MRI were comparable in the diagnosis of HCC, with a slight decrease in specificity for small nodules with both techniques. Sensitivity and specificity were 72.3% and 89.4% for MRI and 71.6% and 93.6% for CT in lesions between 20-30 mm, respectively (94). In lesions between 10-20 mm in size, sensitivity and specificity were 70.6% and 83.2% for MRI and 67.9% and 76.8% for CT, respectively (94).

Contrast-enhanced ultrasound (CEUS) is also used to characterize liver nodules in expert centers. The pattern of global APHE followed by washout at CEUS is not specific for HCC and occurs in about 50% of mass-forming cholangiocarcinomas in cirrhosis, thus leading to a risk of misdiagnosis of about 1% of nodules (95,96). However, in the vast majority of cholangiocarcinomas, the onset of washout takes place earlier than 60 seconds after contrast injection and the intensity of washout is more marked than in HCC (97–102). This has led to a refinement in the definition of the typical hallmark for HCC at CEUS: APHE followed by late (>60s) and mild-degree washout (103,104). A very recent large retrospective study in more than 1,000 lesions in cirrhotics showed that this new definition has a positive predictive value for HCC of almost 99%, and a positive likelihood ratio of 15.5, with no cases of misdiagnosis with intrahepatic cholangiocarcinoma (105). Furthermore, in a recent prospective multicenter study, in the 10-20 mm nodules CEUS had the highest specificity compared to CT and MRI (92.9%, 76.8% and 83.2%, respectively) (94). This high specificity (with only a slight drop in sensitivity) is also maintained after a first inconclusive CT or MRI for the 10-20 mm nodules, and for the 20-30 mm nodules (94). However, when CEUS is compared with either CT or

MRI, its sensitivity is significantly lower, especially in 10-20 mm nodules because of a lower detection rate of washout. Despite its accuracy in diagnosing HCC, CEUS is not a panoramic technique because the arterial phase is too short to allow adequate exploration of the entire liver. Therefore, it can be useful in characterizing one or few nodules visible at baseline US, particularly when both CT and MRI are contraindicated or are inconclusive for HCC diagnosis (106), but it is not recommended as first-line imaging technique in terms of cost-effectiveness, because CT or MRI will be needed for staging.

In patients with HCC developing in a non-cirrhotic liver, imaging features are not different from those found in cirrhotic patients. Nevertheless, the pre-test probability of having HCC and specificity of the imaging hallmark (APHE followed by washout in portal venous and/or delayed phases) is lower than in cirrhosis, because alternative diagnosis (e.g., hepatocellular adenoma, hypervascular metastasis) are more common. Therefore, the diagnosis of HCC in non-cirrhotic patients requires liver biopsy (63). Establishing a histologic diagnosis in patients with small nodules can be challenging, but combining immunostaining markers (glypican 3, heat shock protein 70, and glutamine synthetase) increase diagnostic accuracy (107).

STAGING AND PROGNOSTIC SYSTEMS

Compared to other tumors, HCC is peculiar since it usually arises in the context of liver cirrhosis that affects patient survival and complicates clinical management. Not only tumor burden and aggressiveness, but also residual liver function and general health status affect prognosis of patients with HCC (108). Number and size of liver nodules, vascular invasion and extrahepatic spread define tumor burden. AFP, which could be considered a surrogate marker of cancer aggressiveness, has been included in some prognostic scores. Liver function is usually evaluated through the inclusion of multiparametric liver function scores such as Child-Pugh score (109), Model of End-stage Liver

Disease (MELD) (110) or albumin-bilirubin (ALBI) score (111). The Eastern-Cooperative Oncology Group (ECOG) performance status (112) or the Karnofsky index (113) are used to describe the general health status of the patient.

Over the last decades, several prognostic scores and staging systems have been proposed to estimate the prognosis of HCC patients. These staging systems/prognostic scores can be classified in three main categories according to the methodology by which they were created (108):

1. Prognostic score, derived from real cohort populations;

2. Staging systems, derived from literature review made by experts;

3. Combined prognostic systems, based on literature data but weighted in real populations, and with the possibility to be used both as prognostic scores and as staging systems for treatment selection.

Prognostic scores

The most important data-based prognostic scores are described in Table 2. The four main one are represented by the Okuda staging (114), the CLIP score (115), the JIS score (116) and the MESH score (117).

Table 2. Main HCC prognostic scores and their variables.

Score	Year	n° patients	PS	Liver function	HCC number	HCC size	AFP	Vascular invasion	Metastasis	Other
Okuda (114)	1984	600	No	Ascites Albumin Bilirubin	No	Yes	No	No	No	/
CLIP (115)	1998	435	No	CPS	Yes	Yes	Yes	Yes	No	/
GRETCH (118)	1999	761	Karnofsky	Bilirubin	No	No	Yes	Yes	No	ALP
CUPI (119)	2002	926	Symptoms	Ascites Bilirubin	Yes	Yes	Yes	Yes	Yes	ALP
JIS (116)	2003	Review	No	CPS	Yes	Yes	No	Yes	Yes	/
Tokyo (120)	2005	403	No	Albumin Bilirubin	Yes	Yes	No	No	No	/
TIS (121)	2010	2030	No	CPS	TTV	TTV	Yes	No	No	/
MESIAH (122)	2012	477	No	MELD Albumin	Yes	Yes	Yes	Yes	Yes	Age
MESH (117)	2016	3182	ECOG	CPS	Yes	Yes	Yes	Yes	Yes	ALP

AFP: alpha fetoprotein; ALP: phosphatase alkaline; CLIP: Cancer of the Liver Italian Program; CPS: Child Pugh Score; GRETCH: GRoupe d'Etude et de Traitement du Carcinome Hépatocellulaire; MELD: Mayo End stage Liver Disease; MESH: Model to Estimate Survival for HCC; MESIAH: Model to Estimate Survival in Ambulatory HCC; JIS Japanese Integrated Staging; PVT: Portal Vein Thrombosis; TIS: Taipei Integrated Scoring System.

The Okuda staging, developed in 1984, represents the first attempt to stage HCC combining tumor burden parameters (≤ or >50% liver involvement) with liver function variables (albumin, bilirubin, presence of ascites), acknowledging the contribution of cirrhosis in determining the prognosis of patients (114). Despite its historical importance, nowadays the Okuda staging has essentially been abandoned due to its classification of tumor burden, that makes this score not useful in modern clinical practice since the majority of HCCs are diagnosed before they involve more than 50% of liver volume.

The CLIP score was developed through a retrospective cohort study, it was externally validated and it has been considered a valuable prognostic score (115). However, the CLIP score does not consider the patient clinical status and it is not sensitive in stratifying early-stage HCCs, amenable to potentially curative therapies. Therefore, also this score is not used in clinical practice.

Japanese authors proposed the JIS score (116), that combine Japanese TNM and Child-Pugh classifications. This score lacks a strong external validation in Western Countries and it is used mostly in Japan.

The MESH score was recently proposed using data of 3182 prospectively enrolled patients (117). This score (ranging from 0 to 6 points) combines Milan criteria, presence of vascular invasion and metastases, Child-Pugh score, performance status and laboratory parameters (AFP, alkaline phosphatase). MESH score was externally validated in European/North American countries (123). Like all other prognostic scores, this system does not provide treatment recommendations.

Staging systems

TNM and Barcelona Clinic Liver Cancer (BCLC) staging systems are the main examples of evidence-based systems. Recently, also the CNLC staging system (124–126) has been proposed.

As for other cancers, TNM is only based on tumor pathological features (127), and it does not consider residual liver function and patients general health conditions for the stratification of patients. Therefore, it is not useful in prognostic estimation of patients with HCC.

The BCLC classification, proposed for the first time in 1999 (128), was the first system combining tumor burden with liver function and patient health status assessment. It classifies patients in five subgroups (0, A, B, C and D), and for each group a specific treatment is recommended (Figure 2). This classification can be considered as an evidence-based system, as it was generated analyzing data from randomized controlled studies evaluating a specific treatment versus placebo in patients with comparable tumor characteristics and liver function. The BCLC system has been endorsed by the American Association for the Study of Liver Diseases (AASLD), the American Gastroenterology Association (AGA), the European Association for the Study of Liver (EASL) and the European Association for research and Treatment of Cancer (EORTC), and it is currently the recommended staging system.

However, the BCLC system suffers from the fact that it was not created and weighted in “real-world” HCC populations, and as a result its performance in prognostic prediction is generally lower than that of data-based prognostic scores (129,130). In addition, some potential limits of the BCLC system that could affect its prognostic power are (108): 1) the absence of a size cut-off for monofocal HCC in the early stage; 2) the high heterogeneity of intermediate and advanced stage; 3) the absence of a distinction between intra- and extra-hepatic vascular tumor invasion; 4) the absence of a prognostic biomarker; 5) the excessive prognostic weight given to performance status 1 (which per se makes the tumor as advanced); 6) the poor prognostic stratification of liver dysfunction degree (i.e., a simple distinction between Child-Pugh C and Child-Pugh A-B classes is proposed in the original BCLC scheme).

Very recently, the last updated version of the BCLC staging system (2022 update) has been published (Figure 2) (131). Major changes from previous versions concern liver function evaluation, which should be refined using also MELD and ALBI in addition to Child-Pugh class, and the introduction of AFP as a prognostic parameter. In addition, treatment recommendation has been updated according to advancements in knowledge.

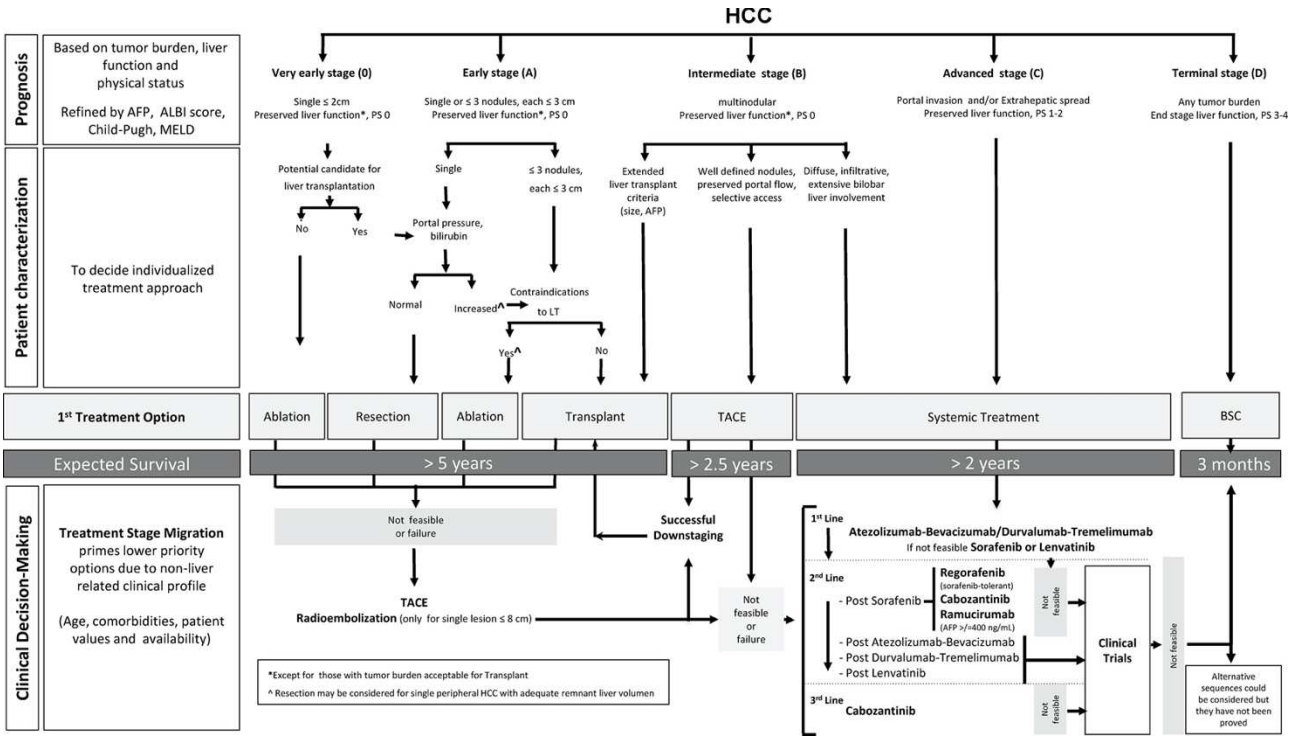

Figure 2. Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy in 2022 (131)

The main advantage of staging systems, such as BCLC, is that they offer a potential linkage between HCC stage and treatment. In fact, in the BCLC system a therapeutic choice is recommended for every stage (treatment decision is determined by the stage of the disease). Even though the BCLC system is very appealing for clinicians due to its simplicity, it has been accused of "rigidity" (i.e., for every stage or substage only one treatment is generally recommended) and this limits its utility in real-life clinical scenarios (81,132). Indeed, in real-life clinical practice poor adherence to BCLC algorithm has been demonstrated (133). Moreover, the trust in BCLC therapeutic indications has been challenged by several studies which demonstrated that patients undergoing treatments with potentially higher

efficiency compared to the BCLC standard of care exhibited better outcomes compared to those treated according to the BCLC algorithm (133,134,143–145,135–142).

In the attempt of increase the plasticity of the "stage hierarchy" approach, the concept of "treatment stage migration" was introduced in the last European guidelines (63). This is defined as a therapeutic choice by which a treatment theoretically recommended for a different stage is selected as best first-line treatment option. Usually, it is applied with a left-to-right direction (i.e., offering the treatment option recommended for the subsequent more advanced tumor stage rather than that forecasted for that specific stage). However, in highly selected patients, a right-to-left migration strategy (i.e., a therapy recommended for earlier stages) could be prescribed. Nevertheless, "treatment stage migration" represents an adjustment for narrowing the gap that separates the real-world clinical requirements from the rigid therapeutic decisions. Moreover, it maintains a "stage-dictated" rather than a "patient-tailored" vision of HCC management.

Combined staging systems

The Hong Kong Liver Cancer (HKLC) (146) and the ITA.LI.CA (147) prognostic systems are the two main combined prognostic systems.

The HKLC prognostic system was developed in 2014, in a cohort of predominantly HBV-infected HCC patients (146). Based on literature review, the variables included in the score were performance status, Child-Pugh score, tumor burden (according to Milano criteria), intra- and extra-hepatic vascular invasion or metastases. Subsequently, these variables were weighted in a real-life population in order to give to each of them a prognostic power. The HKLC system can be used both as prognostic score and as staging system, to help treatment selection. Compared to BCLC, it has better ability to stratify patients in intermediated and advanced stage HCC, who can therefore benefit of more aggressive treatments than those recommended by the BCLC system. Nevertheless, HKLC system has not a solid external validation in non-Asian populations.

The ITA.LI.CA prognostic score (147), created in 2016 through a multicenter retrospective analysis, is a prognostic model able to predict efficiently the outcome of patients with HCC. It includes variables related to tumor burden, liver functional reserves and other patient-related variables. It resembles BCLC classification regarding the stratification of tumor characteristics in different stages, but provides a better definition of intermediate stage (based on literature evidence (146,148)), which has been divided in three subgroups. A size cut-off (5 cm) was introduced for monofocal tumors in order to distinguish between stage A and B1. Moreover, intra- and extra-hepatic vascular invasion were identified as separate entities, also considering that HCC with intra-hepatic vascular invasion is amenable to treatment with curative intent (140,149). Patient functional status was evaluated with the Child-Pugh score and the ECOG performance status. Lastly, AFP was included as a marker of cancer aggressiveness able to provide important prognostic informations.

The ITA.LI.CA prognostic score was created attributing points to each variable, in order to capture their different impact in determining prognosis and correctly weight their prognostic influence (Table 3). The lowest score (0 points) corresponds to the best prognosis, while the highest (13 points) is associated with the worst outcome.

Table 3. ITA.LI.CA prognostic score

Score	**0**	**1**		**2**		**3**		**4**		**5**
Tumor staging	**0**	**A**		**B1**		**B2**		**B3**		**C**
Diameter (cm)	≤2	≤3	2-5	≤5	>5	>5	≤5	>5	Any	Any
n° nodules	1	2-3	1	2-3	1	2-3	>3	>3	Any	Any
Vascular invasion or metastases	No	No	No	No	No	No	No	No	Intrahep	Extrahep
Functional score										
CPS	5	6	7	8	9	10-15				
ECOG-PS	0	1	2	-		3-4				
AFP (ng/mL)	≤1000	-		>1000						

AFP, alpha-fetoprotein; CPS, Child-Pugh score; ECOG-PS, Eastern Cooperative Oncology Group performance status

In the original study (147), the prognostic score was internally and externally validated in a large Taiwanese cohort. More recently, another study externally validated the ITA.LI.CA score in a large

independent multicenter cohort including 1508 patients (150). Not only the ITA.LI.CA prognostic score was shown to perform better than other scores at the time of first patient evaluation (108,147,150), but also in restaging patients at the time of HCC recurrence before treatment decisions (151). Moreover, the ITA.LI.CA prognostic score can also be converted in the simple ITA.LI.CA staging system to assist treatment allocation (152) (Table 4). As an alternative to the "stage hierarchy" concept, this innovative staging system proposes therapeutic options for each stage based on the so called "therapeutic hierarchy" philosophy (153). "Therapeutic hierarchy" implies complete or partial independence of the treatment choice from the tumor stage, and recommends a hierarchical use of a scale of therapies developed according to the survival benefit observed in clinical practice. In other words, in each patient, independently of tumor stage, the therapy with the highest survival benefit should be always offered firstly, whenever feasible.

Table 4. ITA.LI.CA simplified staging for treatment allocation.

Stages	**0**	**A**		**B1**		**B2**		**B3**		**C**	**D**
Tumor staging	**0**	**A**		**B1**		**B2**		**B3**		**C**	**0-C**
Diameter (cm)	≤2	≤3	2-5	≤5	>5	>5	≤5	>5	Any	Any	Any
n° nodules	1	2-3	1	2-3	1	2-3	>3	>3	Any	Any	Any
Vascular invasion or metastases	No	No	No	No	No	No	No	No	Intrahep	Extrahep	Any
Functional score*	**≤ 2**										**> 2**

* Functional score: ≤2=CPS AB and PST 0 or CPS ≤ 7 and PST ≤ 2; >2 CPS 8-9 and PST 1-2, CPS C or PST > 2. CPS, Child-Pugh score; ECOG, Eastern Cooperative Oncology Group; ITA.LI.CA, Italian Liver Cancer; PST, performance status

A lot of comparative studies have been conducted in order to evaluate the best prognostic system in patients with HCC (108). Nevertheless, it is rather difficult to identify a system that could be universally accepted as the best prognostic scheme for all HCC patients. This is mainly because the prognostic accuracy of a staging system relies on several different variables, including time period, the geographical location of the study, numbers and type of patient population, modality of comparison and type of HCC treatment mainly adopted in the analyzed population. Indeed, therapeutic management greatly affect the stratification power of a prognostic score, so that the

best staging system for HCC patients undergoing liver resection may not be the same showing the best performances in patients managed with palliative therapies or best supportive care. In order to obtain an accurate prognostic estimation after that the treatment decision is taken, specific prognostic scores for each treatment should be used (151). Whatever the best prognostic system, all have a suboptimal performance in prognosis prediction, suggesting that substantial improvements are needed (108). Probably, the inclusion of biomarkers measuring cancer biological aggressiveness is the keystone that will allow to reach an optimal estimation of HCC patients survival.

TREATMENT

Surgical therapies

Surgery (liver resection [LR] and liver transplantation [LT]) is the mainstay of HCC treatment, leading to the best survival outcomes compared to all the other available therapies.

The ideal candidates for liver resection are patients with solitary tumors (BCLC stage 0 or A), in whom the performance status is good, liver function is well preserved, and there is no clinically significant portal hypertension (144). For these patients, resection is associated with a survival above 60% at 5 years, with low post-operative mortality (<3%). Nevertheless, about 70% of patients have tumor recurrence at 5 years (154).

Even though these patients remain the optimal candidates for surgical resection, in the last few years surgical technical advancements, better pre-resection imaging planning and intensive post-resection management, allowed the use of LR in patients exceeding these criteria in experienced centers. This is particularly important in the light of the vast amount of evidence demonstrating that patients approached with LR in intermediate-advanced stages achieved better survival results that those managed with the standard of care treatment (i.e., transarterial chemoembolization or

systemic therapy) (137–140,145,149,153,155). Even in patients with portal vein tumor invasion (particularly if intra-hepatic and segmentary) both Oriental (138,139) and Western series (156–158) demonstrated that LR can offer a longer survival outcome compared to non-surgical treatments. Indeed, in real-life clinical practice, LR is widely applied also beyond the criteria established by guidelines (BCLC 0-A), as demonstrated by a large multicenter surgical series showing that >70% of patients underwent LR beyond the BCLC guideline (144).

In patient selection for LR, at least three variables should be considered, measured and combined: the residual liver function, the presence of portal hypertension and the extent of hepatectomy and surgical invasiveness. In addition, as for any other surgical intervention, also patients' general conditions, performance status and comorbidities should be evaluated before LR.

Considering the high rate of tumor recurrence (70% at 5 years), several strategies to prevent recurrence have been tested (159–161). However, no adjuvant therapies demonstrated to reduce recurrence. In particular, a recent randomized controlled trial (STORM trial) evaluating sorafenib vs. placebo as adjuvant therapy after LR or ablation failed to demonstrated any positive effect (162).

HCC is generally the only accepted indication for solid organ transplantation in cancer. In addition to removing the tumor, LT has the advantage to cure the underlying liver disease. Approximately 30-35% of the waiting list population in Europe have HCC in cirrhosis, and this is the fastest growing indication for LT worldwide (163). Milan criteria (single tumor ≤5 cm or ≤3 tumors ≤3 cm in size, without vascular invasion), developed in 1996 (164), still represent the benchmark in the selection for LT of patients with HCC and the basis for comparison with other suggested criteria. The expected 5-year survival rate of patients meeting these criteria is 65-80%, and a survival advantage is maintained compared to patients beyond these boundaries (165).

Over time, several proposals of extending the selection criteria have been made with the goal of maintaining an acceptable risk of HCC recurrence and survival. Indeed, even if survival benefit of LT

as compared to alternative therapies has been demonstrated regardless of tumor burden (provided that macroscopic vascular invasion and extrahepatic spread are absent) (166), in the selection of the optimal candidate patients with HCC not only transplant benefit, but also transplant utility (i.e., the selection of patients with low post-LT recurrence risk) should be considered (167). Traditionally, selection criteria rely only on the evaluation of size and number of tumors (164). More modern criteria based on size/number alone, such as UCSF criteria (single nodule ≤6.5 cm or 2–3 nodules ≤4.5 cm and total tumor diameter ≤8 cm) (168,169) and Up-to-7 criteria (HCC having the number 7 as the sum of the diameter (cm) of the largest tumor and the number of tumor) (170), even though expanding the boundaries of transplantability, have demonstrated comparable survival results. Since serum biomarkers, considered both as static and dynamic variables, may provide accurate information on tumor biology and thus on post-transplant recurrence risk, their evaluation has been included in the more recently developed pre-transplant prognostic models. Among these selection criteria, total tumor volume (TTV) + AFP (TTV <115 cm^3 and AFP <400 ng/mL) (171,172) and the AFP-French model (point system based on tumor size, number and AFP cut-off levels at 100 ng/mL and 1,000 ng/mL) (173) have been externally validated. The recently proposed metroticket 2.0 model consider AFP as a continuous variable, and its variations along with tumor morphology can be used as an accurate predictor of tumor-related death after liver transplantation (174).

Although expanded selection criteria have claimed no significant differences compared to the Milano criteria in terms of post-LT survival, increasing experience demonstrates a concept that is nowadays widely accepted: the further outside the Milano criteria, the greater the risk of recurrence (168–170,174). In considering the expansion of criteria for LT, a minimum survival threshold must be reached to justify expansion, while not harming non-HCC patients on the waiting list (175). Interestingly, for patients with tumor burden beyond the Milano criteria, successful downstaging to within these boundaries is associated with a rate of HCC recurrence and survival comparable to

those meeting Milano criteria without downstaging (176). Thus far, considering that patients who progress despite locoregional therapies exhibit worse post-LT outcomes (177–179), the strategy to consider tumor burden with assessment of response to locoregional therapies as a marker of favorable tumor biology has gained broader acceptance as an additional risk stratification tool (180,181). A recent randomized controlled trial demonstrated that, in patients with HCC beyond the Milano criteria, after successful downstaging liver transplantation improved tumor event-free survival and overall survival (OS) compared to non-transplantation therapies (182).

Local ablation

Several methods for chemical or thermal tumor destruction have been developed in the last decades (183). The seminal ablation technique is percutaneous ethanol injection (PEI), which induces coagulative necrosis of the lesion. Subsequently, thermal ablative therapies emerged. These can be classified as either hyper-thermic treatments (radiofrequency, microwave and laser ablation) or cryoablation. Most of these procedures are performed percutaneously, but in some instances ablation during laparoscopy is recommended.

Thermal ablation with radiofrequency (RFA) is the standard of care in patients with very-early and early HCCs (BCLC 0 and A), who are not candidates for surgery (63,81). Heat produces coagulative necrosis of the tumor and creates a “safety-ring” of necrosis in the surrounding liver tissue, which might eliminate small undetected satellites. RFA, as first-line therapy for early HCC, achieved a 5-year OS and recurrence-free survival (RFS) of 67.9% and 25.9%, respectively (184). Similarly, a metanalysis demonstrated a 3-year OS of 76% in patients with single HCCs <3cm, with a recurrence-free rate of 46% (185). Considered that the extent of tumor necrosis is negatively correlated with tumor size, the only independent predictive factor of local tumor progression, which is approximately 30% at 3 years, is tumor size with a clear threshold at 2 cm in diameter (184,186–189). Indeed, compared with resection, ablation has fewer complications, but provides worse local

control for largest tumors. On the contrary, for small tumors (<2 cm) RFA demonstrated to be at least equal to surgical treatment (185,190), competing with resection as recommended option for frontline treatment in these patients. Selected patients with tumors larger than 3 cm, oligo-nodular multiple (>3 nodules <3 cm) tumors or advanced compensated liver failure (Child-Pugh B not clinically decompensated) can be reasonably treated with RFA on an individual basis. Although these treatments provide good results, they are unable to achieve response rates and outcomes comparable to those observed in small HCC.

Microwave ablation (MWA) showed promising results in local tumor control and survival, but the majority of the studies are small and retrospective. MWA might have some advantages compared to RFA, such as being its efficacy less affected by vessels located in the proximity of the tumor. However, recent metanalysis indicate a similar efficacy between the two percutaneous techniques, with one study showing a possible advantage of MWA in larger tumors (191,192). Laser ablation and cryoablation have also been proposed for local ablation in HCC, but at present only few studies evaluated their efficacy compared to RFA (193–195).

Many studies reported on the efficacy and tolerability of different techniques of external beam radiotherapy (196), but no well conducted prospective trial is currently available to consider radiotherapy a proven option in HCC management.

Transarterial therapies

Transarterial chemoembolization (TACE) is the most widely used primary treatment for unresectable HCC (133,197). According to guidelines, it is the recommended first-line therapy for intermediate stage HCC (63), but it is also widely used outside this specific setting, representing a major part of the daily clinical practice in patients with HCC worldwide (133,198). Indeed, in real-life, approximately 40% of TACE are performed in either early or, more rarely, advanced HCC (199–203).

The rationale behind TACE relies in the intense arterial neo-angiogenetic activity of HCC during its progression. The intra-arterial infusion of a cytotoxic drug followed by the embolization of the tumor-feeding blood vessels will result in a strong cytotoxic and ischemic effect. The damage is targeted to the tumor, which tends to become entirely fed by arterial blood flow, while adjacent non-tumoral liver tissue is generally protected from TACE receiving the majority of inflow from portal system.

The best candidates for TACE are patients with uni- or pauci-nodular disease without vascular invasion or metastases, who are asymptomatic and have a Child-Pugh score ≤B7. In those selected patients, modern series reported a median survival of 40-50 months (199–201,204). General contraindications to TACE are severe hepatic decompensation (Child-Pugh C and Child-Pugh B decompensated cirrhosis), compromised performance status (ECOG-PS ≥2), tumor liver occupation >50% and macrovascular invasion of the main portal branches or the main portal vein (197,205,206). The conventional TACE procedure involves the intra-arterial delivery of chemotherapeutic drug emulsioned with Lipiodol (an oily contrast medium), followed by vascular occlusion achieved with particle embolization. The most commonly used drugs used during conventional TACE, either alone or in combination, are doxorubicin or epirubicin, cisplatin or miriplatin (207). Two randomized controlled trials (206,208) and a metanalysis which included positive and negative studies (209) demonstrated the survival benefits with TACE as compared to best supportive care. Recently, a systematic review and metanalysis including 101 studies (with a total of 10,108 patients) showed an objective response rate of 52.5% (95% CI 43.6-61.5), and an OS of 70.3% at one year, 51.8% at two years, 40.4% at three years, and 32.4% at five years with a median OS of 19.4 months (95% CI 16.2–22.6) (207). Moreover, the mortality associated with TACE was below 1%, with most deaths due to liver failure. This underscores the importance of an adequate patient selection for this therapy and the fact that decompensated cirrhosis is a contraindication to TACE.

Among strategies to improve antitumoral activity and clinical benefits of the treatment, TACE with drug eluting beads (DEB-TACE) has been developed. In this technique embolic microspheres that have the ability to sequester chemotherapeutic agents and release them in a controlled mode over a one-week period are used. Although the use of doxorubicin-carrying microspheres has shown more selective and sustained drug delivery and permanent embolization (210), conventional TACE and DEB-TACE demonstrated equivalent results in terms of survival and tumor response (211–213). As far as the safety is concerned, the PRECISION V study demonstrated some advantages of DEB-TACE in terms of toxicity and radiologic tumor response, particularly in fragile subgroups such as Child-Pugh B patients, performance status >0, bilobar or recurrent tumors (211). Another randomized controlled trial demonstrated that the incidence and severity of adverse events was similar between the two techniques, except for post-procedural pain, which was more frequent and severe after conventional TACE (212). By contrast, a retrospective study showed that biliary injuries, intrahepatic bilioma and global hepatic damage was significantly higher following DEB-TACE, especially in patients with advanced cirrhosis (214). At present, there is insufficient evidence to recommend one TACE technique over another and the choice is left to the operator. Whatever the technique chosen, the treatment should be super-selective in order to increase treatment efficacy and minimize the ischemic insult to non-tumoral tissue.

Regarding treatment schedule, there are still no solid data to suggest that TACE performed at regular intervals irrespective of tumor response is more or less effective at improving patient survival compared to on demand TACE. However, the repetition of TACE according to an aggressive schedule (e.g., every two months) might induce liver function impairment in a high percentage of patients, who are in most instances cirrhotics (215). Therefore, the policy to repeat TACE regardless of the outcome of the first session has been substantially abandoned, and nowadays the recommendation is to retreat with TACE only when residual viable HCC is documented by imaging.

Deciding whether retreating a patient or interrupting TACE is complex, and in recent years several scores have been proposed to guide clinicians in this choice (216–218). Certainly, treatment with TACE should be stopped when substantial necrosis is not achieved after two session or when subsequent treatment fails to induce marked necrosis at sites that have progressed after an initial tumor response (63). In addition, TACE should not be repeated upon "untreatable progression" defined as either major progression (extensive liver involvement, macrovascular invasion, extrahepatic spread) or minor intrahepatic progression associated with impaired liver function and performance status.

The local hypoxia and ischemic necrosis achieved by TACE result in an activation of neoangiogenesis. This leads to the evaluation of antiangiogenetic agents in combination with TACE. Unfortunately, neither sorafenib (which inhibits the vascular endothelial growth factor receptors [VEGFR]) nor brivanib (an inhibitor of VEGFR2 and the fibroblast growth factor receptor) demonstrated to be able to improve survival in TACE-treated patients (204,219,220).

Selective internal radiation therapy (SIRT), or radioembolization, is another transarterial treatment that consists in the infusion of microspheres containing yttrium-90 (Y90) (221). Because of the minimally embolic effect of Y90 microspheres, SIRT can be safely used in patient with portal vein thrombosis (221). Studies reporting on long-term outcome after SIRT showed a median survival of 16.9-17.2 months in patients with intermediate stage HCC and 10-12 months in patients at advanced stages with portal vein invasion (222–225). Objective response rates range from 35% to 50% (222,223,225). Around 20% of patients present liver-related toxicity and 3% treatment-related death (223).

No phase 3 RCTs have compared SIRT and TACE with respect to survival, but several retrospective studies indicated that SIRT induces less toxicity (possibly because the better selection of patients), provides significantly longer time to progression, and maintains higher quality of life, although it

does not prolong survival (226–228). One of the mor common indication of SIRT is the treatment of locally advanced HCC and some trials have compared this therapy with sorafenib. The SARAH trial (229) and SIRveNIB trial (230) were designed for superiority of SIRT over sorafenib. In both studies, tumor response rate was significantly higher with SIRT, although this finding did not translate into longer survival. The added value of SIRT in patients treated with sorafenib was evaluated in the palliative cohort of the SORAMIC trial (231) in which patients were randomized to receive SIRT + sorafenib vs. sorafenib alone. Even this trial failed in its primary endpoint, not demonstrating an improvement of OS with the addition of SIRT to sorafenib. Considered these results, at present the survival benefit of SIRT compared to sorafenib in advanced HCC is still not proved.

Systemic therapies

HCC is recognized as among the most chemo-resistant tumor types, and until 2007 no systemic drug was recommended for patients with advanced tumors. In 2007, sorafenib, a multi-targeted tyrosine kinase inhibitor (TKI) became the first systemic agent to demonstrate a survival benefit. In the pivotal SHARP trial, survival increased from 7.9 months with placebo to 10.7 months with sorafenib (HR, 0.69; 95% CI 0.55–0.87; p = 0.00058), representing a 31% decrease in the relative risk of death (232). The safety and efficacy of sorafenib was then confirmed in the Asia-Pacific population, with a similar magnitude of survival benefit (median OS 6.9 months with sorafenib vs. 4.2 with placebo) (233). Sorafenib is well tolerated, and the most common grade 3 drug-related adverse events observed in the SHARP trial were diarrhea and hand-foot skin reaction, which occurred in 8-9% and 8-16% of patients, respectively (232). As a result, sorafenib received the approval from regulatory agencies in 2007 for the frontline treatment of HCC patients.

Most agents and other treatment approaches subsequently tested in phase 3 trials failed to improve on or parallel the efficacy of sorafenib as first-line treatment. They also did not increased survival, as compared with placebo, in second-line. These agents and treatments include erlotinib (234),

brivanib (235,236), sunitinib (237), linifanib (238), everolimus (239), pegylated arginine deiminase (ADI-PEG20) (240), hepatic arterial infusion chemotherapy (241), and FOLFOX (fluorouracil, leucovorin [folinic acid] and oxaliplatin) (242) as well as tivantinib in patients with overexpression of MET (243). Lack of effectiveness, toxicity in the context of cirrhosis and inadequate patient selection have been proposed as reasons for these failures.

Sorafenib remained the only effective option for frontline therapy until the TKI lenvatinib demonstrated its effectiveness in the non-inferiority REFLECT trial (244). This open label trial randomized patients to continuous treatment with sorafenib (400 mg bid) or a weight-adjusted dose of lenvatinib (8 mg/day if ≤60 kg or 12 mg/day if >60kg). No differences in OS between lenvatinib and sorafenib were demonstrated. However, lenvatinib therapy resulted in a slightly longer OS (13.6 months vs. 12.3 months), a significantly longer progression-free survival (PFS) (HR = 0.64; 95% CI 0.55-0.76) and a significantly higher objective response rate (OR = 5.01; 95%CI: 3.59-7.01). In addition, despite the fact that lenvatinib-treated patients were more likely to discontinue treatment due to adverse events, their median duration of treatment was longer (5.7 months vs. 3.7 months) (244). Grade 3 or 4 adverse events with lenvatinib included hypertension (in 23% of patients vs. 14% receiving sorafenib), decreased weight (8% vs. 3%) and palmar-plantar erythrodysesthesia (3% vs. 11%).

In recent years, substantial progress has been made also in second-line therapies. For patients that progress or do not tolerate sorafenib (and eventually other systemic therapies), regorafenib (245), cabozantinib (246), and ramucirumab (247), a monoclonal antibody that inhibits ligand activation of VEGFR2, demonstrated to prolong OS compared to placebo.

RESORCE was a double-blind phase 3 trial randomizing patients with documented radiological progression after sorafenib in a 2:1 ratio to receive regorafenib (160 mg/day for the first 3 weeks of a 4 weeks cycle) or placebo (245). In this trial, patients who discontinued sorafenib for intolerance

were not included. Regorafenib resulted in a longer median OS (10.6 vs. 7.8 months), and decreased the risk of death by 37% compared to placebo. Grade 3 or 4 treatment-related adverse events were experienced by 46% of patients, and 10% discontinued the therapy because of adverse events. Overall response rate was 7% and median duration of response was 3.5 months. Following these results, regorafenib was the first drug approved by regulatory agencies for the treatment of advanced HCC in second-line.

CELESTIAL was a double-blind phase 3 trial that randomized patients who discontinue sorafenib for intolerance or progression in a 2:1 ratio to cabozantinib (60 mg/day) or placebo (246). Eligible patients had received prior sorafenib and had disease progression after at least 1 and up to 2 systemic treatments for HCC. Cabozantinib resulted in a longer median OS compared to placebo (10.2 vs. 8.0 months in the entire cohort and 11.3 vs. 7.2 months in second-line patients), with 68% of patients experiencing grade 3 or 4 adverse events (mostly hypertension and palmar-plantar erythrodysesthesia) and 16% of patients discontinuing therapy for adverse events.

REACH-2 is a double-blind phase 3 trial that randomized patients in a 2:1 ratio to ramucirumab (8 mg/kg every 2 weeks) or placebo (247). Based on a post-hoc analysis of a previous trial that suggested efficacy in patients with high AFP level (248), in this study only patients with AFP ≥400 ng/mL and sorafenib as the only prior systemic therapy (discontinued because of intolerance or tumor progression) were enrolled. Ramucirumab improved survival over placebo (8.5 vs. 7.3 months), with 35% of patients experiencing serious adverse events of any causality and 11% discontinuing therapy for treatment-related adverse events.

Very recently, several studies evaluating the role of immunotherapy in HCC have been conducted and more are currently ongoing. Immune-checkpoint inhibitors (ICIs) are designed to target programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) or cytotoxic T-lymphocyte- associated antigen 4 (CTLA-4), all fundamental negative regulators of T cell function

(249). These drugs act through the stimulation of an effective antitumor immune response, allowing immune system to recognize and destroy cancer cells. Following positive results of phase 2 clinical trials (Checkmate 040 (250) and Keynote 224 (251)), the Food and Drug Administration granted conditional approval the PD-1 inhibitors nivolumab and pembrolizumab in second-line after sorafenib. Unfortunately, a recent phase 3 study in sorafenib-experienced patients failed to show that pembrolizumab is superior to placebo in terms of OS (with a HR of 0.78; 95% CI 0.61–0.99) (252). No phase 3 data on nivolumab in second-line are available at this time, but the OS of nivolumab treated patients in first-line was not significantly longer compared to those receiving sorafenib in the Checkmate 459 phase 3 trial (HR of 0.85; 95% CI 0.72–1.02) (253).

A significant step forward in the management of advanced HCC and a robust proof of the effectiveness of immunotherapy has been achieved very recently, following the positive results of the IMbrave150 trial (254). More than 10 years after sorafenib approval, the combination of atezolizumab (anti-PD-L1) and bevacizumab (anti-vascular endothelial growth factor (VEGF) monoclonal antibody) proved to be superior over sorafenib in first line. IMbrave150 was an open label phase 3 trial that randomized patients in a 2:1 ratio to either the combination of a flat dose of atezolizumab (1,200 mg) plus a weight-based dose of bevacizumab (15 mg/kg) given every 3 weeks or the standard dose of sorafenib (400 mg bid). This study confirmed that atezolizumab + bevacizumab combination was superior to sorafenib in prolonging both OS (HR of 0.58, 95% CI 0.42-0.79) and PFS (HR of 0.59, 95% CI 0.47-0.76) (254). The combination also resulted in a more frequent (27.3% vs. 11.9%) and more durable (duration >6 months in 87.6% vs. 59.1%) objective remission, and a longer time until deterioration of health-related quality of life (median time 11.2 vs. 3.6 months) despite an increased number of patients with serious adverse events (38.0% vs. 38.8%) and adverse events leading to discontinuation of any agent (15.5% vs. 10.3%). The trial was interrupted at the first interim analysis after a short follow-up (8.6 months), when median OS was not reached

in patients treated with atezolizumab + bevacizumab. After a longer follow-up, a median OS of 19.2 months in the combination group compared to 13 months in the sorafenib arm was demonstrated (255). In addition, network metanalyses showed that the combination of atezolizumab + bevacizumab is superior also over lenvatinib and nivolumab (256,257).

Very recently tremelimumab + durvalumab (HYMALAYA phase 3 trial) was announced to provide a statistically significant survival benefit versus sorafenib in first-line (258). This adds another option for first line treatment.

The landscape of the systemic therapies for HCC is rapidly evolving, and in recent years we have witnessed a significant expansion of treatment possibilities. Sorafenib has been for more than 10 years the standard treatment for advanced HCC and the comparator for other drugs. Following the groundbreaking results of the IMbrave150 trial, there has been a change in the treatment sequence and the combination of atezolizumab + bevacizumab has now become the standard of care frontline therapy (259). Nevertheless, there is a major need to evaluate if the available second-line alternatives maintain their effectiveness in patients initially receiving this treatment. Moreover, it needs to be evaluated if sorafenib and lenvatinib should be considered as “de facto” second-line options or if their effectiveness could be modified after atezolizumab + bevacizumab (131,259). No robust information is available, but several ongoing trials may clarify some current uncertainties, increase the first-line alternatives and/or change the current sequential treatment schedule (131,259).

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA. Cancer J. Clin. 2021;71:209–249.
2. Villanueva A. Hepatocellular Carcinoma. N. Engl. J. Med. 2019;380:1450–1462.
3. Arnold M, Abnet CC, Neale RE, Vignat J, Giovannucci EL, McGlynn KA, et al. Global Burden of 5 Major Types of Gastrointestinal Cancer. Gastroenterology. 2020;159:335-349.e15.
4. Petrick JL, Florio AA, Znaor A, Ruggieri D, Laversanne M, Alvarez CS, et al. International trends in hepatocellular carcinoma incidence, 1978-2012. Int. J. cancer. 2020;147:317–330.
5. Petrick JL, McGlynn KA. The changing epidemiology of primary liver cancer. Curr. Epidemiol. reports. 2019;6:104–111.
6. NCD Risk Factor Collaboration (NCD-RisC) F. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. Lancet (London, England). 2016;387:1377–1396.
7. NCD Risk Factor Collaboration (NCD-RisC) F. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet (London, England). 2016;387:1513–1530.
8. Akinyemiju T, Abera S, Ahmed M, Alam N, Alemayohu MA, Allen C, et al. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level. JAMA Oncol. 2017;98121:1683–1691.
9. Sangiovanni A, Prati GM, Fasani P, Ronchi G, Romeo R, Manini M, et al. The natural history of compensated cirrhosis due to hepatitis C virus: A 17-year cohort study of 214 patients. Hepatology. 2006;43:1303–1310.
10. Ioannou GN, Splan MF, Weiss NS, McDonald GB, Beretta L, Lee SP. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. 2007;5:938–45, 945.e1–4.
11. Chang M-H, You S-L, Chen C-J, Liu C-J, Lee C-M, Lin S-M, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. J. Natl. Cancer Inst. 2009;101:1348–1355.
12. Chang M-H, You S-L, Chen C-J, Liu C-J, Lai M-W, Wu T-C, et al. Long-term Effects of Hepatitis B Immunization of Infants in Preventing Liver Cancer. Gastroenterology. 2016;151:472-480.e1.
13. Yuen M-F, Chen D-S, Dusheiko GM, Janssen HLA, Lau DTY, Locarnini SA, et al. Hepatitis B virus infection. Nat. Rev. Dis. Prim. 2018;4:18035.
14. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature. 1991;350:427–428.
15. Levrero M, Zucman-Rossi J. Mechanisms of HBV-induced hepatocellular carcinoma. J. Hepatol. 2016;64:S84–S101.
16. Nahon P, Sutton A, Rufat P, Ziol M, Akouche H, Laguillier C, et al. Myeloperoxidase and superoxide dismutase 2 polymorphisms comodulate the risk of hepatocellular carcinoma and death in alcoholic cirrhosis. Hepatology. 2009;50:1484–1493.
17. Mancebo A, González-Diéguez ML, Cadahía V, Varela M, Pérez R, Navascués CA, et al. Annual incidence of hepatocellular carcinoma among patients with alcoholic cirrhosis and identification of risk groups. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. 2013;11:95–101.
18. Perlmutter DH. Pathogenesis of chronic liver injury and hepatocellular carcinoma in alpha-1-antitrypsin deficiency. Pediatr. Res. 2006;60:233–238.
19. Deugnier YM, Guyader D, Crantock L, Lopez JM, Turlin B, Yaouanq J, et al. Primary liver cancer in genetic hemochromatosis: a clinical, pathological, and pathogenetic study of 54 cases. Gastroenterology. 1993;104:228–234.
20. Fracanzani AL, Conte D, Fraquelli M, Taioli E, Mattioli M, Losco A, et al. Increased cancer risk in a cohort of 230 patients with hereditary hemochromatosis in comparison to matched control patients with non-iron-related

chronic liver disease. Hepatology. 2001;33:647–651.

21. Schlesinger S, Aleksandrova K, Pischon T, Jenab M, Fedirko V, Trepo E, et al. Diabetes mellitus, insulin treatment, diabetes duration, and risk of biliary tract cancer and hepatocellular carcinoma in a European cohort. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 2013;24:2449–2455.

22. Tsilidis KK, Kasimis JC, Lopez DS, Ntzani EE, Ioannidis JPA. Type 2 diabetes and cancer: umbrella review of meta-analyses of observational studies. BMJ. 2015;350:g7607.

23. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N. Engl. J. Med. 2003;348:1625–1638.

24. Dyson J, Jaques B, Chattopadyhay D, Lochan R, Graham J, Das D, et al. Hepatocellular cancer: The impact of obesity, type 2 diabetes and a multidisciplinary team. J. Hepatol. 2014;60:1–2.

25. Kanwal F, Kramer JR, Duan Z, Yu X, White D, El-Serag HB. Trends in the Burden of Nonalcoholic Fatty Liver Disease in a United States Cohort of Veterans. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. 2016;14:301–302.

26. Younossi ZM, Otgonsuren M, Henry L, Venkatesan C, Mishra A, Erario M, et al. Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009. Hepatology. 2015;62:1723–1730.

27. Younossi ZM, Blissett D, Blissett R, Henry L, Stepanova M, Younossi Y, et al. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. Hepatology. 2016;64:1577–1586.

28. Estes C, Anstee QM, Arias-Loste MT, Bantel H, Bellentani S, Caballeria J, et al. Modeling NAFLD disease burden in China, France, Germany, Italy, Japan, Spain, United Kingdom, and United States for the period 2016-2030. J. Hepatol. 2018;69:896–904.

29. Marchesini G, Day CP, Dufour JF, Canbay A, Nobili V, Ratziu V, et al. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J. Hepatol. 2016;64:1388–1402.

30. Degasperi E, Colombo M. Distinctive features of hepatocellular carcinoma in non-alcoholic fatty liver disease. lancet. Gastroenterol. Hepatol. 2016;1:156–164.

31. Piscaglia F, Svegliati-Baroni G, Barchetti A, Pecorelli A, Marinelli S, Tiribelli C, et al. Clinical patterns of hepatocellular carcinoma in nonalcoholic fatty liver disease: A multicenter prospective study. Hepatology. 2016;63:827–838.

32. Marrero JA, Fontana RJ, Fu S, Conjeevaram HS, Su GL, Lok AS. Alcohol, tobacco and obesity are synergistic risk factors for hepatocellular carcinoma. J. Hepatol. 2005;42:218–224.

33. Trichopoulos D, Bamia C, Lagiou P, Fedirko V, Trepo E, Jenab M, et al. Hepatocellular carcinoma risk factors and disease burden in a European cohort: a nested case-control study. J. Natl. Cancer Inst. 2011;103:1686–1695.

34. Ioannou GN, Bryson CL, Weiss NS, Miller R, Scott JD, Boyko EJ. The prevalence of cirrhosis and hepatocellular carcinoma in patients with human immunodeficiency virus infection. Hepatology. 2013;57:249–257.

35. Pelizzaro F, Cardin R, Sartori A, Imondi A, Penzo B, Farinati F. Coffee and hepatocellular carcinoma: epidemiologic evidence and biologic mechanisms. Hepatoma Res. 2021;7:29.

36. Niederau C, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, et al. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N. Engl. J. Med. 1996;334:1422–1427.

37. Papatheodoridis G V, Chan HL-Y, Hansen BE, Janssen HLA, Lampertico P. Risk of hepatocellular carcinoma in chronic hepatitis B: assessment and modification with current antiviral therapy. J. Hepatol. 2015;62:956–967.

38. Varbobitis I, Papatheodoridis G V. The assessment of hepatocellular carcinoma risk in patients with chronic hepatitis B under antiviral therapy. Clin. Mol. Hepatol. 2016;22:319–326.

39. Papatheodoridis G V, Idilman R, Dalekos GN, Buti M, Chi H, van Boemmel F, et al. The risk of hepatocellular carcinoma decreases after the first 5 years of entecavir or tenofovir in Caucasians with chronic hepatitis B. Hepatology. 2017;66:1444–1453.

40. Su T-H, Hu T-H, Chen C-Y, Huang Y-H, Chuang W-L, Lin C-C, et al. Four-year entecavir therapy reduces hepatocellular carcinoma, cirrhotic events and mortality in chronic hepatitis B patients. Liver Int. Off. J. Int. Assoc. Study Liver. 2016;36:1755–1764.

41. Morgan RL, Baack B, Smith BD, Yartel A, Pitasi M, Falck-Ytter Y. Eradication of hepatitis C virus infection and the development of hepatocellular carcinoma: a meta-analysis of observational studies. Ann. Intern. Med. 2013;158:329–337.

42. Kanwal F, Kramer J, Asch SM, Chayanupatkul M, Cao Y, El-Serag HB. Risk of Hepatocellular Cancer in HCV Patients Treated With Direct-Acting Antiviral Agents. Gastroenterology. 2017;153:996-1005.e1.

43. Romano A, Angeli P, Piovesan S, Noventa F, Anastassopoulos G, Chemello L, et al. Newly diagnosed hepatocellular carcinoma in patients with advanced hepatitis C treated with DAAs: A prospective population study. J. Hepatol. 2018;69:345–352.

44. Calvaruso V, Cabibbo G, Cacciola I, Petta S, Madonia S, Bellia A, et al. Incidence of Hepatocellular Carcinoma in Patients With HCV-Associated Cirrhosis Treated With Direct-Acting Antiviral Agents. Gastroenterology. 2018;155:411-421.e4.

45. Ioannou GN, Green PK, Berry K. HCV eradication induced by direct-acting antiviral agents reduces the risk of hepatocellular carcinoma. J. Hepatol. 2017;

46. Nahon P, Layese R, Bourcier V, Cagnot C, Marcellin P, Guyader D, et al. Incidence of Hepatocellular Carcinoma After Direct Antiviral Therapy for HCV in Patients With Cirrhosis Included in Surveillance Programs. Gastroenterology. 2018;155:1436-1450.e6.

47. Kanwal F, Kramer JR, Asch SM, Cao Y, Li L, El-Serag HB. Long-Term Risk of Hepatocellular Carcinoma in HCV Patients Treated With Direct Acting Antiviral Agents. Hepatology. 2020;71:44–55.

48. Yang B, Zhang B, Xu Y, Wang W, Shen Y, Zhang A, et al. Prospective study of early detection for primary liver cancer. J. Cancer Res. Clin. Oncol. 1997;123:357–360.

49. Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. J. Cancer Res. Clin. Oncol. 2004;130:417–422.

50. Trevisani F, Santi V, Gramenzi A, Di Nolfo MA, Del Poggio P, Benvegnu L, et al. Surveillance for early diagnosis of hepatocellular carcinoma: is it effective in intermediate/advanced cirrhosis? Am. J. Gastroenterol. 2007;102:2448–57.

51. Singal A, Volk ML, Waljee A, Salgia R, Higgins P, Rogers MAM, et al. Meta-analysis: Surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment. Pharmacol. Ther. 2009;30:37–47.

52. Barbara L, Benzi G, Gaiani S, Fusconi F, Zironi G, Siringo S, et al. Natural history of small untreated hepatocellular carcinoma in cirrhosis: A multivariate analysis of prognostic factors of tumor growth rate and patient survival. Hepatology. 1992;16:132–137.

53. Thompson Coon J, Rogers G, Hewson P, Wright D, Anderson R, Jackson S, et al. Surveillance of cirrhosis for hepatocellular carcinoma: A cost-utility analysis. Br. J. Cancer. 2008;98:1166–1175.

54. Sheu JC, Sung JL, Chen DS, Yang PM, Lai MY, Lee CS, et al. Growth rate of asymptomatic hepatocellular carcinoma and its clinical implications. Gastroenterology. 1985;89:259–266.

55. Trinchet JC, Chaffaut C, Bourcier V, Degos F, Henrion J, Fontaine H, et al. Ultrasonographic surveillance of hepatocellular carcinoma in cirrhosis: A randomized trial comparing 3- and 6-month periodicities. Hepatology. 2011;54:1987–1997.

56. Santi V, Trevisani F, Gramenzi A, Grignaschi A, Mirici-Cappa F, Del Poggio P, et al. Semiannual surveillance is superior to annual surveillance for the detection of early hepatocellular carcinoma and patient survival. J. Hepatol. 2010;53:291–297.

57. Santagostino E, Colombo M, Rivi M, Rumi MG, Rocino A, Linari S, et al. A 6-month versus a 12-month surveillance for hepatocellular carcinoma in 559 hemophiliacs infected with the hepatitis C virus. Blood. 2003;102:78–82.

58. Singal AG, Pillai A, Tiro J. Early Detection, Curative Treatment, and Survival Rates for Hepatocellular Carcinoma Surveillance in Patients with Cirrhosis: A Meta-analysis. PLoS Med. 2014;11:e1001624.

59. Poustchi H, Farrell GC, Strasser SI, Lee AU, Mccaughan GW, George J. Feasibility of conducting a randomized control trial for liver cancer screening: Is a randomized controlled trial for liver cancer screening feasible or still needed? Hepatology. 2011;54:1998–2004.

60. Sarasin FP, Giostra E, Hadengue A. Cost-effectiveness of screening for detection of small hepatocellular carcinoma in western patients with Child-Pugh class A cirrhosis. Am. J. Med. 1996;101:422–434.

61. Sherman M. Surveillance for hepatocellular carcinoma. Best Pract. Res. Clin. Gastroenterol. 2014;28:783–793.

62. Díaz-González Á, Forner A. Surveillance for hepatocellular carcinoma. Best Pract. Res. Clin. Gastroenterol. 2016;30:1001–1010.

63. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.

64. Papatheodoridis G, Dalekos G, Sypsa V, Yurdaydin C, Buti M, Goulis J, et al. PAGE-B predicts the risk of developing hepatocellular carcinoma in Caucasians with chronic hepatitis B on 5-year antiviral therapy. J. Hepatol. 2016;64:800–806.

65. Lok AS, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, Curto TM, et al. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. Gastroenterology. 2009;136:138–148.

66. Paradis V, Zalinski S, Chelbi E, Guedj N, Degos F, Vilgrain V, et al. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: a pathological analysis. Hepatology. 2009;49:851–859.

67. Ertle J, Dechêne A, Sowa J-P, Penndorf V, Herzer K, Kaiser G, et al. Non-alcoholic fatty liver disease progresses to hepatocellular carcinoma in the absence of apparent cirrhosis. Int. J. cancer. 2011;128:2436–2443.

68. Kanwal F, Kramer JR, Mapakshi S, Natarajan Y, Chayanupatkul M, Richardson PA, et al. Risk of Hepatocellular Cancer in Patients With Non-Alcoholic Fatty Liver Disease. Gastroenterology. 2018;155:1828-1837.e2.

69. Stine JG, Wentworth BJ, Zimmet A, Rinella ME, Loomba R, Caldwell SH, et al. Systematic review with meta-analysis: risk of hepatocellular carcinoma in non-alcoholic steatohepatitis without cirrhosis compared to other liver diseases. Aliment. Pharmacol. Ther. 2018;48:696–703.

70. Zhang B, Yang B. Combined alpha fetoprotein testing and ultrasonography as a screening test for primary liver cancer. J. Med. Screen. 1999;6:108–110.

71. Tzartzeva K, Obi J, Rich NE, Parikh ND, Marrero JA, Yopp A, et al. Surveillance Imaging and Alpha Fetoprotein for Early Detection of Hepatocellular Carcinoma in Patients With Cirrhosis: A Meta-analysis. Gastroenterology. 2018;154:1706-1718.e1.

72. Pocha C, Dieperink E, McMaken KA, Knott A, Thuras P, Ho SB. Surveillance for hepatocellular cancer with ultrasonography vs. computed tomography -- a randomised study. Aliment. Pharmacol. Ther. 2013;38:303–312.

73. Kim SY, An J, Lim Y-S, Han S, Lee J-Y, Byun JH, et al. MRI With Liver-Specific Contrast for Surveillance of Patients With Cirrhosis at High Risk of Hepatocellular Carcinoma. JAMA Oncol. 2017;3:456–463.

74. Gupta S, Bent S, Kohlwes J. Test Characteristics of α-Fetoprotein for Detecting Hepatocellular Carcinoma in Patients with Hepatitis C: A Systematic Review and Critical Analysis. Ann. Intern. Med. 2003;139:46–50.

75. Di Bisceglie AM, Sterling RK, Chung RT, Everhart JE, Dienstag JL, Bonkovsky HL, et al. Serum alpha-fetoprotein levels in patients with advanced hepatitis C: Results from the HALT-C Trial. J. Hepatol. 2005;43:434–441.

76. Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res. 2008;68:1451–1461.

77. Villanueva A, Minguez B, Forner A, Reig M, Llovet JM. Hepatocellular carcinoma: novel molecular approaches for diagnosis, prognosis, and therapy. Annu. Rev. Med. 2010;61:317–328.

78. Hoshida Y, Nijman SMB, Kobayashi M, Chan JA, Brunet J-P, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res. 2009;69:7385–7392.

79. Biselli M, Conti F, Gramenzi A, Frigerio M, Cucchetti A, Fatti G, et al. A new approach to the use of α-fetoprotein as surveillance test for hepatocellular carcinoma in patients with cirrhosis. Br. J. Cancer. 2015;112:69–76.

80. Nathani P, Gopal P, Rich N, Yopp A, Yokoo T, John B, et al. Hepatocellular carcinoma tumour volume doubling time: a systematic review and meta-analysis. Gut. 2021;70:401–407.

81. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68:723–750.

82. Omata M, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, et al. Asia–Pacific clinical practice guidelines on the

management of hepatocellular carcinoma: a 2017 update. Hepatol. Int. 2017;11:317–370.

83. Kokudo N, Takemura N, Hasegawa K, Takayama T, Kubo S, Shimada M, et al. Clinical practice guidelines for hepatocellular carcinoma: The Japan Society of Hepatology 2017 (4th JSH-HCC guidelines) 2019 update. Hepatol. Res. 2019;49:1109–1113.

84. Matsui O, Kobayashi S, Sanada J, Kouda W, Ryu Y, Kozaka K, et al. Hepatocelluar nodules in liver cirrhosis: hemodynamic evaluation (angiography-assisted CT) with special reference to multi-step hepatocarcinogenesis. Abdom. Imaging. 2011;36:264–272.

85. Burrel M, Llovet JM, Ayuso C, Iglesias C, Sala M, Miquel R, et al. MRI angiography is superior to helical CT for detection of HCC prior to liver transplantation: an explant correlation. Hepatology. 2003;38:1034–1042.

86. Forner A, Vilana R, Ayuso C, Bianchi L, Solé M, Ayuso JR, et al. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: Prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. Hepatology. 2008;47:97–104.

87. Bolondi L, Sofia S, Siringo S, Gaiani S, Casali A, Zironi G, et al. Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. Gut. 2001;48:251–259.

88. Sangiovanni A, Manini MA, Iavarone M, Romeo R, Forzenigo L V, Fraquelli M, et al. The diagnostic and economic impact of contrast imaging techniques in the diagnosis of small hepatocellular carcinoma in cirrhosis. Gut. 2010;59:638–644.

89. Roberts LR, Sirlin CB, Zaiem F, Almasri J, Prokop LJ, Heimbach JK, et al. Imaging for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. Hepatology. 2018;67:401–421.

90. Chernyak V, Fowler KJ, Kamaya A, Kielar AZ, Elsayes KM, Bashir MR, et al. Liver Imaging Reporting and Data System (LI-RADS) Version 2018: Imaging of Hepatocellular Carcinoma in At-Risk Patients. Radiology. 2018;289:816–830.

91. Darnell A, Forner A, Rimola J, Reig M, García-Criado Á, Ayuso C, et al. Liver Imaging Reporting and Data System with MR Imaging: Evaluation in Nodules 20 mm or Smaller Detected in Cirrhosis at Screening US. Radiology. 2015;275:698–707.

92. Chou R, Cuevas C, Fu R, Devine B, Wasson N, Ginsburg A, et al. Imaging Techniques for the Diagnosis of Hepatocellular Carcinoma: A Systematic Review and Meta-analysis. Ann. Intern. Med. 2015;162:697–711.

93. Lee YJ, Lee JM, Lee JS, Lee HY, Park BH, Kim YH, et al. Hepatocellular carcinoma: diagnostic performance of multidetector CT and MR imaging-a systematic review and meta-analysis. Radiology. 2015;275:97–109.

94. Aubé C, Oberti F, Lonjon J, Pageaux G, Seror O, N'Kontchou G, et al. EASL and AASLD recommendations for the diagnosis of HCC to the test of daily practice. Liver Int. Off. J. Int. Assoc. Study Liver. 2017;37:1515–1525.

95. Vilana R, Forner A, Bianchi L, García-Criado A, Rimola J, de Lope CR, et al. Intrahepatic peripheral cholangiocarcinoma in cirrhosis patients may display a vascular pattern similar to hepatocellular carcinoma on contrast-enhanced ultrasound. Hepatology. 2010;51:2020–2029.

96. Galassi M, Iavarone M, Rossi S, Bota S, Vavassori S, Rosa L, et al. Patterns of appearance and risk of misdiagnosis of intrahepatic cholangiocarcinoma in cirrhosis at contrast enhanced ultrasound. Liver Int. Off. J. Int. Assoc. Study Liver. 2013;33:771–779.

97. Li R, Zhang X, Ma K-S, Li X-W, Xia F, Zhong H, et al. Dynamic enhancing vascular pattern of intrahepatic peripheral cholangiocarcinoma on contrast-enhanced ultrasound: the influence of chronic hepatitis and cirrhosis. Abdom. Imaging. 2013;38:112–119.

98. de Sio I, Iadevaia MD, Vitale LM, Niosi M, Del Prete A, de Sio C, et al. Optimized contrast-enhanced ultrasonography for characterization of focal liver lesions in cirrhosis: A single-center retrospective study. United Eur. Gastroenterol. J. 2014;2:279–287.

99. Yuan MX, Li R, Zhang XH, Tang CL, Guo YL, Guo DY, et al. Factors Affecting the Enhancement Patterns of Intrahepatic Cholangiocarcinoma (ICC) on Contrast-Enhanced Ultrasound (CEUS) and their Pathological Correlations in Patients with a Single Lesion. Ultraschall Med. 2016;37:609–618.

100. Wildner D, Bernatik T, Greis C, Seitz K, Neurath MF, Strobel D. CEUS in hepatocellular carcinoma and intrahepatic cholangiocellular carcinoma in 320 patients - early or late washout matters: a subanalysis of the DEGUM

multicenter trial. Ultraschall Med. 2015;36:132–139.

101. Liu G-J, Wang W, Lu M-D, Xie X-Y, Xu H-X, Xu Z-F, et al. Contrast-Enhanced Ultrasound for the Characterization of Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. Liver cancer. 2015;4:241–252.

102. Wildner D, Pfeifer L, Goertz RS, Bernatik T, Sturm J, Neurath MF, et al. Dynamic contrast-enhanced ultrasound (DCE-US) for the characterization of hepatocellular carcinoma and cholangiocellular carcinoma. Ultraschall Med. 2014;35:522–527.

103. Piscaglia F, Kudo M, Han KH, Sirlin C. Diagnosis of Hepatocellular Carcinoma with Non-Invasive Imaging: a Plea for Worldwide Adoption of Standard and Precise Terminology for Describing Enhancement Criteria. Ultraschall Med. 2017;38:9–11.

104. Piscaglia F, Wilson SR, Lyshchik A, Cosgrove D, Dietrich CF, Jang H-J, et al. American College of Radiology Contrast Enhanced Ultrasound Liver Imaging Reporting and Data System (CEUS LI-RADS) for the diagnosis of Hepatocellular Carcinoma: a pictorial essay. Ultraschall Med. 2017;38:320–324.

105. Terzi E, Iavarone M, Pompili M, Veronese L, Cabibbo G, Fraquelli M, et al. Contrast ultrasound LI-RADS LR-5 identifies hepatocellular carcinoma in cirrhosis in a multicenter restropective study of 1,006 nodules. J. Hepatol. 2018;68:485–492.

106. Furlan A, Marin D, Cabassa P, Taibbi A, Brunelli E, Agnello F, et al. Enhancement pattern of small hepatocellular carcinoma (HCC) at contrast-enhanced US (CEUS), MDCT, and MRI: intermodality agreement and comparison of diagnostic sensitivity between 2005 and 2010 American Association for the Study of Liver Diseases (AASLD). Eur. J. Radiol. 2012;81:2099–2105.

107. Tremosini S, Forner A, Boix L, Vilana R, Bianchi L, Reig M, et al. Prospective validation of an immunohistochemical panel (glypican 3, heat shock protein 70 and glutamine synthetase) in liver biopsies for diagnosis of very early hepatocellular carcinoma. Gut. 2012;61:1481–1487.

108. Vitale A, Farinati F, Finotti M, Di Renzo C, Brancaccio G, Piscaglia F, et al. Overview of Prognostic Systems for Hepatocellular Carcinoma and ITA.LI.CA External Validation of MESH and CNLC Classifications. Cancers (Basel). 2021;13:1673.

109. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br. J. Surg. 1973;60:646–649.

110. Kamath PS, Kim WR. The model for end-stage liver disease (MELD). Hepatology. 2007;45:797–805.

111. Johnson PJ, Berhane S, Kagebayashi C, Satomura S, Teng M, Reeves HL, et al. Assessment of liver function in patients with hepatocellular carcinoma: a new evidence-based approach-the ALBI grade. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 2015;33:550–558.

112. Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am. J. Clin. Oncol. 1982;5:649–655.

113. Ohya T, Kikuchi S. [Clinical evaluation of chemotherapeutic agents in the treatment of primary liver cancer]. Gan To Kagaku Ryoho. 1982;9:1623–1627.

114. Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer. 1985;56:918–928.

115. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. Hepatology. 1998;28:751–755.

116. Kudo M, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). J. Gastroenterol. 2003;38:207–215.

117. Liu P-H, Hsu C-Y, Hsia C-Y, Lee Y-H, Huang Y-H, Su C-W, et al. Proposal and validation of a new model to estimate survival for hepatocellular carcinoma patients. Eur. J. Cancer. 2016;63:25–33.

118. Chevret S, Trinchet JC, Mathieu D, Rached AA, Beaugrand M, Chastang C. A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. J. Hepatol. 1999;31:133–141.

119. Leung TWT, Tang AMY, Zee B, Lau WY, Lai PBS, Leung KL, et al. Construction of the Chinese University Prognostic Index for hepatocellular carcinoma and comparison with the TNM staging system, the Okuda staging system,

and the Cancer of the Liver Italian Program staging system: a study based on 926 patients. Cancer. 2002;94:1760–1769.

120. Tateishi R, Yoshida H, Shiina S, Imamura H, Hasegawa K, Teratani T, et al. Proposal of a new prognostic model for hepatocellular carcinoma: an analysis of 403 patients. Gut. 2005;54:419–425.

121. Hsu C-Y, Huang Y-H, Hsia C-Y, Su C-W, Lin H-C, Loong C-C, et al. A new prognostic model for hepatocellular carcinoma based on total tumor volume: the Taipei Integrated Scoring System. J. Hepatol. 2010;53:108–117.

122. Yang JD, Kim WR, Park KW, Chaiteerakij R, Kim B, Sanderson SO, et al. Model to estimate survival in ambulatory patients with hepatocellular carcinoma. Hepatology. 2012;56:614–621.

123. Heinrich S, Sprinzl M, Schmidtmann I, Heil E, Koch S, Czauderna C, et al. Validation of prognostic accuracy of MESH, HKLC, and BCLC classifications in a large German cohort of hepatocellular carcinoma patients. United Eur. Gastroenterol. J. 2020;8:444–452.

124. Zhou J, Sun H-C, Wang Z, Cong W-M, Wang J-H, Zeng M-S, et al. Guidelines for Diagnosis and Treatment of Primary Liver Cancer in China (2017 Edition). Liver cancer. 2018;7:235–260.

125. Xie D-Y, Ren Z-G, Zhou J, Fan J, Gao Q. 2019 Chinese clinical guidelines for the management of hepatocellular carcinoma: updates and insights. Hepatobiliary Surg. Nutr. 2020;9:452–463.

126. Zhou J, Sun H, Wang Z, Cong W, Wang J, Zeng M, et al. Guidelines for the Diagnosis and Treatment of Hepatocellular Carcinoma (2019 Edition). Liver cancer. 2020;9:682–720.

127. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann. Surg. Oncol. 2010;17:1471–1474.

128. Llovet JM, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin. Liver Dis. 1999;19:329–338.

129. Otto G, Pitton MB, Hoppe-Lotichius M, Weinmann A. Liver transplantation and BCLC classification: Limitations impede optimum treatment. Hepatobiliary Pancreat. Dis. Int. 2021;20:6–12.

130. Cho YK, Chung JW, Kim JK, Ahn YS, Kim MY, Park YO, et al. Comparison of 7 staging systems for patients with hepatocellular carcinoma undergoing transarterial chemoembolization. Cancer. 2008;112:352–361.

131. Reig M, Forner A, Rimola J, Ferrer-Fábrega J, Burrel M, Garcia-Criado A, et al. BCLC strategy for prognosis prediction and treatment recommendation Barcelona Clinic Liver Cancer (BCLC) staging system. The 2022 update. J. Hepatol. 2021;

132. Chapiro J, Geschwind J-F. Hepatocellular carcinoma: have we finally found the ultimate staging system for HCC? Nat. Rev. Gastroenterol. Hepatol. 2014;11:334–336.

133. Park JW, Chen M, Colombo M, Roberts LR, Schwartz M, Chen PJ, et al. Global patterns of hepatocellular carcinoma management from diagnosis to death: The BRIDGE Study. Liver Int. 2015;35:2155–2166.

134. Giannini EG, Bucci L, Garuti F, Brunacci M, Lenzi B, Valente M, et al. Patients with advanced hepatocellular carcinoma need a personalized management: A lesson from clinical practice. Hepatology. 2018;67:1784–1796.

135. Tabrizian P, Jibara G, Shrager B, Schwartz M, Roayaie S. Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis. Ann. Surg. 2015;261:947–955.

136. Sangiovanni A, Triolo M, Iavarone M, Forzenigo L V., Nicolini A, Rossi G, et al. Multimodality treatment of hepatocellular carcinoma: How field practice complies with international recommendations. Liver Int. 2018;38:1624–1634.

137. Yin L, Li H, Li AJ, Lau WY, Pan ZY, Lai ECH, et al. Partial hepatectomy vs. transcatheter arterial chemoembolization for resectable multiple hepatocellular carcinoma beyond Milan criteria: A RCT. J. Hepatol. 2014;61:82–88.

138. Kokudo T, Hasegawa K, Matsuyama Y, Takayama T, Izumi N, Kadoya M, et al. Survival benefit of liver resection for hepatocellular carcinoma associated with portal vein invasion. J. Hepatol. 2016;65:938–943.

139. Kokudo T, Hasegawa K, Matsuyama Y, Takayama T, Izumi N, Kadoya M, et al. Liver resection for hepatocellular carcinoma associated with hepatic vein invasion: A Japanese nationwide survey. Hepatology. 2017;66:510–517.

140. Zhang XP, Gao YZ, Chen ZH, Chen MS, Li LQ, Wen TF, et al. An Eastern Hepatobiliary Surgery Hospital/Portal Vein Tumor Thrombus Scoring System as an Aid to Decision Making on Hepatectomy for Hepatocellular Carcinoma Patients With Portal Vein Tumor Thrombus: A Multicenter Study. Hepatology. 2019;69:2076–2090.

141. Kim KM, Sinn DH, Jung SH, Gwak GY, Paik YH, Choi MS, et al. The recommended treatment algorithms of the BCLC and HKLC staging systems: does following these always improve survival rates for HCC patients? Liver Int. 2016;36:1490–1497.

142. Lombardi G, Zustovich F, Farinati F, Cillo U, Vitale A, Zanus G, et al. Pegylated liposomal doxorubicin and gemcitabine in patients with advanced hepatocellular carcinoma: Results of a phase 2 study. Cancer. 2011;117:125–133.

143. Cillo U, Vitale A, Volk ML, Frigo AC, Grigoletto F, Brolese A, et al. The survival benefit of liver transplantation in hepatocellular carcinoma patients. Dig. Liver Dis. 2010;42:642–649.

144. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology. 2015;62:440–451.

145. Pecorelli A, Lenzi B, Gramenzi A, Garuti F, Farinati F, Giannini EG, et al. Curative therapies are superior to standard of care (transarterial chemoembolization) for intermediate stage hepatocellular carcinoma. Liver Int. 2017;37:423–433.

146. Yau T, Tang VYF, Yao T-J, Fan S-T, Lo C-M, Poon RTP. Development of Hong Kong Liver Cancer staging system with treatment stratification for patients with hepatocellular carcinoma. Gastroenterology. 2014;146:1691–700.e3.

147. Farinati F, Vitale A, Spolverato G, Pawlik TM, Huo T, Lee Y-H, et al. Development and Validation of a New Prognostic System for Patients with Hepatocellular Carcinoma. PLoS Med. 2016;13:e1002006.

148. Bolondi L, Burroughs A, Dufour J-F, Galle PR, Mazzaferro V, Piscaglia F, et al. Heterogeneity of patients with intermediate (BCLC B) Hepatocellular Carcinoma: proposal for a subclassification to facilitate treatment decisions. Semin. Liver Dis. 2012;32:348–359.

149. Lu J, Zhang XP, Zhong BY, Lau WY, Madoff DC, Davidson JC, et al. Management of patients with hepatocellular carcinoma and portal vein tumour thrombosis: comparing east and west. Lancet Gastroenterol. Hepatol. 2019;4:721–730.

150. Borzio M, Dionigi E, Rossini A, Marignani M, Sacco R, De Sio I, et al. External validation of the ITA.LI.CA prognostic system for patients with hepatocellular carcinoma: A multicenter cohort study. Hepatology. 2018;67:2215–2225.

151. Vitale A, Farinati F, Noaro G, Burra P, Pawlik TM, Bucci L, et al. Restaging Patients With Hepatocellular Carcinoma Before Additional Treatment Decisions: A Multicenter Cohort Study. Hepatology. 2018;68:1232–1244.

152. Vitale A, Farinati F, Pawlik TM, Frigo AC, Giannini EG, Napoli L, et al. The concept of therapeutic hierarchy for patients with hepatocellular carcinoma: A multicenter cohort study. Liver Int. 2019;39:1478–1489.

153. Vitale A, Trevisani F, Farinati F, Cillo U. Treatment of Hepatocellular Carcinoma in the Precision Medicine Era: From Treatment Stage Migration to Therapeutic Hierarchy. Hepatology. 2020;72:2206–2218.

154. Ishizawa T, Hasegawa K, Aoki T, Takahashi M, Inoue Y, Sano K, et al. Neither multiple tumors nor portal hypertension are surgical contraindications for hepatocellular carcinoma. Gastroenterology. 2008;134:1908–1916.

155. Vitale A, Burra P, Frigo AC, Trevisani F, Farinati F, Spolverato G, et al. Survival benefit of liver resection for patients with hepatocellular carcinoma across different Barcelona Clinic Liver Cancer stages: a multicentre study. J. Hepatol. 2015;62:617–624.

156. Pawlik TM, Poon RT, Abdalla EK, Ikai I, Nagorney DM, Belghiti J, et al. Hepatectomy for hepatocellular carcinoma with major portal or hepatic vein invasion: results of a multicenter study. Surgery. 2005;137:403–410.

157. Roayaie S, Jibara G, Taouli B, Schwartz M. Resection of hepatocellular carcinoma with macroscopic vascular invasion. Ann. Surg. Oncol. 2013;20:3754–3760.

158. Pesi B, Ferrero A, Grazi GL, Cescon M, Russolillo N, Leo F, et al. Liver resection with thrombectomy as a treatment of hepatocellular carcinoma with major vascular invasion: results from a retrospective multicentric study. Am. J. Surg. 2015;210:35–44.

159. Mazzaferro V, Romito R, Schiavo M, Mariani L, Camerini T, Bhoori S, et al. Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. Hepatology. 2006;44:1543–1554.

160. Shen Y-C, Hsu C, Chen L-T, Cheng C-C, Hu F-C, Cheng A-L. Adjuvant interferon therapy after curative therapy for hepatocellular carcinoma (HCC): a meta-regression approach. J. Hepatol. 2010;52:889–894.

161. Samuel M, Chow PK-H, Chan Shih-Yen E, Machin D, Soo K-C. Neoadjuvant and adjuvant therapy for surgical resection of hepatocellular carcinoma. Cochrane database Syst. Rev. 2009;2009:CD001199.

162. Bruix J, Takayama T, Mazzaferro V, Chau G-Y, Yang J, Kudo M, et al. Adjuvant sorafenib for hepatocellular carcinoma after resection or ablation (STORM): a phase 3, randomised, double-blind, placebo-controlled trial. Lancet. Oncol. 2015;16:1344–1354.

163. Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Harper AM, et al. OPTN/SRTR 2016 Annual Data Report: Liver. Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg. 2018;18 Suppl 1:172–253.

164. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N. Engl. J. Med. 1996;334:693–699.

165. Mazzaferro V, Bhoori S, Sposito C, Bongini M, Langer M, Miceli R, et al. Milan criteria in liver transplantation for hepatocellular carcinoma: an evidence-based analysis of 15 years of experience. Liver Transplant. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 2011;17 Suppl 2:S44-57.

166. Vitale A, Morales RR, Zanus G, Farinati F, Burra P, Angeli P, et al. Barcelona Clinic Liver Cancer staging and transplant survival benefit for patients with hepatocellular carcinoma: a multicentre, cohort study. Lancet. Oncol. 2011;12:654–662.

167. Cillo U, Vitale A, Polacco M, Fasolo E. Liver transplantation for hepatocellular carcinoma through the lens of transplant benefit. Hepatology. 2017;65:1741–1748.

168. Yao FY, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, et al. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. Hepatology. 2001;33:1394–1403.

169. Yao FY, Xiao L, Bass NM, Kerlan R, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: validation of the UCSF-expanded criteria based on preoperative imaging. Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg. 2007;7:2587–2596.

170. Mazzaferro V, Llovet JM, Miceli R, Bhoori S, Schiavo M, Mariani L, et al. Predicting survival after liver transplantation in patients with hepatocellular carcinoma beyond the Milan criteria: a retrospective, exploratory analysis. Lancet Oncol. 2009;10:35–43.

171. Toso C, Asthana S, Bigam DL, Shapiro AMJ, Kneteman NM. Reassessing selection criteria prior to liver transplantation for hepatocellular carcinoma utilizing the Scientific Registry of Transplant Recipients database. Hepatology. 2009;49:832–838.

172. Toso C, Meeberg G, Hernandez-Alejandro R, Dufour J-F, Marotta P, Majno P, et al. Total tumor volume and alpha-fetoprotein for selection of transplant candidates with hepatocellular carcinoma: A prospective validation. Hepatology. 2015;62:158–165.

173. Duvoux C, Roudot-Thoraval F, Decaens T, Pessione F, Badran H, Piardi T, et al. Liver transplantation for hepatocellular carcinoma: a model including alpha-fetoprotein improves the performance of Milan criteria. Gastroenterology. 2012;143:985–986.

174. Mazzaferro V, Sposito C, Zhou J, Pinna AD, De Carlis L, Fan J, et al. Metroticket 2.0 Model for Analysis of Competing Risks of Death After Liver Transplantation for Hepatocellular Carcinoma. Gastroenterology. 2018;154:128–139.

175. Volk ML, Vijan S, Marrero JA. A novel model measuring the harm of transplanting hepatocellular carcinoma exceeding Milan criteria. Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg. 2008;8:839–846.

176. Yao FY, Mehta N, Flemming J, Dodge J, Hameed B, Fix O, et al. Downstaging of hepatocellular cancer before liver transplant: long-term outcome compared to tumors within Milan criteria. Hepatology. 2015;61:1968–1977.

177. Otto G, Herber S, Heise M, Lohse AW, Mönch C, Bittinger F, et al. Response to transarterial chemoembolization as a biological selection criterion for liver transplantation in hepatocellular carcinoma. Liver Transplant. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 2006;12:1260–1267.

178. Millonig G, Graziadei IW, Freund MC, Jaschke W, Stadlmann S, Ladurner R, et al. Response to preoperative

chemoembolization correlates with outcome after liver transplantation in patients with hepatocellular carcinoma. Liver Transplant. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 2007;13:272–279.

179. Lai Q, Avolio AW, Graziadei I, Otto G, Rossi M, Tisone G, et al. Alpha-fetoprotein and modified response evaluation criteria in solid tumors progression after locoregional therapy as predictors of hepatocellular cancer recurrence and death after transplantation. Liver Transplant. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 2013;19:1108–1118.

180. Vitale A, D'Amico F, Frigo AC, Grigoletto F, Brolese A, Zanus G, et al. Response to therapy as a criterion for awarding priority to patients with hepatocellular carcinoma awaiting liver transplantation. Ann. Surg. Oncol. 2010;17:2290–2302.

181. Cucchetti A, Cescon M, Bigonzi E, Piscaglia F, Golfieri R, Ercolani G, et al. Priority of candidates with hepatocellular carcinoma awaiting liver transplantation can be reduced after successful bridge therapy. Liver Transplant. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 2011;17:1344–1354.

182. Mazzaferro V, Citterio D, Bhoori S, Bongini M, Miceli R, De Carlis L, et al. Liver transplantation in hepatocellular carcinoma after tumour downstaging (XXL): a randomised, controlled, phase 2b/3 trial. Lancet. Oncol. 2020;21:947–956.

183. Lencioni R, Crocetti L. Local-regional treatment of hepatocellular carcinoma. Radiology. 2012;262:43–58.

184. Lee DH, Lee JM, Lee JY, Kim SH, Yoon JH, Kim YJ, et al. Radiofrequency ablation of hepatocellular carcinoma as first-line treatment: Long-term results and prognostic factors in 162 patients with cirrhosis. Radiology. 2014;270:900–909.

185. Cucchetti A, Piscaglia F, Cescon M, Colecchia A, Ercolani G, Bolondi L, et al. Cost-effectiveness of hepatic resection versus percutaneous radiofrequency ablation for early hepatocellular carcinoma. J. Hepatol. 2013;59:300–307.

186. Livraghi T, Meloni F, Di Stasi M, Rolle E, Solbiati L, Tinelli C, et al. Sustained complete response and complications rates after radiofrequency ablation of very early hepatocellular carcinoma in cirrhosis: Is resection still the treatment of choice? Hepatology. 2008;47:82–89.

187. Lencioni R, Cioni D, Crocetti L, Franchini C, Pina C Della, Lera J, et al. Early-stage hepatocellular carcinoma in patients with cirrhosis: long-term results of percutaneous image-guided radiofrequency ablation. Radiology. 2005;234:961–967.

188. Choi D, Lim HK, Rhim H, Kim Y-S, Lee WJ, Paik SW, et al. Percutaneous radiofrequency ablation for early-stage hepatocellular carcinoma as a first-line treatment: long-term results and prognostic factors in a large single-institution series. Eur. Radiol. 2007;17:684–692.

189. Sala M, Llovet JM, Vilana R, Bianchi L, Solé M, Ayuso C, et al. Initial response to percutaneous ablation predicts survival in patients with hepatocellular carcinoma. Hepatology. 2004;40:1352–1360.

190. Cho YK, Kim JK, Kim WT, Chung JW. Hepatic resection versus radiofrequency ablation for very early stage hepatocellular carcinoma: a Markov model analysis. Hepatology. 2010;51:1284–1290.

191. Poulou LS, Botsa E, Thanou I, Ziakas PD, Thanos L. Percutaneous microwave ablation vs radiofrequency ablation in the treatment of hepatocellular carcinoma. World J. Hepatol. 2015;7:1054–1063.

192. Facciorusso A, Di Maso M, Muscatiello N. Microwave ablation versus radiofrequency ablation for the treatment of hepatocellular carcinoma: A systematic review and meta-analysis. Int. J. Hyperth. Off. J. Eur. Soc. Hyperthermic Oncol. North Am. Hyperth. Gr. 2016;32:339–344.

193. Di Costanzo GG, Tortora R, D'Adamo G, De Luca M, Lampasi F, Addario L, et al. Radiofrequency ablation versus laser ablation for the treatment of small hepatocellular carcinoma in cirrhosis: a randomized trial. J. Gastroenterol. Hepatol. 2015;30:559–565.

194. Francica G, Petrolati A, Di Stasio E, Pacella S, Stasi R, Pacella CM. Effectiveness, safety, and local progression after percutaneous laser ablation for hepatocellular carcinoma nodules up to 4 cm are not affected by tumor location. AJR. Am. J. Roentgenol. 2012;199:1393–1401.

195. Wang C, Wang H, Yang W, Hu K, Xie H, Hu K-Q, et al. Multicenter randomized controlled trial of percutaneous cryoablation versus radiofrequency ablation in hepatocellular carcinoma. Hepatology. 2015;61:1579–1590.

196. Nabavizadeh N, Mitin T, Dawson LA, Hong TS, Thomas CRJ. Stereotactic body radiotherapy for patients with

hepatocellular carcinoma and intermediate grade cirrhosis. Lancet. Oncol. 2017;18:e192.

197. Raoul J-L, Sangro B, Forner A, Mazzaferro V, Piscaglia F, Bolondi L, et al. Evolving strategies for the management of intermediate-stage hepatocellular carcinoma: available evidence and expert opinion on the use of transarterial chemoembolization. Cancer Treat. Rev. 2011;37:212–220.

198. Bargellini I, Florio F, Golfieri R, Grosso M, Lauretti DL, Cioni R. Trends in utilization of transarterial treatments for hepatocellular carcinoma: results of a survey by the Italian Society of Interventional Radiology. Cardiovasc. Intervent. Radiol. 2014;37:438–444.

199. Takayasu K, Arii S, Kudo M, Ichida T, Matsui O, Izumi N, et al. Superselective transarterial chemoembolization for hepatocellular carcinoma. Validation of treatment algorithm proposed by Japanese guidelines. J. Hepatol. 2012;56:886–892.

200. Burrel M, Reig M, Forner A, Barrufet M, de Lope CR, Tremosini S, et al. Survival of patients with hepatocellular carcinoma treated by transarterial chemoembolisation (TACE) using Drug Eluting Beads. Implications for clinical practice and trial design. J. Hepatol. 2012;56:1330–1335.

201. Malagari K, Pomoni M, Moschouris H, Bouma E, Koskinas J, Stefaniotou A, et al. Chemoembolization with doxorubicin-eluting beads for unresectable hepatocellular carcinoma: five-year survival analysis. Cardiovasc. Intervent. Radiol. 2012;35:1119–1128.

202. Brown KT, Do RK, Gonen M, Covey AM, Getrajdman GI, Sofocleous CT, et al. Randomized Trial of Hepatic Artery Embolization for Hepatocellular Carcinoma Using Doxorubicin-Eluting Microspheres Compared With Embolization With Microspheres Alone. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 2016;34:2046–2053.

203. Terzi E, Golfieri R, Piscaglia F, Galassi M, Dazzi A, Leoni S, et al. Response rate and clinical outcome of HCC after first and repeated cTACE performed "on demand". J. Hepatol. 2012;57:1258–1267.

204. Kudo M, Han G, Finn RS, Poon RTP, Blanc J-F, Yan L, et al. Brivanib as adjuvant therapy to transarterial chemoembolization in patients with hepatocellular carcinoma: A randomized phase III trial. Hepatology. 2014;60:1697–1707.

205. de Baere T, Arai Y, Lencioni R, Geschwind J-F, Rilling W, Salem R, et al. Treatment of Liver Tumors with Lipiodol TACE: Technical Recommendations from Experts Opinion. Cardiovasc. Intervent. Radiol. 2016;39:334–343.

206. Lo C-M, Ngan H, Tso W-K, Liu C-L, Lam C-M, Poon RT-P, et al. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. Hepatology. 2002;35:1164–1171.

207. Lencioni R, de Baere T, Soulen MC, Rilling WS, Geschwind J-FH. Lipiodol transarterial chemoembolization for hepatocellular carcinoma: A systematic review of efficacy and safety data. Hepatology. 2016;64:106–116.

208. Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. Lancet (London, England). 2002;359:1734–1739.

209. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. Hepatology. 2003;37:429–442.

210. Varela M, Real MI, Burrel M, Forner A, Sala M, Brunet M, et al. Chemoembolization of hepatocellular carcinoma with drug eluting beads: efficacy and doxorubicin pharmacokinetics. J. Hepatol. 2007;46:474–481.

211. Lammer J, Malagari K, Vogl T, Pilleul F, Denys A, Watkinson A, et al. Prospective randomized study of doxorubicin-eluting-bead embolization in the treatment of hepatocellular carcinoma: results of the PRECISION V study. Cardiovasc. Intervent. Radiol. 2010;33:41–52.

212. Golfieri R, Giampalma E, Renzulli M, Cioni R, Bargellini I, Bartolozzi C, et al. Randomised controlled trial of doxorubicin-eluting beads vs conventional chemoembolisation for hepatocellular carcinoma. Br. J. Cancer. 2014;111:255–264.

213. Gao S, Yang Z, Zheng Z, Yao J, Deng M, Xie H, et al. Doxorubicin-eluting bead versus conventional TACE for unresectable hepatocellular carcinoma: a meta-analysis. Hepatogastroenterology. 2013;60:813–820.

214. Monier A, Guiu B, Duran R, Aho S, Bize P, Deltenre P, et al. Liver and biliary damages following transarterial chemoembolization of hepatocellular carcinoma: comparison between drug-eluting beads and lipiodol emulsion. Eur. Radiol. 2017;27:1431–1439.

215. A comparison of lipiodol chemoembolization and conservative treatment for unresectable hepatocellular

carcinoma. N. Engl. J. Med. 1995;332:1256–1261.

216. Sieghart W, Hucke F, Pinter M, Graziadei I, Vogel W, Müller C, et al. The ART of decision making: retreatment with transarterial chemoembolization in patients with hepatocellular carcinoma. Hepatology. 2013;57:2261–2273.

217. Hucke F, Sieghart W, Pinter M, Graziadei I, Vogel W, Müller C, et al. The ART-strategy: sequential assessment of the ART score predicts outcome of patients with hepatocellular carcinoma re-treated with TACE. J. Hepatol. 2014;60:118–126.

218. Adhoute X, Penaranda G, Naude S, Raoul JL, Perrier H, Bayle O, et al. Retreatment with TACE: the ABCR SCORE, an aid to the decision-making process. J. Hepatol. 2015;62:855–862.

219. Lencioni R, Llovet JM, Han G, Tak WY, Yang J, Guglielmi A, et al. Sorafenib or placebo plus TACE with doxorubicin-eluting beads for intermediate stage HCC: The SPACE trial. J. Hepatol. 2016;64:1090–1098.

220. Meyer T, Fox R, Ma YT, Ross PJ, James MW, Sturgess R, et al. Sorafenib in combination with transarterial chemoembolisation in patients with unresectable hepatocellular carcinoma (TACE 2): a randomised placebo-controlled, double-blind, phase 3 trial. lancet. Gastroenterol. Hepatol. 2017;2:565–575.

221. Sangro B, Salem R. Transarterial chemoembolization and radioembolization. Semin. Liver Dis. 2014;34:435–443.

222. Mazzaferro V, Sposito C, Bhoori S, Romito R, Chiesa C, Morosi C, et al. Yttrium-90 radioembolization for intermediate-advanced hepatocellular carcinoma: a phase 2 study. Hepatology. 2013;57:1826–1837.

223. Salem R, Lewandowski RJ, Mulcahy MF, Riaz A, Ryu RK, Ibrahim S, et al. Radioembolization for hepatocellular carcinoma using Yttrium-90 microspheres: a comprehensive report of long-term outcomes. Gastroenterology. 2010;138:52–64.

224. Sangro B, Carpanese L, Cianni R, Golfieri R, Gasparini D, Ezziddin S, et al. Survival after yttrium-90 resin microsphere radioembolization of hepatocellular carcinoma across Barcelona clinic liver cancer stages: a European evaluation. Hepatology. 2011;54:868–878.

225. Hilgard P, Hamami M, Fouly A El, Scherag A, Müller S, Ertle J, et al. Radioembolization with yttrium-90 glass microspheres in hepatocellular carcinoma: European experience on safety and long-term survival. Hepatology. 2010;52:1741–1749.

226. Salem R, Lewandowski RJ, Kulik L, Wang E, Riaz A, Ryu RK, et al. Radioembolization results in longer time-to-progression and reduced toxicity compared with chemoembolization in patients with hepatocellular carcinoma. Gastroenterology. 2011;140:497-507.e2.

227. Salem R, Gilbertsen M, Butt Z, Memon K, Vouche M, Hickey R, et al. Increased quality of life among hepatocellular carcinoma patients treated with radioembolization, compared with chemoembolization. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. 2013;11:1358-1365.e1.

228. Salem R, Gordon AC, Mouli S, Hickey R, Kallini J, Gabr A, et al. Y90 Radioembolization Significantly Prolongs Time to Progression Compared With Chemoembolization in Patients With Hepatocellular Carcinoma. Gastroenterology. 2016;151:1155-1163.e2.

229. Vilgrain V, Pereira H, Assenat E, Guiu B, Ilonca AD, Pageaux G-P, et al. Efficacy and safety of selective internal radiotherapy with yttrium-90 resin microspheres compared with sorafenib in locally advanced and inoperable hepatocellular carcinoma (SARAH): an open-label randomised controlled phase 3 trial. Lancet. Oncol. 2017;18:1624–1636.

230. Chow PKH, Gandhi M, Tan S-B, Khin MW, Khasbazar A, Ong J, et al. SIRveNIB: Selective Internal Radiation Therapy Versus Sorafenib in Asia-Pacific Patients With Hepatocellular Carcinoma. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 2018;36:1913–1921.

231. Ricke J, Klümpen HJ, Amthauer H, Bargellini I, Bartenstein P, de Toni EN, et al. Impact of combined selective internal radiation therapy and sorafenib on survival in advanced hepatocellular carcinoma. J. Hepatol. 2019;71:1164–1174.

232. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J-F, et al. Sorafenib in advanced hepatocellular carcinoma. N. Engl. J. Med. 2008;359:378–390.

233. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-

controlled trial. Lancet Oncol. 2009;10:25–34.

234. Zhu AX, Rosmorduc O, Evans TRJ, Ross PJ, Santoro A, Carrilho FJ, et al. SEARCH: A Phase III, Randomized, Double-Blind, Placebo-Controlled Trial of Sorafenib Plus Erlotinib in Patients With Advanced Hepatocellular Carcinoma. J. Clin. Oncol. 2015;33:559–566.

235. Johnson PJ, Qin S, Park J-W, Poon RTP, Raoul J-L, Philip PA, et al. Brivanib Versus Sorafenib As First-Line Therapy in Patients With Unresectable, Advanced Hepatocellular Carcinoma: Results From the Randomized Phase III BRISK-FL Study. J. Clin. Oncol. 2013;31:3517–3524.

236. Llovet JM, Decaens T, Raoul JL, Boucher E, Kudo M, Chang C, et al. Brivanib in patients with advanced hepatocellular carcinoma who were intolerant to sorafenib or for whom sorafenib failed: Results from the randomized phase III BRISK-PS study. J. Clin. Oncol. 2013;31:3509–3516.

237. Cheng A-L, Kang Y-K, Lin D-Y, Park J-W, Kudo M, Qin S, et al. Sunitinib Versus Sorafenib in Advanced Hepatocellular Cancer: Results of a Randomized Phase III Trial. J. Clin. Oncol. 2013;31:4067–4075.

238. Cainap C, Qin S, Huang W-T, Chung IJ, Pan H, Cheng Y, et al. Linifanib Versus Sorafenib in Patients With Advanced Hepatocellular Carcinoma: Results of a Randomized Phase III Trial. J. Clin. Oncol. 2015;33:172–179.

239. AX Z, Kudo M, Assenat E, Al E. Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: The evolve-1 randomized clinical trial. JAMA. 2014;312:57–67.

240. Abou-Alfa GK, Qin S, Ryoo B-Y, Lu S-N, Yen C-J, Feng Y-H, et al. Phase III randomized study of second line ADI-PEG 20 plus best supportive care versus placebo plus best supportive care in patients with advanced hepatocellular carcinoma. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 2018;29:1402–1408.

241. Kudo M, Ueshima K, Yokosuka O, Ogasawara S, Obi S, Izumi N, et al. Sorafenib plus low-dose cisplatin and fluorouracil hepatic arterial infusion chemotherapy versus sorafenib alone in patients with advanced hepatocellular carcinoma (SILIUS): a randomised, open label, phase 3 trial. Lancet Gastroenterol. Hepatol. 2018;3:424–432.

242. Qin S, Bai Y, Lim HY, Thongprasert S, Chao Y, Fan J, et al. Randomized, multicenter, open-label study of oxaliplatin plus fluorouracil/leucovorin versus doxorubicin as palliative chemotherapy in patients with advanced hepatocellular carcinoma from Asia. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 2013;31:3501–3508.

243. Rimassa L, Assenat E, Peck-Radosavljevic M, Pracht M, Zagonel V, Mathurin P, et al. Tivantinib for second-line treatment of MET-high, advanced hepatocellular carcinoma (METIV-HCC): a final analysis of a phase 3, randomised, placebo-controlled study. Lancet Oncol. 2018;19:682–693.

244. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018;391:1163–1173.

245. Bruix J, Qin S, Merle P, Granito A, Huang YH, Bodoky G, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017;389:56–66.

246. Abou-Alfa GK, Meyer T, Cheng A-L, El-Khoueiry AB, Rimassa L, Ryoo B-Y, et al. Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. N. Engl. J. Med. 2018;379:54–63.

247. Zhu AX, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, et al. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2019;20:282–296.

248. Zhu AX, Park JO, Ryoo B-Y, Yen C-J, Poon R, Pastorelli D, et al. Ramucirumab versus placebo as second-line treatment in patients with advanced hepatocellular carcinoma following first-line therapy with sorafenib (REACH): a randomised, double-blind, multicentre, phase 3 trial. Lancet Oncol. 2015;16:859–870.

249. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. Am. J. Clin. Oncol. 2016;39:98–106.

250. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. Lancet. 2017;389:2492–2502.

251. Zhu AX, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, et al. Pembrolizumab in patients with advanced

hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. Lancet. Oncol. 2018;

252. Finn RS, Ryoo BY, Merle P, Kudo M, Bouattour M, Lim HY, et al. Pembrolizumab As Second-Line Therapy in Patients With Advanced Hepatocellular Carcinoma in KEYNOTE-240: A Randomized, Double-Blind, Phase III Trial. J. Clin. Oncol. 2020;38:193–202.

253. Yau T, Park J-W, Finn RS, Cheng A-L, Mathurin P, Edeline J, et al. Nivolumab versus sorafenib in advanced hepatocellular carcinoma (CheckMate 459): a randomised, multicentre, open-label, phase 3 trial. Lancet. Oncol. 2021;

254. Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim T-Y, et al. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. N. Engl. J. Med. 2020;382:1894–1905.

255. Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim T-Y, et al. IMbrave150: Updated overall survival (OS) data from a global, randomized, open-label phase III study of atezolizumab (atezo) + bevacizumab (bev) versus sorafenib (sor) in patients (pts) with unresectable hepatocellular carcinoma (HCC). J. Clin. Oncol. 2021;39:267.

256. Sonbol MB, Riaz I Bin, Naqvi SAA, Almquist DR, Mina S, Almasri J, et al. Systemic Therapy and Sequencing Options in Advanced Hepatocellular Carcinoma: A Systematic Review and Network Meta-analysis. JAMA Oncol. 2020;6.

257. Vogel A, Rimassa L, Sun H-C, Abou-Alfa GK, El-Khoueiry AB, Pinato DJ, et al. Clinical value of atezolizumab + bevacizumab for first-line unresectable hepatocellular carcinoma (HCC): A network meta-analysis. J. Clin. Oncol. 2020;38:4585.

258. https://www.astrazeneca.com/content/astraz/media-centre/press-releases/2021/imfinzi-and-tremelimumab-improved-os-in-liver-cancer.html.

259. Bruix J, Chan SL, Galle PR, Rimassa L, Sangro B. Systemic treatment of hepatocellular carcinoma: An EASL position paper. J. Hepatol. 2021;75:960–974.

CHAPTER 3

Liquid biopsy in hepatocellular carcinoma: where are we now?

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related death worldwide. Diagnostic, prognostic and predictive biomarkers are urgently needed in order to improve patient survival. Indeed, the most widely used biomarkers, such as alpha-fetoprotein (AFP), have limited accuracy as both diagnostic and prognostic test. Liver biopsy provides an insight on the biology of the tumor, but it is an invasive procedure, not routinely used, and not representative of the whole neoplasia due to the demonstrated intra-tumoral heterogeneity. In recent years, liquid biopsy, defined as the molecular analysis of cancer by-products, released by the tumor in the bloodstream, emerged as an appealing source of new biomarkers. Several studies focused on evaluating extracellular vesicles, circulating tumor cells, cell-free DNA and non-coding RNA as novel reliable biomarkers. In this review, we aimed to provide a comprehensive overview on the most relevant available evidences on novel circulating biomarkers for early diagnosis, prognostic stratification and therapeutic monitoring. Liquid biopsy seems to be a very promising instrument and, in the near future, some of these new non-invasive tools will probably change the clinical management of HCC patients.

INTRODUCTION

According to the International Agency for Research on Cancer, in 2018 primary liver tumors ranked as the sixth most common cancer and the fourth leading cause of cancer-related death worldwide (1). These figures are predicted to increase in the coming decades and it is estimated that more than 1 million people will die duo to liver cancer in 2030 (2). Hepatocellular carcinoma (HCC) account for 85% of all primary hepatic malignancies. The majority of HCC cases occur in patients with underlying liver diseases, mainly due to chronic hepatitis B or C virus (HBV and HCV) infections, alcohol abuse, aflatoxin exposure or non-alcoholic liver disease (NAFLD) (3). Despite the recommendation of all available guidelines to apply a regular surveillance in patients at risk, HCC is often diagnosed in advanced stages when curative therapies are no longer feasible. As a consequence, despite the remarkable progresses in therapy, the prognosis of HCC patients remains dismal, with a 5-years survival rate ranging around 20% (4).

Currently, according to guidelines, liver biopsy has a limited role in the management of HCC patients. This is due to the fact that, in patients with liver cirrhosis, a non-invasive diagnosis in the presence of typical imaging features (hypervascularity in the arterial phase and wash-out in portal venous and/or delayed phases) has high specificity. On the other hand, biopsy is indicated for patients without cirrhosis or for cirrhotics with lesions not showing the peculiar and specific radiologic appearance (5). In most cases liver biopsy, which is associated with a small but still present risk of bleeding and tumor seeding, is unnecessary. Nevertheless, the debate on a more widespread use of liver biopsy is still open (6), with the expansion in recent years of therapeutic possibilities and in consideration of the identification of molecular markers of susceptibility to available systemic treatments, in an attempt of tailoring first and subsequent lines of therapy (7). However, a high degree of spatial and temporal heterogeneity is present in HCC. Some somatic mutations occur early during tumorigenesis and propagate in many clones, whereas later mutations are present only in

some clones (spatial heterogeneity) (8). Moreover, different therapies select rare mutants and treatment-resistant clones, leading to the development of several genetic backgrounds at different times (temporal heterogeneity) (9,10). Therefore, a single biopsy is unlikely to represent the entire biology of the tumor, thus limiting the utility of tissue sampling beyond in confirming the diagnosis (11).

The European Association for the Study of the Liver (EASL) recognizes as an urgent unmet need the identification of reliable biomarkers, for risk stratification and early HCC detection, prediction of prognosis and of response to therapy (in particular to systemic treatments) (5). Despite its unsatisfactory performance in early diagnosis and prognostication (12–16), alpha-fetoprotein (AFP) is still the most widely used biomarker in the clinical management of patients with HCC. Other protein biomarkers, such as des-γ-carboxyprothrombin (17), glypican-3 (18), osteopontin (19), Golgi protein-73 (20) and squamous cell carcinoma antigen (21–23) have been evaluated, with erratic results. In the spectrum of circulating molecules derived from the primary tumor ("HCC circulome"), other biomarkers emerged as appealing tools in overcoming the limitations of conventional biomarkers and of tissue biopsy in diagnosis and prognosis. Liquid biopsy is defined as the molecular analysis of circulating cancer by-products, such as extracellular vesicles (EVs), circulating tumor cells (CTCs) and circulating tumor nucleic acids (Figure 1). In recent years, large evidence has been published, paving the way for the use of liquid biopsy as a source of reliable biomarkers for early tumor detection, prognostic stratification, disease monitoring and evaluation of response to treatment. Considering that these non-invasive biomarkers will probably revolutionize the management of patients with HCC in the near future, with this review we aimed to provide a comprehensive overview of the most relevant available data on the role of liquid biopsy in HCC.

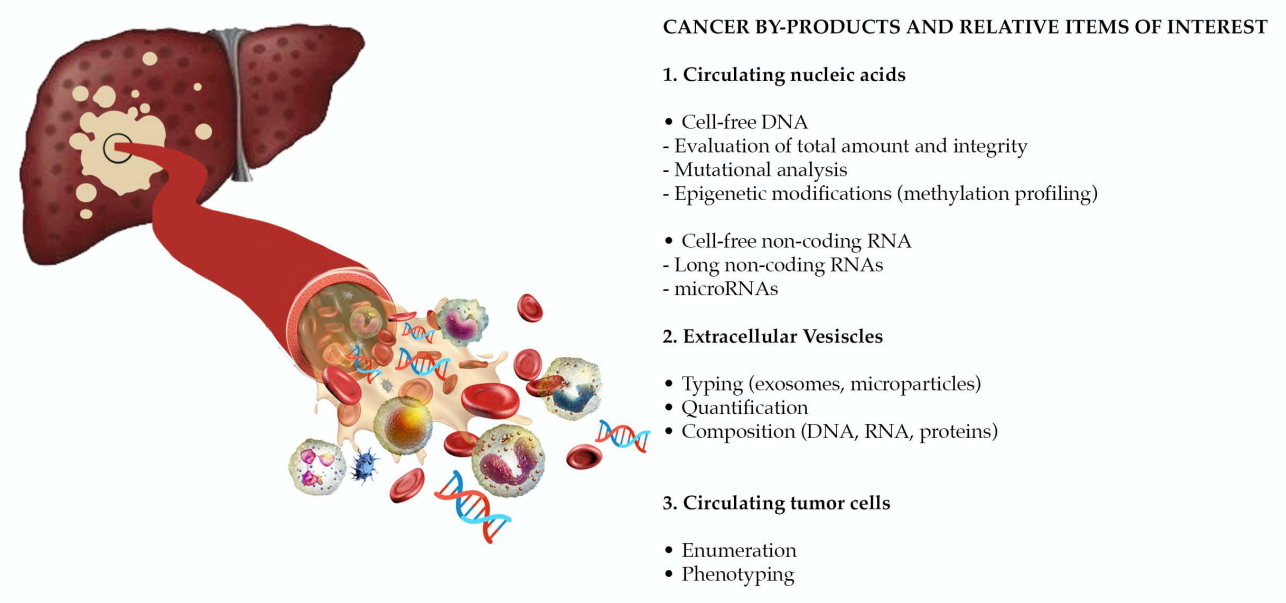

Figure 1. Liquid biopsy is the molecular analysis of cancer by-products released in the bloodstream. Novel potential biomarkers are represented by circulating nucleic acids, extracellular vesicles (EVs), and circulating tumor cells (CTCs). (Adapted from Labgaa et al. (24))

CIRCULATING NUCLEIC ACIDS

Circulating nucleic acids, released in the bloodstream through active secretion or following apoptosis, necrosis or lysis of tumor cells and circulating tumor cells, can be subgrouped in “cell-free DNA” (cfDNA) and “cell-free RNA” (cfRNA). cfDNA can be found in circulation as short nucleosome-associated fragments or long fragments incapsulated in EVs, while cfRNA is usually detected in association with proteins, proteolipid complexes and EVs due to its relative instability (25).

The analysis of circulating nucleic acids represents a very promising liquid biopsy strategy for getting informations on liver tumor. Beyond the utility in risk prediction, early detection and monitoring treatment response, cfDNA and cfRNA are optimal candidates for tumor molecular profiling. Unlike tumor biopsy, their ability to mirror tumor heterogeneity represents a powerful tool to identify point mutations, aberrant methylation and chromosomal aberrations conferring drug resistance and guiding molecular target therapy (26).

Cell-free DNA

The original discovery of cfDNA from sera of healthy individuals dates back to 1948. Following the demonstration of high serum concentration of cfDNA in patients with gastrointestinal cancers (27), its potential role as tumor marker emerged when KRAS mutations were identified in cfDNA from patients with colorectal and pancreatic cancers (28–30). From this starting point, a large number of studies has been conducted focusing on the utility of cfDNA analysis also in HCC (Table 1).

Cell-free DNA amount and integrity

The easiest way to use circulating DNA as a biomarker is through the evaluation of its total amount, since a high level of cfDNA in blood reflect cancer growth and tumor burden (31–38). In 2006, Iizuka et al. (31) demonstrated that cfDNA was able to identify HCC in a cohort of HCV positive patients with a sensitivity of 69.2% and a specificity of 93.3% (AUC=0.90), both higher than those of AFP. These early results are in line with previous data from our research group: the total amount of cfDNA achieved a sensitivity of 91%, a specificity of 43% and an AUC of 0.69 in discriminating HCC from chronic liver disease (CLD) and cirrhotic patients (34). Since cfDNA is not specific for liver cancer, several studies reported an increased diagnostic accuracy when its determination was combined with other biomarkers (i.e. AFP) (32,33,38). cfDNA have an average size of ~180 base pairs and its fragmentation is a nonrandom process, since liver cfDNA has been found to end at specific genomic coordinates (39). Interestingly, shorter cfDNA was found in HCC patients compared to non-cancer patients, probably reflecting that not only apoptosis, but also necrosis of tumor cells contributes to the pool of circulating DNA (40,41). Some researchers demonstrated that the evaluation of length and integrity of cfDNA achieved a diagnostic accuracy comparable to that of AFP (36,42). The measure of cfDNA total amount or integrity may also be useful as prognostic biomarker. In their seminal study, Tokuhisa et al. (43) demonstrated that higher levels of cfDNA after liver resection in patients with HCV-related HCC were associated with an increased risk of metastases (adjusted hazard ratio [HR]=4.5, 95% CI 1.3-14.9) and poorer overall survival (OS) (adjusted HR=3.4, 95% CI

1.5-7.6). Several other subsequent studies confirmed that patients with high levels of cfDNA had a worse prognosis after different treatments (liver transplantation, liver resection and sorafenib) (34,44,45). Moreover, a poorer OS was also demonstrated in patients with decreased cfDNA integrity (adjusted HR=1.86, 95% CI 1.20-2.88) in the study by El-Shazly et al. (36).

When dealing with cfDNA amount as a cancer biomarker, it should be noted that the circulating DNA does not derive only from tumor cells. More precisely, the fraction of cfDNA directly attributable to the presence of cancer is named circulating tumor DNA (ctDNA) (46). Although patients with cancer have higher cfDNA levels compared to healthy subjects, ctDNA represent a small proportion of the total amount and its level depend on disease burden, stage, cellular turnover and treatment response (47). Moreover, high quantities of cfDNA are not cancer specific being also elevated in inflammatory and autoimmune diseases (cirrhosis, chronic hepatitis, systemic lupus erythematous and rheumatoid arthritis), in pregnancy and after physical exercise (27,47). This low specificity may scale back the role of whole cfDNA quantification as diagnostic biomarkers. Nevertheless, a remarkable study demonstrated that the cell and tissue of origin of cfDNA could be inferred by the analysis of the position of nucleosomes (48). Snyder et al. demonstrated that since nucleosomes, the basic unit of chromatin, are placed in different positions depending on the cell type, nucleosome footprint in cfDNA could be useful to determine the relative contribution of cancer cells to the total circulating DNA pool (48).

Mutations

The majority of studies on cfDNA focused on mutational analysis and epigenetic characteristics, such as its methylation signature. HCC, when compared to other solid tumors, has a lower mutational burden (49). The main driver somatic mutations affect telomere integrity (TERT promoter, 44%), cell cycle (TP53, 31%) and WNT signaling (CTNNB1, 27%) (50). Less commonly AXIN1, ARID1A, ARID2, BAP1, RB1 and KEAP1 are mutated (5-10%) (50). In addition, genetic alterations may be present,

including broad chromosome gains and losses with high-level DNA amplifications of chromosomes 6p21 and 11q13, loci corresponding to VEGFA and CCND1/FGF19 respectively (49). A relevant proportion of the mutations found in HCC biopsies is also detectable in cfDNA (43-83%) (45,51). According to Howell et al. (52), all the mutations found in the plasma cfDNA matched with tissue mutations, while only 71% of mutations on tumor tissue were found in circulating DNA. When dealing with mutational analysis of cfDNA, we must keep in mind that mutations are more easily identified in advanced disease. In a recent study, at least one mutation in cfDNA was found in almost all (6/7) patients with a tumor ≥5 cm or with metastases, while only 9% of mutations were detected in the cfDNA of patients with smaller, not metastatic HCC (53). Others reported that, in 48 patients, at least one type of mutation among TP53 (c.747G>T), CTNNB1 (c.121A>G, c.133T>C) or TERT (c.1-124C>T) was documented in 56.3% of patients; only 22.2% of patients had matched mutations in HCC tissue, while none of these mutations was found in non-tumoral liver tissue or in peripheral mononuclear cells (54). In parallel to what found in HCC tissue, TP53 is the most commonly mutated gene in cfDNA (55). In particular, TP53 c.747G>T (p.R249S) mutation appear to be highly specific, since Cohen et al. (56) found it in approximately 20% of HCC blood samples and, conversely, in only 3-4% of pancreatic and stomach cancer samples and in none of more than 800 healthy controls. Although confirming a very high specificity (100%), another study showed a very poor sensitivity (7.6%) for the analysis of TP53 R249S mutation alone in cfDNA (57). In order to overcome this limitation, the accuracy of TP53 mutation in association with other mutations in a diagnostic panel was evaluated (58–60). Qu et al. demonstrated that a score including several cfDNA mutations (TP53, TERT, CTNNB1 and AXIN1, and HBV integrations), in combination with protein biomarkers (AFP and DCP), age and gender efficiently identified early-stage HCC in a high-risk HBsAg-seropositive population (60). Sensitivity and specificity, 85% and 93% in the training cohort, were even better in the validation cohort (100% and 94%, respectively) (60). Moreover, the positivity of

TP53 R249S mutation in cfDNA proved to be useful also as prognostic biomarker in a large study involving 895 HCC patients, being a predictor of poorer OS and shorter progression-free survival (PFS) in patients with or without liver resection (61).

The human telomerase reverse transcriptase (TERT) gene encodes for the catalytic subunit of telomerase, which acts together with multiple molecules to maintain telomere homeostasis and chromosomal integrity (62). The mutations found in TERT promoter lead to TERT reactivation and cell immortalization. Male patients with HCV and/or alcoholic related cirrhosis have a higher prevalence of TERT promoter mutations both in tumor tissue and in cfDNA (63), providing the rationale for TERT promoter mutations analysis in cfDNA for early detection in some populations at risk of developing HCC. In addition, presence of TERT promoter mutation in cfDNA has been associated with poor prognosis after different treatments (58,63–65).

Methylation/epigenetics

Changes in DNA methylation, particularly in the CpG islands of tumor suppressor genes, have been demonstrated to be pivotal in HCC development (66). Analysis of the methylation pattern of cfDNA may have a value as diagnostic and prognostic biomarker, and might reveal information about tumor size, risk of metastatic spread and recurrence (67). Alterations in DNA methylation patterns in HCC tumor tissue after liver resection have been described for many genes. In particular, hypermethylation was found in p15, CDKN2A (encoding for p16), glutathione S-transferase (GSTP1), Ras association domain family 1A (RASSF1A), APC, SOCS1, SOCS3, TIMP3, blood vessel epicardial substance (BVES), and Homeobox A9 (HOXA9) genes, while hypomethylation in long interspersed element-1 (LINE-1) repetitive sequence (67–73). However, only a proportion of cfDNA carried the same methylation patterns: hypermethylation of GSTP1 and RASSF1A was found in 50% and in 70-93% of cases respectively, while hypomethylation of LINE-1 in approximately 67% of cases (71,72). Nevertheless, a large number of studies investigated the diagnostic accuracy of the methylation

patterns in several different genes, demonstrating a diagnostic accuracy comparable or even superior to that of AFP. A very high diagnostic accuracy could be obtained with methylation scores, which combine methylation pattern in different genes. Wen et al. (74) demonstrated that a methylation score derived from the analysis of more than 10 genes achieve a sensitivity of 94% and a specificity of 89%. Lu et al. (75) obtained an AUC of 0.87 analyzing the methylation of APC, COX2, RASSF1A and miR-203, compared to an AUC of 0.56 for AFP. In another study, the methylation of RASSF1A, BVES and HOXA9 achieved a 73.5% sensitivity and a 91.1%, specificity, with an AUC of 0.83 (70). A very high diagnostic accuracy in distinguish HCC patients from cirrhotics (sensitivity/specificity 95%/86%, AUC=0.93) was reported by Kiesel et al. for a score composed by the analysis of HOXA1, EMX1, ECE1, AK055957, PFKP and CLEC11A methylation in a discovery, phase I pilot and phase II clinical validation cohort study (76). Cai et al. developed and validated a non-invasive diagnostic model based on Genome-wide mapping of 5-hydroxymethylcytosines in cfDNA achieving an AUC of 0.85 in distinguish early HCC from CLD, thus outperforming AFP (AUC=0.69) (77). The methylation analysis of cfDNA demonstrated to be useful also in predicting prognosis. RASSF1A methylation was positively correlated with tumor size, while LINE-1 hypomethylation was associated with HCC progression and patients' survival. The combination of these two genes methylation status was able to predict tumor recurrence after liver resection (71). The role of LINE-1 hypomethylation in predicting poor prognosis was also confirmed by other researchers (78,79).

In a very interesting recent study including 1098 HCC patients and 835 controls, the authors constructed a diagnostic model with 10 methylation markers in cfDNA, achieving a sensitivity of 85.7% and a specificity of 94.3% in the training cohort (560 normal samples and 715 HCC) (80). In the validation cohort (275 normal samples and 383 HCC) the model demonstrated a sensitivity of 83.3% and a specificity of 90.5%, thereby differentiating HCC patients from normal controls with an AUC of 0.966 (80). In the same study, the prognostic score, which was based on the evaluation of

the methylation profile of 8 different genes, was associated with higher mortality both in the training (HR=2.41, 95% CI 1.90-3.03) and in the validation cohort (HR=1.55, 95% CI 1.25-1.92) (80).

In the chapter of epigenetic biomarkers, also nucleosomes and extracellular histones are emerging. Nucleosomes, beyond being fundamental for genome compaction in the nucleus, may regulate genes expression through their composition and post-translational modifications (81). Their circulating levels are increased in stroke, trauma and sepsis (82). In addition, circulating nucleosome demonstrated a remarkable diagnostic and prognostic performance in several human malignancies, including pancreatic (83), lung (84), colorectal (85) and breast cancers (86). Moreover, circulating histones have been demonstrated to be key mediators of lethal sepsis (87) and liver inflammatory injury (88). Some studies demonstrated an involvement of macro histone variants (in particular macroH2A1) in modulating HCC progression and stem cell differentiation (89,90). There is still poor evidence about circulating nucleosomes and cell-free histones/histone complexes as liquid biopsy biomarkers in HCC. Nevertheless, some interesting results have been achieved in obesity and metabolic dysfunction-associated fatty liver disease (MAFLD), both risk factors for HCC development. A strong correlation between fatty liver index (a predictor of MAFLD based on BMI, waist circumference, triglycerides and GGT) and high levels of circulating nucleosomes have been found in obese patients with MAFLD (91). Moreover, a circulating histone signature (depletion of histone variants macroH2A1.1 and macroH2A1.2, individually or in complex with H2B) identified the severity of steatosis in subjects with lean MAFLD (92). These encouraging results, together with the simple methodology of the determination (ELISA), could pave the way to the evaluation of circulating nucleosomes and cell-free histones/histone complexes as diagnostic and prognostic biomarkers in HCC.

Overall, a large body of evidence has been produced supporting the great potential of cfDNA as diagnostic and prognostic biomarker in HCC. However, it should be considered that current data

largely derive from proof-of-concept retrospective studies, lacking adequate controls (not always including patients at risk of developing HCC, i.e. cirrhotics) and including only a minority of cases with early-stage HCC, which would be candidates for curative treatment options. Moreover, an additional concern regards the lack of standardized protocols for pre-analytical sample preparation, purification and analysis. Although the use of cfDNA as a liquid biopsy currently presents several limitations in the early detection of HCC, due to the very low amount of cfDNA in the early stages, these approaches may probably dramatically change HCC surveillance. Indeed, a study published more than 10 years ago demonstrated that aberrant methylation of cfDNA fragments was detected up to 9 years before the diagnosis achieved with standard methodology (93).

Table 1. Studies on cell-free DNA (cfDNA) as biomarker in HCC patients.

Diagnosis				
Study	**cfDNA property**	**Number of patients**	**Comparator**	**Main findings (sensitivity/specificity, AUC)**
cfDNA amount or integrity				
Iizuka et al, 2006 (31)	Total amount	52 HCC 30 CLD (HCV) 16 healthy subjects	AFP (cut-off 10.2 ng/mL) DCP (cut-off 29.5 ng/mL)	AFP: 69.2%/72.7% (0.79) DCP: 73.1%/75% (0.73) cfDNA: 69.2%/93.3% (0.90); p<0.05 vs. both AFP and DCP
Ren et al, 2006 (35)	Total amount and chromosome 8p allelic imbalance (D8S258 or D8S264)	79 HCC 20 LC 20 healthy subjects	AFP (cut-off 20 ng/mL)	Total amount of cfDNA: HCC vs. healthy subjects: 52%/95%; 0.80 Allelic imbalance at D8S258 in the plasma of 62% of patients Allelic imbalance at D8S264 in the plasma of 60% of patients High cfDNA concentration + allelic imbalance abnormal in 8/24 patients with low AFP
El-Shazly et al, 2010 (36)	Total amount and integrity	25 HCV-related HCC 25 CLD (HCV) 15 healthy subjects	AFP (cut-off 20 ng/mL)	HCC vs. CLD cfDNA amount: 72%/68%, 0.57 cfDNA integrity: 88%/92%, 0.75
Huang et al, 2012 (32)	Total amount	72 HCC 37 LC or CLD 41 healthy subjects	NR	HCC vs. healthy subjects: 90.3%/90.2%; 0.949 HCC vs. CLD: 59.7%/78.4%; 0.705 cfDNA + AFP (HCC vs. healthy subjects): 95.1%/94.4%; 0.974
Piciocchi et al, 2013 (34)	Total amount	66 HCC 35 LC 41 CLD (HCV)	AFP (cut-off 14 ng/mL)	HCC vs. LC+CLD: cfDNA: 91%/43%; 0.69 AFP: 45%/83%; 0.64
Chen et al, 2013 (33)	Total amount	39 HCC 45 healthy subjects	NR	ctDNA: 56.4%/95.6%; 0.742 AFP: 53.8%/91.1% cfDNA + AFP: 71.8%/86.7% (p<0.05 vs. ctDNA + AFP + AFU group) cfDNA + AFP + AFU: 89.7%/64.4% (p<0.05 vs. ctDNA + AFP)
Huang et al, 2016 (42)	ctDNA integrity	53 HCC 15 benign liver diseases 22 healthy subjects	AFP (cut-off 20 ng/mL)	cfDNA integrity: 43.4%/100%; 0.705 AFP: 50.9%/100%; 0.605 cfDNA integrity + AFP: 79.2%/100%
Marchio et al, 2018 (37)	Total amount, TP53 R249S mutation by digital droplet PCR	149 HCC 164 CLD 49 healthy	AFP (cut-off 10 ng/mL)	cfDNA amount: AUC = 0.585 AFP: AUC = 0.805 Proportion of droplets with TP53 R249S: AUC = 0.827 (p>0.05 vs. AFP)
Yan et al, 2018 (38)	Total amount	24 HCC 62 CLD (HBV)	AFP (cut-off 80.5 ng/mL)	cfDNA amount: 62.5%/93.6%; 0.82 AFP: 47.8%/93.2%; 0.67 cfDNA + AFP + age: 87%/100%; 0.98
Mutations				
Igetei et al, 2008 (57)	TP53 R249S mutation	85 HCC 77 healthy subjects	AFP (cut-off 400 ng/mL)	Sensitivity/specificity: 7.6%/100% Patients with HCC and AFP measurements: 16.7% overall, 20% without increased AFP (p>0.05)

Xu et al, 2015 (94)	Copy number variation: gain in 1q, 7q and 19q; loss in 1p, 9q and 14q	31 HCC 8 LC or CLD	AFP (cut-off 10 ng/mL)	Copy number variation score: All HCCs: 83.9%/100% (AUC = 0.95) HCCs ≤5 cm: 68.8%/100% Low AFP: 7/10 positive
Liao et al, 2016 (58)	TERT, CTNNB1 or TP53 mutations	41 HCC 10 healthy subjects	AFP (cut-off 20 ng/mL)	Sensitivity 23% and 13% in high vs. low AFP group, respectively (p=0.70) Specificity 90%
An et al, 2019 (95)	ctDNA mutations (139 somatic mutations)	26 HCC 10 LC 10 CLD	NR	cfDNA: AUC = 0.917 Mutation number: AUC = 0.876 cfDNA (cfDNA concentration times variant allele frequency): AUC = 0.871 Maximal variant allele frequency: AUC = 0.802 AFP: AUC = 0.783
Cai et al, 2019 (96)	Fraction of single nucleotide or copy number variants	34 HCC	NR	cfDNA: sensitivity, 100% AFP: sensitivity, 56% AFP-L3: sensitivity, 50% DCP: sensitivity, 82%
Qu et al, 2019 (60)	HCCscreen: mutations in ctDNA (HVB integrations, TP53, CTNNB1, AXIN1 and TERT promoter), AFP, DCP, age and sex	Training: 65 HCC, 70 CLD Validation: 24 HCC, 307 CLD	None	Training cohort (AFP or US positive suspected individuals): 85%/93%, 0.928 Validation cohort (AFP and US negative individuals): 24/331 patients tested positive and eventually 4/24 develop HCC. None of the negative patients develop HCC. Sensitivity/specificity: 100%/94%
Xiong et al, 2019 (59)	Mutations in TP53, ARID1A, FLCN, SETD2, PTEN, BUB1B, CTNNB1, JAK1, AXIN1, EPS15 or CACNA2D4	37 HCC 6 healthy subjects	AFP (cut-off 400 ng/mL)	cfDNA mutations overall: 65%/100%, 0.92 AFP negative: 73%/100%, 0.96 AFP positive: 53%/100%, 0.86
Methylation/epigenetics				
Chu et al, 2004 (97)	p16 methylation	46 HCC 23 LC	AFP (cut-off 20 ng/mL)	Overall cohort (sensitivity/specificity): 48%/83% Normal AFP (sensitivity): 44%
Yeo et al, 2005 (98)	RASSF1A methylation	40 HCC 10 healthy subjects	AFP (cut-off 20 ng/mL)	Overall (sensitivity/specificity): 43%/100% Low AFP (sensitivity): 36%
Chan et al, 2008 (99)	RASSF1A methylation	63 HCC 63 CLD (HBV) 50 healthy subjects	AFP (cut-off 20 ng/mL)	RASSF1A methylation detected in: 93% HCC (50% among normal AFP); 58% CLD; 8% healthy subjects
Iizuka et al, 2011 (100)	SPINT2 and SRD5A2 methylation	Training cohort: 108 HCC, 56 CLD Validation cohort:112 HCC, 146 CLD	AFP (cut-off 20 ng/mL) DCP (cut-off 40 mAU/mL)	Methylation of SPINT2 and SRD5A2 + AFP + DCP (sensitivity/specificity): 82.4%/82.1% (training cohort); 73.2%/87.7% (validation cohort) AUC = 0.72 for ≥5 cm HCC and 0.89 for >5 cm HCC AFP alone (sensitivity/specificity): 57.4%/85.7% (training cohort) DCP alone (sensitivity/specificity): 60.2%/89.3% (training cohort)
Sun et al, 2013 (101)	TFPI2 methylation	43 HCC 24 CLD (HBV) 26 healthy subjects	AFP (cut-off 400 μg/L)	TFPI2 methylation (sensitivity/specificity): HCC vs. healthy: 46.5%/80.8% HCC vs. CLD: 46.5%/83.3% AFP alone (sensitivity): 54% TFPI2 + AFP (sensitivity): 61%

Han et al, 2014 (102)	TGR5 promoter methylation	160 HCC 88 CLD (HBV) 45 healthy subjects	AFP (cut-off 20, 200 and 400 ng/mL)	TGR5 methylation frequency: HCC 48%, CLD 14% and healthy subjects 4% HCC vs. CLD (sensitivity/specificity) TGR5 alone: 48.1%/86.4% TGR5 methylation + AFP (200 ng/mL): 68.1%/78.4% AFP (200 ng/mL): 30.6%/92.1%
Huang et al, 2014 (103)	INK4A promoter methylation	66 HCC 43 CLD	AFP (cut-off 200 ng/mL)	INK4A methylation: sensitivity, 74.2% AFP: sensitivity, 45.5% INK4A methylation + AFP: sensitivity, 80.3% (p<0.05 vs. AFP)
Ji et al, 2014 (104)	MT1M and MT1G methylation	121 HCC 37 CLD (HBV) 31 healthy subjects	AFP (cut-off 20 ng/mL)	MT1M or MT1G methylation: HCC vs. CLD: 90.0%/81.1%, 0.86 HCC vs. healthy: 90.9%/83.9%, AUC=NR AFP alone: HCC vs. CLD: 56.0%/62.1%
Kuo et al, 2014 (105)	HOXA9 methylation	40 HCC 34 healthy subjects	AFP (cut-off 10 ng/mL)	HOXA9: 73.3%/97.1%, 0.835 HOXA9 or AFP: 94.6%/97.1%
Li et al, 2014 (106)	IGFBP7 promoter methylation	136 HCC 46 CLD (HBV) 35 healthy subjects	AFP (cut-off 20 ng/mL)	IGFBP7: 65%/83%, 0.740 AFP: 57%/52%, 0.618 IGFBP7 + AFP: 85%/41% (p<0.05 vs. AFP)
Kanekiyo et al, 2015 (107)	RASSF1A, CCND2, CFTR, SPINT2, SRD5A2 and/or BASP1 methylation	125 HCC (HCV)	AFP (cut-off 20 ng/mL) DCP (cut-off 40 ng/mL)	Serum methylation score: Positive in 41% high vs. 48% low AFP Positive in 42% high vs. 46% low DCP (p>0.05 for both)
Wen et al, 2015 (74)	Methylation score: RGS10, ST8SIA6, RUNX2, VIM, CACNA1C, TBX2, SOX9 5'end), NEDD4L intron), ALX3, ZNF683 (3' end), KCNQ4 (i), ERG, PTPN18 (intron), SYN2, LINC00682 (3' end), CPLX1 (intron), FLJ42709, UBD (3' end), SNX10 (3' end), TRPS1 (intron)	36 HCC 17 CLD 38 healthy subjects	AFP (cut-off 20 ng/mL)	Two cfDNA methylation scores, either score positive (sensitivity/specificity): Training set: 93%/91% Validation set: 100%/80% Combined cohort: 94%/89% Sensitivity 100% in patients with low AFP (n=10)
Dou et al, 2016 (108)	CDH1, DNMT3b or ESR1 promoter methylation	183 HCC 47 LC 126 CLD (HBV) 50 healthy subjects	NR	Methylation frequency: HCC: CDH1 31%, DNMT3b 41%, ESR1 31% CLD: <10% for all 3 genes Healthy subjects: 0% HCC vs. CLD Methylation of any gene (AUC): 0.75; AFP (AUC): 0.63 HCC vs. LC Methylation of any gene (AUC): 0.73; AFP (AUC): 0.62
Hu et al, 2017 (109)	UBE2Q1 hypomethylation	80 HCC 40 LC 40 CLD (HBV)	AFP (cut-off 20, 200 and 400 ng/mL)	UBE2Q1 methylation: 66.3%/57.5%, 0.619 AFP alone: 53.8%/87.5%, 0.668 UBE2Q1 methylation + AFP: 53.8%/87.5%, 0.760

		20 healthy subjects		
Lu et al, 2017 (75)	Methylation score: APC, COX2, RASSF1A and miR-203	203 HCC 104 CLD 50 healthy subjects	AFP (cut-off 20 ng/mL)	In HBV-related HCC: Methylation score: 84.1%/83.0%, 0.87 AFP: 50.9%/62.1%, 0.56
Xu et al, 2017 (80)	Methylation score: cg10428836, cg26668608, cg25754195, cg05205842, cg11606215, cg24067911, cg18196829, cg23211949, cg17213048, cg25459300	1098 HCC 835 healthy subjects	AFP (cut-off 25 ng/mL)	Training set: 85.7%/94.3%, 0.97 Validation set: 83.3%/90.5%, 0.94 AFP, AUC 0.82 (p<0.05 vs. cfDNA)
Dong et al, 2017 (70)	RASSF1A, APC, BVES, TIMP3, GSTP1, HOXA9 methylation	98 HCC 75 LC 90 CLD (HBV) 80 healthy subjects	AFP (cut-off 20 ng/mL)	HCC vs. CLD RASSF1A, BVES and HOXA9 methylation: 73.5%/91.1%, 0.834 RASSF1A, BVES and HOXA9 methylation + AFP: 83.7%/78.9%, 0.852
Oussalah et al, 2018 (110)	SEPT9 methylation	Derivation cohort: 51 HCC 135 CLD Validation cohort: 47 HCC 56 CLD	NR	Derivation cohort: SEPT9 methylation: 94.1%/84.4%, 0.94 Validation cohort: SEPT9 methylation: 85.1%/87.9%, 0.93 AFP alone (AUC): 0.85 (p=0.002 vs. SEPT9 methylation)
Kisiel et al, 2019 (76)	Methylation score: HOXA1, EMX1, ECE1, AK055957, PFKP, CLEC11A	116 HCC 80 CLD 98 healthy subjects	AFP (cut-off 10 ng/mL)	HCC vs. LC: 95%/86%, AUC 0.93 (no improvement with addition of AFP) HCC vs. healthy: 95%/95% Sensitivity based on cancer stage: 75% (BCLC stage 0), 93% (A/B), 100% (C/D)
Cai et al, 2019 (77)	5-hmC modifications in ctDNA	1204 HCC 392 LC or CLD 958 healthy subjects	AFP (cut-off 20 ng/mL)	Early-stage HCC vs. CLD (AUC): 5-hmC based score: 0.873 (training cohort) and 0.846 (validation cohort) AFP: 0.793 (training cohort) and 0.692 (validation cohort)
Prognosis				
Study	**cfDNA property**	**HCC patients**	**Stage /Treatment**	**Main findings**
cfDNA amount/integrity				
Ren et al, 2006 (35)	Total amount and chromosome 8p allelic imbalance (D8S258 or D8S264)	N = 79	TNM stage I+II/III+IV: 62%/38% Treatment: NR	Better 3-years DFS associated with low cfDNA (p=0.008), allelic imbalance at D8S258 (p=0.004), allelic imbalance at D8S264 (p=0.01). Better 3-years OS associated with low cfDNA (p<0.0001) and allelic imbalance at D8S258 (p=0.02). AI at D8S258 + higher cfDNA associated with better 3-year DFS (p<0.0001) and 3-year OS (p<0.0001).
Tokuhisa et al, 2007 (43)	Total amount	N = 87	TNM stage I/II/III+IV: 46%/44%/10% Treatment: LR	High cfDNA associated with: Poorer OS: HR=3.4 (1.5-7.6) adjusted for tumor size Higher recurrence in distant organs: HR=4.5 (1.3-14.9) adjusted for tumor grade Similar DFS (p=0.19)
El-Shazly et al, 2010 (36)	Total amount, integrity	N = 25	TNM stage I/II/III/IV: 12%/32%/48%/8% Treatment: NR	OS: cfDNA amount: adjusted HR=0.54 (0.20-1.60) cfDNA integrity: adjusted HR=1.86 (1.20-2.88)

Piciocchi et al, 2013 (34)	Total amount	N = 66	Stage: 59% Milan in Treatment: NR	Patients with high cfDNA levels showed a significantly shorter OS (24 vs. 37 months; p=0.03). cfDNA was also an independent predictor of survival (HR= NR; p=0.02)
Ono et al, 2015 (45)	Total amount	N = 46	Stage: T1/T2/T3/T4 24%/39%/33%/4% (all N0/M0) Treatment: LR or LT	Presence of cfDNA associated with: Increased recurrence (p=0.01) Increased extrahepatic metastases (p=0.04) Similar OS (p=0.07) Increased risk of microscopic vascular invasion: adjusted HR=6.10 (1.11-33.33)
Park et al, 2018 (111)	Total amount	N = 55	TNM stage I/II/III/IV: 23%/23%/27%/27% Treatment: radiotherapy	Higher post-RT cfDNA levels associated with: Similar OS (p=0.15) Similar PFS (p=0.26) Increased hepatic failure: adjusted HR=2.41 (1.06-5.46) Decreased local control: adjusted HR=1.96 (0.57-6.81)
Oh et al, 2019 (44)	Total amount, genomic instability and VEGFA amplification	N = 151	BCLC stage B/C: 3.3%/96.7% Treatment: sorafenib	Higher amount of cfDNA associated with: Shorter TTP: HR=1.17 (1-20-2.44), adjusted for AFP Shorter OS: HR=3.50 (2.36-5.20), adjusted for AFP and MVI Genomic instability associated with: Shorter TTP: HR=2.09 (1.46-3.00), adjusted for AFP Shorter OS: HR=3.35 (2.24-5.01), adjusted for AFP and MVI
Mutations				
Liao et al, 2016 (58)	TERT, CTNNB1 or TP53 mutations	N = 41	Stage: 42% >5 cm, 27% multiple tumors, 61% vascular invasion Treatment: LR	Presence of mutations associated with: Lower recurrence-free survival (p<0.001); unadjusted analysis only. This was confirmed also in patients with vascular invasion (p=0.003).
Jiao et al, 2018 (63)	TERT mutations	N = 218	TNM stage I/II/III+IV: 41.3%/23.4%/35.3% Treatment: NR	Decreased OS in patients with TERT mutations (p=0.006), but not significant association (p=0.19) after adjustment for tumor stage. In patients with HCC on LC, trend toward significance after adjustment for tumor stage (p=0.051)
An et al, 2019 (95)	Any mutation	N = 26	TNM stage I/II+III Treatment: LR	Presence of cfDNA post-resection associated with shorter DFS (8.3 months vs. unreached; HR=7.66, p<0.0001). Improved DFS in patients with high vs. low clearance rate (17.5 vs. 6.7 months; HR=3.16, p=0.02). Portal vein tumor thrombosis was the other independent prognostic factor.
Cai et al, 2019 (96)	Fraction of single nucleotide or copy number variants	N = 34	Stage: NR Treatment: LR	Presence of mutated cfDNA postoperatively: Decrease relapse-free survival (p<0.0001) Decrease OS (p<0.0001) Combination of cfDNA and DCP further increased predictive power
Oversoe et al, 2020 (64)	TERT promoter mutations	N = 95	BCLC stage A/B/C/D: 9%/5%/74%/12% Treatment: variable	TERT promoter mutation associated with: Higher mortality: adjusted HR=2.16 (1.20-3.88). No difference in survival when the analysis was restricted to sorafenib treated patients.

Hirai et al, 2020 (65)	TERT promoter mutations	N = 130	TNM stage II+III/IV: 41%/59% Treatment: systemic therapy (66%), TACE (34%)	Presence of TERT promoter mutations associated with: Poorer OS: adjusted HR=1.94 (1.18-3.24) The worse survival was demonstrated even considering patients treated with systemic therapy and TACE separately
Shen et al, 2020 (61)	TP53 R249S mutation	N = 895	TNM stage I+II/III+IV: 67%/33% (cohort 2) Treatment: with (cohort 2) or without (cohort 3) LR	TP53 R249S mutation associated with: Cohort 2 Poorer OS: adjusted HR=1.79 (1.27-2.52) Poorer PFS: adjusted HR=1.74 (1.24-2.45) Cohort 3 Poorer OS: adjusted HR=1.63 (1.30-2.06) Poorer PFS: adjusted HR=2.03 (1.60-2.59)
Kim et al, 2020 (112)	Total amount and MLH1 single-nucleotide variant	N = 107	BCLC stage 0+A/B+C+D: 48%/52% Treatment: variable	Patients with low cfDNA + MLH1 wild-type had the longest OS, while patients with high cfDNA + MLH1 mutated had the shortest OS.
von Felden et al, 2020 (113)	PI3K/mTOR pathway mutations	N = 61	BCLC stage B/C: 30%/70% Treatment: CPI or TKI	Mutations in PI3K/mTOR pathway associated with: Poorer PFS (adjusted p=0.01) in TKI treated patients No association with outcome following CPI
Methylation/epigenetics				
Tangkijvanich et al, 2007 (78)	LINE-1 hypomethylation	N = 85	CLIP score 0-2/3-5: 48%/52% Treatment: NR	LINE-1 hypomethylation associated with poorer OS: adjusted HR=1.74 (1.09-2.79)
Huang et al, 2011 (114)	APC or RASSF1A methylation	N = 72	TNM stage I+II/III+IV: 24%/76% Treatment: NR	RASSF1A methylation: adjusted HR=3.26 (1.48-7.21) APC methylation: poorer OS on univariate analysis, but p=n.s. after adjustment
Kanekiyo et al, 2015 (107)	RASSF1A, CCND2, CFTR, SPINT2, SRD5A2 and/or BASP1 methylation	N = 125	TNM stage I+II/III+IV: 46%/54% Treatment: LR	Methylation of ≥3 genes: Decreased OS: adjusted HR=2.18 (p<0.001) Decreased DFS: adjusted HR=4.20 (p<0.001)
Liu et al, 2017 (71)	LINE-1 hypomethylation and RASSF1A promoter hypermethylation	N = 75	Stage: 47% ≥5 cm (reported only in 49 patients), 16% portal vein thrombosis, 15% lymph node metastases Treatment: LR	LINE-1 hypomethylation associated with: Higher DFS (unadjusted p=0.002) and OS (unadjusted p=0.01) RASSF1A hypermethylation no associated with DFS (p=0.41) and OS (p=0.83) LINE-1 hypomethylation + RASSF1A hypermethylation associated with: Shorter DFS (p=0.0001) and OS (p=0.05). LINE-1 hypomethylation independently associated with poor OS (p=0.045)
Xu et al, 2017 (80)	Methylation of 8 genes: SH3PXD2A, C11orf9, PPFIA1, chromosome 17:78, SERPINB5, NOTCH3, GRHL2, and TMEM8B	N = 1049 680 in validation set 39 in training set	TNM stage I/II/III/IV: 16%/16%/52%/12% Treatment: NR	High risk prognostic score associated with poorer OS: Training set: adjusted HR=2.41 (1.90-3.03) Validation set: adjusted HR=1.55 (1.25-1.92)

Yeh et al, 2017 (79)	LINE-1 hypomethylation	N = 172	BCLC stage 0+A/B+C: 36%/64% Treatment: NR	LINE-1 hypomethylation was associated with: Shorter OS: adjusted HR=1.77 (1.12-2.79)
Li et al, 2018 (115)	IGFBP7 promoter methylation	N = 155	TNM stage I+II/III+IV: 63%/37% Treatment: LR	Methylation of IGFBP7 associated with: Increased recurrence: adjusted HR=4.99 (1.51-16.47) Poorer OS: adjusted HR=3.86 (2.07-7.20)
Chen et al, 2020 (116)	CTCFL hypomethylation	N = 43 (+ 347 HCC from TCGA Atlas)	Stage: 63% size <5 cm, 91% single tumor, 5% metastases Treatment: NR	CTCFL hypomethylation associated with: Higher risk of postoperative recurrence (p=0.03) Poorer OS (p=0.006)

Abbreviations: AFP, alpha-fetoprotein; AFU, α-L-fucosidase; AUC, area under the curve; BCLC, Barcelona Clinic Liver Cancer; CLD, chronic liver disease; CPI, checkpoint inhibitors; CT, computed tomography; DCP, des-γ-carboxyprothrombin; DFS, disease-free survival; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; LC, liver cirrhosis; LR, liver resection; MVI, macroscopic vascular invasion; NR, not reported; OS, overall survival; PFS, progression-free survival; RFA, radiofrequency ablation; TACE, transarterial chemoembilization; TARE, transarterial radioembolization; TCGA, The Cancer Genome Atlas; TKI, tyrosine kinase inhibitors; TNM stage, tumor, nodes, metastases stage; TTP, time to progression; 5-hmC, 2-hydroxymethylcytosine.

Cell-free non-coding RNA

Long and short species of RNA are present in the cell-free non-coding RNA group, both with an extensive involvement in gene expression regulation. The RNA molecules with a length of >200 base pairs are classified as long non-coding RNAs (lncRNAs), several of which are involved in cancer progression. HULC, MEG3, HOTAIR, HOTTIP, MALAT-1 and MVIH are deregulated in HCC, and may be useful as biomarkers (117,118,127,128,119–126). lncRNA-CTBP, in a panel with other RNA-based biomarkers, showed high sensitivity and specificity in differentiating HCC from cirrhosis and healthy controls (129). Circulating levels of LINC00152, XLOC014172 and RP11-160H22.5 were able to distinguish HCC patients from cirrhotics, chronic hepatis and healthy subjects, with very high accuracy when combined with AFP (AUC of 0.986 for HCC vs. chronic hepatitis and 0.985 for HCC vs. healthy controls) (130). lncRNAs may also be useful as prognostic biomarkers, since they have been shown to predict recurrence after liver transplantation, development of metastases, recurrence-free and overall survival (122–126,128).

Among short non-coding RNAs, which are generally ~28 base pairs long, microRNAs (miRNAs) are the most extensively studied biomarkers in HCC in recent years, with a role in the diagnosis and in prognosis prediction. miRNAs generally bind to 3'UTR of the target mRNA resulting in down-regulation of gene expression through translational repression and/or mRNA degradation. More than 70 miRNAs have already been proposed as candidate biomarkers (25). Table 2 summarizes the most relevant studies on miRNAs as HCC biomarkers.

In the diagnostic setting, highly expressed miRNAs (miR-21, miR-199 and miR-122) seem to be the most promising for the diagnosis of HCC when considered individually, due to their elevated specificity (131). For instance, Tomimaru et al. (132) demonstrated that miR-21 yielded an AUC of 0.773 with 61.1% sensitivity and 83.3% specificity in differentiating HCC from chronic hepatitis, and an AUC of 0.953 with 87.3% sensitivity and 92.0% specificity in differentiating HCC from healthy

volunteers (in both cases superior to AFP). However, in the long run the diagnostic power of a single miRNA turned out to be limited and various panels consisting of more than one circulating miRNA have been evaluated. Lin et al. (133) demonstrated that a seven miRNAs classifier (miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505) had a greater AUC compared to AFP in identifying small size and early-stage HCC, detecting also AFP-negative tumors. Another panel consisting of miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801 was able to distinguish with high accuracy between HCC and healthy controls (AUC=0.941), chronic hepatitis B (AUC=0.842) and liver cirrhosis (AUC=0.884) (134). Interestingly enough, in a recent study the determination of eight miRNAs showed a sensitivity of 97.7% and a specificity of 94.7% in identifying the presence of HCC among patients at risk, with almost all cancers (98%) diagnosed in early stages (135).

Moreover, circulating miRNAs have a prognostic and predictive significance. Low levels of circulating miR-1, miR-122, miR-26a, miR-29a and miR-223-3p and high levels of miR-155, miR-96 and miR-193-5p were associated with poor prognosis (136–141). In a recent study, the whole miRNome profile was evaluated in 116 patients with HCC and six miRNAs were identified as prognostic factors; in particular, low levels of miR-424-5p and miR-101-3p and high levels of miR-128, miR-139-5p, miR-382-5p and miR-410 were associated with lower survival rates (142). After surgical resection, miR-224 and miR-500 levels decreased (143,144), miR-148a was up-regulated (145), and increased levels of serum miR-1246 could predict early tumor recurrence (<12 months) (146). High expression of miR-122 as well as low levels of miR-26a and miR-29a have been found to be poor prognostic marker in patients undergoing radiofrequency ablation (138,147) and some authors found that miRNAs evaluation could predict response to TACE (148,149) or sorafenib (150,151). Recently, a study evaluating plasma samples from participants to the registrative trial of regorafenib (RESORCE)

identified 9 plasma miRNAs (miR-30a, miR-122, miR-125b, miR-200a, miR-374b, miR-15b, miR-107, miR-320 and miR-645) whose levels were significantly associated with OS (152).

Table 2. Studies on use of microRNAs (miRNAs) as biomarkers in HCC.

Diagnosis				
Study	**Type of miRNA**	**Number of patients**	**Comparator**	**Main findings (sensitivity/specificity, AUC)**
Zhou et al, 2011 (134)	miRNA panel: miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801	Training phase: 204 HCC, 60 LC, 75 CLD (HBV), healthy subjects 68 Validation phase: 196 HCC, 56 LC, 72 CLD (HBV), 66 healthy subjects	AFP (cut-off 400 ng/mL)	Training cohort: 68.6%/90.1%, 0.864 Validation cohort: 81.8%/83.5%, 0.888 AUC according to BCLC stage: 0 = 0.888, A = 0.888, B = 0.901, and C = 0.881 In AFP <400 ng/mL: 77.7%/84.5%, 0.879 In AFP ≥400 ng/mL: 87.7%/83.5%, 0.910 HCC vs. healthy subjects: 83.2%/93.9%, 0.941 HCC vs. CLD: 79.1%/76.4%, 0.842 HCC vs. LC: 75%/91.1%, 0.884
Tomimaru et al, 2012 (132)	miR-21	126 HCC 30 CLD 50 healthy subjects	AFP (cut-off 19 ng/mL in HCC vs. CLD and 6 ng/mL in HCC vs. healthy subjects)	HCC vs. CLD: miR-21: 61.1%/83.3%, 0.773 AFP: 59.5%/83.3%, 0.743 miR-21 + AFP: 81%/76.7%, 0.823 HCC vs. healthy subjects: miR-21: 87.3%/92%, 0.953 AFP: 77.8%/96%, 0.882 miR-21 + AFP: 92.9%/90%, 0.971
Lin et al, 2015 (133)	miRNA classifier: miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505	Training cohort: 108 HCC, 47 LC, 51 CLD (HBV), 51 healthy subjects Validation cohort: 2020 HCC, 181 CLD + LC, 108 healthy subjects	AFP (cut-off 20 ng/mL)	Validation cohort 1: miRNA classifier: 74.5%/88.9%, 0.817 AFP: 56.9%/84.9%, 0.709 (p<0.05 vs. miRNA classifier) Validation cohort 2: miRNA classifier: 85.7%/91.1%, 0.884 AFP: 59.2%/100%, 0.796 (p<0.05 vs. miRNA classifier) miRNA classifier vs. AFP (AUC): Small HCC: 0.833 vs. 0.727 (p<0.05) Early-stage HCC: 0.824 vs. 0.754 (p<0.05) miRNA classifier in AFP negative patients (AUC): 0.825
El-Tawdi et al, 2016 (129)	lncRNA-CTBP +miR-16-2 + miR-21-5p + LAMP2	78 HCC 36 CLD (HCV) 44 healthy subjects	NR	HCC vs. CLD + healthy subjects: 79.5%/100%, 0.938
Amr et al, 2016 (153)	miR-21 and miR-199a	23 HCC 17 CLD	AFP	miR-21: 100%/81.2%, 0.943 miR-199a: 54.5%/100%, 0.856 AFP: 100%/69.2%, 0.832
Okajima et al, 2016 (143)	miR-224	87 HCC 55 healthy subjects	AFP (cut-off 20 ng/mL) DCP (cut-off 40 mAU/mL)	93.1%/80.0%, 0.908 Early-stage HCC (TNM stage I) vs. healthy subjects: AUC=0.0899 miR-224 in the detection of tumor <18 mm (sensitivity, AUC): First cohort: 80%, 0.802 (DCP 45%, 0.741; AFP 50%, 0.475) Second cohort: 63.6%, 0.731 (DCP 50%, 0.595; AFP 20%, 0.726)
Zhuang et al, 2016 (154)	miR-21, miR-26a and miR-101	52 HCC 42 CLD	AFP (cut-off NR)	HCC vs. healthy subjects: miR-21 + miR-26a + miR-101: 88.2%/58.5%, 0.803

		43 healthy subjects		miR-21 + miR-26a + miR-101 + AFP: 87%/78%, 0.914 HCC vs. CLD: miR-26a + miR-101: 70.6%/80.2%, 0.822 miR-26a + miR-101 + AFP: 72.5%/86.7%, 0.854
Zekri et al, 2016 (155)	Several miRNAs	192 HCC 96 LC 96 CLD (HCV) 95 healthy subjects	AFP (cut-off NR)	HCC vs. healthy subjects: miR-122 + miR-885-5p + miR-29b + AFP: AUC = 1 HCC vs. LC: miR-122 + miR-885-5p + miR-221 + miR-22 + AFP: AUC = 0.982 HCC vs. CLD: miR-22 + miR-199a-3p + AFP: AUC = 0.988
Shi et al, 2017 (156)	miR-106b	25 HCC 310 non-HCC	NR	Patients with HCC had higher serum miR-106b levels. 90%/66.7% (0.855)
Guo et al, 2017 (157)	miR-21	175 HCC 64 CLD (HBV) 78 LC 136 healthy subjects	AFP (cut-off 16.42 ng/mL)	HCC vs. LC: miR-21: 80.8%/72.9%, 0.814 AFP: 70.4%/71.5%, 0.686 HCC vs. CLD: miR-21: 76.9%/85.7%, 0.789 AFP: 59.3%/69.7%, 0.634
Zhang et al, 2017 (158)	miRNA panel: miR-92-3p, miR-107 and miR-3126-5p	115 HCC 40 healthy subjects	AFP (cut-off NR)	miR-3126-5p: AUC = 0.881 miR-107: AUC = 0.730 miR-92a-3p: AUC = 0.705 miRNA panel: AUC = 0.969 vs. AFP: AUC=0.848 miRNA panel + AFP: AUC = 0.994
Moshiri et al, 2018 (159)	miR-106b-3p, miR-101-3p and miR-1246	62 HCC 41 LC 25 healthy subjects	NR	HCC vs. healthy subjects: 100%/100%, 1.00 HCC vs. LC: 100%/92.9%, 0.99s
An et al, 2018 (160)	miR-375, miR-10a, miR-122 and miR-423	84 HCC 84 normal controls	NR	miR-375: AUC = 0.918 miR-10a: AUC =0.838 miR-122: AUC = 0.871 miR-423: AUC =0.898 miR-375, miR-10a, miR-122 and miR-423: AUC = 0.995
Han et al, 2019 (145)	miR-148a	155 HCC 96 LC 95 healthy subjects	AFP (cut-off NR)	HCC vs. healthy subjects: 97.9%/92.9%, 0.980 HCC vs. LC: AFP: 88.4%/84.4%, 0.941 miR-148a: 89.6%/89%, 0.919 (in patients with low AFP: 90.6%/92.6%, 0.949)
Weis et al, 2019 (161)	miR-122-5p, miR-486-5p and miR-142-3p	20 HCC 20 LC 20 CLD	AFP (cut-off 20 ng/mL)	miR-122 + miR-486 + miR-142: 80%/95%, 0.94 AFP: 25%/90%, 0.64 (p=0.06 vs. miRNA panel) miR-122 + miR-486 + miR-142 + AFP: 0.94
Yamamoto et al, 2020 (135)	8 miRNA panel: miR-320b, miR-663a, miR-4448, miR-4651,	353 HCC 93 LC 46 CLD 1033 healthy subjects	AFP (cut-off 10 ng/mL) DCP (cut-off 40 mAU/mL)	HCC vs. healthy subjects: 97.7%/98.4%, 1.00 HCC vs. CLD + LC: 97.7%/94.7%, 0.99 In TNM stage I, the 8 miRNAs panel: 100%/94.7%, 1.00

	miR-4749-5p, miR-6724-5p, miR-6877-5p, and miR-6885-5p			
Prognosis				
Study	**Type of miRNA**	**HCC patients**	**Stage /Treatment**	**Main findings**
Koberle et al, 2013 (136)	miR-1 and miR-122	N = 195	BCLC stage A/B/C/D: 24%/39%/30%/7% Treatment: LR 9%, ABL+IAT 53%, SOR 24%, LT 11%	At univariate analysis, longer survival in: High miR-1 group: HR = 0.44 (0.23-0.83) High miR-122 group: HR = 0.49 (0.25-0.96) High miR-1 was an independent predictor of better OS: adjusted HR = 0.45 (0.24-0.86)
Xu et al, 2015 (162)	miR-122	N = 122	Stage: NR Treatment: NR	High miR-122 associated with longer OS: adjusted HR = 0.26 (0.14-0.47)
Cho et al, 2015 (163)	miR-122	N = 120	TNM stage I+II/III+IV: 73.3%/26.7% Treatment: LR 52.5%, ABL 47.5%	miR-122 levels were not associated with OS in the entire cohort and in LR patients. In ABL patients, high miR-122 associated with shorter OS: adjusted HR = 2.67 (1.12-6.35).
Okajima et al, 2016 (143)	miR-224	N = 87	TNM stage I/II-IV: 71%/29% Treatment: LR	miR-224 was significantly reduced in post-LR samples (p=0.006). Tumor >2 cm, advanced stage and presence of recurrences correlated with high levels of miR-224 (p=0.0005, 0.04 and 0.003, respectively).
Cho et al, 2017 (138)	miR-21, miR-26a and miR-29a	N = 120	TNM stage I+II/III+IV: 73.3%/26.7% Treatment: LR 52.5%, ABL 47.5%	Poorer DFS was demonstrated for: Low miR-26a levels: adjusted HR = 1.72 (1.04-2.83) Low miR-29a levels: adjusted HR = 1.75 (1.04-2.94) Shorter LT-free survival was demonstrated for: Low miR-26a: adjusted HR = 3.41 (1.32-8.82) Low miR-29a: adjusted HR = 2.75 (1.10-6.85)
Fornari et al, 2017 (150)	miR-221	N = 90 (50 in training set and 43 in validation set)	Stage: advanced HCC without extrahepatic metastases Treatment: sorafenib	Both in training and in validation cohorts: Higher pre-treatment miR-221 levels in non-responders vs. responders. After 2 months of treatment: increase of miR-221 in responders; non-significant decrease in non-responders.
Nishida et al, 2017 (151)	miR-181a-5p and miR-339-5p	N = 53	BCLC stage A/B/C: 15%/28.3%/56.6% Treatment: sorafenib	miR-181a-5p independently predicted DC: adjusted HR = 0.14 (0.01–0.66). High miR-181a-5p was associated with longer OS: adjusted HR = 0.27 (0.07-0.82).
Kim et al, 2018 (149)	miR-21, miR-26a and miR-29a-3p	N = 198	BCLC stage: A-B/C-D: 73.2%/26.8% Treatment: TACE	High miR-21, high miR-26a and low miR-29a-3p levels associated with overall TACE refractoriness (no after adjustment for confounders). miRNA combination panel (high miR-21 and miR-26a and low miR-29a-3p) independently predict early TACE refractoriness: adjusted HR = 2.32 (1.08-4.99).
Chuma et a, 2019 (146)	miR-1246	N = 121	BCLC stage 0/A/B: 27.3%/67.8%/5%	miR-1246 levels higher in patients with early tumor recurrence. High levels of miR1246 associated with:

			Treatment: LR	Early tumor recurrence: adjusted HR = 3.42 (1.33-8.82) Shorter DFS (p<0.001) Shorter OS (p<0.001); adjusted HR = 2.78 (1.53-5.07)
Ali et al, 2019 (148)	miR-133b, miR-26a, miR-107 and miR-106	N = 51	Stage: single tumor 57% Treatment: TACE	Baseline miR-106b, miR-107 and miR-133b elevated in TACE-responders; miR-26a in non-responders (all p<0.001). Prediction of TACE response (sensitivity/specificity, AUC): miR-26a: 100%/100%, 1.00 in CR vs. NR; 94%/83%, 0.958 in CR vs. PR miR-133b: 100%/94%, 0.997 in CR vs. NR; 93%/88%, 0.919 in CR vs. PR; 94%/83%, 0.935 in PR vs. NR miR-26a + miR-133 (AUC): 1.00 in CR vs. NR; 0.997 in PR vs. NR; 0.919 in CR vs. PR; 0.998 in CR+PR vs. NR
Ning et al, 2019 (139)	miR-155, miR-96 and miR-99a	N = 30	TNM stage I+II/III+IV: 40%/60% Treatment: LR	Decreased OS in patients with: High miR-155 levels (p=0.004) High miR-96 levels (p=0.02) miR-99a levels were not significantly associated with OS.
Jin et al, 2019 (164)	miR-128, miR-139-5p, miR-382-5p, miR-410, miR-424-5p and miR-101-3p	N = 116	Stage: multifocal 29% Treatment: NR	Higher expression of miR-128, miR-139-5p, miR-382-5p and miR-410 and lower levels of miR-424-5p and miR-101-3p associated with worse prognosis.
Han et al, 2019 (145)	miR-148a	N = 155	Stage: metastases 30% Treatment: TACE or ABL	miR-148a increased significantly after treatment (p<0.0001).
Teufel et al, 2019 (152)	miR-30a, miR-122, miR-125b, miR-200a, miR-374b, miR-15b, miR-107, miR-320 and miR-645	N = 243	BCLC stage A/B/C: <1%/14%/86% (in RESORCE trial) Treatment: regorafenib	Increased miR-30a, miR-122, miR-125b, miR-200a, and MIR374B, decreased miR-15b, miR-107, and miR-320b, and absence of miR-645 predictive of survival benefit. miR-15b, miR-320b, and miR-200a were prognostic for OS (p<0.05)
Loosen et al, 2020 (140)	miR-193a-5p	N = 41	TNM stage T1-2/T3-4: 75%/25% Treatment: LR 19.5%, LT 80.5%	High levels of miR-193a-5p independently associated with poorer OS: adjusted HR = 3.71 (1.35-10.16)
Pratedrat et al, 2020 (141)	miR-223-3p	N = 70	BCLC stage 0-A/B/C-D: 30%/34.3%/35.7% Treatment: NR	High levels of miR-223-3p independently associated with poorer OS: adjusted HR = 6.61 (2.36-18.55)

Abbreviations: ABL, ablation; AFP, alpha-fetoprotein; AUC, area under the curve; BCLC, Barcelona Clinic Liver Cancer; CLD, chronic liver disease; CR, complete response; DC, disease control; DCP, des-γ-carboxyprothrombin; DFS, disease-free survival; HCC, hepatocellular carcinoma; HR, hazard ratio; IAT, intrarterial therapies; LC, liver cirrhosis; LR, liver resection; LT, liver transplantation; miRNA, microRNA; NR, not reported; OS, overall survival; PR, partial response; SOR, sorafenib; TACE, trans-arterial chemoembolization.

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are small membrane vesicles released by cells in extracellular environment in normal physiology and in pathological conditions (165). EVs transport a variety of bioactive molecules, including mRNA, miRNAs, proteins and lipids, that can be transferred among cells both in the environment in which they are released, as well as at distant sites, regulating various cell responses (165,166). Considered their ability of altering intracellular pathways (167–170), cancer cells can use EVs to take advantage in proliferation (171).

EVs are generally classified in small (exosomes) and large EVs (ectosomes, also called microparticles (MPs) or microvesicles) (172). Although small and large EVs may be distinguished by some of the expressed markers, such as CD63, HSP70, CD9, CD81 and integrins (173,174), the border between these two entities is not sharp (25). The growing number of studies providing evidence for a key pathophysiological role of EVs in various aspects of liver diseases and the fact that EVs are released in the systemic circulation, where they are remarkably stable, provide the background to consider their assessment and quantification in blood as a novel form of liquid liver biopsy (66). Several studies demonstrated a potential role of EVs as biomarkers in HCC patients (Table 3).

First reports showed that HCC patients had a higher level of circulating EVs compared to controls (175) and the determination of total amount of EVs provided slightly better sensitivity and specificity compared to alpha-fetoprotein (AFP) in HCC diagnosis (176). A specific form of large EVs expressing surface AnnexinV, EpCAM, ASGPR1 and CD133 was identified by Julich-Haertel et al. (177) as a marker able to distinguish HCC and cholangiocarcinoma from other cancer types, cancer-free cirrhotic patients and healthy subjects. Sensitivity, positive predictive value and area under the curve (AUC) in the distinction between HCC and cirrhosis were 80%, 73% and 0.744, respectively (177).

Going beyond the simple determination of the total amount of EVs, the researchers subsequently focused on analyzing their content. Arbelaiz et al. (178) demonstrated that galectin-3-binding protein (LG3BP) and polymeric immune receptor (PIGR) had higher diagnostic accuracy (AUC of 0.904 and 0.837, respectively) compared to AFP (AUC=0.802). Other promising molecules are exosomal AFP and GPC3 mRNA (179), hnRNPH1 mRNA (180) and long non-coding RNAs (lncRNAs) (181–184). In particular, Xu et al. (182) obtained AUCs of 0.894 and 0.885 in derivation and validation cohorts, respectively, with the combination of two lncRNAs (ENSG00000258332.1 and LINC00635). In another study, a machine learning based score ("HCC classifier") with 8 lncRNAs markers showed very promising AUCs (0.953 in training cohort, 0.983 in validation cohort and 0.963 in testing cohort) (183). Several other researchers focused their attention on exosomal miRNAs (185–189). Some studies found similar diagnostic accuracies for AFP and EVs miRNAs (185,187), while others demonstrated the superiority of the latter (186,188).

A lower number of studies investigating EVs in the prognostic field are available, and most of them focused on the evaluation of exosomal miRNAs, in particular after surgical therapies (liver resection or liver transplantation) (189–195). The only miRNA included in more than one study was miR-21, and its high levels have been repetitively associated with increased risk of disease progression and poorer survival (194,196,197). Other studies demonstrated that low levels of exosomal miR-718, miR-125b, miR-638 and miR-320a (189–191,193) and high exosomal miR-665 and miR-10b (192,194) were associated with worse prognosis.

EVs and their content are promising candidate biomarkers in patients with HCC for diagnosis and prognosis prediction. Nevertheless, additional larger prospective studies should be conducted to definitely establish their role as liquid biopsy.

Table 3. Studies on extracellular vesicles (EVs) as biomarkers in HCC patients.

Diagnosis				
Study	**EVs property**	**Number of patients**	**Comparator**	**Main findings (sensitivity/specificity, AUC)**
Wang et al, 2013 (176)	Total amount	55 HCC; 40 LC; 21 healthy subjects	AFP (cut-off 20 ng/mL)	Sensitivity/specificity: 88.9%/62.6% for EVs and 85.7%/40.0% for AFP TNM stage I vs. cirrhosis: AUC = 0.83 (p<0.01 vs. AFP) TNM stage II vs. cirrhosis: AUC = 0.94 (p<0.01 vs. AFP)
Cheng et al, 2015 (175)	Total amount	12 HCC; 11 CLD; 6 healthy subjects	NR	EVs concentration higher in HCC patients vs. healthy controls or cirrhotics. No differences in EVs concentration based on AFP levels.
Julich-Haertel et al, 2017 (177)	Tumor-associated MPs	Explorative study: 22 HCC, 26 CCA, 5 LC, 18 IH, 53 CLD, 18 controls. Validation study: 86 HCC, 38 CCA, 49 LC, 10 NSCLC, 19 CRC, 26 IH, 173 CLD, 58 controls.	NR	Explorative study. HCC vs. controls AnnexinV +, EpCAM + taMPs: 81.8%/66.7%, 0.833 AnnexinV +, EpCAM +, CD147 + taMPs: 72.7%/82.3%, 0.739 Validation study. HCC vs. controls AnnexinV +, EpCAM + taMPs: 76.5%/63.3%, 0.769 AnnexinV +, EpCAM +, CD133 + taMPs: 69.8%/41.4%, 0.626 AnnexinV +, EpCAM +, ASGPR1 +, CD133 + taMPs: 80.0%/50.0%, 0.744 Validation study. Cirrhosis vs. HCC AnnexinV +, EpCAM +, ASGPR1 + taMPs: 81.4%/46.9%, 0.732
Arbelaiz et al, 2017 (178)	EV proteins (LG3BP and PIGR)	29 HCC; 43 CCA; 30 PSC; 32 healthy subjects	AFP	HCC vs. controls LG3BP: 96.6%/71.8%, 0.904 PIGR: 82.8%/71.8%, 0.837 AFP: 82.1%/64.0%, 0.802
Abd El Gwad et al, 2018 (181)	lncRNA-RP11-513I15.6, miR-1262 and RAB11A	60 HCC; 42 CLD; 18 healthy subjects	NR	96.7%/95.0% for lncRNA-RP11-513I15.6 95.0%/80.0% for miR-1262 75.0%/73.3% for RAB11A mRNA 100.0%/76.7% for lncRNA-RP11-513I15.6 + miR-1262 + AFP
Pu et al, 2018 (185)	miR-21-5p and miR-144-3p	24 HCC; 16 CLD; 17 healthy subjects	NR	miR-21-5p: AUC = 0.442 miR-144-3p: AUC = 0.747 miR-144.3p/miR-21-5p ratio: AUC = 0.780 AFP: AUC = 0.626
Wang et al, 2018 (179)	AFP and GPC3 mRNA	40 HCC; 38 healthy subjects	AFP (cut-off 20 ng/mL)	EV AFP mRNA: AUC = 0.947 EV GPC3 mRNA: AUC = 0.979 AFP protein: AUC = 0.936 AFP and GPC3 mRNA: AUC = 0.995
Wang et al, 2018 (186)	miR-122, miR-148a and miR-1246	68 HCC; 53 LC; 50 CLD; 64 healthy subjects	AFP	Cirrhosis vs. HCC (all stages). AUC: miR-122: AUC = 0.816 miR-148a: AUC = 0.891 miR-1246: AUC = 0.785 AFP: AUC = 0.712 miR-122 + miR-148a + AFP: AUC = 0.931

Xu et al, 2018 (182)	lncRNAs (ENSG00000258332.1 and LINC00635)	60 HCC (+55 in validation cohort); 85 LC; 96 CLD (+60 in validation cohort); 60 healthy subjects (+60 in validation cohort)	AFP (cut-off 20 μg/L)	HCC vs. CLD First cohort: ENSG00000258332.1: 71.6%/83.4%, 0.719 LINC00635: 76.2%/77.7%, 0.750 AFP: 54.7%/75.3%, 0.666 All 3 markers: 83.6%/87.7%, 0.894 Second cohort: ENSG00000258332.1: 73.5%/80.5%, 0.718 LINC00635: 79.6%/75.2%, 0.731 AFP: 52.5%/74.1%, 0.634 All 3 markers: 84.5%/85.3%, 0.885
Xu et al, 2018 (180)	hnRNPH1 mRNA	88 HCC; 67 LC; 68 CLD; 68 healthy subjects	AFP (cut-off 20 ng/mL)	HCC vs. CLD hnRNPH1 mRNA: 85.2%/76.5%, 0.865 AFP: 69.3%/87.9%, 0.785 hnRNPH1 + AFP: 87.5%/84.8%, 0.891 HCC vs. cirrhosis hnRNPH1 mRNA: 86.4%/54.0%, 0.647 AFP: 46.6%/88.3%, 0.674 hnRNPH1 + AFP: 50.3%/91.0%, 0.749
Zhang et al, 2019 (187)	miR-212	78 HCC; 95 LC; 58 CLD; 70 healthy subjects	NR	HBV-related HCC vs. healthy subjects miR-212: 70.0%/95.0%, 0.89 AFP: 0.85 Non-HBV-related HCC vs. healthy subjects miR-212: 89.0%/62.0%, 0.79 AFP: 0.84
Li et al, 2019 (183)	lncRNAs	71 HCC; 37 CLD; 94 healthy subjects	AFP (cut-off 10 ng/mL)	Support vector machine model (HCC classifier with 8 markers) Training cohort: 84%/94%, 0.953 Validation cohort: 89%/91%, 0.983 Testing cohort: 85%/95%, 0.963
Lu et al, 2020 (184)	lncRNAs: ENSG00000248932.1 ENST00000440688.1 ENST00000457302.2	200 HCC; 200 CLD; 200 healthy controls	NR	Three lncRNAs: AUC = 0.96/0.53 in training/validation cohorts Three lncRNAs + AFP: AUC = 0.97/0.87 in training/validation cohorts
Sorop et al, 2020 (188)	miR-21-5p and miR-92a-3p	48 HCC; 38 LC; 20 healthy subjects	AFP	AFP alone: AUC = 0.72 miR-21-5p + miR-92a-3p + AFP: AUC = 0.85 ($p<0.05$ vs. AFP)
Hao et al, 2020 (189)	miR-320a	104 HCC; 55 CLD; 50 healthy subjects	NR	HCC vs. healthy subjects: 77.9%/80.0%, 0.86 HCC vs. CLD: 76.1%/81.8%, 0.83
Prognosis				
Study	**EVs property**	**Number of patients**	**Stage /Treatment**	**Main findings**

Sugimachi et al, 2015 (190)	miR-718 and miR-1246	N = 66 (6 in exploratory and 59 in validation analysis)	Stage: 34% beyond Milan criteria Treatment: LT	Recurrence post-LT: 6/42 in the low and 0/11 in the high miR-718 groups (p=n.s.). Patients with tumor diameter ≥3 cm: greater recurrence with high miR-718 (p=0.0002). No association with recurrence for miR-1246
Liu et al, 2017 (191)	miR-125b	N= 128	TNM stage I/II-III: 37.5%/62.5% Treatment: LR	Low miR-125b associated with: Lower time-to-recurrence: HR=0.14 (0.08-0.27); p<0.001 Poorer OS: HR=0.33 (0.18-0.62); p<0.001
Qu et al, 2017 (192)	miR-665	N= 30	TNM stage I-II/III-IV: 20%/80% Treatment: LR	Patients with high miR-665 showed lower OS (p<0.05; HR not reported)
Shi et al, 2018 (193)	miR-638	N = 126	TNM stage I+II/III+IV: 53%/47% Treatment: LR	Low miR-638 levels associated with: Poorer OS (adjusted HR=2.80, 1.24-4.31; p=0.01)
Suehiro et al, 2018 (196)	miR-122 and miR-21	N = 75 (57 with LC)	Stage: NR Treatment: TACE	miR-21 and miR-122 not associated with survival in the entire cohort. In LC group, high miR-122 ratio (after/before TACE) associated with poorer OS: adjusted HR=2.72 (1.04-8.02); p=0.04
Abd El Gwad et al, 2018 (181)	RAB11A mRNA	N = 60	BCLC stage early: 90% Treatment: NR	Low levels of RAB11A mRNA are associated with longer recurrence-free survival: adjusted HR=0.36 (0.15-0.88), p=0.03
Lee et al, 2019 (197)	miR-21 and lncRNA-ATB	N = 79	TNM stage I-II/III-IV:40.5%/59.5% Treatment: 10 LR, 5 LT, 24 ABL, 9 TACE, 17 SOR and 14 BSC	High miR-21 and lncRNA-ATB independent predictors of mortality (HR=2.87 and 2.17, respectively; all p<0.05). High miR-21 and lncRNA-ATB independent predictors of disease progression (HR=2.53 and 2.55, respectively; all p<0.05).
Tian et al, 2019 (194)	miR-21 and miR-10b	N = 124	Stage: 79% monofocal, 35% ≤3 cm Treatment: LR	Poorer disease-free survival with: High miR-21: adjusted HR=2.45 (1.25-4.78); p=0.009 High miR-10b: adjusted HR=2.55 (1.30-4.99); p=0.006
Hao et al, 2020 (189)	miR-320a	N = 104	TNM stage: 37.5%/62.5% Treatment: LR (+/- adjuvant chemotherapy)	Low miR-320a associated with poorer OS and DFS. Low miR-320a independent predictor of mortality: adjusted HR=2.97 (1.56-4.63); p=0.008
Luo et al, 2020 (195)	circAKT3	N = 124	TNM stage I-II/III-IV: 44%/37% Treatment LT/LR: 19/81%	Patients with high circAKT3 have: Higher tumor recurrence rates (HR 3.14, 1.29-6.21; p=0.01) Higher mortality (HR 1.89, 1.04-3.01; p=0.048)

Abbreviations: AFP, alpha-fetoprotein; AUC, area under the curve; BCLC, Barcelona Clinic Liver Cancer; CCA, cholangiocarcinoma; CLD, chronic liver disease; CRC, colorectal carcinoma; DFS, disease-free survival; EVs, extracellular vesicles; HCC, hepatocellular carcinoma; HR, hazard ratio; IH, inguinal hernia; LC, liver cirrhosis; lncRNA, long non-coding RNA; LR, liver resection; LT, liver transplantation; miR, microRNA; MPs, microparticles; NSCLC, non-small cell lung carcinoma; NR, not reported; OS, overall survival; PSC, primary sclerosing cholangitis; TACE, transarterial chemoembolization; taMPs, tumor-associated microparticles; TNM stage, tumor, nodes, metastases stage.

CIRCULATING TUMOR CELLS

Metastatization is a complex and largely unknown process requiring the ability for cancer cells to escape from primary tumor, survive in the circulation and then settle in a new organ. Circulating tumor cells (CTCs) are key players in cancer dissemination. Considering that CTCs are present in the order of one per billion of blood cells in patients with metastatic disease, there have been some initial obstacles in their study (198). Nevertheless, technical and methodological advances in the last years led to a significant expansion of publications aimed at investigating their role as candidate biomarkers (Table 4).

Platforms for the detection of CTCs are based on their known biological and physical properties, and can grossly be divided in immunoaffinity-based and biophysical property-based enrichment (199). Immunoaffinity-based CTCs enrichment techniques use antibodies against cell surface markers bounded to the device or a magnetic substance. The enrichment can be positive when CTCs are captured with antibodies against tumor specific antigens expressed on CTC surface, or negative when hematopoietic cells in the background are depleted by using antibodies against CD45 (200). The CellSearch™ system (Veridex) captures CTCs using an immunomagnetic separation system with antibodies against EpCAM and cytokeratin coated onto ferrofluid beads and has been approved by the US Food and Drug Administration for use in patients with breast, prostate and colorectal cancers (201,202). Other developed detection systems include CTC-Chip™ (203), CTC-iChip™ (204) and NanoVelcro™ (205). These methods rely on tumor expression of the target proteins and their role is limited for cancers that do not typically express them. Only about one third of CTCs in HCC are positive for EpCAM and cytokeratin (206,207), and even if CellSearch™ became the most popular detection system, it could be of limited application in HCC. Moreover, given that epithelial markers such as EpCAM are often downregulated or lost during epithelial-to-mesenchymal transition (EMT) (208), CTC with EMT phenotype which have strong metastatic potential could not be detected by

positive enrichment methods that target epithelial markers. Therefore, strategies targeting stem cell markers (CD133), mesenchymal markers (vimentin) and cancer specific antigens (such as HER2, PSMA, ASGPR, Hepar 1 and carbamoyl phosphate synthetase 1) have been developed (209,210). The biophysical methods to isolate CTCs rely on their typical features such as large size, mechanical plasticity and dielectric mobility properties, employing centrifugation and filters or flow devices with channels of varying size or nature. Although the advantage of avoiding the challenges of targeting numerous tumor specific epitopes, these methods may be less cancer-specific.

As far as the diagnostic value of CTCs analysis is considered, published studies showed that CTCs may have a role in differentiating HCC from controls. A major concern when dealing with CTCs analysis as diagnostic biomarker is the fact that, since their levels correlate with tumor burden (211), the sensitivity in early-stage disease may be too low. Nevertheless, Guo et al. (212) in a large study investigating a CTC-derived PCR score (quantifying the expression of cancer-related genes in blood), demonstrated a sensitivity of 72.5%, a specificity of 95% and an AUC of 0.88 (compared with 57%, 90% and 0.77 of AFP at a cut-off of 20 ng/mL). In addition, this score performed well also in patients with early-stage HCC (AUC of 0.92 in BCLC stage 0 and 0.86 in BCLC stage A).

CTCs evaluation combined with AFP provided incremental performance with respect to AFP alone in identifying HCC patients. In a study, the AUC in the discrimination of CLD and HCC patients was 0.67 for AFP (cut-off 400 ng/mL), 0.77 for CTCs (detected with CanPatrol™) and 0.82 for the combination of both (213). Guo et al. reported that CTCs, defined by the expression of EpCAM mRNA, had a sensitivity of 42.6% and an AUC of 0.70 in discriminating HCC from CLD and healthy controls, while AFP (cut-off 400 ng/mL) demonstrated a lower sensitivity (39.5%; AUC not reported); the combination of CTCs and AFP increased sensitivity to 73% and the AUC to 0.86 (214).

Considering that CTCs are extremely rare in the circulation and that their number tends to be proportional to tumor volume, which make their detection in early-stage disease challenging (211),

they are probably more useful for prognostication rather than for early diagnosis. Indeed, several evidences emerged linking CTCs enumeration with prognosis of HCC patients. A landmark study in 2004 demonstrated that the presence and number of CTCs, identified and enumerated based on their morphology, were associated with shorter survival (215). Subsequent studies using CellSearch™ showed that the detection of EpCAM positive CTCs was associated with an higher tumor recurrence rate after liver resection (216) and with a worse prognosis (217,218). The independent prognostic value of CTCs amount was confirmed even with other CTCs enrichment technologies, such as ImageStream flow cytometry, which uses a panel of markers and generates high resolution images of isolated CTCs (207,219). Beyond simple enumeration, other reports have investigated the prognostic impact of subgroups of CTCs, divided according to cell surface markers, RNA expression or genomic aberrations. The identification of CTCs with cancer stem cell-like or mesenchymal surface markers is useful to predict tumor recurrence (220–223). Other studies demonstrated that CTCs with detectable AFP mRNA were associated with higher risk of metastatic dissemination (224), whereas CTCs with aneuploid chromosome 8 predicted shorter survival in patients treated with surgical resection (225). The interesting study by Ha et al. (226) introduced the concept of ΔCTC, which is defined as the variation in CTCs enumeration after surgery, and is an independent factor of lower survival and recurrence after hepatectomy.

Cancer cell dissemination seems to be promoted by treatment, in particular surgical therapies. Liver manipulation is associated with a release of CTCs (227) and the anterior as compared to the conventional surgical approach is associated with a lower release of CTCs as well as better survival (228). In liver transplantation for HCC, five steps to minimize CTCs dissemination and thereby reduce the risk of recurrence have been described (229). This approach in transplantation assumes even more importance as an association between CTCs detection and recurrence after transplantation has been demonstrated (230,231). Overall, data consistently reported that the number of CTCs is a

surrogate of poor prognosis, predicting higher recurrence and lower survival. A recent metanalysis and data from experimental models led to the same conclusions (232,233).

Considered that CTCs detection methods are costly and time consuming, the application of CTCs enumeration in clinical practice requires a clear advantage to be established. Probably, this is an unrealistic goal and therefore phenotypic characterization of CTCs may be more useful, since tissue-based biomarkers that could be of help in treatment choice and monitoring are currently lacking. Moreover, it is clear from several studies that CTCs are a heterogeneous population and that they may reflect tumoral heterogeneity better than a tissue biopsy (199,207). The CTC pERK/pAkt phenotype has recently been reported to predict sensitivity to sorafenib (234), while the presence of CTCs positive for PD-L1 is associated with response to checkpoint inhibitors (235). Considered that result, it could be imagined that phenotyped CTCs will be useful surrogates for guiding enrichment trials with molecular targeted therapies. Moreover, methods for collecting living CTCs from HCC patients and culture them into three-dimensions spheroid-like structures have also been developed, with the possibility to bring personalized medicine to a new level. In this scenario, Zhang et al. (236) explored individual sensitivity to sorafenib and oxaliplatin after collecting and culturing CTCs, and the evaluation of multiple therapeutic candidates in patients' CTC-derived xenografts may become a future reality (66).

Even if the use of CTCs analyses as biomarkers in guiding clinical decisions has huge potential, perhaps the most innovative and relevant contribution of CTC studies will be in advancing our understanding of the biology of metastatic disease as well as the development of treatment strategies. The analysis of CTCs at a molecular level, facilitated by the advancements in sequencing technologies, may lead to the identification of new mutations responsible for tumor metastatization and resistance to drugs (237). Moreover, other insights in metastatic spread have been achieved analyzing the spatial distribution of CTCs in the bloodstream. An interesting study analyzed and

compared CTCs collected in HCC patients from different vessels (peripheral veins and arteries, portal vein and hepatic veins). The greatest number of CTCs was demonstrated in hepatic veins, with a dramatic reduction in peripheral vessels after passage through the lungs. Moreover, there was a phenotypic heterogeneity in CTCs isolated from different sites, being predominantly epithelial into the hepatic vein and EMT-transformed when isolated in peripheral vessels (238). The CTC burden and the presence of CTC clusters in both hepatic and peripheral veins predicted lung and liver metastases.

Although the rapid evolution in technologies supporting CTCs detection, isolation and characterization and the very promising results in the studies so far published, the clinical application of CTCs as biomarkers is hindered by the different methodologies applied by single researchers. Indeed, few studies have been reproduced by more than one research group. Before the incorporation of CTCs evaluation in trials and clinical practice, standardized protocols with reproducible results, currently lacking in HCC, are needed.

Table 4. Studies on use of circulating tumor cells (CTCs) as biomarkers in HCC patients.

Diagnosis				
Study	**CTCs definition**	**Number of patients**	**Comparator**	**Main findings (sensitivity/specificity, AUC)**
Yao et al, 2005 (239)	CD45 (-) EpCAM (+) then AFP mRNA	49 HCC 36 CLD or LC 18 healthy subjects	AFP (cut-off 20 ng/mL)	AFP mRNA (sensitivity/specificity): 72.1%/66.7% Low AFP: sensitivity, 75% High AFP: sensitivity, 71% (p>0.05)
Guo et al, 2007 (240)	CD45 (-) and EpCAM (+), then AFP mRNA	44 HCC 7 healthy subjects	AFP (20 ng/mL)	AFP mRNA (sensitivity): 72.7%; 50% in patients with AFP <20 ng/mL and 86.7% in patients with AFP >1000 ng/mL (p<0.05)
Xu et al, 2011 (241)	ASGPR (+)	85 HCC 37 CLD or benign liver diseases 20 healthy subjects	AFP (cut-off 20 or 100 ng/mL)	CTCs (sensitivity/specificity): 81%/100% No significant differences in CTCs level according to AFP values
Liu et al, 2013 (222)	CD45 (-) and ICAM-1 (+)	60 HCC	AFP (cut-off 20 ng/mL)	High levels of CTCs in 83.3% of AFP + and 16.7% of AFP negative patients (p=0.14)
Sun et al, 2013 (216)	CellSearch™	123 HCC 5 CLD 10 healthy subjects	AFP (cut-off 400 ng/mL)	≥2 CTCs/7.5 mL: Overall (sensitivity/specificity): 41.5%/100% High AFP: sensitivity, 54.7% Low AFP: sensitivity, 31.4% (p=0.009)
Bahnassy et al, 2014 (242)	CD45 (-) and either CK19, CD90 or CD133 (+)	70 HCC 30 CLD (HCV) 33 healthy subjects	AFP ratio (undefined)	CTCs have poorer performances compared to AFP. HCC vs. CLD: AFP ratio: 95.7%/90.5%, 0.99 CK19 (+) CTCs: 87.1%/82.5% CD90 (+) CTCs: 81%/89.6% CD133 (+) CTCs: 40%/6.3%
Fang et al, 2014 (243)	CellSearch™	42 HCC 10 CLD 10 healthy subjects	AFP (cut-off 40 ng/mL)	CTCs (sensitivity/specificity): 74%/100% Sensitivity 89% among patients with high AFP and 61% among those with low AFP (p=0.08)
Guo et al, 2014 (214)[†]	CellSearch™ and quantitative PCR for EpCAM in CD45 (-) cells	122 HCC 25 CLD or LC (HBV) 24 benign tumors 71 healthy subjects	AFP (cut-off NR)	HCC vs. other groups: EpCAM-mRNA (+) CTCs: 42.6%/96.7%, 0.70 EpCAM-mRNA (+) CTCs + AFP: 73%/93.4%, 0.86
Kelley et al, 2015 (206)	CellSearch™	20 HCC 10 CLD	AFP (400 ng/mL)	CTC detection in 7 of 20 (35%) HCC patients and 0 of 9 CLD (p=0.04). AFP≥400 ng/mL: sensitivity 70% AFP<400 ng/mL: sensitivity 10% (p=0.008)
Zhou et al, 2016 (244)	CD45 (-) EpCAM-mRNA (+)	49 HCC	AFP (cut-off 400 ng/mL)	Any CTCs (sensitivity): Overall: 34.6% Low vs. high AFP: 28.2% vs. 60% (p=0.06)
Kalinich et al, 2017 (245)	PCR assay: AFP, AHSG, ALB, APOH, FABP1, FGB, FGG, GPC3, RBP and TF	63 HCC 31 CLD 26 healthy subjects	AFP (cut-off 100 ng/mL)	PCR score +: 9 of 16 (56%) untreated HCC patients, 1 of 31 (3%) CLD and 2 of 26 (7.6%) healthy subjects. 15 patients with both PCR score and AFP available: 4 (27%) PCR score +, 1 (7%) AFP +, 5 (33%) PCR score + and AFP +. 6 patients within Milan criteria: 2 (33%) PCR score + and 0 (0%) AFP +.

Bhan et al, 2018 (246)	CD45 (-) and hydrodynamics, followed by HCC score based on gene expression	54 HCC 39 CLD 10 healthy subjects	AFP (cut-off 20 ng/mL)	HCC score outperformed AFP in identifying HCC vs. CLD (sensitivity/specificity): HCC score: 85%/95% AFP: 55%/100%
Guo et al, 2018 (212)[†]	CTC detection panel: PCR for EpCAM, CD133, CD90 and CK19	Training and validation cohorts: 395 HCC 301 CLD and LC (HBV) 210 healthy subjects	AFP (cut-off 20 ng/mL)	Validation cohort (sensitivity/specificity, AUC): HCC vs. other groups: CTC detection panel: 72.5%/95%, 0.88 AFP: 57%/90%, 0.77 CTC detection panel + AFP: 76%/95%, 0.89 Early-stage HCC vs. other groups: CTC detection panel: 71.8%/95%, 0.87 AFP: 53.4%/90%, 0.74 CTC detection panel + AFP: 80.9%/87%, 0.88 AUC in different stages: 0.92 (BCLC 0), 0.86 (BCLC A), 0.91 (BCLC B), 0.86 (BCLC C) In AFP negative patients: 77.7%/95%, 0.89
Xue et al, 2018 (247)	CellSearch™ and iFISH (either CD45 (-) CK (+) DAPI (+) and hybridization signal for CEP8 ≥2 or CD45 (-) CK (-) DAPI (+) and hybridization signal for CEP8 >2)	30 HCC 10 healthy subjects	AFP (400 IU/mL)	CTCs measured by CellSearch™ (sensitivity/specificity): 26.7%/100% CTCs measured by iFISH (sensitivity/specificity): 70/100% Low AFP: sensitivity, 90% High sensitivity, 30% (p=0.002)
Yin et al, 2018 (248)	CanPatrol™	80 HCC 10 healthy subjects	AFP (cut-off 20 ng/mL)	Overall cohort (sensitivity/specificity): Any CTCs: 77.5%/100% Twist (+) CTCs: 67.5%/100% Low AFP: sensitivity, 35.3% or 17.7% for any CTCs or Twist (+) CTCs, respectively (p<0.001) High AFP: sensitivity, 88.9% or 71.8%for any CTCs or Twist (+) CTCs, respectively (p<0.001)
Cheng et al, 2019 (213)	CanPatrol™	113 HCC 57 benign liver lesions	AFP (cut-off 400 μg/L)	CTCs outperformed and provided incremental benefit to AFP. AFP: 44.3%/89.5%, 0.67 Total CTCs (≥3): 62%/89.5%, 0.77 Total CTCs or AFP: AUC = 0.82
Prognosis				
Study	**CTCs definition**	**HCC patients**	**Stage /Treatment**	**Main findings**
Vona et al, 2004 (215)	Size (diameter>25 μm)	N = 44	Stage: 39% multinodular, 39% tumor ≤3 cm, 45% PVT, no EHS Treatment: NR	Patients with CTCs/circulating tumor microemboli had poorer OS (p=0.01) No significant association with survival in multivariate analysis.
Fan et al, 2011 (220)	CD45 (-) CD90 (+) CD44 (+)	N = 82	TNM stage I/II/III/IV: 5%/34%/34%/27%	CTCs predicted recurrence (sensitivity/specificity): 65.9%/80.5% CTCs (>0.01%) independently associated with poorer: Median recurrence-free survival (6.0 vs. >46.5 months)

			Treatment: LR	2-years recurrence-free survival (22.7% vs. 64.2%) 2-year OS (58.5% vs. 94.1%) (p<0.001 for all)
Liu et al, 2013 (222)	CD45 (-) ICAM-1 (+)	N = 60	Stage: tumor size >5 cm 72%, multifocal 12% Treatment: LR	High proportion of ICAM-1 (+) CTCs associated with: Poorer DFS: adjusted HR = 7.15 (2.99-17.05) No independent association with OS: adjusted HR = 2.28 (0.95-7.82)
Nel et al, 2013 (249)	CTCs: CD45 (-), DAPI (+), EpCAM (+), ASGPR1 (+) Mesenchymal: either N-cadherin (+) or vimentin (+) Epithelial: pan-CK (+) Mixed: CK (+) and either N-cadherin (+) or vimentin (+)	N = 11	Stage: NR Treatment: various (SIRT in 45%)	Vimentin (+)/CK (+) ratio >0.5 associated with a longer TTP: 1 vs 15 months (p=0.03) N-cadherin (+)/CK (+) ratio <0.1 associated with a shorter TTP: 1 vs 15 months (p=0.03)
Sun et al, 2013 (216)	CellSearch™	N = 123	BCLC stage 0-A/B-C: 82%/18% Treatment: LR	Presence of CTCs (>2/7.5 mL) before surgery associated with: Increased risk of recurrence: adjusted HR = 5.20 (2.65-10.21)
Cheng et al, 2013 (221)	Magnetic cell sorting and PCR for Lin28B	N = 96	BCLC stage A/B-C: 55%/45% Treatment: LR	Lin28B positive CTCs associated with: Decreased RFS: adjusted HR = 2.25 (1.01-4.99) Early recurrence (< 1 year): adjusted HR = 2.65 (1.02-6.86); also true in earlier stages
Schulze et al, 2013 (217)	CellSearch™	N = 59	BCLC stage A/B/C: 15%/53%/32% Treatment: NR	Detection of CTCs was associated with lower OS at the Kaplan-Meier analysis (p=0.02)
Guo et al, 2014 (214)	CellSearch™ and quantitative PCR for EpCAM in CD45 (-) cells	N = 299	Stage: NR Treatment: LR 53%, TACE 25%, RT 22%	EpCAM mRNA (+) CTCs associated with worse outcomes Surgery: shorter TTR; adjusted HR = 2.9 (1.6-5.3) TACE: shorter PFS; unadjusted HR = 3.8 (1.4-10) RT: shorter PFS; unadjusted HR =5.1 (1.4-18.5)
Nel et al, 2014 (250)	CD45 (-), EpCAM (+), DAPI (+), pan-CK (+) and IGFBP1 mRNA (+)	N = 25	TNM stage II/III/IV: 28%/48%/24% Treatment: SIRT	Low expression of IGFBP1 mRNA in CTCs discriminate progression from disease control (sensitivity 80%, specificity 80%, AUC = 0.8). Low IGFBP1 mRNA in CTCs correlated with shorter TTP (p=0.04)
Li et al, 2016 (234)	Density-based, CD45 (-), pan-CK (+) and either pAkt1/2/3 or pERK1/2 (+)	N = 109	Stage: advanced Treatment: sorafenib	High proportion of pERK (+) pAkt (-) CTCs associated with longer PFS: adjusted HR = 9.39 (3.24-27.19)
Ogle et al, 2016 (207)	CD45 (-), morphology, size	N = 69	BCLC stage A/B/C/D: 16%/7%/73%/4% Treatment: LT 6%, LR 4%, ABL 10%, IAT 39%, sorafenib 13%, BSC 28%	Presence of CTCs (>1/4 mL) at any time (N=69): Shorter OS: adjusted HR = 2.34 (1.015.43) Shorter TTP (p=0.006) Presence of CTCs post-treatment (N=29): Shorter OS: adjusted HR = 6.16 (1.71-22.33) Shorter TTP (p=0.002)
Zhou et al, 2016 (244)	EpCAM mRNA (+)	N = 49	BCLC stage 0-A/B-C: 90%/10% Treatment: LR	High EpCAM mRNA (+) CTCs associated with increased risk of recurrence: adjusted HR = 6.69 (1.94-22.88)

von Felden et al, 2017 (218)	CellSearch™	N = 57	BCLC stage A/B: 92%/8% Treatment: LR	CTCs status was independently associated with increased risk of recurrence: adjusted HR = 3.1 (1.0-9.4)
Guo et al, 2018 (212)	CTC detection panel: PCR for EpCAM, CD133, CD90 and CK19	N = 395	Training: BCLC stage 0-A: 66% Treatment: LR 98%, TACE 2% Validation: BCLC stage 0-A: 48% Treatment: LR 67%, TACE 33%	CTC detection panel was associated with shorter TTR: Training cohort: adjusted HR = 2.69 (1.62-4.48) Validation cohort: adjusted HR = 3.13 (1.36-7.19) Association remained significant in patients with negative AFP and with early-stage (BCLC 0-A) tumor
Qi et al, 2018 (223)	Can Patrol™	N = 112	BCLC stage 0/A/B/C: 10%/39%/21%/30% Treatment: LR	CTCs associated with HCC recurrence: CTC count: adjusted HR = 1.02 (1.01-1.04) Mesenchymal CTC percentage: adjusted HR = 1.02 (1.01-1.03) Mesenchymal > epithelial CTC percentage: adjusted HR = 1.00 (0.99-1.02) Mesenchymal = epithelial CTC percentage, mesenchymal < epithelial CTC percentage, epithelial CTC percentage not associated with recurrence at univariate analysis.
Sun et al, 2018 (238)	CellSearch™	N = 73	BCLC stage 0-A/B-C: 77%/23% Treatment: LR	Presence of CTCs in different vascular sites. Association with intrahepatic recurrence: Peripheral veins: adjusted HR = 0.77 (0.14-5.19) Peripheral arteries: adjusted HR = 2.54 (0.87-7.42) Peripheral veins CTCs with clusters: adjusted HR = 3.48 (1.40-8.61) Association with lung metastasis: Hepatic vein CTCs: adjusted HR = 0.59 (0.04-9.54) Intrahepatic inferior vena cava CTCs: adjusted HR = 0.67 (0.10-4.40) Hepatic vein CTCs with clusters: adjusted HR = 42.2 (3.73-477.8)
Wang et al, 2018 (251)	CanPatrol™	N = 62	BCLC stage 0-A/B-C: 37%/63% Treatment: LR	Association with early recurrence: Total CTCs: unadjusted HR = 2.95 (1.18-7.35); NS after adjustment Mesenchymal CTCs: unadjusted HR = 4.74 (2.04-11.01); adjusted HR = 3.45 (1.39-8.56) Mixed CTCs: unadjusted HR = 2.94 (1.31-6.59); NS after adjustment
Yu et al, 2018 (227)	CellSearch™	N = 139	BCLC stage 0+A/B+C: 40%/60% Treatment: LR	4 categories: 1) persistently (+); 2) preoperatively (+) but postoperatively (-); 3) preoperatively (-) but postoperatively (+); 4) persistently (-). For a 1-point increase in category: DFS: adjusted HR = 0.53 (0.41-0.68) OS: adjusted HR = 0.48 (0.36-0.66)
Ye et al, 2018 (252)	CanPatrol™	N = 42	BCLC stage A-B/C-D: 81%/19% Treatment: LR	Pre-operative CTC count not associated with OS and PFS Post-operative CTC count (>5): Poorer PFS: adjusted HR = 6.89 (1.64-29.0) No independent association with OS: adjusted HR = 15.65 (0.80-304.64)

				Increase of post-operative CTC count: Poorer PFS: adjusted HR = 39.58 (4.22-371.64)
Wang et al, 2018 (225)	SE-iFISH	N = 14	Stage: NR Treatment: NR	Detection of small CTCs with triploid chromosome 8 showed shorter DFS (p=0.007); HR not reported
Court et al, 2018 (253)	NanoVelcro™	N = 80	BCLC stage A/B/C/D: 18%/28%/43%/11% Treatment: ABL, TACE, SIRT, systemic therapy, BSC	Total CTCs were associated with: Shorter TTR in patients with early stage: univariate HR = 9.7 (2.08-45.19); no significant association in multivariate. Shorter PFS in patients with advanced disease: univariate HR = 2.09 (1.11-3.96); multivariate HR =2.09 (1.11-3.96) Vimentin (+) CTCs independently associated with: Poorer OS: adjusted HR = 2.21 (1.38-3.56) Poorer PFS in patients with advanced disease: adjusted HR = 2.16 (1.33-4.42) Trend toward fast TTR in patients with early stage: adjusted HR = 2.45 (0.91-6.57)
Shen et al, 2018 (254)	CellSearch™	N = 97	BCLC stage A-B/C: 56%/44% Treatment: TACE	CTC count independently predicted OS: High vs. low level group: adjusted HR = 2.82 (1.22-6.53) Intermediate vs. low group: adjusted HR = 1.30 (0.63-2.69)
Ha et al, 2019 (226)	Tapered slit platform (detection based on size and morphology)	N = 105	BCLC stage 0/A: 19%/81% Treatment: LR	Presence of pre- and post-operative CTCs not associated with recurrence. Positive ΔCTC (increase of CTC after surgery): Shorter RFS: adjusted HR = 2.28 (1.06-4.90) No associations with OS
Hamaoka et al, 2019 (255)	Glypican-3 (+)	N = 85	Stage: median tumor number 1 and median size 25 mm Treatment: LR	CTCs associated with: Higher risk of microscopic portal vein invasion: adjusted OR = 14.6 (3.3-106.0) Shorter DFS (p=0.02) Shorter OS (p=0.047)
Wu et al, 2019 (256)	CD45 (-) and abnormal chromosome 8 amplification by FISH	N = 155	BCLC stage A/B/C: 38%/14%/48% Treatment: TACE	Presence of pre-TACE CTCs associated with poorer OS: adjusted HR = 2.84 (1.41-5.73)
Chen et al, 2020 (230)	CD45 (-) and imFISH	N = 50	TNM stage I/II/III/IV: 8%/32%/58%2% Treatment: LT	CTCs detection was associated with recurrence post-LT: adjusted HR = 5.41 (1.13-25.87)
Zhou et al, 2020 (257)	Size and deformability	N = 137	BCLC stage 0-A/B-C: 57%/43% Treatment: LR	Presence of CTCs: Independently associated with microvascular invasion: adjusted HR = 1.76 (1.34-2.30) Shorter OS (19.2 months vs. not reached; p=0.005)
Winograd et al, 2020 (235)	CD45 (-), DAPI (+), CK (+), PD-L1 (+)	N = 87	BCLC stage A/B/C/D: 25%/25%/41%/8% Treatment: various; checkpoint inhibitors 14.3%	Detection of CTCs expressing PD-L1: Associated with poorer OS (≥4 PD-L1 (+) CTCs): adjusted HR = 3.22 (1.33-7.79) Predicted response to checkpoint inhibitors

Wang et al, 2020 (258)	CellSearch™	N = 344	BCLC stage 0-A/B-C: 73.8%/26.2% Treatment: LR ± adjuvant TACE	After propensity score matching, in CTC positive patients adjuvant TACE provide benefits in: TTR (45.8 vs. 9.8 months, p<0.001) OS (not reached vs. 36.4 months; p<0.001)
Wang et al, 2020 (231)	ChimeraX ®-i120 platform	N = 193	Stage: Milan-in 60% Treatment: LT	Post-operative CTC count ≥1 independently associated with tumor recurrence: adjusted HR = 2.67 (1.50-4.74)

[†] Cohort of Guo et al, 2014 (214) and Guo et al, 2018 (212) may overlap.

Abbreviations: AFP, alpha-fetoprotein; ABL, ablation; AUC, area under the curve; BCLC, Barcelona Clinic Liver Cancer; BSC, best supportive care; DC, disease control; DFS, disease-free survival; EHS, extrahepatic spread; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; IAT, intra-arterial therapies; LR, liver resection; LT, liver transplantation; OS, overall survival; OR, odds ratio; NS, not significant; NR, not reported; PFS, progression-free survival; PVT, portal vein thrombosis; RFS, recurrence-free survival; RT, radiotherapy; SIRT, selective internal radiation therapy;TACE, transarterial chemoembolization; TTP, time to progression; TTR, time to recurrence.

CONCLUSIONS AND FUTURE PERSPECTIVES

The identification of reliable non-invasive biomarkers that could allow a personalized management of HCC patients has become a key priority in the last years. Circulating markers that can integrate or eventually replace percutaneous liver biopsy, overcoming its limitations, are crucial. In addition, HCC detection at early-stages, when it is susceptible to potentially curative treatments, and prediction of response to therapy are critical to improve patient survival. Although fewer data are available for HCC compared to other malignancies, numerous recent publications demonstrated very interesting and promising results regarding liquid biopsy role in diagnosis, prognosis and prediction of response to treatment. cfDNA, cfRNA, EVs and CTCs emerged as attractive liquid biopsy candidates because they fulfil many of the major characteristics of an ideal biomarker. To date, the approach closest to the introduction in clinical practice, after the necessary large and prospective studies, is cfDNA methylation profiling for early detection of HCC in patients at risk. Mutational profiling of cfDNA and CTCs analyses are dependent on tumor burden and therefore likely more useful in intermediate or advanced settings as prognostic and predictive tools. Even tough fewer data are currently available, the analysis of EVs could provide biomarkers at every HCC stage and has the advantage to provide functional information (e.g., interactions between cancer cells and tumor microenvironment or distant cells).

Although the large amount of encouraging data collected in recent years predict a bright future for liquid biopsy in HCC, its widespread clinical application is yet not on the horizon. The majority of data supporting its utility derives from proof-of-concept studies, mainly retrospective, and not validated by different researchers. The main limitation that hinders the routine application of liquid biopsy is the lack of standardization, absence of accepted standard operating procedures and the lack of comparability between existing approaches (47). The standardization of pre-analytical, analytical and post-analytical variables should be addressed. Considering for instance cfDNA

analysis, the avoidance of white blood cells (WBC) lysis during blood collection and processing is important to prevent dilution of tumor circulating fragments with non-tumoral DNA (pre-analytical phase). Moreover, transportation, processing and storage temperature are also critical, impacting on WBC stability and cfDNA degradation. Since cfDNA has a short half-life and there are time-dependent changes of DNA in blood collection tubes (because of the degradation from DNase activity), plasma should be isolated within an hour from collection (analytical phase). Considered the relevance of these and others variables on the final results, the standardization of methodological protocols is an essential step to take in order to integrate liquid biopsy in the everyday clinical practice.

With the aim of identifying clinically useful diagnostic biomarkers, studies should include as controls only patients at risk of developing HCC (i.e., cirrhotics or high risk chronic hepatitis patients), who represent the ideal target for surveillance (5). This is not trivial, also considering that it could make more difficult the identification of specific diagnostic biomarkers. In fact, chronic hepatitis and cirrhosis are pre-cancerous conditions in which some of the molecular modifications found in overt HCC are already in place. For instance, during the progression of liver damage the pattern of DNA methylation changes over time in multiple hepatic cell types, and the release of methylated cfDNA from dying hepatocytes has been demonstrated to be a useful approach to evaluate fibrosis grade (259,260). In order to have a chance of being introduced in clinical practice, liquid biopsy biomarkers should be specific enough to distinguish early-stage HCC from simple cirrhosis, a condition in which the molecular pathways leading to cancer may be already at least in part activated. In addition, when tumor burden is low, highly sensitive tests are necessary to overcome the limitation posed by the small amount of circulating cancer by-products. Even though these new liquid biopsy strategies represent very promising tools, another not negligible consideration should be done about their costs. While currently used biomarkers (AFP) are measured with unexpensive and simple methods,

EVs isolation and analysis, cfDNA mutational profiling and epigenetic analysis, and CTCs enrichment methods require devoted personnel and are all costly and time consuming. Nevertheless, such limitations will likely be overcome by advances in technology that will make these determinations easier and accessible to most laboratories.

Once these new generation reliable biomarkers will be developed and validated, the final step will be to determine the optimal way to integrate them in the clinical management of patients with HCC. The replacement of currently used tools in the management of HCC patients by liquid biopsy biomarkers is unrealistic, but they will likely be integrated in the process providing a stronger predictive power. An interesting approach in surveillance, which remains to be evaluated in ad hoc studies, could be the combined evaluation of liquid biopsy biomarkers with the currently used periodic liver ultrasonography. Given the possibility of minimally invasive repeated sampling, liquid biopsies can enable real-time monitoring of disease during therapy and could supplement imaging informations to provide a more careful assessment of the tumor. Hopefully, in the future, the analysis of circulating HCC by-products will also allow personalized molecular targeted therapy. In order to achieve these important goals, not only prospective observational trials should be conducted, to correlate liquid biopsy biomarkers with clinical outcome, but also interventional studies, in which cfDNA, EVs and CTCs analysis will prompt therapeutic decisions, are necessary.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. 2018;68:394–424.
2. Villanueva A. Hepatocellular Carcinoma. N. Engl. J. Med. 2019;380:1450–1462.
3. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, et al. Hepatocellular carcinoma. Nat. Rev. Dis. Prim. 2016;2.
4. Pelizzaro F, Vitale A, Sartori A, Vieno A, Penzo B, Russo FP, et al. Surveillance as determinant of long-term survival in non-transplanted hepatocellular carcinoma patients. Cancers (Basel). 2021;13:1–16.
5. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.
6. Russo FP, Imondi A, Lynch EN, Farinati F. When and how should we perform a biopsy for HCC in patients with liver cirrhosis in 2018? A review. Dig. Liver Dis. 2018;50:640–646.
7. Torbenson M, Schirmacher P. Liver cancer biopsy - back to the future?! Hepatology. 2015;61:431–433.
8. Jamal-Hanjani M, Quezada SA, Larkin J, Swanton C. Translational implications of tumor heterogeneity. Clin. Cancer Res. 2015;21:1258–1266.
9. McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. Cancer Cell. 2015;27:15–26.
10. Yoshida S, Kornek M, Ikenaga N, Schmelzle M, Masuzaki R, Csizmadia E, et al. Sublethal heat treatment promotes epithelial-mesenchymal transition and enhances the malignant potential of hepatocellular carcinoma. Hepatology. 2013;58:1667–1680.
11. Friemel J, Rechsteiner M, Frick L, Böhm F, Struckmann K, Egger M, et al. Intratumor heterogeneity in hepatocellular carcinoma. Clin. Cancer Res. 2015;21:1951–1961.
12. Trevisani F, D'Intino PE, Morselli-Labate AM, Mazzella G, Accogli E, Caraceni P, et al. Serum α-fetoprotein for diagnosis of hepatocellular carcinoma in patients with chronic liver disease: Influence of HBsAg and anti-HCV status. J. Hepatol. 2001;34:570–575.
13. Singal A, Volk ML, Waljee A, Salgia R, Higgins P, Rogers MAM, et al. Meta-analysis: Surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment. Pharmacol. Ther. 2009;30:37–47.
14. Sherman M. Serological Surveillance for hepatocellular carcinoma: Time to quit. J. Hepatol. 2010;52:614–615.
15. Farinati F, Marino D, De Giorgio M, Baldan A, Cantarini M, Cursaro C, et al. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? Am. J. Gastroenterol. 2006;101:524–532.
16. Galle PR, Foerster F, Kudo M, Chan SL, Llovet JM, Qin S, et al. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. Liver Int. 2019;39:2214–2229.
17. Marrero JA, Feng Z, Wang Y, Nguyen MH, Befeler AS, Roberts LR, et al. α-Fetoprotein, Des-γ Carboxyprothrombin, and Lectin-Bound α-Fetoprotein in Early Hepatocellular Carcinoma. Gastroenterology. 2009;137:110–118.
18. Nault JC, Guyot E, Laguillier C, Chevret S, Ganne-Carrie N, N'Kontchou G, et al. Serum proteoglycans as prognostic biomarkers of hepatocellular carcinoma in patients with alcoholic cirrhosis. Cancer Epidemiol. Biomarkers Prev. 2013;22:1343–1352.
19. Shang S, Plymoth A, Ge S, Feng Z, Rosen HR, Sangrajrang S, et al. Identification of osteopontin as a novel marker for early hepatocellular carcinoma. Hepatology. 2012;55:483–490.
20. Mao Y, Yang H, Xu H, Lu X, Sang X, Du S, et al. Golgi protein 73 (GOLPH2) is a valuable serum marker for hepatocellular carcinoma. Gut. 2010;59:1687–1693.
21. Giannelli G, Marinosci F, Trerotoli P, Volpe A, Quaranta M, Dentico P, et al. SCCA antigen combined with alpha-fetoprotein as serologic markers of HCC. Int. J. Cancer. 2005;117:506–509.
22. Pozzan C, Cardin R, Piciocchi M, Cazzagon N, Maddalo G, Vanin V, et al. Diagnostic and prognostic role of SCCA-

IgM serum levels in hepatocellular carcinoma (HCC). J. Gastroenterol. Hepatol. 2014;29:1637–1644.

23. Pelizzaro F, Soldà F, Cardin R, Imondi A, Sartori A, Penzo B, et al. SCCA-IgM in hepatocellular carcinoma patients treated with transarterial chemoembolization: Gender-related differences. Biomark. Med. 2020;14:855–867.

24. Labgaa I, Villanueva A, Dormond O, Demartines N, Melloul E. The role of liquid biopsy in hepatocellular carcinoma prognostication. Cancers (Basel). 2021;13:1–17.

25. Mocan T, Simão AL, Castro RE, Rodrigues CMP, Słomka A, Wang B, et al. Liquid Biopsies in Hepatocellular Carcinoma: Are We Winning? J. Clin. Med. 2020;9:1541.

26. Chan SL, Wong AM, Lee K, Wong N, Chan AKC. Personalized therapy for hepatocellular carcinoma: Where are we now? Cancer Treat. Rev. 2016;45:77–86.

27. Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer. 1983;51:2116–2120.

28. Anker P, Lefort F, Vasioukhin V, Lyautey J, Lederrey C, Xu Qi Chen, et al. K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology. 1997;112:1114–1120.

29. Theodor L, Melzer E, Sologov M, Idelman G, Friedman E, Bar-Meir S. Detection of pancreatic carcinoma: Diagnostic value of K-ras mutations in circulating DNA from serum. Dig. Dis. Sci. 1999;44:2014–2019.

30. Castells A, Puig P, Móra J, Boadas J, Boix L, Urgell E, et al. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: Diagnostic utility and prognostic significance. J. Clin. Oncol. 1999;17:578–584.

31. Iizuka N, Sakaida I, Moribe T, Fujita N, Miura T, Stark M, et al. Elevated levels of circulating cell-free DNA in the blood of patients with hepatitis C virus-associated hepatocellular carcinoma. Anticancer Res. 2006;26:4713–4719.

32. Huang Z, Hua D, Hu Y, Cheng Z, Zhou X, Xie Q, et al. Quantitation of plasma circulating DNA using quantitative PCR for the detection of hepatocellular carcinoma. Pathol. Oncol. Res. 2012;18:271–276.

33. Chen K, Zhang H, Zhang LN, Ju SQ, Qi J, Huang DF, et al. Value of circulating cell-free DNA in diagnosis of hepatocelluar carcinoma. World J. Gastroenterol. 2013;19:3143–3149.

34. Piciocchi M, Cardin R, Vitale A, Vanin V, Giacomin A, Pozzan C, et al. Circulating free DNA in the progression of liver damage to hepatocellular carcinoma. Hepatol. Int. 2013;7:1050–1057.

35. Ren N, Qin LX, Tu H, Liu YK, Zhang BH, Tang ZY. The prognostic value of circulating plasma DNA level and its allelic imbalance on chromosome 8p in patients with hepatocellular carcinoma. J. Cancer Res. Clin. Oncol. 2006;132:399–407.

36. El-Shazly SF, Eid MA, El-Sourogy HA, Attia GF, Ezzat SA. Evaluation of serum DNA integrity as a screening and prognostic tool in patients with hepatitis C virus-related hepatocellular carcinoma. Int. J. Biol. Markers. 2010;25:79–86.

37. Marchio A, Amougou Atsama M, Béré A, Komas NP, Noah Noah D, Atangana PJA, et al. Droplet digital PCR detects high rate of TP53 R249S mutants in cell-free DNA of middle African patients with hepatocellular carcinoma. Clin. Exp. Med. 2018;18:421–431.

38. Yan L, Chen Y, Zhou J, Zhao H, Zhang H, Wang G. Diagnostic value of circulating cell-free DNA levels for hepatocellular carcinoma. Int. J. Infect. Dis. 2018;67:92–97.

39. Jiang P, Sun K, Tong YK, Cheng SH, Cheng THT, Heung MMS, et al. Preferred end coordinates and somatic variants as signatures of circulating tumor DNA associated with hepatocellular carcinoma. Proc. Natl. Acad. Sci. U. S. A. 2018;115:E10925–E10933.

40. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001;61:1659–1665.

41. Jiang P, Chan CWM, Chan KCA, Cheng SH, Wong J, Wong VWS, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc. Natl. Acad. Sci. U. S. A. 2015;112:E1317–E1325.

42. Huang A, Zhang X, Zhou S-L, Cao Y, Huang X-W, Fan J, et al. Plasma Circulating Cell-free DNA Integrity as a Promising Biomarker for Diagnosis and Surveillance in Patients with Hepatocellular Carcinoma. J. Cancer.

2016;7:1798–1803.

43. Tokuhisa Y, Iizuka N, Sakaida I, Moribe T, Fujita N, Miura T, et al. Circulating cell-free DNA as a predictive marker for distant metastasis of hepatitis C virus-related hepatocellular carcinoma. Br. J. Cancer. 2007;97:1399–1403.

44. Oh CR, Kong SY, Im HS, Kim HJ, Kim MK, Yoon KA, et al. Genome-wide copy number alteration and VEGFA amplification of circulating cell-free DNA as a biomarker in advanced hepatocellular carcinoma patients treated with Sorafenib. BMC Cancer. 2019;19.

45. Ono A, Fujimoto A, Yamamoto Y, Akamatsu S, Hiraga N, Imamura M, et al. Circulating Tumor DNA Analysis for Liver Cancers and Its Usefulness as a Liquid Biopsy. CMGH. 2015;1:516–534.

46. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer-A survey. Biochim. Biophys. Acta - Rev. Cancer. 2007;1775:181–232.

47. Siravegna G, Mussolin B, Venesio T, Marsoni S, Seoane J, Dive C, et al. How liquid biopsies can change clinical practice in oncology. Ann. Oncol. 2019;30:1580–1590.

48. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an in Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. Cell. 2016;164:57–68.

49. Nault JC, Villanueva A. Biomarkers for Hepatobiliary Cancers. Hepatology. 2021;73:115–127.

50. Ally A, Balasundaram M, Carlsen R, Chuah E, Clarke A, Dhalla N, et al. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. Cell. 2017;169:1327-1341.e23.

51. Labgaa I, Villacorta-Martin C, D'avola D, Craig AJ, Von Felden J, Martins-Filho SN, et al. A pilot study of ultra-deep targeted sequencing of plasma DNA identifies driver mutations in hepatocellular carcinoma. Oncogene. 2018;37:3740–3752.

52. Howell J, Atkinson SR, Pinato DJ, Knapp S, Ward C, Minisini R, et al. Identification of mutations in circulating cell-free tumour DNA as a biomarker in hepatocellular carcinoma. Eur. J. Cancer. 2019;116:56–66.

53. Ng CKY, Di Costanzo GG, Tosti N, Paradiso V, Coto-Llerena M, Roscigno G, et al. Genetic profiling using plasma-derived cell-free DNA in therapy-naive hepatocellular carcinoma patients: A pilot study. Ann. Oncol. 2018;29:1286–1291.

54. Huang A, Zhang X, Zhou SL, Cao Y, Huang XW, Fan J, et al. Detecting circulating tumor DNA in hepatocellular carcinoma patients using droplet digital PCR is feasible and reflects intratumoral heterogeneity. J. Cancer. 2016;7:1907–1914.

55. Kaseb AO, Sanchez NS, Sen S, Kelley RK, Tan B, Bocobo AG, et al. Molecular profiling of hepatocellular carcinoma using circulating cell-free DNA. Clin. Cancer Res. 2019;25:6107–6118.

56. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science (80-.). 2018;359:926–930.

57. Igetei R, Otegbayo JA, Ndububa DA, Lesi OA, Anumudu CI, Hainaut P, et al. Detection of p53 codon 249 mutation in Nigerian patients with hepatocellular carcinoma using a novel evaluation of cell-free DNA. Ann. Hepatol. 2008;7:339–344.

58. Liao W, Yang H, Xu H, Wang Y, Ge P, Ren J, et al. Noninvasive detection of tumor-associated mutations from circulating cell-free DNA in hepatocellular carcinoma patients by targeted deep sequencing. Oncotarget. 2016;7:40481–40490.

59. Xiong Y, Xie CR, Zhang S, Chen J, Yin ZY. Detection of a novel panel of somatic mutations in plasma cell-free DNA and its diagnostic value in hepatocellular carcinoma. Cancer Manag. Res. 2019;11:5745–5756.

60. Qu C, Wang Y, Wang P, Chen K, Wang M, Zeng H, et al. Detection of early-stage hepatocellular carcinoma in asymptomatic HBsAg-seropositive individuals by liquid biopsy. Proc. Natl. Acad. Sci. U. S. A. 2019;116:6308–6312.

61. Shen T, Li SF, Wang JL, Zhang T, Zhang S, Chen HT, et al. TP53 R249S mutation detected in circulating tumour DNA is associated with Prognosis of hepatocellular carcinoma patients with or without hepatectomy. Liver Int. 2020;40:2834–2847.

62. Piciocchi M, Cardin R, Cillo U, Vitale A, Cappon A, Mescoli C, et al. Differential timing of oxidative DNA damage and telomere shortening in hepatitis C and B virus–related liver carcinogenesis. Transl. Res. 2016;168:122–133.

63. Jiao J, Watt GP, Stevenson HL, Calderone TL, Fisher-Hoch SP, Ye Y, et al. Telomerase reverse transcriptase mutations in plasma DNA in patients with hepatocellular carcinoma or cirrhosis: Prevalence and risk factors. Hepatol. Commun. 2018;2:718–731.

64. Oversoe SK, Clement MS, Pedersen MH, Weber B, Aagaard NK, Villadsen GE, et al. TERT promoter mutated circulating tumor DNA as a biomarker for prognosis in hepatocellular carcinoma. Scand. J. Gastroenterol. 2020;55:1433–1440.

65. Hirai M, Kinugasa H, Nouso K, Yamamoto S, Terasawa H, Onishi Y, et al. Prediction of the prognosis of advanced hepatocellular carcinoma by TERT promoter mutations in circulating tumor DNA. J. Gastroenterol. Hepatol. 2020;

66. Mann J, Reeves HL, Feldstein AE. Liquid biopsy for liver diseases. Gut. 2018;67.

67. Ng CKY, Di Costanzo GG, Terracciano LM, Piscuoglio S. Circulating cell-free DNA in hepatocellular carcinoma: Current insights and outlook. Front. Med. 2018;5.

68. Pezzuto F, Buonaguro L, Buonaguro FM, Tornesello ML. The role of circulating free DNA and microRNA in non-invasive diagnosis of HBV- and HCV-related hepatocellular carcinoma. Int. J. Mol. Sci. 2018;19.

69. Huang W, Li T, Yang W, Chai X, Chen K, Wei L, et al. Analysis of DNA methylation in plasma for monitoring hepatocarcinogenesis. Genet. Test. Mol. Biomarkers. 2015;19:295–302.

70. Dong X, Hou Q, Chen Y, Wang X. Diagnostic Value of the Methylation of Multiple Gene Promoters in Serum in Hepatitis B Virus-Related Hepatocellular Carcinoma. Dis. Markers. 2017;2017.

71. Liu ZJ, Huang Y, Wei L, He JY, Liu QY, Yu XQ, et al. Combination of LINE-1 hypomethylation and RASSF1A promoter hypermethylation in serum DNA is a non-invasion prognostic biomarker for early recurrence of hepatocellular carcinoma after curative resection. Neoplasma. 2017;64:795–802.

72. Liu M, Cui LH, Li CC, Zhang L. Association of APC, GSTP1 and SOCS1 promoter methylation with the risk of hepatocellular carcinoma: A meta-analysis. Eur. J. Cancer Prev. 2015;24:470–483.

73. Wei L, Huang Y, Zhao R, Zhang J, Liu Q, Liang W, et al. Detection of promoter methylation status of suppressor of cytokine signaling 3 (SOCS3) in tissue and plasma from Chinese patients with different hepatic diseases. Clin. Exp. Med. 2018;18:79–87.

74. Wen L, Li J, Guo H, Liu X, Zheng S, Zhang D, et al. Genome-scale detection of hypermethylated CpG islands in circulating cell-free DNA of hepatocellular carcinoma patients. Cell Res. 2015;25:1250–1264.

75. Lu CY, Chen SY, Peng HL, Kan PY, Chang WC, Yen CJ. Cell-free methylation markers with diagnostic and prognostic potential in hepatocellular carcinoma. Oncotarget. 2017;8:6406–6418.

76. Kisiel JB, Dukek BA, V.S.R. Kanipakam R, Ghoz HM, Yab TC, Berger CK, et al. Hepatocellular Carcinoma Detection by Plasma Methylated DNA: Discovery, Phase I Pilot, and Phase II Clinical Validation. Hepatology. 2019;69:1180–1192.

77. Cai J, Chen L, Zhang Z, Zhang X, Lu X, Liu W, et al. Genome-wide mapping of 5-hydroxymethylcytosines in circulating cell-free DNA as a non-invasive approach for early detection of hepatocellular carcinoma. Gut. 2019;68:2195–2205.

78. Tangkijvanich P, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, Mutirangura A. Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. Clin. Chim. Acta. 2007;379:127–133.

79. Yeh CC, Goyal A, Shen J, Wu H chen, Strauss JA, Wang Q, et al. Global Level of Plasma DNA Methylation is Associated with Overall Survival in Patients with Hepatocellular Carcinoma. Ann. Surg. Oncol. 2017;24:3788–3795.

80. Xu RH, Wei W, Krawczyk M, Wang W, Luo H, Flagg K, et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. Nat. Mater. 2017;16:1155–1162.

81. Ramachandran S, Henikoff S. Nucleosome dynamics during chromatin remodeling in vivo. Nucleus. 2016;7:20–26.

82. Holdenrieder S, Stieber P. Clinical use of circulating nucleosomes. Crit. Rev. Clin. Lab. Sci. 2009;46:1–24.

83. Bauden M, Pamart D, Ansari D, Herzog M, Eccleston M, Micallef J, et al. Circulating nucleosomes as epigenetic

biomarkers in pancreatic cancer. Clin. Epigenetics. 2015;7.

84. Holdenrieder S, Stieber P, Von Pawel J, Raith H, Nagel D, Feldmann K, et al. Circulating nucleosomes predict the response to chemotherapy in patients with advanced non-small cell lung cancer. Clin. Cancer Res. 2004;10:5981–5987.

85. Rahier JF, Druez A, Faugeras L, Martinet JP, Géhénot M, Josseaux E, et al. Circulating nucleosomes as new blood-based biomarkers for detection of colorectal cancer. Clin. Epigenetics. 2017;9.

86. Roth C, Pantel K, Müller V, Rack B, Kasimir-Bauer S, Janni W, et al. Apoptosis-related deregulation of proteolytic activities and high serum levels of circulating nucleosomes and DNA in blood correlate with breast cancer progression. BMC Cancer. 2011;11.

87. Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, et al. Extracellular histones are major mediators of death in sepsis. Nat. Med. 2009;15:1318–1321.

88. Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. Hepatology. 2011;54:999–1008.

89. Giallongo S, Re O Lo, Vinciguerra M. Macro histone variants: Emerging rheostats of gastrointestinal cancers. Cancers (Basel). 2019;11.

90. Lo Re O, Douet J, Buschbeck M, Fusilli C, Pazienza V, Panebianco C, et al. Histone variant macroH2A1 rewires carbohydrate and lipid metabolism of hepatocellular carcinoma cells towards cancer stem cells. Epigenetics. 2018;13:829–845.

91. Lo Re O, Maugeri A, Hruskova J, Jakubik J, Kucera J, Bienertova-Vasku J, et al. Obesity-induced nucleosome release predicts poor cardio-metabolic health. Clin. Epigenetics. 2019;12.

92. Buzova D, Maugeri A, Liguori A, Napodano C, Lo Re O, Oben J, et al. Circulating histone signature of human lean metabolic-associated fatty liver disease (MAFLD). Clin. Epigenetics. 2020;12.

93. Zhang YJ, Wu HC, Shen J, Ahsan H, Wei YT, Yang HI, et al. Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. Clin. Cancer Res. 2007;13:2378–2384.

94. Xu H, Zhu X, Xu Z, Hu Y, Bo S, Xing T, et al. Non-invasive analysis of genomic copy number variation in patients with hepatocellular carcinoma by next generation DNA sequencing. J. Cancer. 2015;6:247–253.

95. An Y, Guan Y, Xu Y, Han Y, Wu C, Bao C, et al. The diagnostic and prognostic usage of circulating tumor DNA in operable hepatocellular carcinoma. Am. J. Transl. Res. 2019;11:6462–6474.

96. Cai Z, Chen G, Zeng Y, Dong X, Li Z, Huang Y, et al. Comprehensive liquid profiling of circulating tumor DNA and protein biomarkers in long-term follow-up patients with hepatocellular carcinoma. Clin. Cancer Res. 2019;25:5284–5294.

97. Chu HJ, Heo J, Seo SB, Kim GH, Kang DH, Song GA, et al. Detection of Aberrant p16INK4A Methylation in Sera of Patients with Liver Cirrhosis and Hepatocellular Carcinoma. J. Korean Med. Sci. 2004;19:83–86.

98. Yeo W, Wong N, Wong WL, Lai PBS, Zhong S, Johnson PJ. High frequency of promoter hypermethylation of RASSF1A in tumor and plasma of patients with hepatocellular carcinoma. Liver Int. 2005;25:266–272.

99. Chan KCA, Lai PBS, Mok TSK, Chan HLY, Ding C, Yeung SW, et al. Quantitative analysis of circulating methylated DNA as a biomarker for hepatocellular carcinoma. Clin. Chem. 2008;54:1528–1536.

100. Iizuka N, Oka M, Sakaida I, Moribe T, Miura T, Kimura N, et al. Efficient detection of hepatocellular carcinoma by a hybrid blood test of epigenetic and classical protein markers. Clin. Chim. Acta. 2011;412:152–158.

101. Sun FK, Fan YC, Zhao J, Zhang F, Gao S, Zhao ZH, et al. Detection of TFPI2 methylation in the serum of hepatocellular carcinoma patients. Dig. Dis. Sci. 2013;58:1010–1015.

102. Han LY, Fan YC, Mu NN, Gao S, Li F, Ji XF, et al. Aberrant DNA methylation of G-protein-coupled bile acid receptor gpbar1 (TGR5) is a potential biomarker for hepatitis B virus associated hepatocellular carcinoma. Int. J. Med. Sci. 2014;11:164–171.

103. Huang G, Krocker JD, Kirk JL, Merwat SN, Ju H, Soloway RD, et al. Evaluation of INK4A promoter methylation using pyrosequencing and circulating cell-free DNA from patients with hepatocellular carcinoma. Clin. Chem. Lab. Med. 2014;52:899–909.

104. Ji XF, Fan YC, Gao S, Yang Y, Zhang JJ, Wang K. MT1M and MT1G promoter methylation as biomarkers for

hepatocellular carcinoma. World J. Gastroenterol. 2014;20:4723–4729.

105. Kuo CC, Lin CY, Shih YL, Hsieh CB, Lin PY, Guan SB, et al. Frequent methylation of HOXA9 gene in tumor tissues and plasma samples from human hepatocellular carcinomas. Clin. Chem. Lab. Med. 2014;52:1235–1245.

106. Li F, Fan YC, Gao S, Sun FK, Yang Y, Wang K. Methylation of serum insulin-like growth factor-binding protein 7 promoter in hepatitis B virus-associated hepatocellular carcinoma. Genes Chromosom. Cancer. 2014;53:90–97.

107. Kanekiyo S, Iizuka N, Tsunedomi R, Tokumitsu Y, Hashimoto N, Tokuhisa Y, et al. Preoperative serum methylation signature as prognostic tool after curative hepatectomy in patients with hepatocellular carcinoma. Anticancer Res. 2015;35:997–1007.

108. Dou CY, Fan YC, Cao CJ, Yang Y, Wang K. Sera DNA Methylation of CDH1, DNMT3b and ESR1 Promoters as Biomarker for the Early Diagnosis of Hepatitis B Virus-Related Hepatocellular Carcinoma. Dig. Dis. Sci. 2016;61:1130–1138.

109. Hu N, Fan XP, Fan YC, Chen LY, Qiao CY, Han LY, et al. Hypomethylated ubiquitin-conjugating enzyme2 Q1 (UBE2Q1) gene promoter in the serum is a promising biomarker for hepatitis B virus-associated hepatocellular carcinoma. Tohoku J. Exp. Med. 2017;242:93–100.

110. Oussalah A, Rischer S, Bensenane M, Conroy G, Filhine-Tresarrieu P, Debard R, et al. Plasma mSEPT9: A Novel Circulating Cell-free DNA-Based Epigenetic Biomarker to Diagnose Hepatocellular Carcinoma. EBioMedicine. 2018;30:138–147.

111. Park S, Lee EJ, Rim CH, Seong J. Plasma cell-free DNA as a predictive marker after radiotherapy for hepatocellular carcinoma. Yonsei Med. J. 2018;59:470–479.

112. Kim SS, Eun JW, Choi JH, Woo HG, Cho HJ, Ahn HR, et al. MLH1 single-nucleotide variant in circulating tumor DNA predicts overall survival of patients with hepatocellular carcinoma. Sci. Rep. 2020;10.

113. von Felden J, Craig AJ, Garcia-Lezana T, Labgaa I, Haber PK, D'Avola D, et al. Mutations in circulating tumor DNA predict primary resistance to systemic therapies in advanced hepatocellular carcinoma. Oncogene. 2021;40:140–151.

114. Huang ZH, Hu Y, Hua D, Wu YY, Song MX, Cheng ZH. Quantitative analysis of multiple methylated genes in plasma for the diagnosis and prognosis of hepatocellular carcinoma. Exp. Mol. Pathol. 2011;91:702–707.

115. Li F, Qiao CY, Gao S, Fan YC, Chen LY, Wang K. Circulating cell-free DNA of methylated insulin-like growth factor-binding protein 7 predicts a poor prognosis in hepatitis B virus-associated hepatocellular carcinoma after hepatectomy. Free Radic. Res. 2018;52:455–464.

116. Chen MM, Zhao RC, Chen KF, Huang Y, Liu ZJ, Wei YG, et al. Hypomethylation of CTCFL promoters as a noninvasive biomarker in plasma from patients with hepatocellular carcinoma. Neoplasma. 2020;67:909–915.

117. Du Y, Kong G, You X, Zhang S, Zhang T, Gao Y, et al. Elevation of highly up-regulated in liver cancer (HULC) by hepatitis B virus X protein promotes hepatoma cell proliferation via down-regulating p18. J. Biol. Chem. 2012;287:26302–26311.

118. Wang J, Liu X, Wu H, Ni P, Gu Z, Qiao Y, et al. CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. Nucleic Acids Res. 2010;38:5366–5383.

119. Xie H, Ma H, Zhou D. Plasma HULC as a promising novel biomarker for the detection of hepatocellular carcinoma. Biomed Res. Int. 2013;2013.

120. Braconi C, Kogure T, Valeri N, Huang N, Nuovo G, Costinean S, et al. MicroRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer. Oncogene. 2011;30:4750–4756.

121. Anwar SL, Krech T, Hasemeier B, Schipper E, Schweitzer N, Vogel A, et al. Loss of Imprinting and Allelic Switching at the DLK1-MEG3 Locus in Human Hepatocellular Carcinoma. PLoS One. 2012;7.

122. Yang Z, Zhou L, Wu LM, Lai MC, Xie HY, Zhang F, et al. Overexpression of long non-coding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation. Ann. Surg. Oncol. 2011;18:1243–1250.

123. Geng YJ, Xie SL, Li Q, Ma J, Wang GY. Large intervening non-coding RNA HOTAIR is associated with hepatocellular carcinoma progression. J. Int. Med. Res. 2011;39:2119–2128.

124. Ishibashi M, Kogo R, Shibata K, Sawada G, Takahashi Y, Kurashige J, et al. Clinical significance of the expression

of long non-coding RNA HOTAIR in primary hepatocellular carcinoma. Oncol. Rep. 2013;29:946–950.

125. Quagliata L, Matter MS, Piscuoglio S, Arabi L, Ruiz C, Procino A, et al. Long noncoding RNA HOTTIP/HOXA13 expression is associated with disease progression and predicts outcome in hepatocellular carcinoma patients. Hepatology. 2014;59:911–923.

126. Lai MC, Yang Z, Zhou L, Zhu QQ, Xie HY, Zhang F, et al. Long non-coding RNA MALAT-1 overexpression predicts tumor recurrence of hepatocellular carcinoma after liver transplantation. Med. Oncol. 2012;29:1810–1816.

127. Lin R, Maeda S, Liu C, Karin M, Edgington TS. A large noncoding RNA is a marker for murine hepatocellular carcinomas and a spectrum of human carcinomas. Oncogene. 2007;26:851–858.

128. Yuan SX, Yang F, Yang Y, Tao QF, Zhang J, Huang G, et al. Long noncoding RNA associated with microvascular invasion in hepatocellular carcinoma promotes angiogenesis and serves as a predictor for hepatocellular carcinoma patients' poor recurrence-free survival after hepatectomy. Hepatology. 2012;56:2231–2241.

129. El-Tawdi AHF, Matboli M, Shehata HH, Tash F, El-Khazragy N, Azazy AESM, et al. Evaluation of Circulatory RNA-Based Biomarker Panel in Hepatocellular Carcinoma. Mol. Diagnosis Ther. 2016;20:265–277.

130. Yuan W, Sun Y, Liu L, Zhou B, Wang S, Gu D. Circulating LncRNAs Serve as Diagnostic Markers for Hepatocellular Carcinoma. Cell. Physiol. Biochem. 2017;44:125–132.

131. Ding Y, Yan J-L, Fang A-N, Zhou W-F, Huang L. Circulating miRNAs as novel diagnostic biomarkers in hepatocellular carcinoma detection: a meta-analysis based on 24 articles. Oncotarget. 2017;8:66402–66413.

132. Tomimaru Y, Eguchi H, Nagano H, Wada H, Kobayashi S, Marubashi S, et al. Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J. Hepatol. 2012;56:167–175.

133. Lin XJ, Chong Y, Guo ZW, Xie C, Yang XJ, Zhang Q, et al. A serum microRNA classifier for early detection of hepatocellular carcinoma: A multicentre, retrospective, longitudinal biomarker identification study with a nested case-control study. Lancet Oncol. 2015;16:804–815.

134. Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, et al. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J. Clin. Oncol. 2011;29:4781–4788.

135. Yamamoto Y, Kondo S, Matsuzaki J, Esaki M, Okusaka T, Shimada K, et al. Highly Sensitive Circulating MicroRNA Panel for Accurate Detection of Hepatocellular Carcinoma in Patients With Liver Disease. Hepatol. Commun. 2020;4:284–297.

136. Köberle V, Kronenberger B, Pleli T, Trojan J, Imelmann E, Peveling-Oberhag J, et al. Serum microRNA-1 and microRNA-122 are prognostic markers in patients with hepatocellular carcinoma. Eur. J. Cancer. 2013;49:3442–3449.

137. Xu Y, Bu X, Dai C, Shang C. High serum microRNA-122 level is independently associated with higher overall survival rate in hepatocellular carcinoma patients. Tumor Biol. 2015;36:4773–4776.

138. Cho HJ, Kim SS, Nam JS, Kim JK, Lee JH, Kim B, et al. Low levels of circulating microRNA-26a/29a as poor prognostic markers in patients with hepatocellular carcinoma who underwent curative treatment. Clin. Res. Hepatol. Gastroenterol. 2017;41:181–189.

139. Ning S, Liu H, Gao B, Wei W, Yang A, Li J, et al. MiR-155, miR-96 and miR-99a as potential diagnostic and prognostic tools for the clinical management of hepatocellular carcinoma. Oncol. Lett. 2019;18:3381–3387.

140. Loosen SH, Wirtz TH, Roy S, Vucur M, Castoldi M, Schneider AT, et al. Circulating levels of microRNA193a-5p predict outcome in early stage hepatocellular carcinoma. PLoS One. 2020;15.

141. Pratedrat P, Chuaypen N, Nimsamer P, Payungporn S, Pinjaroen N, Sirichindakul B, et al. Diagnostic and prognostic roles of circulating miRNA-223-3p in hepatitis B virus–related hepatocellular carcinoma. PLoS One. 2020;15.

142. Jin Y, Wong YS, Goh BKP, Chan CY, Cheow PC, Chow PKH, et al. Circulating microRNAs as Potential Diagnostic and Prognostic Biomarkers in Hepatocellular Carcinoma. Sci. Rep. 2019;9.

143. Okajima W, Komatsu S, Ichikawa D, Miyamae M, Kawaguchi T, Hirajima S, et al. Circulating microRNA profiles in plasma: Identification of miR-224 as a novel diagnostic biomarker in hepatocellular carcinoma independent of hepatic function. Oncotarget. 2016;7:53820–53836.

144. Yamamoto Y, Kosaka N, Tanaka M, Koizumi F, Kanai Y, Mizutani T, et al. MicroRNA-500 as a potential diagnostic

marker for hepatocellular carcinoma. Biomarkers. 2009;14:529–538.

145. Han J, Li J, Qian Y, Liu W, Liang J, Huang Z, et al. Identification of plasma miR-148a as a noninvasive biomarker for hepatocellular carcinoma. Clin. Res. Hepatol. Gastroenterol. 2019;43:585–593.

146. Chuma M, Toyoda H, Matsuzaki J, Saito Y, Kumada T, Tada T, et al. Circulating microRNA-1246 as a possible biomarker for early tumor recurrence of hepatocellular carcinoma. Hepatol. Res. 2019;49:810–822.

147. Cho HJ, Kim JK, Nam JS, Wang HJ, Lee JH, Kim BW, et al. High circulating microRNA-122 expression is a poor prognostic marker in patients with hepatitis B virus-related hepatocellular carcinoma who undergo radiofrequency ablation. Clin. Biochem. 2015;48:1073–1078.

148. Ali HEA, Emam AA, Zeeneldin AA, Srour R, Tabashy R, El-Desouky ED, et al. Circulating miR-26a, miR-106b, miR-107 and miR-133b stratify hepatocellular carcinoma patients according to their response to transarterial chemoembolization. Clin. Biochem. 2019;65:45–52.

149. Kim SS, Cho HJ, Nam JS, Kim HJ, Kang DR, Won JH, et al. Plasma MicroRNA-21, 26a, and 29a-3p as Predictive Markers for Treatment Response Following Transarterial Chemoembolization in Patients with Hepatocellular Carcinoma. J. Korean Med. Sci. 2018;33:e6.

150. Fornari F, Pollutri D, Patrizi C, La Bella T, Marinelli S, Casadei Gardini A, et al. In hepatocellular carcinoma miR-221 modulates sorafenib resistance through inhibition of caspase-3–mediated apoptosis. Clin. Cancer Res. 2017;23:3953–3965.

151. Nishida N, Arizumi T, Hagiwara S, Ida H, Sakurai T, Kudo M. MicroRNAs for the Prediction of Early Response to Sorafenib Treatment in Human Hepatocellular Carcinoma. Liver cancer. 2017;6:113–125.

152. Teufel M, Seidel H, Köchert K, Meinhardt G, Finn RS, Llovet JM, et al. Biomarkers Associated With Response to Regorafenib in Patients With Hepatocellular Carcinoma. Gastroenterology. 2019;156:1731–1741.

153. Amr KS, Ezzat WM, Elhosary YA, Hegazy AE, Fahim HH, Kamel RR. The potential role of miRNAs 21 and 199-a in early diagnosis of hepatocellular carcinoma. Gene. 2016;575:66–70.

154. Zhuang C, Jiang W, Huang D, Xu L, Yang Q, Zheng L, et al. Serum miR-21, miR-26a and miR-101 as potential biomarkers of hepatocellular carcinoma. Clin. Res. Hepatol. Gastroenterol. 2016;40:386–396.

155. Zekri ARN, Youssef ASED, El-Desouky ED, Ahmed OS, Lotfy MM, Nassar AAM, et al. Serum microRNA panels as potential biomarkers for early detection of hepatocellular carcinoma on top of HCV infection. Tumor Biol. 2016;37:12273–12286.

156. Shi BM, Lu W, Ji K, Wang YF, Xiao S, Wang XY. Study on the value of serum miR-106b for the early diagnosis of hepatocellular carcinoma. World J. Gastroenterol. 2017;23:3713–3720.

157. Guo X, Lv X, Lv X, Ma Y, Chen L, Chen Y. Circulating miR-21 serves as a serum biomarker for hepatocellular carcinoma and correlated with distant metastasis. Oncotarget. 2017;8:44050–44058.

158. Zhang Y, Li T, Qiu Y, Zhang T, Guo P, Ma X, et al. Serum microRNA panel for early diagnosis of the onset of hepatocellular carcinoma. Med. (United States). 2017;96.

159. Moshiri F, Salvi A, Gramantieri L, Sangiovanni A, Guerriero P, De Petro G, et al. Circulating miR-106b-3p, miR-101-3p and miR-1246 as diagnostic biomarkers of hepatocellular carcinoma. Oncotarget. 2018;9:15350–15364.

160. An Y, Gao S, Zhao WC, Qiu BA, Xia NX, Zhang PJ, et al. Novel serum microRNAs panel on the diagnostic and prognostic implications of hepatocellular carcinoma. World J. Gastroenterol. 2018;24:2596–2604.

161. Weis A, Marquart L, Calvopina DA, Genz B, Ramm GA, Skoien R. Serum microRNAs as biomarkers in hepatitis C: Preliminary evidence of a microRNA panel for the diagnosis of hepatocellular carcinoma. Int. J. Mol. Sci. 2019;20.

162. Xu Y, Bu X, Dai C, Shang C. High serum microRNA-122 level is independently associated with higher overall survival rate in hepatocellular carcinoma patients. Tumour Biol. 2015;36:4773–4776.

163. Cho HJ, Kim JK, Nam JS, Wang HJ, Lee JH, Kim BW, et al. High circulating microRNA-122 expression is a poor prognostic marker in patients with hepatitis B virus-related hepatocellular carcinoma who undergo radiofrequency ablation. Clin. Biochem. 2015;48:1073–1078.

164. Jin Y, Wong YS, Goh BKP, Chan CY, Cheow PC, Chow PKH, et al. Circulating microRNAs as Potential Diagnostic and Prognostic Biomarkers in Hepatocellular Carcinoma. Sci. Rep. 2019;9:10464.

165. Yáñez-Mó M, Siljander PRM, Andreu Z, Zavec AB, Borràs FE, Buzas EI, et al. Biological properties of extracellular

vesicles and their physiological functions. J. Extracell. Vesicles. 2015;4:1–60.

166. Hirsova P, Ibrahim SH, Verma VK, Morton LA, Shah VH, LaRusso NF, et al. Extracellular vesicles in liver pathobiology: Small particles with big impact. Hepatology. 2016;64:2219–2233.

167. Słomka A, Urban SK, Lukacs-Kornek V, Żekanowska E, Kornek M. Large Extracellular Vesicles: Have We Found the Holy Grail of Inflammation? Front. Immunol. 2018;9:2723.

168. Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat. Cell Biol. 2019;21:9–17.

169. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 2007;9:654–659.

170. Théry C, Zitvogel L, Amigorena S. Exosomes: Composition, biogenesis and function. Nat. Rev. Immunol. 2002;2:569–579.

171. Whiteside TL. Exosomes in cancer: Another mechanism of tumor-induced immune suppression. In: Advances in Experimental Medicine and Biology. Springer New York LLC; 2017. p. 81–89.

172. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles. 2018;7.

173. Banales JM, Feldstein AE, Sänger H, Lukacs-Kornek V, Szabo G, Kornek M. Extracellular Vesicles in Liver Diseases: Meeting Report from the International Liver Congress 2018. Hepatol. Commun. 2019;3:305–315.

174. Urban SK, Mocan T, Sänger H, Lukacs-Kornek V, Kornek M. Extracellular Vesicles in Liver Diseases: Diagnostic, Prognostic, and Therapeutic Application. Semin. Liver Dis. 2019;39:70–77.

175. Cheng R, Ban L, Tu T, McCaughan G, Mclennan S, Shackel N. Utility of microvesicles as plasma biomarkers in patients with hepatocellular carcinoma. J. Gastroenterol. Hepatol. 2015;30:5.

176. Wang W, Li H, Zhou Y, Jie S. Peripheral blood microvesicles are potential biomarkers for hepatocellular carcinoma. Cancer Biomarkers. 2013;13:351–357.

177. Julich-Haertel H, Urban SK, Krawczyk M, Willms A, Jankowski K, Patkowski W, et al. Cancer-associated circulating large extracellular vesicles in cholangiocarcinoma and hepatocellular carcinoma. J. Hepatol. 2017;67:282–292.

178. Arbelaiz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Hepatology. 2017;66:1125–1143.

179. Wang X, Kwak KJ, Yang Z, Zhang A, Zhang X, Sullivan R, et al. Extracellular mRNA detected by molecular beacons in tethered lipoplex nanoparticles for diagnosis of human hepatocellular carcinoma. PLoS One. 2018;13.

180. Xu H, Dong X, Chen Y, Wang X. Serum exosomal hnRNPH1 mRNA as a novel marker for hepatocellular carcinoma. Clin. Chem. Lab. Med. 2018;56:479–484.

181. Abd El Gwad A, Matboli M, El-Tawdi A, Habib EK, Shehata H, Ibrahim D, et al. Role of exosomal competing endogenous RNA in patients with hepatocellular carcinoma. J. Cell. Biochem. 2018;119:8600–8610.

182. Xu H, Chen Y, Dong X, Wang X. Serum exosomal long noncoding RNAs ENSG00000258332.1 and LINC00635 for the diagnosis and prognosis of hepatocellular carcinoma. Cancer Epidemiol. Biomarkers Prev. 2018;27:710–716.

183. Li Y, Zhao J, Yu S, Wang Z, He X, Su Y, et al. Extracellular vesicles long RNA sequencing reveals abundant mRNA, circRNA, and lncRNA in human blood as potential biomarkers for cancer diagnosis. Clin. Chem. 2019;65:798–808.

184. Lu Y, Duan Y, Xu Q, Zhang L, Chen W, Qu Z, et al. Circulating exosome-derived bona fide long non-coding RNAs predicting the occurrence and metastasis of hepatocellular carcinoma. J. Cell. Mol. Med. 2020;24:1311–1318.

185. Pu C, Huang H, Wang Z, Zou W, Lv Y, Zhou Z, et al. Extracellular Vesicle-Associated mir-21 and mir-144 Are Markedly Elevated in Serum of Patients With Hepatocellular Carcinoma. Front. Physiol. 2018;9:930.

186. Wang Y, Zhang C, Zhang P, Guo G, Jiang T, Zhao X, et al. Serum exosomal microRNAs combined with alpha-fetoprotein as diagnostic markers of hepatocellular carcinoma. Cancer Med. 2018;7:1670–1679.

187. Zhang Y, Xi H, Nie X, Zhang P, Lan N, Lu Y, et al. Assessment of miR-212 and Other Biomarkers in the Diagnosis

and Treatment of HBV-infection-related Liver Diseases. Curr. Drug Metab. 2019;20:785–798.

188. Sorop A, Iacob R, Iacob S, Constantinescu D, Chitoiu L, Fertig TE, et al. Plasma Small Extracellular Vesicles Derived miR-21-5p and miR-92a-3p as Potential Biomarkers for Hepatocellular Carcinoma Screening. Front. Genet. 2020;11.

189. Hao X, Xin R, Dong W. Decreased serum exosomal miR-320a expression is an unfavorable prognostic factor in patients with hepatocellular carcinoma. J. Int. Med. Res. 2020;48.

190. Sugimachi K, Matsumura T, Hirata H, Uchi R, Ueda M, Ueo H, et al. Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. Br. J. Cancer. 2015;112:532–538.

191. Liu W, Hu J, Zhou K, Chen F, Wang Z, Liao B, et al. Serum exosomal miR-125b is a novel prognostic marker for hepatocellular carcinoma. Onco. Targets. Ther. 2017;10:3843–3851.

192. Qu Z, Wu J, Wu J, Ji A, Qiang G, Jiang Y, et al. Exosomal miR-665 as a novel minimally invasive biomarker for hepatocellular carcinoma diagnosis and prognosis. Oncotarget. 2017;8:80666–80678.

193. Shi M, Jiang Y, Yang L, Yan S, Wang YG, Lu XJ. Decreased levels of serum exosomal miR-638 predict poor prognosis in hepatocellular carcinoma. J. Cell. Biochem. 2018;119:4711–4716.

194. Tian XP, Wang CY, Jin XH, Li M, Wang FW, Huang WJ, et al. Acidic microenvironment up-regulates exosomal mir-21 and mir-10b in early-stage hepatocellular carcinoma to promote cancer cell proliferation and metastasis. Theranostics. 2019;9:1965–1979.

195. Luo Y, Liu F, Gui R. High expression of circulating exosomal circAKT3 is associated with higher recurrence in HCC patients undergoing surgical treatment. Surg. Oncol. 2020;33:276–281.

196. Suehiro T, Miyaaki H, Kanda Y, Shibata H, Honda T, Ozawa E, et al. Serum exosomal microRNA-122 and microRNA-21 as predictive biomarkers in transarterial chemoembolization-treated hepatocellular carcinoma patients. Oncol. Lett. 2018;16:3267–3273.

197. Lee YR, Kim G, Tak WY, Jang SY, Kweon YO, Park JG, et al. Circulating exosomal noncoding RNAs as prognostic biomarkers in human hepatocellular carcinoma. Int. J. Cancer. 2019;144:1444–1452.

198. Miller MC, Doyle G V., Terstappen LWMM. Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. J. Oncol. 2010;2010:1–8.

199. Ahn JC, Teng PC, Chen PJ, Posadas E, Tseng HR, Lu SC, et al. Detection of Circulating Tumor Cells and Their Implications as a Biomarker for Diagnosis, Prognostication, and Therapeutic Monitoring in Hepatocellular Carcinoma. Hepatology. 2021;73:422–436.

200. Ferreira MM, Ramani VC, Jeffrey SS. Circulating tumor cell technologies. Mol. Oncol. 2016;10:374–394.

201. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin. Cancer Res. 2004;10:6897–6904.

202. Negin BP, Cohen SJ. Circulating tumor cells in colorectal cancer: Past, present, and future challenges. Curr. Treat. Options Oncol. 2010;11:1–13.

203. Talasaz AH, Powell AA, Huber DE, Berbee JG, Roh KH, Yu W, et al. Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. Proc. Natl. Acad. Sci. U. S. A. 2009;106:3970–3975.

204. Karabacak NM, Spuhler PS, Fachin F, Lim EJ, Pai V, Ozkumur E, et al. Microfluidic, marker-free isolation of circulating tumor cells from blood samples. Nat. Protoc. 2014;9:694–710.

205. Wang S, Liu K, Liu J, Yu ZTF, Xu X, Zhao L, et al. Highly efficient capture of circulating tumor cells by using nanostructured silicon substrates with integrated chaotic micromixers. Angew. Chemie - Int. Ed. 2011;50:3084–3088.

206. Kelley RK, Magbanua MJ, Butler TM, Collisson EA, Hwang J, Sidiropoulos N, et al. Circulating tumor cells in hepatocellular carcinoma: A pilot study of detection, enumeration, and next-generation sequencing in cases and controls. BMC Cancer. 2015;15.

207. Ogle LF, Orr JG, Willoughby CE, Hutton C, McPherson S, Plummer R, et al. Imagestream detection and

characterisation of circulating tumour cells – A liquid biopsy for hepatocellular carcinoma? J. Hepatol. 2016;65:305–313.

208. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: Acquisition of malignant and stem cell traits. Nat. Rev. Cancer. 2009;9:265–273.

209. Satelli A, Brownlee Z, Mitra A, Meng QH, Li S. Circulating tumor cell enumeration with a combination of epithelial cell adhesion molecule-and cell-surface vimentin-based methods for monitoring breast cancer therapeutic response. Clin. Chem. 2015;61:259–266.

210. Li J, Chen L, Zhang X, Zhang Y, Liu H, Sun B, et al. Detection of circulating tumor cells in hepatocellular carcinoma using antibodies against asialoglycoprotein receptor, carbamoyl phosphate synthetase 1 and pan-cytokeratin. PLoS One. 2014;9.

211. Okajima W, Komatsu S, Ichikawa D, Miyamae M, Ohashi T, Imamura T, et al. Liquid biopsy in patients with hepatocellular carcinoma: Circulating tumor cells and cell-free nucleic acids. World J. Gastroenterol. 2017;23:5650–5668.

212. Guo W, Sun YF, Shen MN, Ma XL, Wu J, Zhang CY, et al. Circulating tumor cells with stem-like phenotypes for diagnosis, prognosis, and therapeutic response evaluation in hepatocellular carcinoma. Clin. Cancer Res. 2018;24:2203–2213.

213. Cheng Y, Luo L, Zhang J, Zhou M, Tang Y, He G, et al. Diagnostic Value of Different Phenotype Circulating Tumor Cells in Hepatocellular Carcinoma. J. Gastrointest. Surg. 2019;23:2354–2361.

214. Guo W, Yang XR, Sun YF, Shen MN, Ma XL, Wu J, et al. Clinical significance of EpCAM mRNA-Positive circulating tumor cells in hepatocellular carcinoma by an optimized negative enrichment and qRT-PCR-Based platform. Clin. Cancer Res. 2014;20:4794–4805.

215. Vona G, Estepa L, Béroud C, Damotte D, Capron F, Nalpas B, et al. Impact of Cytomorphological Detection of Circulating Tumor Cells in Patients with Liver Cancer. Hepatology. 2004;39:792–797.

216. Sun YF, Xu Y, Yang XR, Guo W, Zhang X, Qiu SJ, et al. Circulating stem cell-like epithelial cell adhesion molecule-positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection. Hepatology. 2013;57:1458–1468.

217. Schulze K, Gasch C, Staufer K, Nashan B, Lohse AW, Pantel K, et al. Presence of EpCAM-positive circulating tumor cells as biomarker for systemic disease strongly correlates to survival in patients with hepatocellular carcinoma. Int. J. Cancer. 2013;133:2165–2171.

218. von Felden J, Schulze K, Krech T, Ewald F, Nashan B, Pantel K, et al. Circulating tumor cells as liquid biomarker for high HCC recurrence risk after curative liver resection. Oncotarget. 2017;8:89978–89987.

219. Dent BM, Ogle LF, O'donnell RL, Hayes N, Malik U, Curtin NJ, et al. High-resolution imaging for the detection and characterisation of circulating tumour cells from patients with oesophageal, hepatocellular, thyroid and ovarian cancers. Int. J. Cancer. 2016;138:206–216.

220. Fan ST, Yang ZF, Ho DWY, Ng MNP, Yu WC, Wong J. Prediction of posthepatectomy recurrence of hepatocellular carcinoma by circulating cancer stem cells: A prospective study. In: Annals of Surgery. 2011. p. 569–576.

221. Cheng SW, Tsai HW, Lin YJ, Cheng PN, Chang YC, Yen CJ, et al. Lin28B is an oncofetal circulating cancer stem cell-like marker associated with recurrence of hepatocellular carcinoma. PLoS One. 2013;8.

222. Liu S, Li N, Yu X, Xiao X, Cheng K, Hu J, et al. Expression of intercellular adhesion molecule 1 by hepatocellular carcinoma stem cells and circulating tumor cells. Gastroenterology. 2013;144.

223. Qi LN, Xiang B De, Wu FX, Ye JZ, Zhong JH, Wang YY, et al. Circulating tumor cells undergoing emt provide a metric for diagnosis and prognosis of patients with hepatocellular carcinoma. Cancer Res. 2018;78:4731–4744.

224. Jin J, Niu X, Zou L, Li L, Li S, Han J, et al. AFP mRNA level in enriched circulating tumor cells from hepatocellular carcinoma patient blood samples is a pivotal predictive marker for metastasis. Cancer Lett. 2016;378:33–37.

225. Wang L, Li Y, Xu J, Zhang A, Wang X, Tang R, et al. Quantified postsurgical small cell size CTCs and EpCAM+ circulating tumor stem cells with cytogenetic abnormalities in hepatocellular carcinoma patients determine cancer relapse. Cancer Lett. 2018;412:99–107.

226. Ha Y, Kim TH, Shim JE, Yoon S, Jun MJ, Cho YH, et al. Circulating tumor cells are associated with poor outcomes in early-stage hepatocellular carcinoma: a prospective study. Hepatol. Int. 2019;13:726–735.

227. Yu J jing, Xiao W, Dong S lin, Liang H fang, Zhang Z wei, Zhang B xiang, et al. Effect of surgical liver resection on circulating tumor cells in patients with hepatocellular carcinoma. BMC Cancer. 2018;18.

228. Hao S, Chen S, Tu C, Huang T. Anterior Approach to Improve the Prognosis in HCC Patients Via Decreasing Dissemination of EpCAM+ Circulating Tumor Cells. J. Gastrointest. Surg. 2017;21:1112–1120.

229. Toso C, Mentha G, Majno P. Liver transplantation for hepatocellular carcinoma: Five steps to prevent recurrence. Am. J. Transplant. 2011;11:2031–2035.

230. Chen Z, Lin X, Chen C, Chen Y, Zhao Q, Wu L, et al. Analysis of preoperative circulating tumor cells for recurrence in patients with hepatocellular carcinoma after liver transplantation. Ann. Transl. Med. 2020;8:1067.

231. Wang PX, Xu Y, Sun YF, Cheng JW, Zhou KQ, Wu SY, et al. Detection of circulating tumour cells enables early recurrence prediction in hepatocellular carcinoma patients undergoing liver transplantation. Liver Int. 2021;41:562–573.

232. Cui K, Ou Y, Shen Y, Li S, Sun Z. Clinical value of circulating tumor cells for the diagnosis and prognosis of hepatocellular carcinoma (HCC): A systematic review and meta-analysis. Medicine (Baltimore). 2020;99:e22242.

233. Yan J, Fan Z, Wu X, Xu M, Jiang J, Tan C, et al. Circulating tumor cells are correlated with disease progression and treatment response in an orthotopic hepatocellular carcinoma model. Cytom. Part A. 2015;87:1020–1028.

234. Li J, Shi L, Zhang X, Sun B, Yang Y, Ge N, et al. pERK/pAkt phenotyping in circulating tumor cells as a biomarker for sorafenib efficacy in patients with advanced hepatocellular carcinoma. Oncotarget. 2016;7:2646–2659.

235. Winograd P, Hou S, Court CM, Lee Y, Chen P, Zhu Y, et al. Hepatocellular Carcinoma–Circulating Tumor Cells Expressing PD-L1 Are Prognostic and Potentially Associated With Response to Checkpoint Inhibitors. Hepatol. Commun. 2020;4:1527–1540.

236. Zhang Y, Zhang X, Zhang J, Sun B, Zheng L, Li J, et al. Microfluidic chip for isolation of viable circulating tumor cells of hepatocellular carcinoma for their culture and drug sensitivity assay. Cancer Biol. Ther. 2016;17:1177–1187.

237. Wu CP, Wu P, Zhao HF, Liu WL, Li WP. Clinical Applications of and Challenges in Single-Cell Analysis of Circulating Tumor Cells. DNA Cell Biol. 2018;37:78–89.

238. Sun YF, Guo W, Xu Y, Shi YH, Gong ZJ, Ji Y, et al. Circulating tumor cells from different vascular sites exhibit spatial heterogeneity in epithelial and mesenchymal composition and distinct clinical significance in hepatocellular carcinoma. Clin. Cancer Res. 2018;24:547–559.

239. Yao F, Guo JM, Xu CF, Lou YL, Xiao BX, Zhou WH, et al. Detecting AFP mRNA in peripheral blood of the patients with hepatocellular carcinoma, liver cirrhosis and hepatitis. Clin. Chim. Acta. 2005;361:119–127.

240. Guo J, Yao F, Lou Y, Xu C, Xiao B, Zhou W, et al. Detecting carcinoma cells in peripheral blood of patients with hepatocellular carcinoma by immunomagnetic beads and RT-PCR. J. Clin. Gastroenterol. 2007;41:783–788.

241. Xu W, Cao L, Chen L, Li J, Zhang XF, Qian HH, et al. Isolation of circulating tumor cells in patients with hepatocellular carcinoma using a novel cell separation strategy. Clin. Cancer Res. 2011;17:3783–3793.

242. Bahnassy AA, Zekri ARN, El-Bastawisy A, Fawzy A, Shetta M, Hussein N, et al. Circulating tumor and cancer stem cells in hepatitis C virus-associated liver disease. World J. Gastroenterol. 2014;20:18240–18248.

243. Fang ZT, Zhang W, Wang GZ, Zhou B, Yang GW, Qu XD, et al. Circulating tumor cells in the central and peripheral venous compartment - Assessing hematogenous dissemination after transarterial chemoembolization of hepatocellular carcinoma. Onco. Targets. Ther. 2014;7:1311–1318.

244. Zhou Y, Wang B, Wu J, Zhang C, Zhou Y, Yang XR, et al. Association of preoperative EpCAM Circulating Tumor Cells and peripheral Treg cell levels with early recurrence of hepatocellular carcinoma following radical hepatic resection. BMC Cancer. 2016;16.

245. Kalinich M, Bhan I, Kwan TT, Miyamoto DT, Javaid S, LiCausi JA, et al. An RNA-based signature enables high specificity detection of circulating tumor cells in hepatocellular carcinoma. Proc. Natl. Acad. Sci. U. S. A. 2017;114:1123–1128.

246. Bhan I, Mosesso K, Goyal L, Philipp J, Kalinich M, Franses JW, et al. Detection and Analysis of Circulating Epithelial Cells in Liquid Biopsies From Patients With Liver Disease. Gastroenterology. 2018;155:2016-2018.e11.

247. Xue F, Shi S, Zhang Z, Xu C, Zheng J, Qin T, et al. Application of a novel liquid biopsy in patients with hepatocellular

carcinoma undergoing liver transplantation. Oncol. Lett. 2018;15:5481–5488.

248. Yin LC, Luo ZC, Gao YX, Li Y, Peng Q, Gao Y. Twist expression in circulating hepatocellular carcinoma cells predicts metastasis and prognoses. Biomed Res. Int. 2018;2018.

249. Nel I, Baba HA, Ertle J, Weber F, Sitek B, Eisenacher M, et al. Individual profiling of circulating tumor cell composition and therapeutic outcome in patients with hepatocellular carcinoma. Transl. Oncol. 2013;6:420–428.

250. Nel I, Baba HA, Weber F, Sitek B, Eisenacher M, Meyer HE, et al. IGFBP1 in epithelial circulating tumor cells as a potential response marker to selective internal radiation therapy in hepatocellular carcinoma. Biomark. Med. 2014;8:687–698.

251. Wang Z, Luo L, Cheng Y, He G, Peng B, Gao Y, et al. Correlation Between Postoperative Early Recurrence of Hepatocellular Carcinoma and Mesenchymal Circulating Tumor Cells in Peripheral Blood. J. Gastrointest. Surg. 2018;22:633–639.

252. Ye X, Li G, Han C, Han Q, Shang L, Su H, et al. Circulating tumor cells as a potential biomarker for postoperative clinical outcome in HBV-related hepatocellular carcinoma. Cancer Manag. Res. 2018;10:5639–5647.

253. Court CM, Hou S, Winograd P, Segel NH, Li QW, Zhu Y, et al. A novel multimarker assay for the phenotypic profiling of circulating tumor cells in hepatocellular carcinoma. Liver Transplant. 2018;24:946–960.

254. Shen J, Wang WS, Zhu XL, Ni CF. High Epithelial Cell Adhesion Molecule–Positive Circulating Tumor Cell Count Predicts Poor Survival of Patients with Unresectable Hepatocellular Carcinoma Treated with Transcatheter Arterial Chemoembolization. J. Vasc. Interv. Radiol. 2018;29:1678–1684.

255. Hamaoka M, Kobayashi T, Tanaka Y, Mashima H, Ohdan H. Clinical significance of glypican-3-positive circulating tumor cells of hepatocellular carcinoma patients: A prospective study. PLoS One. 2019;14.

256. Wu X, Yang C, Yu H, Cao F, Shan Y, Zhao W. The predictive values of serum dickkopf-1 and circulating tumor cells in evaluating the efficacy of transcatheter arterial chemoembolization treatment on hepatocellular carcinoma. Med. (United States). 2019;98.

257. Zhou J, Zhang Z, Zhou H, Leng C, Hou B, Zhou C, et al. Preoperative circulating tumor cells to predict microvascular invasion and dynamical detection indicate the prognosis of hepatocellular carcinoma. BMC Cancer. 2020;20.

258. Wang P, Sun Y, Zhou K, Cheng J, Hu B, Guo W, et al. Circulating tumor cells are an indicator for the administration of adjuvant transarterial chemoembolization in hepatocellular carcinoma: A single-center, retrospective, propensity-matched study. Clin. Transl. Med. 2020;10.

259. Hardy T, Zeybel M, Day CP, Dipper C, Masson S, McPherson S, et al. Plasma DNA methylation: A potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. Gut. 2017;66:1321–1328.

260. Yiğit B, Boyle M, Özler O, Erden N, Tutucu F, Hardy T, et al. Plasma cell-free DNA methylation: a liquid biomarker of hepatic fibrosis. Gut. 2018;67:1907–1908.

CHAPTER 4

Aims of the thesis

Filippo Pelizzaro

AIMS OF THE THESIS

The primary aim of the thesis was to investigate some molecules as circulating prognostic biomarkers in patients with hepatocellular carcinoma (HCC). In particular, were evaluated as biomarkers:

- Squamous Cell Carcinoma Antigen (SCCA)-IgM, evaluating gender-related differences in its prognostic role;

- Angiogenesis molecules, namely HIF-1α and VEGF;

- microRNAs (miRNAs), with particular attention to miR-21 and miR-122;

- Prostaglandin E_2 (PGE_2), investigating also the activation of the monoacylglycerol lipase (MAGL)/cyclooxygenase 2 (COX-2)/PGE_2 pathway in HCC patients as compared to cirrhotics;

- Inflammatory-based scores, namely platelets-to-lymphocytes ratio (PLR) and neutrophils-to-lymphocytes ratio (NLR).

All these biomarkers, with the exception of inflammatory-based scores which were investigated in HCC patients regardless of treatment received, were studied in patients treated with transarterial chemoembolization. For the evaluation of each biomarker a correspondent project was designed and the results are here reported in the form of five original articles. Moreover, a study regarding changes of miR-21 and HIF-1α circulating levels in chronic liver disease and HCC, and the correlation of the miRNA with liver fibrosis and liver function laboratory tests was included.

After studying prognostic biomarkers, some clinical factors associated with prognosis of HCC patients (surveillance of at-risk patients, tumor staging and treatment) were evaluated. In particular, in the second part of the thesis, were investigated:

- surveillance as determinant of long-term survival in non-transplanted HCC patients;

- the comparison between 3- and 6-months surveillance in viral cirrhotic patients in terms of tumor stage at diagnosis, possibility of potentially curative treatments and survival benefit;

- the appropriate staging and treatment for large monofocal HCC;

- temporal trends and survival outcome of patients treated with transarterial chemoembolization;

- capecitabine efficacy and safety in advanced HCC patients.

Even in the case of these secondary aims, for each topic a specific study was designed and is here presented.

CHAPTER 5

SCCA-IgM in hepatocellular carcinoma patients treated with transarterial chemoembolization: gender-related differences

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ABSTRACT

Aims. SCCA-IgM is a useful but not completely satisfactory biomarker of hepatocellular carcinoma (HCC). Considered its gender-specific behavior in pre-clinical models, we investigated gender-related differences of SCCA-IgM as a prognostic marker in HCC.

Patients & methods. 208 prospectively recruited patients treated with transarterial chemoembolization in a single tertiary care hospital were retrospectively evaluated. Correlations between SCCA-IgM levels, clinical characteristics and survival were assessed according to gender.

Results. In advanced disease, SCCA-IgM was higher in males and lower in females. Levels below 130 AU/mL predicted a significantly longer survival in males (p=0.007) and a shorter one in females (p=0.01).

Conclusions. In predicting prognosis of HCC patients, the interpretation of SCCA-IgM should consider gender as a relevant variable.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the 5° most frequent malignancy in men and the 9° in women, with approximately 597,000 and 245,000 new cases/year, respectively (1). It is a major cause of death in cirrhotics and, in 2015, it became the leading indication for liver transplantation in the United States (2). HCC is more frequent in males, with a male-to-female ratio for age-standardized incidence rate of 2.5 (3).

Alpha-fetoprotein (AFP) is the serological biomarker most frequently used in clinical practice. Its prognostic role was confirmed in patients in the waiting list for liver transplantation (4) and in predicting response in patients undergoing loco-regional treatments (5). However, AFP has a limited usefulness in defining the patient's prognosis at an individual level (6).

In 2004 Squamous Cell Carcinoma Antigen (SCCA), a member of serine-protease inhibitors (serpins), was found to be expressed in HCC (7). SCCA overexpression is an early event in hepatocarcinogenesis, being detectable not only in overt HCC, but also in pre-neoplastic lesions (dysplastic nodules) (8). Despite a correlation between serologic levels of SCCA and expression in neoplastic tissue being absent (9,10), SCCA proved to be a useful marker when determined in serum of HCC patients, in particular when assessed as an immune-complex with immunoglobulin M (IgM) (11–13). Indeed, the diagnostic accuracy of serum biomarkers in HCC proved to be higher when determined as immune-complexes (AFP-IgM and SCCA-IgM) (14,15). An increase over time of SCCA-IgM levels in cirrhotics has been shown to herald the development of HCC (16,17). It is also suggested that serpin hyperexpression is a marker of poor prognosis, despite studies in the literature not being conclusive. High levels of the marker in tumor tissues of surgically treated patients are associated with a lower recurrence-free survival (18), and SCCA-IgM proved to be useful in predicting the response to treatment, progression-free and overall survival (OS) in HCC patients treated with loco-regional and systemic therapies (19,20).

Biomarkers are of particular interest in HCC and last version of European guidelines (21) identified the development of new reliable markers in predictive and prognostic setting as an unmet need. To date, there is no biomarker confirmed as useful in all HCC patients, probably because the many clinical characteristics involved (underlying liver disease and neoplastic variables). In the precision medicine era, not only the treatments, but also the diagnostic and predictive tools, should be tailored on patient's characteristics. Stimulated by the pre-clinical data showing a different biological behavior of serpins according to gender at a molecular level (22), we hypothesized that SCCA-IgM might have different role according to gender, particularly regarding its ability in prognostic prediction. In HCC, no studies investigating the gender differences of this marker from the clinical point of view have been published. We therefore aimed to evaluate any gender-related differences in SCCA-IgM levels in HCC considering a relatively homogeneous group of patients treated with transarterial chemoembolization (TACE), in particular regarding its prognostic role, and in comparison with AFP, the routine HCC biomarker.

PATIENTS AND METHODS

We retrospectively evaluated prospectively collected serum samples from 208 consecutive HCC patients treated with TACE at the Gastroenterology Unit of Padova University Hospital (from January 2010 to December 2018). Patients provided informed consent to participate in this study, that was approved by the Padova Hospital Ethic Committee (Protocol n° 46093).

The diagnosis of HCC was defined according to the available European guidelines (21,23), using non-invasive radiological criteria as appropriate (24). A blood sample was collected immediately before TACE (t_0) for every patient and in 149 of the 208 patients (72%) a second sample was obtained four weeks after treatment (t_1), at the same time of an abdomen computed tomography (or magnetic resonance) performed to evaluate its efficacy. The following parameters were recorded: gender,

age, etiology of the liver disease, presence of clinically relevant portal hypertension (defined either as splenomegaly, varices, ascites on imaging or platelet count < 100,000/mL), Child-Pugh class, MELD (Model for End Stage Liver Disease) and MELD-Na (MELD sodium) scores. Also, number and diameter of liver nodules, presence of portal vein thrombosis (PVT) and extra-hepatic spread (EHS), Eastern Cooperative Oncology Group performance status (ECOG-PS), BCLC (Barcelona Clinic Liver Cancer) stage, ITA.LI.CA (Italian Liver Cancer) prognostic score (25), type of TACE (conventional TACE or c-TACE, Drug Eluting Beads TACE or DEB-TACE), previous and subsequent treatments, AFP levels before TACE and at the time of control imaging were assessed. mRECIST (modified Response Evaluation Criteria In Solid Tumors) criteria (26) were used to evaluate response to treatment. OS was calculated from the study entry to death for any reason and time to progression (TTP) from the study entry to progression of the disease after therapy. Survival data were censored on 1st June 2019.

After TACE, 156 patients (75%) overall and 110 patients (73.8%) of the 149 patients with the marker sampled in two timepoints were re-treated (no one in the first month following TACE). Of the latter subgroup, after a mean time of 7.5 months from the first TACE, 17 (15.4%) were resected or ablated, 73 (66.4%) underwent another TACE and 20 (18.2%) started sorafenib.

To evaluate the association between any variation of the marker levels and response to treatment/survival, ΔSSCA-IgM (delta SCCA-IgM) was arbitrarily defined as positive or negative if there was an increase/decrease of the marker levels after TACE >25% with respect to the baseline level; the marker was considered otherwise stable. ΔAFP (delta AFP) was similarly defined.

SCCA-IgM assay

SCCA-IgM levels were determined in serum using a commercial ELISA Kit (Hepa-IC, Xeptagen, Xeptagen SpA, Marghera, Venice, Italy) according to the manufacturer's instructions. Arbitrary units

(AU)/mL were used to express the SCCA-IgM immune complexes amount, with a calibrator as reference. More details regarding the SCCA-IgM assay have been previously described (19).

Statistical analysis

Quantitative data were summarized with median and interquartile range (IQR), while categorical data with absolute and relative frequency. Mann-Whitney or Wilcoxon matched-pairs signed rank tests and Kruskal-Wallis test were used to compare quantitative variables, as appropriate. For comparing categorical data, χ^2 test or Fischer's exact test were used. The SCCA-IgM prognostic cut-off was fixed at the level of 130 AU/mL, as already validated in literature (19). Survival curves were estimated with the Kaplan-Meier method and compared by using the log-rank test. Univariate and multivariate Cox analyses were used to identify predictors of survival, inserting in the multivariate model only the variables with a statistically significant association with survival at univariate analysis. A p-value <0.05 (two-tail) was considered significant. All the statistical analyses were carried out with IBM SPSS Statistics (Version 25.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 8.3.1 (GraphPad Software, La Jolla, California, USA).

RESULTS

Baseline characteristics (Table 1)

Of the 208 patients enrolled in the study, 166 (80%) were males and 42 (20%) females. Beyond sample size difference, males differed significantly from females for age, etiology, MELD/MELD-Na, number of nodules, presence of metastases and AFP levels. Moreover, females had an earlier stage according to the BCLC (0/A in 76% vs. 50% in males).

Table 1. Baseline patients' characteristics

	Total population 208 (100)	**Males 166 (80)**	**Females 42 (20)**	**p***
Age (years)	70 (63-76)	69 (63-74)	74 (70-79)	0.0002
Cirrhosis	200 (96)	159 (96)	41 (98)	0.58

Viral etiology		127 (61)	92 (56)	35 (83)	0.0009
CRPH		149 (72)	120 (72)	29 (68)	0.68
Child-Pugh class A		170 (82)	133 (80)	37 (88)	0.23
MELD score		8 (7-10)	9 (8-11)	8 (7-10)	0.005
MELD-Na score		10 (9-12)	10 (9-12)	9 (7-11)	0.002
Number of liver nodules		2 (1-4)	3 (1-4)	2 (1-3)	0.01
Diameter of the largest nodule (cm)		2.5 (1.6-4.0)	2.5 (1.8-4.0)	2.5 (1.3-4.0)	0.78
PVT		6 (3)	6 (4)	0 (0)	0.60
EHS		4 (2)	1 (0.6)	3 (7)	0.03
ECOG-PS 0		198 (95)	158 (95)	40 (97)	0.99
AFP (ng/mL)	< 20 20 – 200 > 200	106 (51) 58 (28) 44 (21)	91 (55) 43 (26) 32 (19)	15 (36) 15 (36) 12 (28)	0.04
BCLC stage	0 A B C	31 (15) 83 (40) 79 (38) 15 (7)	23 (14) 59 (36) 72 (43) 12 (7)	8 (19) 24 (57) 7 (17) 3 (7)	0.01
ITA.LI.CA prognostic score	0 – 1 2 – 3 4 – 5 > 5	65 (31) 79 (38) 56 (27) 8 (4)	46 (28) 65 (39) 48 (29) 7 (4)	19 (46) 14 (33) 8 (19) 1 (2)	0.17
Type of TACE	c-TACE DEB-TACE	85 (41) 123 (59)	68 (41) 98 (59)	17 (40) 25 (60)	0.99
Radiological response (mRECIST)	CR PR SD PD	77 (37) 58 (28) 25 (12) 48 (23)	65 (39) 46 (28) 18 (11) 37 (22)	12 (28.5) 12 (28.5) 7 (17) 11 (26)	0.53
Pre-TACE treatments		146 (70)	119 (72)	27 (64)	0.35
Post-TACE treatments		156 (75)	126 (76)	30 (71)	0.55

Continuous variables are expressed as median and interquartile range; categorical variables are expressed as absolute and relative frequencies.

* Mann-Whitney test, c^2 test and Fischer's exact test, as appropriate.

Abbreviations: CRPH = clinically relevant portal hypertension; MELD = Model of End stage Liver Disease; MELD-Na = Model of End stage Liver Disease-Sodium; PVT = portal vein thrombosis; EHS = extra-hepatic spread; ECOG-PS = Eastern Cooperative Oncology Group performance status; AFP = alpha-fetoprotein; BCLC = Barcelona Clinic Liver Cancer; ITALICA = Italian Liver Cancer; TACE = transarterial chemoembolization; c-TACE = conventional transarterial chemoembolization; DEB-TACE = Drug Eluting Beads transarterial chemoembolization; mRECIST = modified Response Evaluation Criteria In Solid Tumor; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.

Levels of SCCA-IgM according to clinical and tumoral characteristics

Overall, SCCA-IgM levels were associated with etiology of liver disease and with MELD score. Patients with a viral etiology had higher levels of the marker (median 173.0 AU/mL [117.3 – 356.4] vs. 125.0 AU/mL [79.4 – 181.4]; p<0.0001). Patients with MELD ≤9 (median value of MELD) had a median level of SCCA-IgM of 138.0 AU/mL (76.1 – 262.0) vs. 171.3 AU/mL (111.8 – 302.8) in patients with MELD >9 (p=0.03). There was a clear trend towards higher levels of SCCA-IgM in patients with smaller nodules (146.4 AU/mL in patients with nodules ≤5 cm vs. 106.9 AU/mL in >5 cm; p=0.06) and earlier BCLC stages (155.1 AU/mL in BCLC 0-A vs. 143.8 AU/mL in BCLC B-C; p=0.06). No

association of SCCA-IgM levels with all the remaining characteristics was recorded including patients' gender, since males and females did not differ for SCCA-IgM serum levels.

The associations with etiology and MELD were maintained in males but not in females (178.5 AU/mL in viral vs. 127.3 AU/mL in non-viral male patients, p=0.0003; 138.0 AU/mL in MELD ≤9 vs. 180.8 AU/mL in MELD >9, p=0.02). Moreover, males with an ITA.LI.CA prognostic score ≤3 points had significantly lower SCCA-IgM levels: 138.0 AU/mL vs. 184.6 AU/mL (p=0.028) (Figure 1A). In contrast, females with ITA.LI.CA prognostic score ≤3 had higher levels of SCCA-IgM, despite the difference not being statistically significant (186.6 AU/mL vs. 113.7 AU/mL; p=0.086) (Figure 1B).

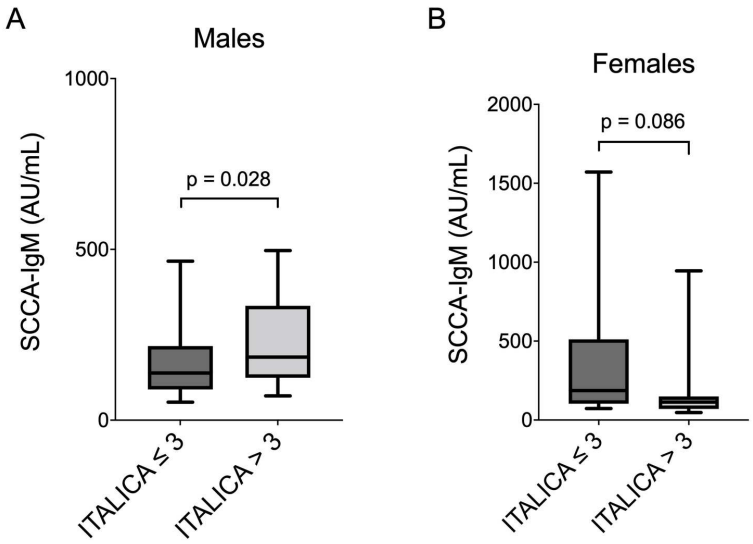

Figure 1. Box and whiskers plots showing differential levels of SCCA-IgM in males and females according to Italian Liver Cancer (ITA.LI.CA) prognostic score (the box indicate the upper and lower quartile, with a line at median; whiskers indicate the 10°-90° percentile range). Males with an ITA.LI.CA prognostic score ≤ 3 points had significantly lower SCCA-IgM levels (138.0 AU/mL vs. 184.6 AU/mL; p=0.028) (Figure 1A). Females with lower ITA.LI.CA prognostic score (≤ 3) had higher levels of SCCA-IgM, despite the difference not being statistically significant (186.6 AU/mL vs. 113.7 AU/mL; p=0.086) (Figure 1B).

Evaluation of radiological response to TACE

SCCA-IgM levels before (t_0) and after TACE (t_1) did not differ significantly and there was no association between the levels of SCCA-IgM before TACE and radiological response. Patients with complete (CR) or partial response (PR) had median SCCA-IgM levels of 142.4 AU/mL (95.3 – 269.7), as high as those with stable (SD) or progressive disease (PD) (142.4 AU/mL [97.0 – 238.0]). The same

was true for SCCA-IgM levels post-TACE. Subgrouping patients according to gender, no association between radiological response and SCCA-IgM levels (before and after TACE) or as ΔSCCA-IgM was detected.

By contrast, AFP levels before and after TACE differed significantly: median AFP at t_0 was 18.0 ng/mL (5.7 – 90.7) vs. 14.2 ng/mL (4.9 – 59.0) at t_1 (p=0.005). AFP levels measured before or after TACE were not associated with radiological response, in overall study population and in females; however, in males the median level of AFP after TACE was 9.6 ng/mL (3.9 – 50.5) for patients with CR+PR and 19.3 ng/mL (7.2 – 190.2) for patients with SD+PD (p=0.048).

Patients with negative or stable ΔAFP had more frequently a CR or PR compared to patients with positive ΔAFP (χ^2=7.9; p=0.005), but this association was confirmed only in males (χ^2=6.0; p=0.01) (Table 2).

Table 2. Association between DAFP (delta AFP) and radiological response in the overall population, in males and in females.

Overall population			
	CR + PR	**SD + PD**	
ΔAFP negative/stable	85	34	χ^2 = 7.9
ΔAFP positive	9	13	p = 0.005
Males			
	CR + PR	**SD + PD**	
ΔAFP negative/stable	67	27	χ^2 = 6.0
ΔAFP positive	8	11	p = 0.01
Females			
	CR + PR	**SD + PD**	
ΔAFP negative/stable	18	7	χ^2 = 1.8
ΔAFP positive	1	2	p = 0.18

AFP after TACE (and consequently ΔAFP) was available only for 141 patients.
Abbreviations: ΔAFP= delta alpha-fetoprotein; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.

Survival analysis

The median OS of the entire population was 19.9 months (95% CI 10.5 – 37.2), with a 5-year survival rate of 8.7%. Females had higher median OS compared to males (37.4 vs. 22.9 months), despite the difference not being statistically significant (p=0.2).

At the cut-off chosen (130 AU/mL) (19), SCCA-IgM was not able to distinguish between short and long-term survivors in the entire population, as patients with SCCA-IgM <130 AU/mL had a median OS of 26.6 months while patients with SCCA-IgM ≥130 AU/mL of 22.6 months (p=0.21). However, SCCA-IgM predicted prognosis differently according to gender: in males, high levels of the marker were significantly associated with worse prognosis, meanwhile in females, high levels of SCCA-IgM were significantly associated with better survival. Males with SCCA-IgM <130 AU/mL had a median OS of 35.7 months vs. 20.8 months in those with SCCA-IgM ≥130 AU/mL (p=0.007) (Figure 2A). Conversely, females with SCCA-IgM levels <130 AU/mL had a median OS of 15.7 months compared to 36.4 months of those with SCCA-IgM levels ≥130 AU/mL (p=0.01) (Figure 2B).

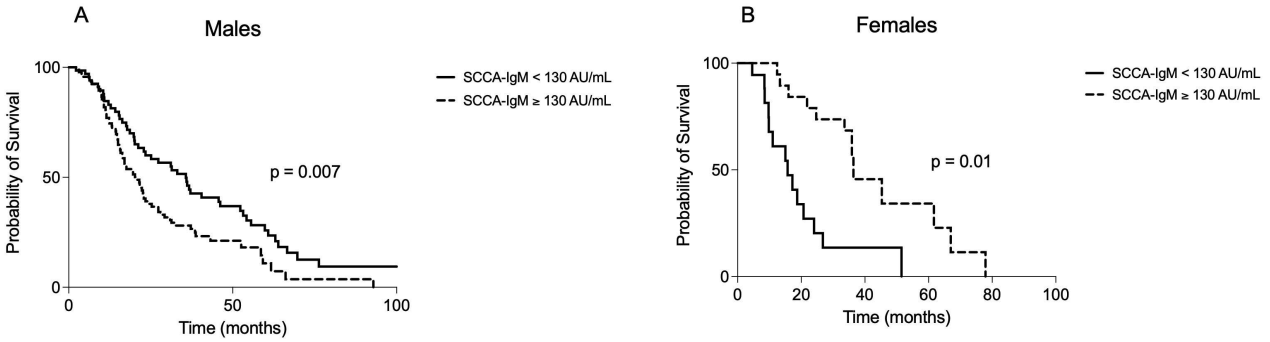

Figure 2. Kaplan-Meier curves for overall survival in males and females according to SCCA-IgM levels. In males, patients with SCCA-IgM < 130 AU/mL have better survival compared to those with SCCA-IgM ≥ 130 AU/mL (Figure 2A). In females, patients with SCCA-IgM < 130 AU/mL have shorter survival compared to those with SCCA-IgM ≥ 130 AU/mL (Figure 2B).

At a cut-off of 200 ng/mL, AFP proved to be useful in predicting patient prognosis: those with AFP levels below the cut-off had a median OS of 29.2 months, compared to 15.7 months of patients with AFP levels above the cut-off (p=0.0003). This was confirmed in males (29.2 months with AFP <200 ng/mL vs. 15.5 months in AFP ≥200 ng/mL; p=0.0003) but not in females, despite a clear trend being observed (33.6 months with AFP <200 ng/mL vs. 20.7 months in AFP ≥200 ng/mL; p=0.46). Also ΔAFP predicted prognosis, since patients with negative or stable ΔAFP had a median OS of 27.3 months vs. 13.1 months of patients with positive ΔAFP (p=0.001) (Figure 3A). The

predictive capacity of ΔAFP was maintained considering males and females separately (Figure 3B and 3C).

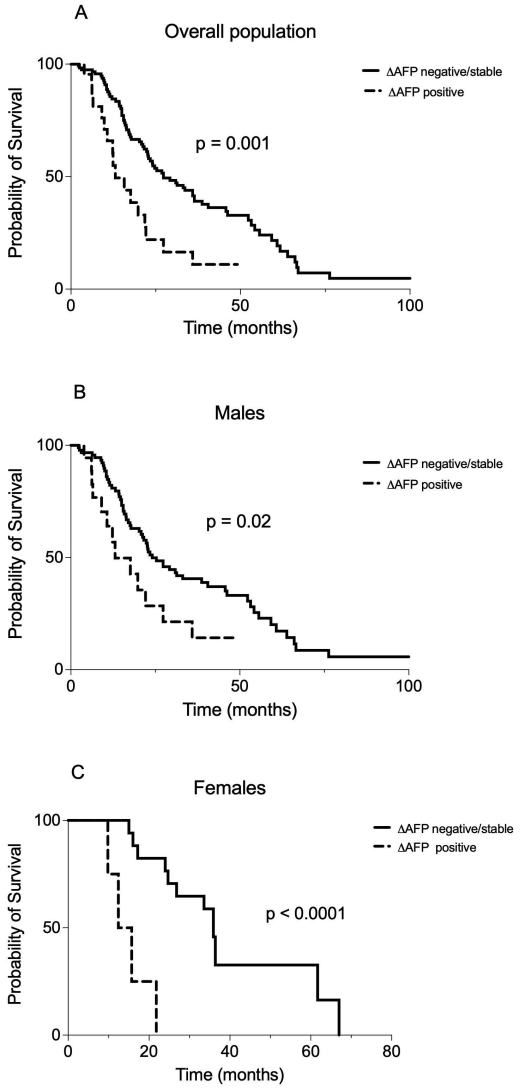

Figure 3. Kaplan-Meier curves for overall survival in the entire population, in males and in females according to ΔAFP (delta AFP). In the overall population, patients with ΔAFP negative or stable had better survival than patients with ΔAFP positive (Figure 3A). In males, patients with ΔAFP negative or stable had better survival than those with ΔAFP positive (Figure 3B). In females, patients with ΔAFP negative or stable had better survival compared to those with ΔAFP positive (Figure 3C).

Combining the two markers together, a significant gradient in survival was shown: patients with levels of both markers below the respective cut-offs (SCCA-IgM <130 AU/mL and AFP <200 ng/mL) had the highest survival (35.7 months), while patients with levels of both markers above the cut-offs showed the lowest (15.5 months) (p=0.0004) (Figure 4).

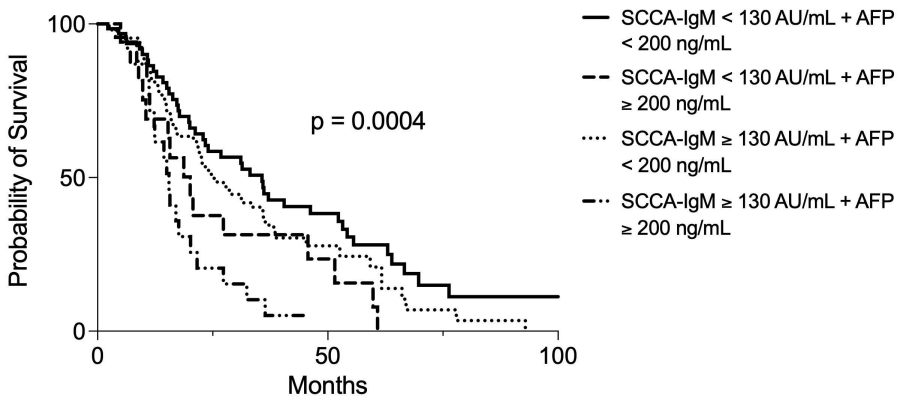

Figure 4. Kaplan-Meier curves showing overall survival of patients divided according to the combined SCCA-IgM and AFP levels. Patients with SCCA-IgM < 130 AU/mL and AFP < 200 ng/mL had a median OS of 35.7 months; patients with SCCA-IgM ≥ 130 AU/mL and AFP < 200 ng/mL had a median OS of 24.7 months; patients with SCCA-IgM < 130 AU/mL and AFP ≥ 200 ng/mL had a median OS of 20.1 months; patients with SCCA-IgM ≥ 130 AU/mL and AFP ≥ 200 ng/mL had a median OS of 15.5 months (p=0.0004).

SCCA-IgM measured at t_0 did not predict TTP overall, but its opposite behavior according to gender was maintained, despite the difference not being statistically significant: better TTP was shown in males with SCCA-IgM levels <130 AU/mL (6.6 vs. 5.2 months; p=0.74) and in females with SCCA-IgM levels above the cut-off (5.2 vs. 1.2 months; p=0.39). AFP was able to predict TTP in the overall population and in males, but not in females (data not shown).

Univariate and multivariate analysis

In the entire population of patients enrolled in the study, parameters associated with OS at univariate analysis are shown in Table 3. The independent predictors of survival at the Cox multivariate analysis were: radiological response (p=0.02), Child-Pugh class (p=0.03), MELD (p=0.01), ITA.LI.CA prognostic score (p=0.02) and additional treatments after TACE (p<0.0001).

Parameters associated with OS at univariate analysis in males and females are shown in Table 4. Independent predictors of overall survival were: radiological response (p=0.02), Child-Pugh class (p=0.03), MELD (p=0.001), ITA.LI.CA prognostic score (p=0.008) and treatments post-TACE (p<0.0001) in males and ΔAFP (p=0.01) in females.

Table 3. Cox univariate and multivariate analysis for the predictors of overall survival in the overall population.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
Sex, Male vs. Female	1.01 (0.67 – 1.53)	0.95		
Age, < 65 vs. ≥ 65 (years)	0.83 (0.57 – 1.19)	0.31		
c-TACE vs. DEB-TACE	1.05 (0.74 – 1.48)	0.78		
SCCA-IgM, < 130 vs. ≥ 130 AU/mL	1.24 (0.89 – 1.73)	0.21		
ΔSCCA-IgM, Neg/Stable vs. Pos	1.13 (0.47 – 2.93)	0.23		
AFP, < 200 vs. ≥ 200 ng/mL	2.06 (1.38 – 3.06)	0.0003	1.38 (0.76 – 2.49)	0.29
ΔAFP, Neg/Stable vs. Pos	2.44 (1.42 – 4.20)	0.001	1.70 (0.79 – 3.63)	0.17
Radiological response, CR+PR vs. SD+PD	1.99 (1.42 – 2.79)	< 0.0001	2.43 (1.39 – 4.25)	0.02
Cirrhosis, Yes vs. No	0.48 (0.00 – 5.84)	0.22		
Etiology, Viral vs. Not viral	0.89 (0.63 – 1.24)	0.48		
CRPH, Yes vs. No	1.02 (0.70 – 1.48)	0.93		
Child-Pugh, A vs. B	3.24 (2.18 – 4.81)	< 0.0001	2.17 (1.08 – 4.39)	0.03
MELD score, ≤ 9 vs. > 9	1.93 (1.37 – 2.71)	< 0.0001	3.19 (1.61 – 6.31)	0.001
MELD-Na score, ≤ 10 vs. > 10	1.74 (1.24 – 2.46)	0.001	1.50 (0.75 – 3.02)	0.26
N° of nodules, ≤ 3 vs. > 3	1.10 (0.77 – 1.58)	0.59		
Diameter, ≤ 5 vs > 5 cm	1.93 (1.21 – 3.10)	0.006	0.74 (0.34 – 1.62)	0.45
PVT, Yes vs. No	0.37 (0.16 – 0.86)	0.02	0.39 (0.12 – 1.19)	0.09
EHS, Yes vs. No	0.32 (0.99 – 1.01)	0.05		
ECOG-PS, 0 vs. 1	3.04 (1.31 – 7.03)	0.01	1.95 (0.71 – 5.37)	0.19
BCLC stage, 0+A vs. B+C	1.51 (1.09 – 2.10)	0.01	0.59 (0.27 – 1.28)	0.18
ITA.LI.CA score, ≤ 3 vs. > 3	2.29 (1.61 – 3.26)	< 0.0001	4.49 (1.73 – 11.64)	0.002
Post-TACE treatment, Yes vs. No	3.39 (2.26 – 5.10)	< 0.0001	4.28 (2.20 – 8.32)	< 0.0001

Abbreviations: HR = hazard ratio; CI = confidence interval; c-TACE = conventional transarterial chemoembolization; DEB-TACE = Drug Eluting Beads transarterial chemoembolization; SCCA-IgM = Squamous Cell Carcinoma Antigen; DSCCA-IgM = delta SCCA-IgM; AFP = alpha-fetoprotein; DAFP = delta AFP; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease; CRPH = clinically relevant portal hypertension; MELD = Model of End stage Liver Disease; MELD-Na = Model for End Stage Liver Disease – Sodium; PVT = portal vein thrombosis; EHS = extra-hepatic spread; ECOG-PS = Eastern Cooperative Oncology Group performance status; BCLC = Barcelona Clinic Liver Cancer; ITA.LI.CA = Italian Liver Cancer; ITA.LI.CA = Italian Liver Cancer.

Table 4. Cox univariate and multivariate analysis for predictors of overall survival in males and females.

Males				
	Univariate analysis		**Multivariate analysis**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
SCCA-IgM, < 130 vs. ≥ 130 AU/mL	1.67 (1.15 – 2.44)	0.007	1.33 (0.63 – 2.81)	0.46
AFP, < 200 vs. ≥ 200 ng/mL	2.24 (1.43 – 3.49)	0.0003	1.28 (0.64 – 2.55)	0.49
ΔAFP, Neg./Stable vs. Positive	2.04 (1.11 – 3.76)	0.02	1.28 (0.55 – 2.98)	0.58
Radiological response, CR+PR vs. SD+PD	2.14 (1.47 – 3.12)	< 0.0001	2.72 (1.47 – 5.05)	0.01
Child-Pugh, A vs. B	3.34 (2.17 – 5.15)	< 0.0001	2.20 (0.99 – 4.87)	0.52
MELD score, ≤ 9 vs. > 9	2.0 (1.36 – 2.93)	< 0.0001	2.92 (1.35 – 6.30)	0.007
MELD-Na score, ≤ 10 vs. > 10	1.90 (1.29 – 2.79)	0.001	1.93 (0.92 – 4.07)	0.83
Diameter, ≤ 5 vs > 5 cm	1.82 (1.09 – 3.03)	0.02	0.81 (0.33 – 2.0)	0.65
PVT, No vs. Yes	0.38 (0.17 – 0.88)	0.02	0.40 (0.13 – 1.29)	0.13
ECOG-PS, 0 vs. 1	2.93 (1.26 – 6.84)	0.01	1.92 (0.69 – 5.37)	0.22
BCLC stage, 0+A vs. B+C	1.55 (1.07 – 2.24)	0.02	0.55 (0.24 – 1.28)	0.17
ITA.LI.CA score, ≤ 3 vs. > 3	2.62 (1.75 – 3.91)	< 0.0001	4.03 (1.43 – 11.35)	0.008
Post-TACE treatments, Yes vs. No	3.48 (2.21 – 5.49)	< 0.0001	4.71 (2.16 – 10.28)	< 0.0001
Females				
	Univariate analysis		**Multivariate analysis**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
SCCA-IgM, < 130 vs. ≥ 130 AU/mL	0.27 (0.12 – 0.61)	0.02	0.85 (0.19 – 3.77)	0.84

ΔAFP, Neg./Stable vs. Positive	12.55 (2.71 – 58.25)	0.001	44.4 (4.59 – 429.35)	0.01
Diameter, ≤ 5 vs > 5 cm	3.84 (1.02 – 14.49)	0.047	0.02 (0 – 9.75)	0.98
EHS, Yes vs. No	0.23 (0.06 – 0.87)	0.03	0 (0 - 0.17)	0.96
Post-TACE treatments, Yes vs. No	3.18 (1.28 – 7.92)	0.01	3.01 (0.64 – 14.06)	0.16

Abbreviations: HR = hazard ratio; CI = confidence interval; SCCA-IgM = Squamous Cell Carcinoma Antigen; AFP = alpha-fetoprotein; DAFP = delta AFP; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease; MELD = Model for End Stage Liver Disease; MELD-Na = Model for End Stage Liver Disease – Sodium; PVT = portal vein thrombosis; ECOG-PS = Eastern Cooperative Oncology Group performance status; BCLC = Barcelona Clinic Liver Cancer; ITA.LI.CA = Italian Liver Cancer; TACE = transarterial chemoembolization; EHS = extra-hepatic spread.

DISCUSSION

No biomarker is completely satisfactory in the management of HCC and AFP, although still being the most widely used, is not efficient enough to be recommended in the management of HCC patients (21,27). TACE is classified as a “palliative” therapy, but it is the most widely adopted treatment in HCC management (21). According to guidelines, TACE is the recommended therapy in BCLC stage B patients (21,28), but it proved to be useful also in other settings, such as bridge to liver transplantation (29,30).

Several studies demonstrated the expression of serpins in HCC (7,8). Tumor growth, inhibition of apoptosis and epithelial to mesenchymal transition are promoted by these molecules, leading to cancer development and progression (31). SCCA overexpression is an early event in hepatocarcinogenesis: its expression progressively increase from cirrhosis to dysplastic nodules and HCC (8). In cirrhotics, the increase in serum serpin levels heralds HCC development (16,17) and several studies demonstrated their usefulness as biomarkers in HCC (11–14). SCCA overexpression seems to be associated with poor prognosis in HCC patients (18). Moreover, serum SCCA-IgM levels predicted response to treatment and prognosis after loco-regional and systemic therapies (19,20). However, the studies published so far on the prognostic role of serpins are not completely conclusive.

There are some reports in the literature suggesting a potential differential role of serpins according to gender in animal models. Mice transgenic for SERPINB3 have a greater survival compared to wild-type, with male transgenic mice surviving longer than females. Moreover, the biologic mechanisms

underlying this differential survival are also gender-specific, since male animals are characterized by an up-regulation of mTOR, while females by a down-regulation of p53 (22). The authors speculated on a role of sex hormones in modulating the serpin's ability to up or down-regulate specific genetic pathways. A different behavior of the marker according to the patients' gender in HCC, modulated by the sex hormones, is an intriguing hypothesis, but at the moment there are no studies specifically addressing this point. In addition, to the best of our knowledge, currently there are no clinical data on the differential ability of SCCA in predicting HCC patient prognosis according to gender.

There is a continuous search for reliable biomarkers in HCC, not only in the diagnostic setting, but also in predicting response to treatment and prognosis. The concept of "liquid biopsy" has recently emerged as an important tool to detect HCC, monitor response to treatment and predict survival (32). Moreover, in recent years several others technologies have been developed and implemented for the detection of new biomarkers. Among these innovative approaches, multi-omics analysis, in particular when combined with artificial intelligence tools, seems to be very promising (33,34). These approaches are of paramount importance in personalized medicine, allowing individualized patients profiling and eventually personalization of care (35,36). With the background on SCCA-IgM role as biomarker in mind and in the light of the need of moving more and more toward a personalized approach in HCC management, we evaluated if the intriguing pre-clinical findings on a different behavior of serpins at a molecular level (22) could be translated in a clinical application. Therefore, we aimed to investigate if the interpretation of serum SCCA-IgM in HCC patients treated with TACE, the most widely used treatment in HCC, should be personalized according to gender.

Female sex has been suggested to be a favorable prognostic factor in HCC (37). Whether real biological differences exist or whether females had a higher compliance to diagnostic and therapeutic process (e.g., in surveillance) is still debatable. In this study, females were significantly older than males, but achieved a longer median OS (despite not statistically significant). Regarding

age, there are data indeed showing that treatments for HCC are equally effective in old and young patients, and the survival is not affected by age (38). Therefore, despite the older age of females could be perceived as a confounding, we believe that this difference has not biased our results on survival predictors between males and females. Moreover, the gender-related inverse behavior of SCCA-IgM is not influenced by the age in the two subgroups. Females had more frequently a viral etiology, a better-preserved liver function (better MELD and MELD-Na) and less advanced tumors, with lower number of liver nodules and an earlier BCLC stage. Overall, SCCA-IgM levels were higher in patients with viral etiology. Studies already published suggested a correlation between serpins expression and viral liver disease, in particular when HCV-related (15,39), an association consistent with a direct viral role in modulating serpins expression. Moreover, our data documented a trend towards higher levels of SCCA-IgM in patients with smaller nodules and earlier BCLC stages, as already reported (9).

In our study, SCCA-IgM levels were not able to predict response to treatment, with no difference between the CR+PR and the SD+PD groups, both at t_0 and t_1, and also the changes in the marker levels after TACE (ΔSSCA-IgM) did not correlate with radiological response. By contrast, AFP was more useful in predicting radiological response, with a significant drop of the levels four weeks after the procedure. Specifically, patients with a decrease or a stability of the marker after TACE had more frequently a complete or a partial response to TACE compared to patients with a positive ΔAFP. On these bases, SCCA-IgM seems not to be useful in predicting radiological response after TACE. Our findings are not completely in agreement with previous reports suggesting a correlation between a SCCA-IgM reduction after TACE and a radiological response (CR+PR) (19,20).

We did not even confirm previous data showing a role of SCCA-IgM in predicting prognosis of HCC patients treated with TACE (19), with no correlation between SCCA-IgM levels and OS. On the contrary, patients with higher levels of AFP had a significantly shorter survival, even though it was

not an independent predictor of survival at the Cox multivariate analysis. ΔAFP was also associated with survival: patients with a decrease or stability of the marker had longer OS compared to patients with an increasing AFP.

Males and females did not differ in SCCA-IgM levels. As in the overall population, SCCA-IgM levels were higher in males with viral etiology, in those with MELD >9 and in ITA.LI.CA prognostic score >3. The ITA.LI.CA prognostic score is a recently developed prognostic system that includes tumor staging, Child-Pugh score, ECOG-PS and AFP (25). It shows strong ability in predicting individual survival and it has a better discriminating ability compared to the BCLC staging system. In our study, the ITA.LI.CA prognostic score confirmed to be an independent predictor of prognosis. A gender-related difference in the levels of SCCA-IgM emerged in relation to the ITA.LI.CA score: among patients with an advanced ITA.LI.CA score, males had significantly higher levels of SCCA-IgM while females had lower levels of the marker, indirectly confirming the ability of serpins in predicting prognosis differently in males and in females.

Males with SD or PD had significantly higher levels of AFP at t_1 and ΔAFP correlated with radiological response. Moreover, ΔAFP was able to predict prognosis both in males and in females: patients with a decrease or a stability of the marker after TACE had a longer survival compared to patients with an increase.

SCCA-IgM levels proved to be a prognostic predictor in males and in females considered separately, and the marker showed an opposite behavior according to gender. Males with higher serpin levels had significantly shorter survival, the opposite was true for females. These findings are difficult to explain without knowing exactly the molecular pathway in which serpins are involved and their different regulation in males and females. We could speculate on an involvement of sex hormones in this differential correlation with survival: different hormones may regulate serpins expression differently and probably more aggressive tumors, in the two genders, regulated these molecules

and activate their pathways in an opposite way. It must be kept in mind that we measured serpin levels in serum and we cannot be sure that what we detect in the circulating blood reflects exactly what is going on in the neoplastic tissue. Two considerations must be done. Firstly, in the studies published so far, no correlation between tissue expression and serum levels of these molecules was detected (9,10). This is due to the fact that SCCA is not actively secreted by the neoplastic cells, but it is released passively as a consequence of cell lysis (40). Secondly, being SCCA-IgM measured in serum in the form of an immunocomplex, its levels are not only function of serpin expression but also of individual immune response.

SCCA-IgM was not able to predict TTP neither in males nor in females, but the opposite behavior of the marker seemed to be confirmed: longer median TTP was shown in males with SCCA-IgM <130 AU/mL and in females with SCCA-IgM ≥130 AU/mL (despite these differences not being statistically significant).

Our study has several limitations, the principal being the larger sample size in males compared to females (80% vs. 20%) that might have introduced unintentional biases. However, the difference in size of the two groups reflects the epidemiology of HCC in our area. Moreover, despite this study being retrospective in nature, patients were collected consecutively and prospectively, and this could have reduced the selection bias.

CONCLUSIONS

In conclusion, SCCA-IgM proved to be useful in predicting prognosis of HCC patients treated with transarterial chemoembolization. In recent years individualized patients profiling and personalized medicine have become increasingly important in the management of HCC patients. Despite not confirming its role in the entire population of patients, we demonstrated a gender-specific role of SCCA-IgM in predicting prognosis. It seems to accurately predict prognosis differently in males and

in females, with a better survival for males with lower levels and for females with higher levels of the marker. In addition, the same gender-specific differential effects was observed for the ITA.LI.CA score, with higher levels in males with advanced and in females with early disease and, as a trend, for TTP. In accordance with the dictates of precision medicine, SCCA-IgM has not the same value as prognostic biomarker in males and females and should be interpreted in an opposite way. Despite additional confirmation studies specifically focused on the point are needed, our findings could contribute to improve the management of HCC patients, identifying more precisely their expected survival.

FUTURE PERSPECTIVE

As pointed out in the recently updated EASL guidelines (21), in the future, research should focus on the development of biomarkers for surveillance and prediction of patients prognosis. SCCA-IgM is a useful biomarker in HCC but probably its role is not the same in every clinical situation. We believe that gender of the patient is a relevant variable that has to be considered in the clinical application of this biomarker and in the near future other studies will enable to elucidate the different role of serpins in males and females, the mechanisms underlying this different behavior and the precise setting of applicability of SCCA-IgM as marker, as for precision medicine. Moreover, in performing the task of the search of new biomarkers for a personalized and “patient-tailored” HCC management, liquid biopsy and recently developed technologies, such as multi-omics analysis, will certainly help.

REFERENCES

1. Fact Sheets by Population - Globocan - IARC n.d. [Internet]. [cited 2020 Oct 16];Available from: https://gco.iarc.fr/today/fact-sheets-populations
2. Yang JD, Larson JJ, Watt KD, Allen AM, Wiesner RH, Gores GJ, et al. Hepatocellular Carcinoma Is the Most Common Indication for Liver Transplantation and Placement on the Waitlist in the United States. Clin. Gastroenterol. Hepatol. 2017;15:767-775.e3.
3. Akinyemiju T, Abera S, Ahmed M, Alam N, Alemayohu MA, Allen C, et al. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level. JAMA Oncol. 2017;98121:1683–1691.
4. Vibert E, Azoulay D, Hoti E, Iacopinelli S, Samuel D, Salloum C, et al. Progression of alphafetoprotein before liver transplantation for hepatocellular carcinoma in cirrhotic patients: a critical factor. Am. J. Transplant. 2010;10:129–137.
5. Riaz A, Ryu RK, Kulik LM, Mulcahy MF, Lewandowski RJ, Minocha J, et al. Alpha-fetoprotein response after locoregional therapy for hepatocellular carcinoma: Oncologic marker of radiologic response, progression, and survival. J. Clin. Oncol. 2009;27:5734–5742.
6. Farinati F, Marino D, De Giorgio M, Baldan A, Cantarini M, Cursaro C, et al. Diagnostic and prognostic role of α-fetoprotein in hepatocellular carcinoma: Both or neither? Am. J. Gastroenterol. 2006;101:524–532.
7. Pontisso P, Calabrese F, Benvegnù L, Lise M, Belluco C, Ruvoletto MG, et al. Overexpression of squamous cell carcinoma antigen variants in hepatocellular carcinoma. Br. J. Cancer. 2004;90:833–837.
8. Guido M, Roskams T, Pontisso P, Fassan M, Thung SN, Giacomelli L, et al. Squamous cell carcinoma antigen in human liver carcinogenesis. J. Clin. Pathol. 2008;61:445–447.
9. Trerotoli P, Fransvea E, Angelotti U, Antonaci G, Lupo L, Mazzocca A, et al. Tissue expression of Squamous Cellular Carcinoma Antigen (SCCA) is inversely correlated to tumor size in HCC. Mol. Cancer. 2009;8:1–8.
10. Giannelli G, Marinosci F, Sgarra C, Lupo L, Dentico P, Antonaci S. Clinical role of tissue and serum levels of SCCA antigen in hepatocellular carcinoma. Int. J. cancer. 2005;116:579–583.
11. Giannelli G, Marinosci F, Trerotoli P, Volpe A, Quaranta M, Dentico P, et al. SCCA antigen combined with alpha-fetoprotein as serologic markers of HCC. Int. J. Cancer. 2005;117:506–509.
12. Bui Huu H, Ha Thuc N, Thi Le HP, Thi Thanh T Do, Luong Bac A, Tiribelli C, et al. Characterization of SCCA-IgM as a biomarker of liver disease in an Asian cohort of patients. Scand. J. Clin. Lab. Invest. 2018;78:204–210.
13. Liu CH, Gil-Gómez A, Ampuero J, Romero-Gómez M. Diagnostic accuracy of SCCA and SCCA-IgM for hepatocellular carcinoma: A meta-analysis. Liver Int. 2018;38:1820–1831.
14. Beneduce L, Castaldi F, Marino M, Quarta S, Ruvoletto M, Benvegnù L, et al. Squamous cell carcinoma antigen-immunoglobulin M complexes as novel biomarkers for hepatocellular carcinoma. Cancer. 2005;103:2558–2565.
15. Giannelli G, Fransvea E, Trerotoli P, Beaugrand M, Marinosci F, Lupo L, et al. Clinical validation of combined serological biomarkers for improved hepatocellular carcinoma diagnosis in 961 patients. Clin. Chim. Acta. 2007;383:147–152.
16. Pontisso P, Quarta S, Caberlotto C, Beneduce L, Marino M, Bernardinello E, et al. Progressive increase of SCCA-IgM immune complexes in cirrhotic patients is associated with development of hepatocellular carcinoma. Int. J. Cancer. 2006;119:735–740.
17. Biasiolo A, Trotta E, Fasolato S, Ruvoletto M, Martini A, Gallotta A, et al. Squamous cell carcinoma antigen-IgM is associated with hepatocellular carcinoma in patients with cirrhosis: A prospective study. Dig. Liver Dis. 2016;48:197–202.
18. Turato C, Vitale A, Fasolato S, Ruvoletto M, Terrin L, Quarta S, et al. SERPINB3 is associated with TGF-beta1 and cytoplasmic beta-catenin expression in hepatocellular carcinomas with poor prognosis. Br. J. Cancer. 2014;110:2708–2715.
19. Pozzan C, Cardin R, Piciocchi M, Cazzagon N, Maddalo G, Vanin V, et al. Diagnostic and prognostic role of SCCA-IgM serum levels in hepatocellular carcinoma (HCC). J. Gastroenterol. Hepatol. 2014;29:1637–1644.

20. Guarino M, Di Costanzo GG, Gallotta A, Tortora R, Paneghetti L, Auriemma F, et al. Circulating SCCA–IgM complex is a useful biomarker to predict the outcome of therapy in hepatocellular carcinoma patients. Scand. J. Clin. Lab. Invest. 2017;77:448–453.

21. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.

22. Villano G, Ruvoletto M, Ceolotto G, Quarta S, Calabrese F, Turato C, et al. SERPINB3 is associated with longer survival in transgenic mice. Sci. Rep. 2013;3:1–9.

23. Llovet JM, Ducreux M, Lencioni R, Di Bisceglie AM, Galle PR, Dufour JF, et al. EASL-EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2012;56:908–943.

24. Roberts LR, Sirlin CB, Zaiem F, Almasri J, Prokop LJ, Heimbach JK, et al. Imaging for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. Hepatology. 2018;67:401–421.

25. Farinati F, Vitale A, Spolverato G, Pawlik TM, Huo T, Lee Y-H, et al. Development and Validation of a New Prognostic System for Patients with Hepatocellular Carcinoma. PLoS Med. 2016;13:e1002006.

26. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. Semin. Liver Dis. 2010;30:52–60.

27. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68:723–750.

28. Heimbach JK, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, et al. AASLD guidelines for the treatment of hepatocellular carcinoma. Hepatology. 2018;67:358–380.

29. Kulik L, Heimbach JK, Zaiem F, Almasri J, Prokop LJ, Wang Z, et al. Therapies for patients with hepatocellular carcinoma awaiting liver transplantation: A systematic review and meta-analysis. Hepatology. 2018;67:381–400.

30. Chedid M, Scaffaro L, Chedid A, Maciel A, Cerski C, Reis M, et al. Transarterial Embolization and Percutaneous Ethanol Injection as an Effective Bridge Therapy Before Liver Transplantation for Hepatitis C-Related Hepatocellular Carcinoma. Gastroenterol. Res. Pract. 2016;2016:9420274.

31. Quarta S, Vidalino L, Turato C, Ruvoletto M, Calabrese F, Valente M, et al. SERPINB3 induces epithelial-mesenchymal transition. J. Pathol. 2010;221:343–356.

32. Golubnitschaja O, Polivka J, Yeghiazaryan K, Berliner L. Liquid biopsy and multiparametric analysis in management of liver malignancies: new concepts of the patient stratification and prognostic approach. EPMA J. 2018;9:271–285.

33. Gerner C, Costigliola V, Golubnitschaja O. MULTIOMIC patterns in body fluids: Technological challenge with a great potential to implement the advanced paradigm of 3P medicine. Mass Spectrom. Rev. 2019;[Online ahead of print].

34. Chaudhary K, Poirion OB, Lu L, Garmire LX. Deep learning–based multi-omics integration robustly predicts survival in liver cancer. Clin. Cancer Res. 2018;24:1248–1259.

35. Janssens JP, Schuster K, Voss A. Preventive, predictive, and personalized medicine for effective and affordable cancer care. EPMA J. 2018;9:113–123.

36. Lu M, Zhan X. The crucial role of multiomic approach in cancer research and clinically relevant outcomes. EPMA J. 2018;9:77–102.

37. Farinati F, Sergio A, Giacomin A, Di Nolfo MA, Del Poggio P, Benvegnù L, et al. Is female sex a significant favorable prognostic factor in hepatocellular carcinoma? Eur. J. Gastroenterol. Hepatol. 2009;21:1212–1218.

38. Mirici-Cappa F, Gramenzi A, Santi V, Zambruni A, Micoli A Di, Frigerio M, et al. Treatments for hepatocellular carcinoma in elderly patients are as effective as in younger patients: A 20-year multicentre experience. Gut. 2010;59:387–396.

39. Giannini EG, Basso M, Bazzica M, Contini P, Marenco S, Savarino V, et al. Successful antiviral therapy determines a significant decrease in squamous cell carcinoma antigen-associated (SCCA) variants' serum levels in anti-HCV positive cirrhotic patients. J. Viral Hepat. 2010;17:563–568.

40. Uemura Y, Pak SC, Luke C, Çataltepe S, Tsu C, Schick C, et al. Circulating serpin tumor markers SCCA1 and SCCA2 are not actively secreted but reside in the cytosol of squamous carcinoma cells. Int. J. Cancer. 2000;89:368–377.

CHAPTER 6

HIF-1α and VEGF as prognostic biomarkers in hepatocellular carcinoma patients treated with transarterial chemoembolization

Filippo Pelizzaro

ABSTRACT

Background. Neoangiogenesis plays a pivotal role in the development and progression of hepatocellular carcinoma (HCC), and its activation caused by transarterial chemoembolization (TACE) has been called into question in explaining the low effectiveness of this treatment. In this study, we aimed at evaluating Vascular Endothelial Growth Factor (VEGF) and Hypoxia-Inducible Factor-1α (HIF-1α) as biomarkers in HCC patients treated with TACE.

Methods. Blood samples from the 163 patients included in this retrospective study were collected before TACE (t_0) and four weeks after treatment, at the time of the control imaging (t_1). VEGF levels were measured in 149 patients, while HIF-1α levels were assessed in 96 patients.

Results. Compared to t_0, statistically significant higher levels of VEGF after TACE were demonstrated (264.0 [78.7-450.8] vs. 278.6 [95.0-576.6] pg/mL; p<0.0001), while HIF-1α was not increased. Responders to TACE had significantly lower levels of VEGF than non-responders both at t_0 (200.0 [58.9-415.8] vs. 406.6 [181.4-558.6] pg/mL; p=0.006) and at t_1 (257.3 [68.5-528.6] vs. 425.9 [245.2-808.3] pg/mL; p=0.003), and in both groups there was an increase in VEGF compared to the levels measured before treatment (p=0.001 and p=0.005, respectively). In addition, VEGF correlated with tumor burden (higher in patients with multifocal tumors and in intermediate stage). VEGF was not associated with survival, while HIF-1α below the identified cut-off predicted better prognosis (median OS 28.0 months [95% CI 19.7-36.3] vs. 17.0 months [95% CI 11.1-22.9]; p=0.01) and it was identified as an independent prognostic parameter at the Cox multivariate analysis.

Conclusions. VEGF and HIF-1α can be considered useful prognostic biomarkers in HCC patients treated with TACE. VEGF is associated with tumor burden and higher levels are predictive of poor response to treatment, while HIF-1α levels turned out to be a valuable prognostic parameter.

INTRODUCTION

Angiogenesis, one of the hallmarks of cancer (1), plays a pivotal role in the development and progression of hepatocellular carcinoma (HCC) (2,3). HCC displays an intense neoangiogenic activity during its growth and a peculiar vascular derangement occurs during hepatic carcinogenesis, since the tumor tends to be almost entirely fed by arterial inflow, unlike the surrounding parenchyma that receives the majority of blood supply through the portal system (4).

However, liver tumors display marked vascular abnormalities. Aberrant microvasculature typically may seem "arterialized" (tight vessels covered by smooth muscle cells) and/or "capillarized" (capillaries without fenestration and with laminin basement membrane deposition) (2). As a consequence of the abnormal blood flow, although being a highly angiogenic cancer, HCC is characterized by hypoxia (2). Hypoxia may promote tumor growth and progression, and resistance to therapies (5). Indeed, it has been demonstrated that the overactivation of angiogenesis in HCC is associated with worse prognosis. A transcriptomic signature of five genes involved in the angiogenetic process (ANGPT2, NETO2, ESM1, NR4A1, DLL4) was found to accurately identify rapidly growing tumors and was associated with shorter survival (6). In addition, several studies demonstrated that overexpression of Vascular Endothelial Growth Factor (VEGF) and its transcription factor Hypoxia-Inducible Factor (HIF)-1α, the two key mediators of angiogenesis, is a negative prognostic factor, particularly in patients treated with surgery and systemic therapies (7–18).

The release of angiogenic factors, prompted by ischemia caused by embolization, is among the reasons advocated to justify the high risk of recurrence after transarterial chemoembolization (TACE) (3,19). This treatment relies its activity on both local intra-arterial administration of a chemotherapeutic agent and embolization of tumor feeding arteries. Ischemia, hypoxia and necrosis caused by embolization are able to stimulate neo-angiogenesis. However, due to structural

and functional defects, newly formed tumor blood vessels may further aggravate hypoxia and thereby form a vicious cycle leading to tumor recurrence and metastasis (20). Consequently, it may be hypothesized that HIF-1α and VEGF could represent valuable biomarkers in the identification of patients with poor response to TACE and shorter overall survival (OS). Some studies already suggested that high VEGF levels are associated with less effective treatment and poorer prognosis (21,22), while few data are available for circulating HIF-1α in this setting. In this study we aimed to evaluate, in a group of HCC patients treated with TACE, the prognostic accuracy, the ability to predict response to treatment and the correlation with tumoral and clinical parameters of the two most important molecules involved in angiogenesis, HIF-1α and VEGF.

MATERIALS AND METHODS

Blood samples consecutively collected from 163 patients with HCC admitted to the Gastroenterology Unit of Padova University Hospital for TACE treatment from January 2010 to December 2018 were evaluated. Each subject provided written informed consent to participate to the study, which was conducted in accordance to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova University Hospital.

Presence of HCC was defined according to guidelines available at the time of the diagnosis (23,24). HCC was histologically confirmed in 53 patients (32.5%), while in the remaining cases the diagnosis was based on the typical features at imaging (computed tomography [CT] or magnetic resonance imaging [MRI]) (24).

TACE was performed, after super selective catheterization of the tumor-feeding artery, either administering a mixture of chemotherapeutic drug and Lipiodol followed by embolization (conventional TACE) or drug-eluting beads loaded with doxorubicin (DEB-TACE). In all patients, two blood samples were collected: the first immediately before TACE procedure (t_0) and the second after

four weeks, at the time of the control imaging (abdomen CT scan or MRI) routinely performed to evaluate the efficacy of treatment (t_1).

The following clinical and tumoral parameters were recorded: sex, age, etiology of the underlying liver disease, main serological parameters (total bilirubin, INR, albumin and AFP, this latter both at t_0 and at t_1) and Child-Pugh class. In addition, tumor characteristics such as number and size of liver nodules, evaluated before TACE with dynamic CT or MRI, were recorded. Patients were staged according to the Barcelona Clinic Liver Cancer (BCLC) system (24). Tumors were classified as poorly or highly vascularized, by an expert Radiologist at the time of the contrast enhanced imaging before TACE and during angiography. The efficacy of DEB-TACE was evaluated with dynamic CT or MRI performed four weeks after the treatment and the modified Response Evaluation Criteria In Solid Tumors (mRECIST) were used to classify the radiological response in complete (CR), partial (PR), stable disease (SD), or progressive disease (PD) (25). Adverse events to TACE were also registered.

HIF-1α and VEGF assay

Ten millilitres of venous blood were collected from each patient, 5 mL of which were used for serum separation. Samples were preserved at − 20 °C till the assay of biochemical markers.

VEGF (pg/mL) and HIF-1α (ng/mL) were determined on serum by using specific ELISA kits (Human VEGF-A Platinum ELISA, Affymetrix eBioscience; ELISA kit for HIF-1α, Cloud-Clone Corp.). Levels of VEGF-A were determined in 149 patients, while 96 patients had HIF-1α levels measured.

Statistical analysis

Quantitative variables were reported as median and interquartile range (IQR), while categorical variables as absolute frequency and percentage. Mann-Whitney test, Wilcoxon matched-pairs signed rank test, chi-square test or Fischer's exact test were used in the comparison between groups, as appropriate. The Spearman correlation coefficient was calculated in order to establish correlations between quantitative variables.

Survivals were expressed as median and 95% confidence interval (CI). Overall survival (OS) was calculated from the date of TACE to the date of death for any reason, last follow-up evaluation, or data censoring (1st June 2019). The prognostic cut-off of the markers was established using the ROC curve method, taking as threshold value the one with maximal sensitivity and specificity (Youden J test). Survival curves were estimated with the Kaplan-Meier method and the difference between curves was assessed by the log-rank test. Multivariate Cox analysis was used to identify independent prognostic predictors, including in the model only variables significantly or borderline ($p \leq 0.1$) associated with survival at univariate analysis. The p value (two-tail) was considered statistically significant when <0.05. IBM SPSS Statistics (Version 25.0, IBM Corp. Armonk, NY, USA) and GraphPad Prism (version 8.3.1, GraphPad Software, La Jolla, CA, USA) were used for all the calculations in this study.

RESULTS

Baseline characteristics

Baseline characteristics are showed in Table 1. Patients were predominantly males (79.8%), with a median age of 69 years (IQR, 63-75). The majority of patients had a virus-related liver disease (HBV or HCV in 54.6% of patients, HBV/HCV + other causes in 4.3% of patients). Liver function was preserved (Child-Pugh class A) in 79.8% of patients. Tumors were mostly multifocal (71.2%) and approximately half of the patients (53.4%) had a major liver lesion larger than 3 cm. In half of the cases (51.5%), TACE was performed in patients with intermediate stage tumors. Remaining patients were treated with TACE in very early/early stages, and no cases of advanced tumors were present in this cohort.

TACE (c-TACE in 47.2% and DEB-TACE in 52.8%) proved to be an effective treatment with a disease control rate (CR + PR + SD) of 93.9% (153 patients) and an objective response rate (CR+PR) of 78.0%

(127 patients). In addition, it was also a safe procedure with adverse events in 19.6% of cases, in most instances mild and easily manageable (post-embolic syndrome in 9.8% and abdominal pain in 6.8%). Liver abscess, pancreatitis and liver decompensation were rarely registered (0.6% in each case).

Table 1. Baseline characteristics

Variables	Study population (n=163)
Sex - males	130 (79.8)
Age	69 (63-75)
Etiology	
Viral	89 (54.6)
Not viral	67 (41.1)
Viral + other	7 (4.3)
Child-Pugh class A	130 (79.8)
Multifocal tumors	116 (71.2)
Size (cm)	
<3	76 (46.6)
3-5	53 (32.5)
>5	34 (20.9)
AFP (ng/mL)	15.0 (5.5-65.2)
Vascularization grade	
Low	84 (51.5)
High	79 (48.5)
BCLC stage	
0-A	79 (48.5)
B	84 (51.5)
Previously treated with TACE	93 (57.1)
Type of TACE	
c-TACE	77 (47.2)
DEB-TACE	86 (52.8)
Radiological response (mRECIST)	
CR	49 (30.1)
PR	78 (47.9)
SD	26 (15.9)
PD	10 (6.1)
Adverse events	
No	131 (80.4)
Post-embolic syndrome	16 (9.8)
Abdominal pain	11 (6.8)
Cholecystitis	2 (1.2)
Liver abscess	1 (0.6)
Pancreatitis	1 (0.6)
Liver decompensation	1 (0.6)

Data are presented either as median and interquartile range or number and percentage.
Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; c-TACE, conventional transarterial chemoembolization; DEB-TACE, drug-eluting beads transarterial chemoembolization; mRECIST, modified Response Criteria In Solid Tumors; CR, completer response; PR, partial response; SD, stable disease; PD, progressive disease.

VEGF and HIF-1α levels

Significantly higher levels of VEGF were demonstrated at t_1 (278.6 pg/mL, IQR 95.0-576.6) compared to t_0 (264 pg/mL, IQR 78.7-450.8; p<0.0001) (Figure 1a). On the contrary, no statistically significant differences were found between HIF-1α levels at t_0 and t_1 (0.25 ng/mL [IQR 0.11-0.49] vs. 0.23 ng/mL [IQR 0.12-0.38]; p=0.37) (Figure 1b).

A positive correlation was demonstrated between HIF-1α and VEGF levels at t_0 (r=0.47, 95% CI 0.28-0.62; p<0.0001) and at t_1 (r=0.43, 95% CI 0.23-0.58; p<0.0001) (Figure 2a and 2b). In addition, VEGF levels at t_0 were positively correlated with t_1 levels (r=0.86, 95% CI 0.80-0.89; p<0.0001) and HIF-1α levels at t_0 were positively correlated with HIF-1α levels at t_1 (r=0.75, 95% CI 0.64-0.83; p<0.0001) (Figure 2c and 2d).

No statistically significant differences in VEGF levels were demonstrated at t_0 and at t_1 in patients naïve to TACE compared to experienced patients (p=0.11 and p=0.73, respectively). The lack of difference between these two groups of patients was also confirmed for HIF-1α in the two time points (p=0.17 and p=0.70, respectively).

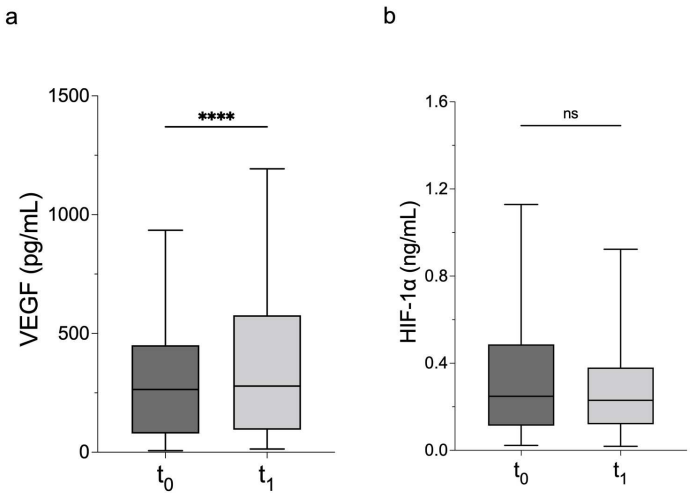

Figure 1. Comparison of VEGF and HIF-1α levels before (t_0) and after (t_1) TACE. (**a**) VEGF levels were significantly higher after TACE compared to the levels measured before the treatment. (**b**) No significant differences were demonstrated in HIF-1α levels before and after TACE.

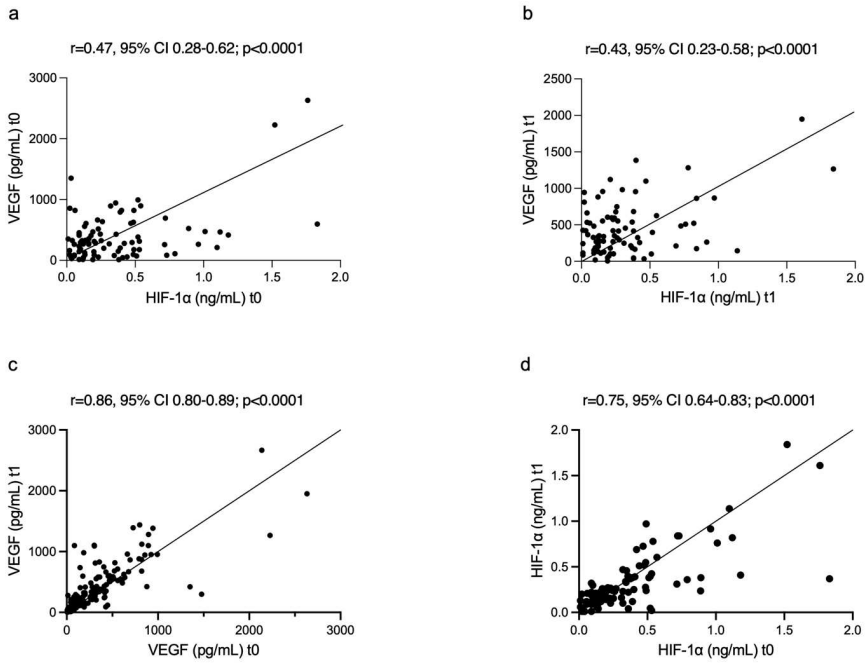

Figure 2. Correlations between VEGF and HIF-1α levels. VEGF levels were positively correlated with HIF-1α levels at t_0 (**a**) and at t_1 (**b**). VEGF levels at t_0 and t_1 (**c**), and HIF-1α levels at t_0 and t_1 (**d**) were also positively correlated.

The objective response rate after TACE was 78.0% (127 patients). VEGF levels were associated with radiological response, as patients with SD and PD showed higher levels of the marker (Figure 3a). At t_0, patients without radiological response to TACE had significantly higher levels of VEGF (406.6 pg/mL, IQR 181.4-558.6) compared to patients with CR or PR (200.0 pg/mL, IQR 58.9-415.8; p=0.006). The same result was obtained when VEGF levels were considered at t_1, with SD or PD patients having a significantly higher levels of the marker compared to responders to treatment (425.9 pg/mL [IQR 245.2-808.3] vs. 257.3 pg/mL [IQR 68.5-528.6]; p=0.003). Both in responders and in non-responders, there was an increase in VEGF compared to the levels measured before treatment (p=0.001 and p=0.005, respectively), with a slightly higher relative increase in patients with CR and PR (Figure 3a). In responders, HIF-1α levels decreased after TACE, although this difference not being statistically significant (0.27 ng/mL [IQR 0.12-0.47] vs. 0.23 ng/mL [IQR 0.12-0.38]; p=0.30). By contrast, HIF-1α levels increased after the treatment in non-responders, but also

in this case the difference is not statistically significant (0.19 ng/mL [IQR 0.09-0.67] vs. 0.26 ng/mL [IQR 0.11-0.42]; p=0.95) (Figure 3b).

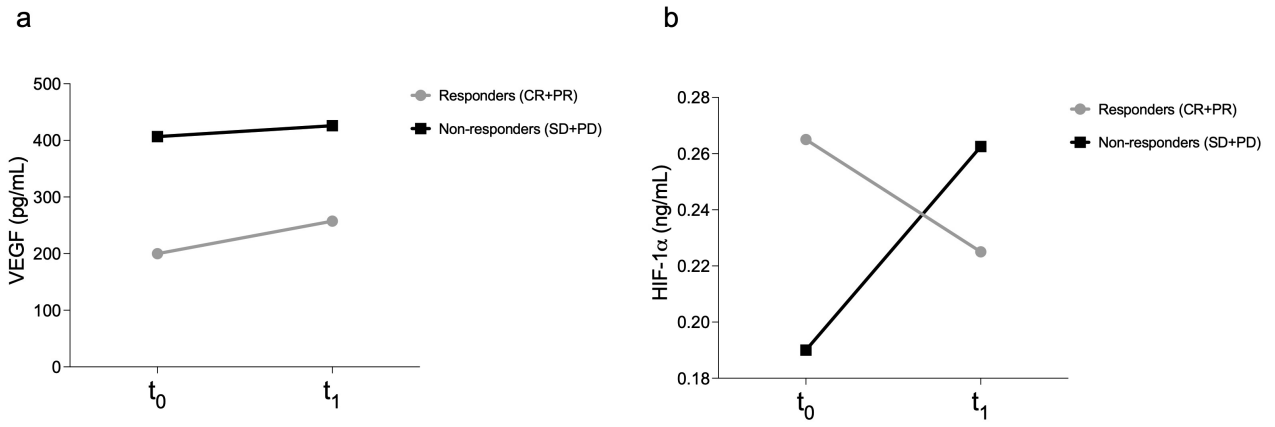

Figure 3. VEGF (**a**) and HIF-1α (**b**) levels in responders (CR+PR) and non-responders (SD+PD) at t_0 and t_1.

With the ROC curve method, the prognostic cut-off for VEGF able to maximizing sensitivity and specificity was identified at a value of 177 pg/mL. The probability of being refractory to TACE was higher in patients with VEGF levels above this cut-off (21.2% vs. 78.8%; p=0.009) (Table 2). The same was demonstrated considering VEGF levels after TACE, with a cut-off of 102 pg/mL (established with the ROC curve method). Even in this case, the probability having SD or PD after TACE was higher in patients with the marker above the cut-off (6.1% vs. 93.9%; p=0.003).

Both VEGF and HIF-1α at t_0 were higher in patients with an adverse event after TACE compared with patients with no adverse events (p<0.0001 and p=0.002, respectively). Also at t_1 the two markers were higher in patients with adverse events (p<0.0001 for VEGF and p=0.0008 for HIF-1α).

Table 2. Radiological response to TACE according to VEGF levels.

VEGF t_0 (n=149)			
	<177 pg/mL	≥177 pg/mL	p
CR+PR	55 (47.4)	61 (52.6)	0.009
SD+PD	7 (21.2)	26 (78.8)	
VEGF t_1 (n=149)			
	<102 pg/mL	≥102 pg/mL	p
CR+PR	37 (31.9)	79 (68.1)	0.003
SD+PD	2 (6.1)	31 (93.9)	

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

VEGF levels at t_0 were associated with the number of liver lesions (Figure 4a), with higher values in multifocal (303.0 pg/mL, IQR 91.-557.8) compared to monofocal HCC (116.6 pg/mL, IQR 18.7-295.0; p<0.0001), but not with tumor size (Figure 4b). Intermediate stage tumors had higher levels of VEGF compared to very early and early stage (308.0 pg/mL [IQR 104.0-586.9] vs. 185.1 pg/mL [IQR 46.7-328.3], respectively; p=0.006) (Figure 4c). Although patients with highly vascularized tumors had higher levels of VEGF (297.9 pg/mL, IQR 86.3-472.4) than patients with a low vascularization grade (203.0, IQR 74.6-418.7), the difference was not statistically significant (p=0.12) (Figure 4d). VEGF levels were not different according to the other variables considered (sex, age, etiology, Child-Pugh class). None of the variables considered showed a correlation with HIF-1α (Figure 4e-h). Nevertheless, despite not statistically significant, HIF-1α levels were higher in patients with multifocal tumors, with lesion ≥ 3 cm in diameter and in BCLC B patients.

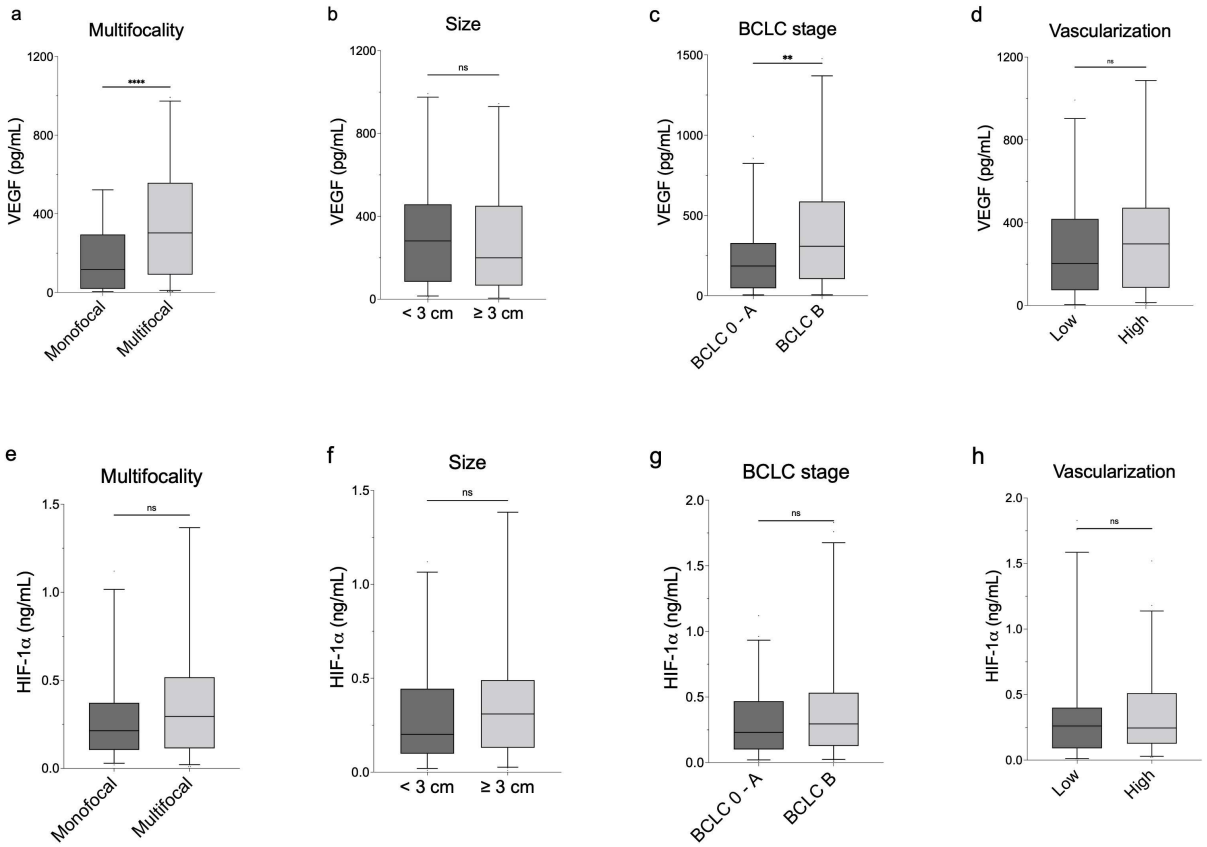

Figure 4. Differential levels of VEGF and HIF-1α according to tumor characteristics. Patients with multifocal tumors had significantly higher levels of VEGF compared to patients with monofocal HCC (**a**), as patients with intermediate stage (BCLC B) compared to patients with earlier stages (**c**). No significant differences were registered according to size of liver lesions (**b**) and vascularization grade (**d**). HIF-1α levels were not significantly different according to tumor number, diameter, stage and vascularization grade (**e**, **f**, **g** and **h**).

Survival analysis

The median OS in the whole patient population was 25.0 months (95% CI 20.6-29.4), with a 3-year survival rate of 33.2%.

According to the cut-off established with the ROC curve method (177 pg/mL), VEGF was not associated with survival. The median OS for patients with VEGF <177 pg/mL was 24.0 months (95% CI 18.9-29.1) compared to 23.0 months (95% CI 15.1-30.9) in patients with VEGF ≥177 pg/mL (p=0.34) (Figure 5a). On the contrary, HIF-1α at t_0 at the identified cut-off of 0.49 ng/mL proved to be a useful prognostic predictor. Patients with levels of HIF-1α below the cut-off had a statistically significant longer OS compared to patients with marker levels above the cut-off (median OS 28.0 months [95% CI 19.7-36.3] vs. 17.0 months [95% CI 11.1-22.9], respectively; p=0.01) (Figure 5b).

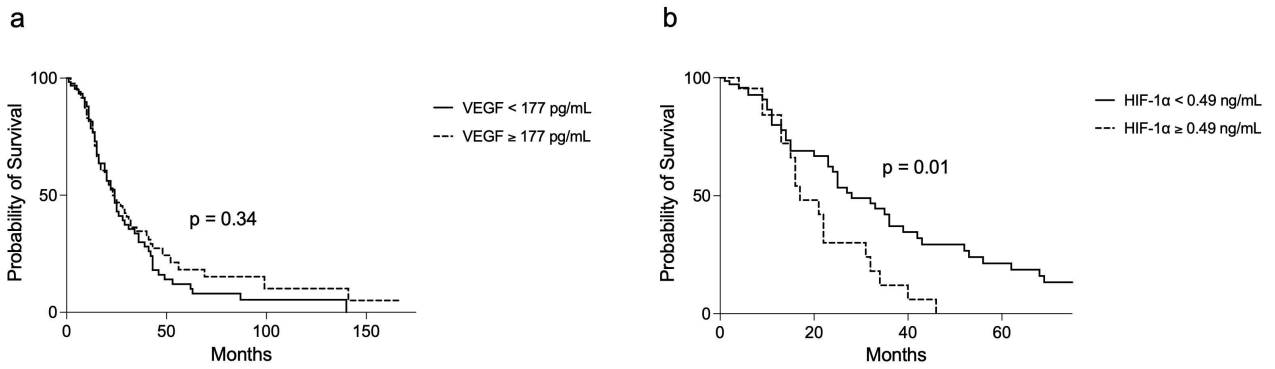

Figure 5. Kaplan-Meier survival curves according to the level of VEGF and HIF-1α. (**a**) No statistically significant differences in survival were demonstrated between patients with levels of VEGF below and above the cut-off (177 pg/mL). (**b**) Patients with levels of HIF-1α below the identified cut-off (0.49 ng/mL) demonstrated significantly longer survival.

The univariate analysis demonstrated that, beyond high HIF-1α level, factors associated with an increased mortality risk were Child-Pugh class B (HR=2.28, 95% CI 1.46-3.56), multifocality (HR=1.60, 95% CI 1.03-2.46), larger tumors (HR=1.73 [95% CI 1.13-2.66] for 3-5 cm nodules and HR=1.66 [95% CI 1.03-2.67] for >5 cm nodules), BCLC-B stage (HR=2.02, 95% CI 1.35-3.03) and high vascularization grade (HR=1.50, 95% CI 1.02-2.21). There were no differences in survival related to type of TACE (c-

TACE vs. DEB-TACE). After adjustment for confounders, HIF-1α was singled out at multivariate Cox analysis as an independent prognostic factor (HR=2.03, 95% CI 1.05-3.94), together with Child-Pugh class (HR=2.97, 95% CI 1.55-5.68) and BCLC stage (HR=1.98, 95% CI 1.04-3.77) (Table 3).

Table 3. Univariate and multivariate Cox models for factors independently associated with survival.

Variable	**Univariate analysis**		**Multivariate analysis**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
Sex				
Male Female	Ref 0.80 (0.49-1.27)	- 0.33	-	-
Age (years)	1.01 (0.99-1.03)	0.21	-	-
Etiology				
Viral Not viral Viral + other	Ref 0.97 (0.66-1.42) 1.28 (0.55-2.95)	- 0.87 0.57	-	-
Child-Pugh class				
A B	Ref 2.28 (1.46-3.56)	- 0.0003	Ref 2.97 (1.55-5.68)	- 0.001
Multifocality				
Monofocal Multifocal	Ref 1.60 (1.03-2.46)	- 0.03	-[a]	-[a]
Diameter (cm)				
<3 cm 3-5 cm >5 cm	Ref 1.73 (1.13-2.66) 1.66 (1.03-2.67)	- 0.01 0.04	-[a]	-[a]
BCLC stage				
0-A B	Ref 2.02 (1.35-3.03)	- 0.001	Ref 1.98 (1.04-3.77)	- 0.04
AFP (ng/mL)				
≤200 >200	Ref 1.19 (0.70-2.03)	- 0.53	-	-
Vascularization				
Low High	Ref 1.50 (1.02-2.21)	- 0.04	Ref 0.97 (0.49-1.90)	- 0.92
Type of TACE				
c-TACE DEB-TACE	Ref 0.86 (0.59-1.27)	- 0.45	-	-
Radiological response (mRECIST)				
CR+PR SD+PD	Ref 1.07 (0.70-1.64)	- 0.76	-	-
VEGF (pg/mL)				
<177 ≥177	Ref 0.83 (0.57-1.22)	- 0.35	-	-
HIF-1α (ng/mL)				
<0.49 ≥0.49	Ref 2.13 (1.17-3.86)	- 0.01	Ref 2.03 (1.05-3.94)	- 0.04

a) not included in the multivariate model in order to avoid collinearity with BCLC stage.

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; c-TACE, conventional transarterial chemoembolization; DEB-TACE, drug-eluting beads transarterial chemoembolization; mRECIST, modified Response Criteria In Solid Tumors; CR, completer response; PR, partial response; SD, stable disease; PD, progressive disease; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia inducible factor 1α.

DISCUSSION

Angiogenesis is of fundamental importance in the development and progression of HCC (2,3). Considering their key role in the angiogenic process, VEGF and HIF-1α have been evaluated as potential prognostic biomarkers, and several studies in the literature demonstrate their usefulness in patients managed with different treatments, including TACE (7–18,22,26). In this study, we provide further evidence on the usefulness of these biomarkers in stratifying prognosis of HCC patients treated with TACE.

Firstly, a statistically significant increase of VEGF levels was observed after TACE compared to the levels measured before treatment. By contrast, no statistically significant differences were demonstrated between t_0 and t_1 levels of HIF-1α. Some previous studies investigated the dynamic changes of these two molecules in serum of patients treated with TACE, showing different results. *Jia et al.* (26) found that the day after TACE both markers reach their peak value and then decline one week after the procedure, although remaining significantly higher than before TACE. According to the paper of *Li et al.* (27), VEGF levels increase significantly the day after treatment and then decrease gradually on the third and seventh day post-TACE. Other studies report a slower increase in the VEGF levels after TACE (28,29). The challenge in many of these studies, including ours, is to measure dynamic changes of HIF-1α and VEGF values in serum following TACE, as these biomarkers are evanescent (19). This may contribute to explain the absence of differences between HIF-1α before TACE and the same marker measured one month after TACE-induced ischemia. However, similarly to previous studies (22,26), we confirmed the sustained increase over time of VEGF after TACE, and the increase of this latter without a correspondent variation in HIF-1α levels after TACE was an unexpected finding, considering that the two molecules are related at a molecular level in

the stimulation of angiogenesis (HIF-1α is a transcription factor of VEGF) (3). Indeed, a strong positive correlation between these two molecules has been demonstrated also in this study.

Another interesting finding was that, although the relative increase of VEGF in non-responders was lower compared to responders to treatment, patients who achieved a complete or a partial response had significantly lower levels of VEGF both before and after TACE. This result, confirming previous studies (21), seems to suggest that treatment is less effective in patients with more activated neoangiogenic process, and supports other reports in the literature showing that increased VEGF levels has an important role in the development of collateral blood vessels nourishing the surviving residual tumor tissue (30). The results achieved with HIF-1α also confirm this hypothesis. Although not demonstrating a statistically significant difference between t_0 and t_1 levels both in responders and non-responders, a decrease in the level of this transcription factor in responders and an increase in non-responders was found. Considered that patients with lower levels of VEGF were more likely to have a radiological response (CR + PR), this marker could be considered as a useful predictor of the response to TACE.

VEGF circulating levels were higher in patients with multifocal tumors and in BCLC B stage, while no differences were demonstrated according to tumor size and vascularization grade. As far as HIF-1α was evaluated in association with tumor burden, no statistically significant differences were found. Nevertheless, multifocality, larger nodule size and intermediate stage has higher levels of the marker. These results suggest that angiogenesis is particularly activated in tumors with more aggressive biology, and this was further confirmed by the association of neoangiogenic molecules with survival. Differently from what has been previously demonstrated in patients treated with TACE (21,22), the prognostic role of VEGF was not confirmed in this study. Nevertheless, HIF-1α levels above the identified cut-off were predictive of poorer survival and this variable maintained its independent prognostic role at the Cox multivariate analysis. HIF-1α expression has been

repeatedly associated with prognosis of HCC patients, and a metanalysis demonstrated that its overexpression correlated with poor OS and disease-free survival (17). However, all these studies considered the tissue expression of HIF-1α and, in the majority of cases, evaluated its ability to predict prognosis after liver resection (7,8,11–17). Our results demonstrated that, also when evaluated as a circulating marker in patients treated with TACE, HIF-1α might provide valuable prognostic informations.

Among the limitations of this study, the most relevant one is its retrospective design that might have introduced unintended biases. Moreover, this study included both patients who were naïve to the treatment, at their first TACE, and patients who had already undergone a number of procedures. This could have introduced a selection bias, but no difference in the baseline levels of any of the markers considered between naïve and experienced patients was observed. Therefore, we can consider that, if any difference between naïve and experienced patients exists, this is minimal.

In conclusion, in this study we confirmed that TACE-induced ischemia is able to activate neoangiogenesis signalling pathways, as demonstrated by the increase of VEGF after treatment. Both VEGF and HIF-1α could be considered useful circulating prognostic biomarkers in patients with HCC treated with TACE. In particular, VEGF levels are increased in patients with greater tumor burden (intermediate stage multifocal tumor) and could be useful for predicting response to TACE, since patients with higher levels are more frequently non-responders to treatment. Moreover, HIF-1α was an accurate predictor of patient survival. With the aim of refining patient prognosis, the evaluation of these biomarkers could be useful, but additional studies, possibly prospective, are needed to confirm these encouraging results.

REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57–70.
2. Zhu AX, Duda DG, Sahani D V, Jain RK. HCC and angiogenesis: possible targets and future directions. Nat. Rev. Clin. Oncol. 2011;8:292–301.
3. Muto J, Shirabe K, Sugimachi K, Maehara Y. Review of angiogenesis in hepatocellular carcinoma. Hepatol. Res. 2015;45:1–9.
4. Matsui O, Kobayashi S, Sanada J, Kouda W, Ryu Y, Kozaka K, et al. Hepatocelluar nodules in liver cirrhosis: hemodynamic evaluation (angiography-assisted CT) with special reference to multi-step hepatocarcinogenesis. Abdom. Imaging. 2011;36:264–272.
5. Wu X-Z, Xie G-R, Chen D. Hypoxia and hepatocellular carcinoma: The therapeutic target for hepatocellular carcinoma. J. Gastroenterol. Hepatol. 2007;22:1178–1182.
6. Villa E, Critelli R, Lei B, Marzocchi G, Camma C, Giannelli G, et al. Neoangiogenesis-related genes are hallmarks of fast-growing hepatocellular carcinomas and worst survival. Results from a prospective study. Gut. 2016;65:861–869.
7. Liu L, Zhu X-D, Wang W-Q, Shen Y, Qin Y, Ren Z-G, et al. Activation of beta-catenin by hypoxia in hepatocellular carcinoma contributes to enhanced metastatic potential and poor prognosis. Clin. cancer Res. an Off. J. Am. Assoc. Cancer Res. 2010;16:2740–2750.
8. Huang G-W, Yang L-Y, Lu W-Q. Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival. World J. Gastroenterol. 2005;11:1705–1708.
9. Zhan P, Qian Q, Yu L-K. Serum VEGF level is associated with the outcome of patients with hepatocellular carcinoma: a meta-analysis. Hepatobiliary Surg. Nutr. 2013;2:209–215.
10. Llovet JM, Pena CEA, Lathia CD, Shan M, Meinhardt G, Bruix J. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. Clin. Cancer Res. 2012;18:2290–2300.
11. Xia L, Mo P, Huang W, Zhang L, Wang Y, Zhu H, et al. The TNF-alpha/ROS/HIF-1-induced upregulation of FoxMI expression promotes HCC proliferation and resistance to apoptosis. Carcinogenesis. 2012;33:2250–2259.
12. Wada H, Nagano H, Yamamoto H, Yang Y, Kondo M, Ota H, et al. Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1 alpha. Liver Int. 2006;26:414–423.
13. Dai C-X, Gao Q, Qiu S-J, Ju M-J, Cai M-Y, Xu Y-F, et al. Hypoxia-inducible factor-1 alpha, in association with inflammation, angiogenesis and MYC, is a critical prognostic factor in patients with HCC after surgery. BMC Cancer. 2009;9:418.
14. Wang D, Zhang X, Lu Y, Wang X, Zhu L. Hypoxia inducible factor 1alpha in hepatocellular carcinoma with cirrhosis: Association with prognosis. Pathol. Res. Pract. 2018;214:1987–1992.
15. Yang S-L, Liu L-P, Jiang J-X, Xiong Z-F, He Q-J, Wu C. The correlation of expression levels of HIF-1alpha and HIF-2alpha in hepatocellular carcinoma with capsular invasion, portal vein tumor thrombi and patients' clinical outcome. Jpn. J. Clin. Oncol. 2014;44:159–167.
16. Liu L-P, Hu B-G, Ye C, Ho RLK, Chen GG, Lai PBS. HBx mutants differentially affect the activation of hypoxia-inducible factor-1alpha in hepatocellular carcinoma. Br. J. Cancer. 2014;110:1066–1073.
17. Zheng S-S, Chen X-H, Yin X, Zhang B-H. Prognostic significance of HIF-1alpha expression in hepatocellular carcinoma: a meta-analysis. PLoS One. 2013;8:e65753.
18. Deli G, Jin C-H, Mu R, Yang S, Liang Y, Chen D, et al. Immunohistochemical assessment of angiogenesis in hepatocellular carcinoma and surrounding cirrhotic liver tissues. World J. Gastroenterol. 2005;11:960–963.
19. Petrillo M, Patella F, Pesapane F, Suter MB, Ierardi AM, Angileri SA, et al. Hypoxia and tumor angiogenesis in the era of hepatocellular carcinoma transarterial loco-regional treatments. Futur. Oncol. 2018;
20. Liu K, Min X-L, Peng J, Yang K, Yang L, Zhang X-M. The Changes of HIF-1α and VEGF Expression After TACE in Patients With Hepatocellular Carcinoma. J. Clin. Med. Res. 2016;8:297–302.

21. Li Z, Xue T-Q, Chen X-Y. Predictive values of serum VEGF and CRP levels combined with contrast enhanced MRI in hepatocellular carcinoma patients after TACE. Am. J. Cancer Res. 2016;6:2375–2385.

22. Sergio A, Cristofori C, Cardin R, Pivetta G, Ragazzi R, Baldan A, et al. Transcatheter arterial chemoembolization (TACE) in hepatocellular carcinoma (HCC): the role of angiogenesis and invasiveness. Am. J. Gastroenterol. 2008;103:914–921.

23. Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J. Hepatol. 2001;35:421–430.

24. Llovet JM, Ducreux M, Lencioni R, Di Bisceglie AM, Galle PR, Dufour JF, et al. EASL-EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2012;56:908–943.

25. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. Semin. Liver Dis. 2010;30:52–60.

26. Jia Z, Jiang G, Feng Y. Serum HIF-1alpha and VEGF levels pre- and post-TACE in patients with primary liver cancer. Chinese Med. Sci. J. = Chung-kuo i hsueh k'o hsueh tsa chih. 2011;26:158–162.

27. Li X, Feng G-S, Zheng C-S, Zhuo C-K, Liu X. Expression of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma and effect of transcatheter arterial chemoembolization therapy on plasma vascular endothelial growth factor level. World J. Gastroenterol. 2004;10:2878–2882.

28. Suzuki H, Mori M, Kawaguchi C, Adachi M, Miura S, Ishii H. Serum vascular endothelial growth factor in the course of transcatheter arterial embolization of hepatocellular carcinoma. Int. J. Oncol. 1999;14:1087–1090.

29. Chao Y, Wu C-Y, Kuo C-Y, Wang JP, Luo J-C, Kao C-H, et al. Cytokines are associated with postembolization fever and survival in hepatocellular carcinoma patients receiving transcatheter arterial chemoembolization. Hepatol. Int. 2013;7:883–892.

30. Li X, Feng G-S, Zheng C-S, Zhuo C-K, Liu X. Influence of transarterial chemoembolization on angiogenesis and expression of vascular endothelial growth factor and basic fibroblast growth factor in rat with Walker-256 transplanted hepatoma: an experimental study. World J. Gastroenterol. 2003;9:2445–2449.

CHAPTER 7

Circulating microRNA-21 and microRNA-122 as prognostic biomarkers in hepatocellular carcinoma patients treated with transarterial chemoembolization

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ABSTRACT

Background: MicroRNAs (miRNAs) have been proposed as biomarkers in hepatocellular carcinoma (HCC). We aim at evaluating miR-21 and miR-122 in HCC patients treated with drug-eluting beads transarterial chemoembolization (DEB-TACE) as prognostic biomarkers and investigating their correlation with hypoxia inducible factor-1α (HIF-1α) serum levels.

Methods: In this retrospective study, 12 healthy subjects, 28 cirrhotics, and 54 HCC patients (tested before and four weeks after DEB-TACE) were included. Whole blood miR-21 and miR-122 levels were measured by quantitative real time (qRT)-PCR, while serum HIF-1α was assessed by an enzyme-linked immunosorbent assay (ELISA) test.

Results: The highest level of miR-21 was found in cirrhotics, while HCC patients had the highest level of miR-122 (which was even higher in "viral" HCC, p=0.006). miR-21 ratio (after/before DEB-TACE) and miR-122 below their respective cut-offs identified patients with longer progression-free survival (p=0.0002 and p=0.02, respectively). The combined assessment of alpha-fetoprotein and miR-21 ratio, both independent prognostic predictors, identified early progressors among patients with complete or partial radiological response. miR-21 levels positively correlated with HIF-1α before (p=0.045) and after DEB-TACE (p=0.035).

Conclusions: miR-21 ratio and miR-122 are useful prognostic markers after DEB-TACE. miR-21 correlates with HIF-1α and probably has a role in modulating angiogenesis in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most relevant cause of cancer-related death worldwide (1). Among all the biomarkers proposed for HCC, only alpha-fetoprotein (AFP) has a worldwide clinical application, despite not being completely satisfactory (2). As a consequence, there is a continuous search for new reliable biomarkers for the management of HCC patients, in particular in the predictive and prognostic settings.

MicroRNAs (miRNAs) are small non-coding single-stranded RNAs (≈22 nucleotides long), extensively involved in the regulation of gene expression. In carcinogenesis, they act on major tumor-related genes, either as oncogenes or as onco-suppressors (3). Data on their role as biomarkers in HCC have been produced especially by Oriental authors in non-Caucasian populations and, in particular, miR-21 and miR-122 seem to be very promising. miR-21 is an onco-miRNA, detectable at high levels in tissue (4–6) and serum (7–9) of HCC patients. High levels of miR-21 after liver resection are predictive of disease-progression (6) and poor prognosis (5,7) while, in patients treated with loco-regional therapies, its role as a prognostic predictor is less clear (10–12). miR-122, on the other hand, the most abundant liver-specific miRNA (13), acts as a tumor-suppressor reducing cancer cell proliferation, promoting apoptosis, and modulating drug resistance, invasion and metastasis (14). Despite its down-regulation in HCC cells, miR-122 levels have been reported to be elevated in the serum of HCC patients compared to healthy controls (15,16), while its potential role in predicting HCC prognosis is still debatable.

Neoangiogenesis is one of the most important molecular pathways involved in HCC progression. miR-21 proved to be a regulator of angiogenesis in prostate, lung, and colorectal cancers, modulating hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) (17–19). miR-122, as recently demonstrated, targets HIF-1α in diet-induced steatohepatitis (20), with some data suggesting an interplay between the two molecules also in HCC (21). Moreover, a very

recent paper found a role of miR-122 in enhancing liver ischemia tolerance in a murine model of hepatic ischemia-reperfusion injury through its induction by HIF-1α (22).

In this study, we aimed at comparing the levels of circulating miR-21 and miR-122 in healthy subjects, cirrhotics, and HCC patients and at evaluating the role of these miRNAs as predictors of progression-free survival (PFS) in a group of Caucasian HCC patients treated with drug-eluting beads transarterial chemoembolization (DEB-TACE). Moreover, we assessed the correlation of miR-21 and miR-122 with the circulating transcription factor HIF-1α before and after the treatment, which is able to profoundly stimulate angiogenesis by the induction of tumor ischemia (23).

MATERIALS AND METHODS

Patients

In this study, blood samples from 12 healthy volunteers, 28 cirrhotics, and 54 HCC patients consecutively collected between July 2019 and April 2020, were retrospectively evaluated. Each subject provided written informed consent to participate to the study, which was conducted in accordance to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova University Hospital (protocol code 46093, 12 August 2016).

Blood samples from cirrhotics were obtained in the outpatient's clinic of the Gastroenterology Unit of the Padova University Hospital from patients with chronic liver disease fulfilling the following criteria: International Normalized Ratio (INR) >1.2, White Blood Cell $<4.4 \times 10^9$/L, Platelets $<150 \times 10^9$/L (at least two out of three), and abdomen ultrasonography (US) showing findings compatible with cirrhosis. All cirrhotic patients were regularly surveilled for the development of HCC with US every six months, and the presence of HCC was ruled out with dynamic computed tomography (CT) or magnetic resonance imaging (MRI) at the time of study entry.

HCC patients included in the study, diagnosed according to guidelines (24,25), were admitted to Gastroenterology Unit of Padova University Hospital for treatment (DEB-TACE). In all patients, chemoembolization was performed using doxorubicin-loaded drug-eluting beads after super selective catheterization of the tumor feeding artery. In this subgroup, two blood samples were collected: the first immediately before DEB-TACE (t_0) and the second four weeks after the procedure (t_1), at the time of the control imaging performed in order to evaluate the efficacy of treatment.

The following clinical and tumor-related variables were recorded: sex, age, etiology, presence of clinically relevant portal hypertension (CRPH), main serological parameters (total bilirubin, INR, creatinine, albumin and AFP, the latter both at t_0 and at t_1 in patients with HCC), Child-Pugh class, Model for End-Stage Liver Disease (MELD) score and Eastern Cooperative Oncology Group performance status (ECOG-PS). CRPH was defined as presence of splenomegaly, esophageal varices or ascites, and platelets count $<100 \times 10^9$/L (26). In HCC patients, number and size of liver nodules, presence of macrovascular invasion (MVI) and/or extrahepatic spread (EHS), evaluated before DEB-TACE with dynamic CT or MRI, were recorded. Patients were staged according to the Barcelona Clinic Liver Cancer (BCLC) system. The efficacy of DEB-TACE was evaluated with dynamic CT or MRI performed four weeks after the treatment and the Modified Response Evaluation Criteria In Solid Tumors (mRECIST) (27) were used to classify the radiological response in complete (CR), partial (PR), stable disease (SD), or progressive disease (PD).

RNA Isolation and miRNAs Analysis

Ten milliliters of venous blood were collected from each patient: 5 mL of whole blood were used for RNA extraction, and the other 5 mL for serum and plasma separation. Samples were preserved at −80 °C till the assays.

Whole blood samples were used for the determination of miRNAs. Total RNA was extracted from 200 µL of whole blood using the Quick-RNA™ Whole Blood extraction kit (Zymo Research, Irvine,

CA, USA). Extraction efficiency was checked through adding synthetic oligonucleotides (UniSp2, UniSp4, UniSp5) at recommended concentrations. Reverse transcription for cDNA synthesis was performed using the miRCURY LNA RT kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription efficiency was checked through adding synthetic oligonucleotides (UniSp6). The expression of miRNAs was evaluated by quantitative real time (qRT)-PCR analysis (miRCURY LNA miRNA PCR Assays and PCR Panels, Qiagen, GmbH, Hilden, Germany), according to the manufacturer's instructions, on a PRISM 7900HT system (Applied Biosystems, Foster City, CA, USA) with miR-93, miR-103a, miR-425 as internal reference controls for normalization (levels of these control miRNAs are shown in Supplementary Table 1). Each miRNA assay was replicated twice. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ (fold-change [fc]) method, using healthy subjects as the reference group for the normalization.

HIF-1α Assay

A commercial ELISA kit (Cloud-Clone Corp., Katy, TX, USA) was used to determine HIF-1α in the serum samples from cirrhotics and HCC, according to the manufacturer's instructions. The amount of HIF-1α (ng/mL) was derived by interpolation of samples absorbance on the calibration curves plotted with calibrators. Briefly, plates precoated with an antibody specific to HIF-1α were incubated with 100 μL of serum. HIF-1α was revealed by the addition of Detection Reagent A and B at 450 nm.

Statistical Analysis

Quantitative variables were reported as median and interquartile range (IQR), while categorical variables as absolute frequency and percentage. Mann–Whitney and Wilcoxon matched-pairs signed rank tests were used to compare quantitative variables. The comparison between categorical data were performed with χ^2 or Fischer's exact tests. The correlations between continuous variables were established calculating the non-parametric Spearman coefficient.

PFS was calculated from the date of DEB-TACE to tumor progression or death, with data censored on 1st February 2021, and it was expressed as median and IQR. In the definition of the prognostic role of miRNAs, not only their values measured before DEB-TACE (t_0), but also miR-21 and miR-122 ratios, defined as the ratio between t_1 and t_0 levels, were considered as potential biomarkers. The prognostic cut-offs of the markers (miR-21, miR-122 and their ratios; HIF-1α; AFP) were established using the receiver operating characteristic (ROC) curve method, taking as threshold the value with maximal sensitivity and specificity (Youden J test). The Kaplan–Meier method and the log-rank test were used to estimate and compare survival curves. The independent predictors of prognosis were assessed with the Cox multivariate regression analysis, including in the model only the variables significantly or borderline ($p \leq 0.10$) associated with PFS in the univariate test. A p-value (two-tails) <0.05 was considered as significant in this study. IBM SPSS Statistics (Version 25.0, IBM Corp. Armonk, NY, USA) and GraphPad Prism (version 8.3.1, GraphPad Software, La Jolla, CA, USA) were used for all the calculations in this study.

RESULTS

Baseline Characteristics

Baseline characteristics of cirrhotics and HCC patients included in the study are shown in Table 1. Cirrhotics and HCC patients were predominantly males, with similar age. Cirrhotics had mostly an alcohol-related liver disease, while HCV was the most frequent etiology in HCC patients (p=0.07). Compared to HCC group, cirrhotics had more frequently CRPH (92.9% vs. 60.4%; p=0.002) and a worse residual liver function (Child-Pugh A in 46.4% vs. 87.0%, p=0.0002; and median MELD of 13 [IQR, 10–19] vs. 8 [7–11], p<0.0001).

In HCC patients, the median number of liver nodules was 2 (1–4) with a median size of 2.2 cm (1.8–3.6). The majority of patients were classified in BCLC stages A (46.3%) and B (37.0%), and 79.6% of

patients had been previously treated, mostly with a combination of curative and intra-arterial therapies. The disease control rate after DEB-TACE was 81.5% (CR in 44.5%, PR in 29.6% and SD in 7.4% of patients).

Table 1. Baseline characteristics of cirrhotic and HCC patients.

Variable		**Cirrhotics (n = 28)**	**HCC (n = 54)**	**p** †
Males–n (%)		20 (71.4)	44 (81.5)	0.40
Age (years)		63.5 (49.3–72.0)	67.0 (61.8–76.0)	0.13
Cirrhosis—n (%)		28 (100)	48 (88.9)	0.09
Etiology—n (%)	HBV	3 (10.7)	6 (11.1)	0.07
	HCV	4 (14.3)	20 (37.1)	
	Alcohol	16 (57.1)	16 (29.6)	
	Other	5 (17.9)	12 (22.2)	
CRPH—n (%)		26 (92.9)	33 (60.4)	0.002
Child-Pugh A—n (%)		13 (46.4)	47 (87.0)	0.0002
MELD score		13 (10–19)	8 (7–11)	<0.0001
Bilirubin (µmol/L)		23.5 (13.4–68.6)	15.0 (10.0–20.0)	0.006
Albumin (g/dL)		3.5 (2.9–4.0)	4.0 (3.5–4.3)	0.007
INR		1.32 (1.15–1.60)	1.12 (1.09–1.21)	0.0003
Number of nodules			2 (1–4)	
Diameter (cm)			2.2 (1.8–3.6)	
MVI and/or EHS—n (%)			4 (7.5)	
BCLC stage—n (%)	0/A		30 (55.6)	
	B/C		24 (44.4)	
Previous treatments—n (%)	LR/ABL		13 (24.0)	
	TACE		9 (16.7)	
	ABL/LR + TACE		21 (38.9)	
	No		11 (20.4)	
Radiological response	CR		24 (44.5%)	
	PR		16 (29.6%)	
	SD		4 (7.4%)	
	PD		10 (18.5%)	

† Mann–Whitney test, χ^2 test and Fischer's exact test, as appropriate.
Continuous data are expressed as median (interquartile range), while categorical data are presented as absolute frequency (percentage).
Abbreviations: HCC, hepatocellular carcinoma; CRPH, clinically relevant portal hypertension; MELD, Model of End Stage Liver Disease; MELD-Na, Model of End Stage Liver Disease–Sodium; INR, international normalized ratio; PLT, platelets; MVI, macrovascular invasion; EHS, extrahepatic spread; BCLC, Barcelona Clinic Liver Cancer; ABL, ablation; LR, liver resection; TACE, transarterial chemoembolization; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Levels of Circulating MiRNAs

Cirrhotic patients had a median level of miR-21 of 1.72 fc (1.13–2.54), significantly higher compared to healthy volunteers (1.03 fc [0.74–1.15]; p=0.009) and HCC patients (1.28 fc [0.78–1.88]; p=0.047).

In HCC, a statistically significant drop of miR-21 after DEB-TACE was observed (1.02 fc [0.69–1.66]; p=0.03), returning to levels comparable to those of healthy individuals (p=0.76) (Figure 1a).

miR-122 showed a progressive increase from 1.22 fc (0.39–2.17) in controls to 1.63 fc (0.51–2.99) in cirrhotics and 2.34 fc (1.36–4.5) in HCC patients. It was significantly higher in HCC patients compared to controls (p=0.02) and cirrhotics (p=0.04). After TACE a further increase in miR-122 was observed, despite not statistically significant (3.41 fc [1.25–7.72]; p=0.48) (Figure 1b).

In HCC patients, no association between circulating levels of miR-21 and any of the characteristics evaluated (sex, age, etiology, Child-Pugh class, MELD, tumor burden, BCLC stage) was observed, while miR-122 levels were associated only with etiology, being significantly higher in patients with a virus-related liver disease (HCV or HBV) compared to patients with alternative etiologies (2.91 fc [1.62–9.82] and 1.76 fc [0.86–2.86], respectively; p=0.006) (Figure 2). No differences in miR-122 levels were found between HBV and HCV patients (2.82 fc [1.74–11.2] vs. 2.79 fc [1.37–9.77], respectively; p=0.64).

AFP in cirrhotics (3.2 ng/mL [2.3–6.95]) was significantly lower than in HCC at t_0 (6.85 ng/mL [3.05–17.23]; p=0.009) and at t_1 (6.1 ng/mL [3.2–26.7]; p=0.003).

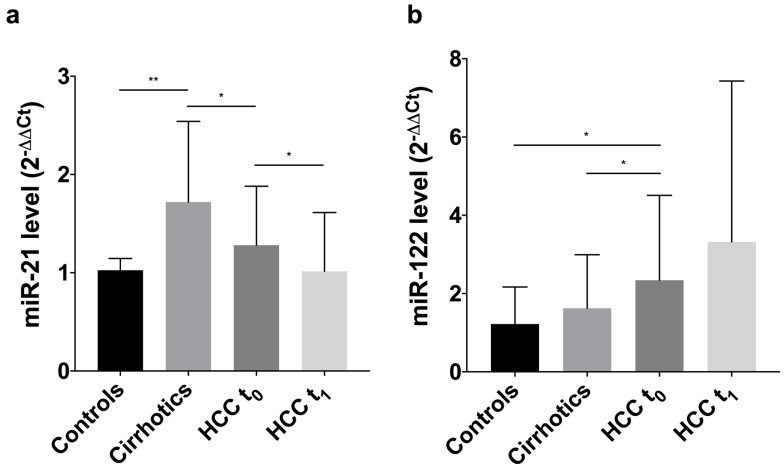


Figure 1. Histograms showing circulating levels of miR-21 and miR-122 in controls, cirrhotics, HCC patients at t_0 and t_1 (representing median with error bar showing the third quartile). (**a**) The median of miR-21 circulating levels is 1.03 fc [0.74–1.15] in controls, 1.72 fc [1.13–2.54] in cirrhotics, and 1.28 fc [0.78–1.88] in patients with HCC at t_0. There is a significant difference in the circulating level between controls and cirrhotics (p = 0.009) and between cirrhotics and HCC patients (p = 0.047). In HCC, the miR-21 levels at t_0 are significantly higher than those measured at t_1 (1.02 fc [0.69–1.66]; p = 0.03); (**b**) the median of miR-122 circulating levels is 1.22 fc [0.39–2.17] in controls, 1.63 fc [0.51–2.99] in cirrhotics, and 2.34 fc [1.36–4.51] in HCC patients at t_0. There is a statistically significant difference in the levels of the

miRNA comparing HCC patients with controls (p = 0.02) and cirrhotics (p = 0.04). In HCC patients, no significant differences are present in t_0 and t_1 levels of miR-122. * $p < 0.05$; ** $p \leq 0.01$

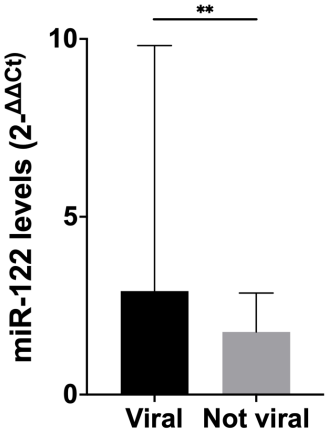


Figure 2. Histograms showing circulating levels of miR-122 according to the etiology of the underlying liver disease (representing median with error bar showing the third quartile). Patients with viral HCC had statistically significantly higher levels of circulating miR-122 compared to patients with alternative etiologies (p = 0.006). The median of miR-122 circulating levels in patients with viral etiology is 2.91 fc [1.62–9.82], a value statistically significant higher compared to the level registered in patients with alternative etiologies (1.76 fc [0.86–2.86]; p = 0.006). ** $p \leq 0.01$

Survival Analysis

HCC patients had a median follow-up of 11.8 months (7.3–16.7) and all except 7 patients were alive at the end of the study. The median PFS was 3.9 months (1.4–8.3).

The ROC curves used to identify the cut-off for miR-21, miR-21 ratio, miR-122, and miR-122 ratio are showed in Supplementary Figure 1. miR-21 quantified before DEB-TACE, at the threshold identified with the ROC curve method (0.73 fc), was not able to discriminate patients according to their PFS (p=0.17). However, patients with miR-21 ratio below its cut-off (1.64 fc) had a statistically significantly longer PFS compared to those with levels above 1.64 fc (median PFS 5.6 months [1.2–10.2] vs 1.4 months [1.1–2.7]; p=0.0002) (Figure 3a).

Unlike miR-21, miR-122 levels measured at t_0 were predictive of PFS: patients with miR-122 below the cut-off (10.22 fc) had a median PFS of 5.6 months (1.4–9.7), significantly longer than the 2.5 months (1.8–3.2) obtained in the comparator group (p=0.02) (Figure 3b). No statistically significant differences in PFS were demonstrated with respect to miR-122 ratio at the cut-off of 0.87 fc, despite

the longer PFS in patients with levels of the marker below the cut-off (5.8 months vs. 3.3 months, respectively; p=0.9).

At the cut-off established with the ROC curve method and the Youden J test (7.5 ng/mL, Supplementary Figure 2), AFP levels at t_0 proved to be predictive of PFS, which was 6.6 months (2.7–13.3) in patients with values ≤7.5 ng/mL and 2.6 months (1.2–4.8) in the other group (p=0.01). By contrast, HIF-1α at t_0 (cut-off of 0.53 ng/mL, Supplementary Figure 3) was not useful in predicting PFS (p=0.26).

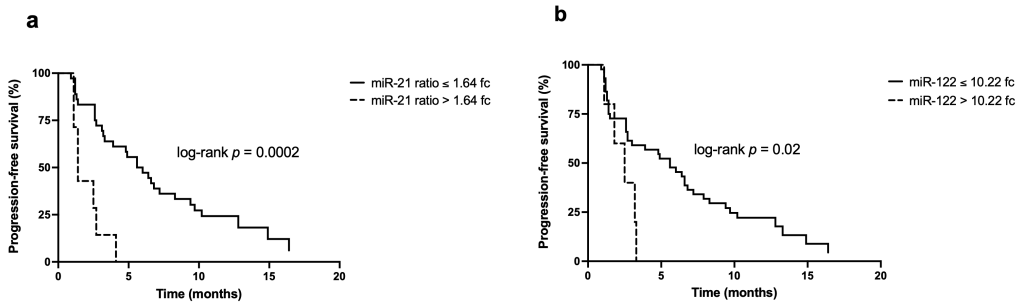


Figure 3. Kaplan–Meier curves for the PFS according to miR-21 ratio and miR-122 levels: (**a**) Patients with miR-21 ratio ≤1.64 fc have a significantly better PFS compared to patients with miR-21 ratio >1.64 fc (5.6 months [1.2–10.2] vs. 1.4 months [0.2–2.7]; *p* = 0.0002); (**b**) patients with miR-122 ≤10.22 fc have a statistically significant higher PFS compared to patients with miR-122 levels >10.22 fc (5.6 months [1.4–9.7] and 2.5 months [0.7–3.2]; *p* = 0.02).

Univariate and Multivariate Analysis

miR-21 ratio, miR-122, AFP, radiological response, number of nodules, tumor size, presence of CRPH, and BCLC stage were associated with PFS at the univariate analysis. In the multivariate model, AFP (hazard ratio [HR] 4.31, 95% CI 1.66–11.20), miR-21 ratio (HR 8.61, 95% CI 2.03–36.47), and radiological response (HR 10.44, 95% CI 2.74–39.79) were singled out as independent predictors of PFS (Table 2).

Considering these results, we also evaluated whether the combined evaluation of miR-21 ratio and AFP was able to sub-stratify patients with a “favorable” radiological response (CR and PR) according to their PFS. We found a statistically significantly longer PFS in patients with both markers below their respective cut-offs (miR-21 ratio ≤1.64 fc and AFP ≤7.5 ng/mL) compared to those with at least

one marker positive (8.3 months [6.4–16.4] and 3.3 months [2.5–6.0], respectively; p=0.001) (Figure 4a). In the subset of patients with CR and PR, the determination of both biomarkers provided an advantage compared to the use of AFP alone, considering that patients with AFP ≤7.5 ng/mL had a longer but not statistically significant different PFS compared to those with AFP above the cut-off (median PFS of 7.2 months [4.1–13.3] vs. 4.1 months [2.6–9.7], respectively; p=0.12) (Figure 4b).

Table 2. Univariate and multivariate Cox analysis for factors independently associated with PFS.

Variables		**Univariate Analysis**		**Multivariate Analysis**	
		HR (95% CI)	***p***	**aHR (95% CI)**	*p*
AFP (ng/mL)	≤7.5 >7.5	Ref 2.52 (1.37–4.66)	- 0.003	Ref 4.31 (1.66–11.20)	- 0.003
miR-21 ratio ($2^{-\Delta\Delta Ct}$)	≤1.64 >1.64	Ref 4.95 (1.93–12.65)	- 0.001	Ref 8.61 (2.03–36.47)	- 0.003
miR-122 ($2^{-\Delta\Delta Ct}$)	≤10.22 >10.22	Ref 2.98 (1.10–8.09)	- 0.03	Ref 2.11 (0.46–9.78)	- 0.3
Radiological response	CR/PR SD/PD	Ref 6.37 (2.91–13.95)	- <0.0001	Ref 10.44 (2.74–39.79)	- 0.001
Number of nodules	≤3 >3	Ref 2.30 (1.24–4.25)	- 0.008	Ref 0.51 (0.12–2.16)	- 0.4
Diameter (cm)	≤5 >5	Ref 2.23 (0.97–5.16)	- 0.06	Ref 1.78 (0.47–6.78)	- 0.4
CRPH	No Yes	Ref 1.71 (0.92–3.17)	- 0.09	Ref 0.56 (0.23–1.35)	- 0.2
BCLC stage	0/A B/C	Ref 2.54 (1.36–4.74)	- 0.003	Ref 3.50 (0.77–15.96)	- 0.1

Abbreviations: HR, hazard ratio; CI; confidence interval; aHR, adjusted hazard ratio; Ref, reference; AFP, alpha-fetoprotein; CR, complete response, PR, partial response; SD, stable disease, PD, progressive disease; CRPH, clinically relevant portal hypertension; BCLC, Barcelona Clinic Liver Cancer.

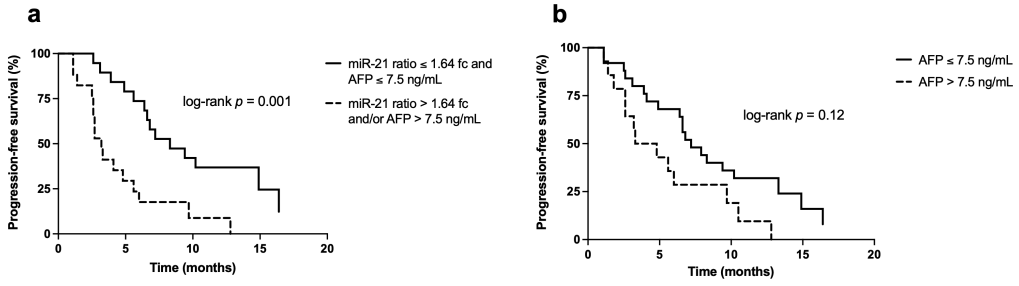


Figure 4. Kaplan–Meier curves for the PFS in patients with favorable radiological response (CR and PR). (**a**) Survival curves according to the combined evaluation of miR-21 ratio and AFP levels. Patients with both markers below their respective cut-offs achieved a statistically significant longer PFS compared to patients with at least one marker above its prognostic cut-off (8.3 months [6.4–16.4] in patients with miR-21 ratio ≤1.64 fc and AFP ≤7.5 ng/mL vs. 3.3 months [2.5–6.0] in the comparator group; *p* = 0.001); (**b**) survival curves of AFP alone in patients with CR and PR. Despite demonstrating a longer median PFS, patients with AFP ≤7.5 ng/mL had not a statistically significant higher survival compared to patients with AFP <7.5 ng/mL (7.2 months [4.1–13.3] vs. 4.1 months [2.6–9.7], respectively; *p* = 0.12).

Correlation between MicroRNAs and HIF-1α

HIF-1α levels were significantly higher in cirrhotics (0.43 ng/mL [0.32–0.54]) than in HCC patients, both before (0.23 ng/mL [0.12–0.49]; p=0.02) and after DEB-TACE (0.23 ng/mL [0.12–0.46]; p=0.009). In HCC patients miR-21, but not miR-122, was positively correlated with HIF-1α both at t_0 (r = 0.34, 95% CI 0.00–0.61; p=0.045) and at t_1 (r = 0.35, 95% CI 0.02–0.61; p=0.035) (Figure 5). In cirrhotics no correlations were found between miRNAs and HIF-1α.

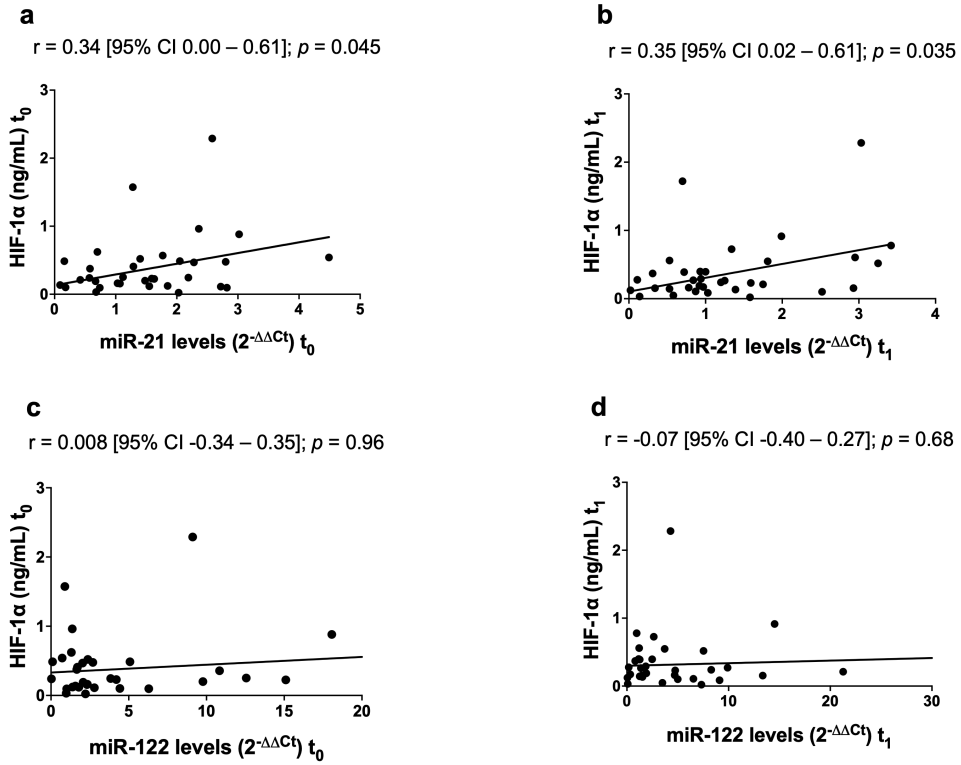


Figure 5. Correlations between the circulating levels of miR-21, miR-122 and HIF-1α in HCC patients. miR-21 was positively correlated with HIF-1α before (**a**) and after DEB-TACE (**b**). No statistically significant correlations were found between miR-122 and HIF-1α, both before (**c**) and after DEB-TACE (**d**).

DISCUSSION

The recently updated European guidelines on HCC management identified as an unmet need the development of useful prognostic and predictive biomarkers (25). Several studies evaluated miR-21 and miR-122 role in promoting HCC development and progression (14,28–33), and, despite the amount of publications about their potential role as biomarkers, data are not conclusive. In

particular, there are conflicting results regarding their prognostic role and the precise clinical and therapeutic setting in which they could be useful is not completely clear (34,35). The majority of data on these miRNAs as biomarkers in HCC come from studies on eastern populations, quite different from the western ones in terms of etiology and severity of liver disease (36). With this in mind, we assessed the prognostic efficiency of miR-21 and miR-122 in a group of Caucasian HCC patients treated with DEB-TACE. Unlike the majority of reports published, in this study circulating miRNAs were measured in whole blood samples rather than in serum or in plasma. The rationale behind this choice is that recently, in pancreatic, ovarian, lung, and gallbladder cancers, miRNAs evaluated in whole blood samples proved to be more accurate (37). Among the advantages on using whole blood samples, there are a higher miRNA yield and fewer errors than when using serum or plasma samples (37).

In our cohort, miR-21 levels were higher in cirrhotics than in controls and in HCC patients, without differences between the latter two. In HCC, miR-21 levels are reported to be higher in comparison to heathy subjects (9,38), but things become less clear when HCC and chronic liver disease are compared. Guo et al. (38) reported higher levels of miR-21 in HCC patients with respect to both chronic hepatitis B and liver cirrhosis patients; in contrast Pu et al. (9) and Xu et al. (15) concluded that miR-21 expression was higher in chronic hepatitis B patients than in HCC patients. In our study, as already reported after TACE (11), miR-21 levels showed a statistically significant decline, returning to levels comparable to those found in controls, confirming its pro-oncogenic role.

We detected higher levels of miR-122 in HCC patients compared to healthy controls, in line with what is already known (15,39), and compared with cirrhotics. On the latter point the published studies are again not concordant: besides studies reporting higher miR-122 in HBV-infected patients compared to HCC (15,39), others claimed no significant differences (16,40) or even higher levels in HCC patients (8). Considering its role as tumor-suppressor and its down-regulation in HCC tissue

compared with adjacent benign liver (14,28,33), the finding of higher circulating miR-122 in HCC patients is not easy to explain. miR-122 levels might reflect liver injury more than the presence of the tumor itself. Indeed, some studies correlated hepatic inflammation and cell death in patients with HBV and HCV chronic hepatitis with serum miR-122 levels (41,42). The mild increase of miR-122 after DEB-TACE found in our study might be explained speculating that its levels at t_1 do not reflect the effectiveness of treatment, but instead the concomitant liver injury. The lack of a statistical significance in this difference is probably due to the long temporal interval between the treatment and the evaluation of miRNA in our experimental setting, with a shorter time more likely resulting in larger differences. Nevertheless, it should be underlined that lower levels of miR-122 seven days after TACE have been reported, in contrast with our results (11).

miR-122 levels were higher in patients with HCC developed on a virus-related liver disease. This was not an unexpected finding, considering that miR-122 is involved in HBV genes expression (43) and it has a role in stimulating HCV replication (44). This association with HBV/HCV etiology was not confirmed in cirrhotic patients, probably because of the small number of patients in this group, who had an alcohol-related liver disease in the majority of cases.

The prognostic role of miR-21 has been extensively studied after surgery (5–8), but in patients undergoing loco-regional treatments data are not conclusive. High plasma miR-21 levels were not found to be associated with survival after TACE from some authors (10,11), while others found an association only at univariate analysis (12). Here, we confirmed that miR-21 is not a predictor of PFS when evaluated at t_0, but when the marker is considered as the ratio before and after treatment, in a dynamic way, it could be predictive of PFS, with Kaplan–Meier curves showing an impressive divergence.

The accuracy of miR-122 as a prognostic marker has not been clearly established. In patients treated with liver resection high serum levels of miR-122 appeared to correlate with longer survival (45),

while the opposite was true for patients treated with radiofrequency ablation (46). In other authors' experience, miR-122 had no prognostic role in general (8) or, specifically, in TACE-treated patients (47). In our study, miR-122 was useful in predicting prognosis when evaluated at t_0, as the patients with lower levels had a significantly longer PFS (again with an important divergence of Kaplan–Meier curves). Conversely, no significant association with PFS was demonstrated for miR-122 ratio, despite the fact that patients with lower values showed a slightly, not statistically significant, longer median survival.

For both miR-21 and miR-122, our results are in contrast to those published by Suehiro et al. (11), who identified only miR-122 ratio as a prognostic marker (longer survival in patients with higher ratio levels). Despite similar experimental designs, the two studies are not completely comparable: in the Suehiro et al. study the second sample was obtained 7 days after TACE, miRNAs levels were measured in extracellular vesicles, patients were treated with conventional and not DEB-TACE, and different internal reference controls were used for normalization.

At the Cox multivariate analysis, radiologic response, miR-21 ratio, and AFP levels were identified as independent predictors of PFS, in this order in terms of HR. In other words, miR-21 ratio had a higher impact than AFP levels. We also wondered whether assessing miR-21 ratio and AFP in combination could be useful to stratify patients with CR/PR according to their PFS and we found that the subgroup of patients with both miR-21 ratio and AFP below their respective cut-offs had significantly longer PFS than patients with at least one marker above the cut-off. Moreover, in this sub-group of patients with favorable radiological response, the combined determination of AFP and miR-21 ratio provided a better stratification according to PFS compared to AFP alone. In fact, we found no statistically significant difference between patients with AFP below and above the cut-off of 7.5 ng/mL. These results strengthen the role of miR-21 ratio in identifying the subgroup of patients with early progressing HCC.

Among the several molecular pathways in which they are involved, miRNAs play a role in modulating neoangiogenesis in human cancers. In prostate, lung, and colorectal cancers, miR-21 regulates the expression of HIF-1α and VEGF (17–19), but this association was not confirmed in human HCC tissue (4). Moreover, HIF-1α proved to be a miR-122 target in diet-induced steatohepatitis (20) and in a mouse model of HCC (21). A very recent paper provided more insights about the interplay between miR-122 and hypoxia-induced pathways in a murine model of ischemia-reperfusion injury (22). With this in mind, we evaluated the correlation between miR-21, miR-122, and HIF-1α in our group of patients treated with DEB-TACE, a treatment that induces liver ischemia and, in turn, overexpression of hypoxia-related genes. A positive mild correlation between miR-21 and HIF-1α in HCC patients both before and after the treatment was found, while no significant associations were found with miR-122. To the best of our knowledge, our study is the first to link miR-21 with HIF-1α in human HCC treated with DEB-TACE. The correlation found does not necessarily imply the causality of the relationship, but our results are consistent with the hypothesis of a miR-21 involvement in regulating the angiogenic pathway also in HCC, as already demonstrated for other malignancies (17–19), and paves the way for additional studies aimed at demonstrating this assumption.

Among the limitations of our study, the most important one is its retrospective nature that might have introduced unintentional biases. However, these biases are mitigated by the fact that patients were consecutively collected. The relatively small sample size (especially looking to cirrhotic patients), and the baseline differences between HCC and cirrhotics (particularly in residual liver function), could have prevented us to reach more definite conclusions in the comparison of miRNAs levels between groups.

There are many differences between our results and those reported in other publications. The comparison of different studies on miRNAs is difficult because a homogeneous methodological protocol in miRNA evaluation has not yet been reached. In particular, the differences between

studies are related to the endless list of confounders that includes samples size and selection, type of biologic samples used for the miRNAs assay (plasma, serum, exosomes, whole blood), RNA extraction procedures, internal controls, control groups for data normalization, methodology to express miRNA levels. Moreover, the majority of data in literature derives from eastern countries in which the leading cause of HCC and chronic liver disease is HBV infection, and with a larger share of tumors developing on a non-cirrhotic background (36). In western countries, by contrast, the vast majority of HCC patients had an underlying cirrhosis, the most frequent etiologies being HCV infection and alcohol.

We selected PFS as end-point because overall survival, the ideal end-point in oncology, was not evaluable considering the very short follow-up of patients enrolled. It must be kept in mind however that PFS could be considered a suitable surrogate of overall survival, particularly in diseases in which multiple lines of active treatment are available, such as HCC (48).

CONCLUSIONS

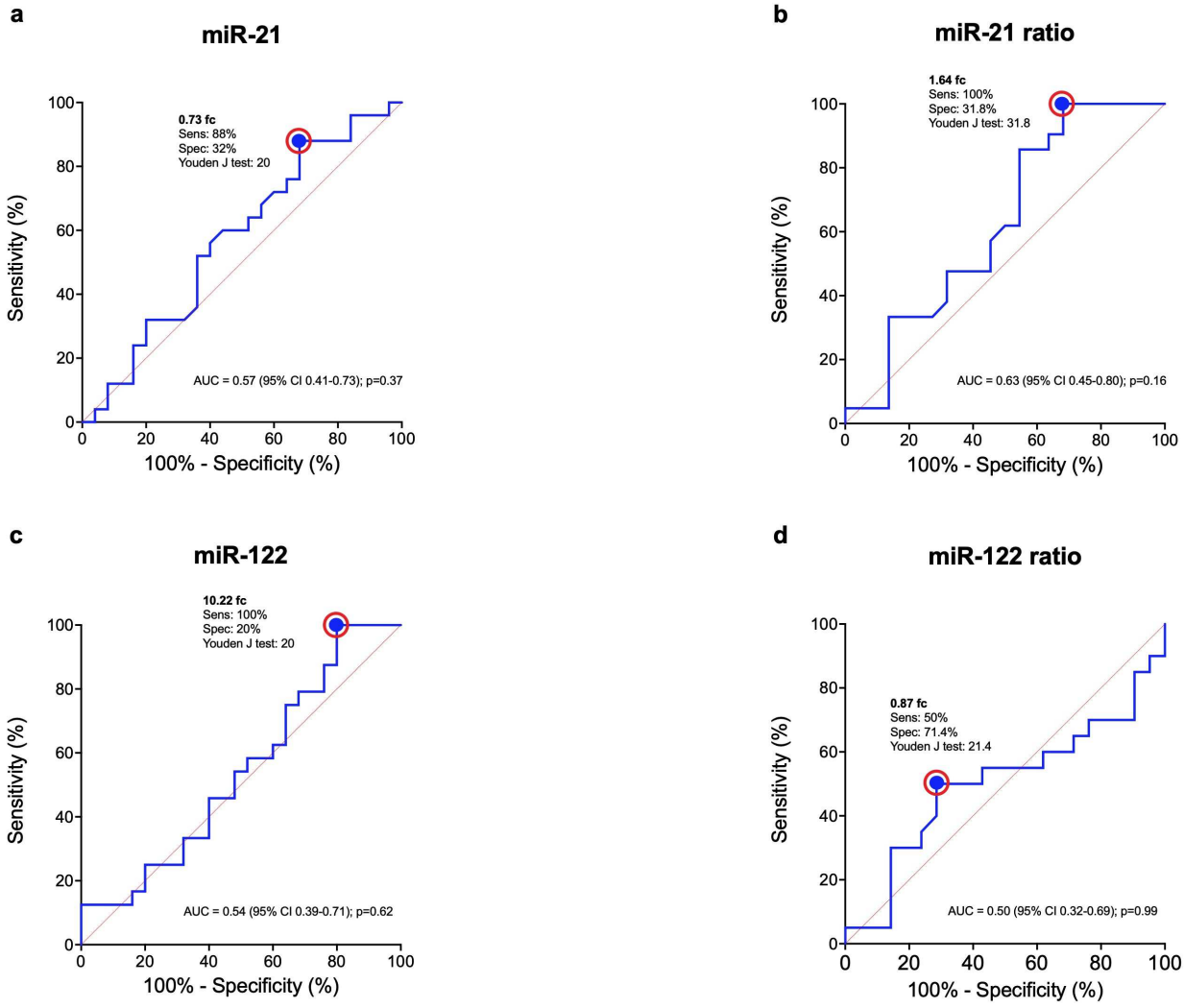
In conclusion, according to our results, miR-21 ratio and miR-122 predict PFS after TACE and propose themselves as useful prognostic markers. In particular, miR-21 ratio is associated to PFS at univariate and multivariate analysis and identifies (together with AFP) early progressors among the patients achieving a radiological response to treatment. In addition, a correlation between circulating HIF-1α and miR-21 expression was found, suggesting a possible role of the latter in modulating angiogenesis also in HCC, as it does in other type of tumors. Additional studies, possibly prospective, and the development of widely shared methodological protocols are necessary before the introduction of miRNAs quantification in clinical practice, but our results look quite promising.

SUPPLEMENTARY MATERIAL

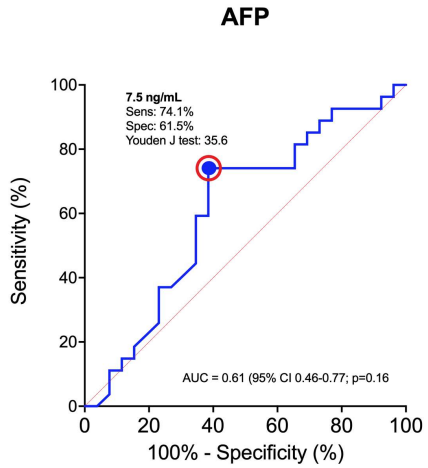
Supplementary Table 1. Levels of miRNAs (miR-93, miR-103a, miR-425) used as internal reference controls for normalization in our study.

	miR-93	miR-103a	miR-425
Healthy subjects	13.60 ± 0.56 ct	13.37 ± 0.62 ct	16.12 ± 0.77 ct
Cirrhotics	13.89 ± 1.41 ct	13.72 ± 1.47 ct	16.14 ± 1.32 ct
HCC pre-TACE	14.79 ± 1.91 ct	14.67 ± 2.02 ct	16.78 ± 1.52 ct
HCC post-TACE	15.11 ± 2.11 ct	15.06 ± 2.30 ct	17.12 ± 1.63 ct

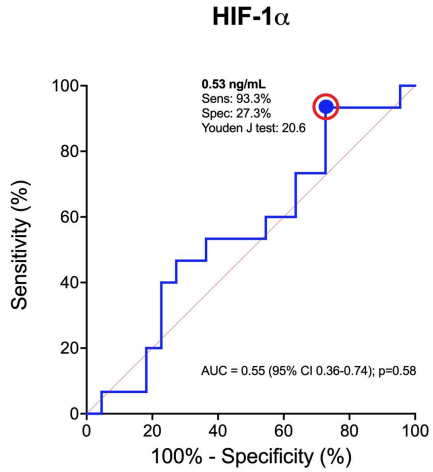
Note: miRNA levels, expressed as threshold cycles (ct), are reported in the table as mean ± standard deviation (SD).



Supplementary Figure 1. ROC curves used to identify the cut-off for miR-21 (a), miR-21 ratio (b), miR-122 (c) and miR-122 ratio (d).



Supplementary Figure 2. ROC curve for the identification of AFP cut-off.



Supplementary Figure 3. ROC curve for the identification of HIF-1α cut-off.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. 2018;68:394–424.
2. Galle PR, Foerster F, Kudo M, Chan SL, Llovet JM, Qin S, et al. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. Liver Int. 2019;39:2214–2229.
3. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat. Rev. Cancer. 2006;6:857–866.
4. Zeng W, van den Berg A, Huitema S, Gouw ASH, Molema G, de Jong KP. Correlation of microRNA-16, microRNA-21 and microRNA-101 expression with cyclooxygenase-2 expression and angiogenic factors in cirrhotic and noncirrhotic human hepatocellular carcinoma. PLoS One. 2014;9:e95826.
5. Huang C-S, Yu W, Cui H, Wang Y-J, Zhang L, Han F, et al. Increased expression of miR-21 predicts poor prognosis in patients with hepatocellular carcinoma. Int. J. Clin. Exp. Pathol. 2015;8:7234–7238.
6. Yoon JS, Kim G, Lee YR, Park SY, Tak WY, Kweon Y-O, et al. Clinical significance of microRNA-21 expression in disease progression of patients with hepatocellular carcinoma. Biomark. Med. 2018;12:1105–1114.
7. Karakatsanis A, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D. Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. Mol. Carcinog. 2013;52:297–303.
8. Bharali D, Banerjee BD, Bharadwaj M, Husain SA, Kar P. Expression Analysis of MicroRNA-21 and MicroRNA-122 in Hepatocellular Carcinoma. J. Clin. Exp. Hepatol. 2019;9:294–301.
9. Pu C, Huang H, Wang Z, Zou W, Lv Y, Zhou Z, et al. Extracellular Vesicle-Associated mir-21 and mir-144 Are Markedly Elevated in Serum of Patients With Hepatocellular Carcinoma. Front. Physiol. 2018;9:930.
10. Kim SS, Cho HJ, Nam JS, Kim HJ, Kang DR, Won JH, et al. Plasma MicroRNA-21, 26a, and 29a-3p as Predictive Markers for Treatment Response Following Transarterial Chemoembolization in Patients with Hepatocellular Carcinoma. J. Korean Med. Sci. 2018;33:e6.
11. Suehiro T, Miyaaki H, Kanda Y, Shibata H, Honda T, Ozawa E, et al. Serum exosomal microRNA-122 and microRNA-21 as predictive biomarkers in transarterial chemoembolization-treated hepatocellular carcinoma patients. Oncol. Lett. 2018;16:3267–3273.
12. Liu M, Liu J, Wang L, Wu H, Zhou C, Zhu H, et al. Association of serum microRNA expression in hepatocellular carcinomas treated with transarterial chemoembolization and patient survival. PLoS One. 2014;9:e109347.
13. Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A. miR-122, a paradigm for the role of microRNAs in the liver. J. Hepatol. 2008;48:648–656.
14. Bai S, Nasser MW, Wang B, Hsu S-H, Datta J, Kutay H, et al. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J. Biol. Chem. 2009;284:32015–32027.
15. Xu J, Wu C, Che X, Wang L, Yu D, Zhang T, et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol. Carcinog. 2011;50:136–142.
16. Jin Y, Wong YS, Goh BKP, Chan CY, Cheow PC, Chow PKH, et al. Circulating microRNAs as Potential Diagnostic and Prognostic Biomarkers in Hepatocellular Carcinoma. Sci. Rep. 2019;9:10464.
17. Liu L-Z, Li C, Chen Q, Jing Y, Carpenter R, Jiang Y, et al. MiR-21 induced angiogenesis through AKT and ERK activation and HIF-1alpha expression. PLoS One. 2011;6:e19139.
18. Donnem T, Fenton CG, Lonvik K, Berg T, Eklo K, Andersen S, et al. MicroRNA signatures in tumor tissue related to angiogenesis in non-small cell lung cancer. PLoS One. 2012;7:e29671.
19. Sabry D, El-Deek SEM, Maher M, El-Baz MAH, El-Bader HM, Amer E, et al. Role of miRNA-210, miRNA-21 and miRNA-126 as diagnostic biomarkers in colorectal carcinoma: impact of HIF-1alpha-VEGF signaling pathway. Mol. Cell. Biochem. 2019;454:177–189.

20. Csak T, Bala S, Lippai D, Satishchandran A, Catalano D, Kodys K, et al. microRNA-122 regulates hypoxia-inducible factor-1 and vimentin in hepatocytes and correlates with fibrosis in diet-induced steatohepatitis. Liver Int. 2015;35:532–541.

21. Ambade A, Satishchandran A, Szabo G. Alcoholic hepatitis accelerates early hepatobiliary cancer by increasing stemness and MIR-122-mediated HIF-1α activation. Sci. Rep. 2016;6:21340.

22. Ju C, Wang M, Tak E, Kim B, Emontzpohl C, Yang Y, et al. Hypoxia-inducible factor-1α-dependent induction of miR122 enhances hepatic ischemia tolerance. J. Clin. Invest. 2021;131.

23. Liu K, Min X-L, Peng J, Yang K, Yang L, Zhang X-M. The Changes of HIF-1α and VEGF Expression After TACE in Patients With Hepatocellular Carcinoma. J. Clin. Med. Res. 2016;8:297–302.

24. Llovet JM, Ducreux M, Lencioni R, Di Bisceglie AM, Galle PR, Dufour JF, et al. EASL-EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2012;56:908–943.

25. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.

26. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology. 2015;62:440–451.

27. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. Semin. Liver Dis. 2010;30:52–60.

28. Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. Oncogene. 2009;28:3526–3536.

29. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology. 2007;133:647–658.

30. Yin D, Wang Y, Sai W, Zhang L, Miao Y, Cao L, et al. HBx-induced miR-21 suppresses cell apoptosis in hepatocellular carcinoma by targeting interleukin-12. Oncol. Rep. 2016;36:2305–2312.

31. Li C, Wang Y, Wang S, Wu B, Hao J, Fan H, et al. Hepatitis B virus mRNA-mediated miR-122 inhibition upregulates PTTG1-binding protein, which promotes hepatocellular carcinoma tumor growth and cell invasion. J. Virol. 2013;87:2193–2205.

32. Nassirpour R, Mehta PP, Yin M-J. miR-122 regulates tumorigenesis in hepatocellular carcinoma by targeting AKT3. PLoS One. 2013;8:e79655.

33. Tsai W-C, Hsu PW-C, Lai T-C, Chau G-Y, Lin C-W, Chen C-M, et al. MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. Hepatology. 2009;49:1571–1582.

34. Yi PS, Li JS. High expression of miR-21 is not a predictor of poor prognosis in all patients with hepatocellular carcinoma. Mol. Clin. Oncol. 2018;8:733–739.

35. Zhang Y, Li Y, Jiang W, Li Q, Lan Y. The clinical significance of microRNA-122 in predicting the prognosis of patients with hepatocellular carcinoma: A meta-analysis validated by the Cancer Genome Atlas dataset. Medicine (Baltimore). 2019;98:e14810.

36. Zhu RX, Seto WK, Lai CL, Yuen MF. Epidemiology of hepatocellular carcinoma in the Asia-Pacific region. Gut Liver. 2016;10:332–339.

37. Long XR, Zhang YJ, Zhang MY, Chen K, Zheng XFS, Wang HY. Identification of an 88-microRNA signature in whole blood for diagnosis of hepatocellular carcinoma and other chronic liver diseases. Aging (Albany. NY). 2017;9:1565–1584.

38. Guo X, Lv X, Lv X, Ma Y, Chen L, Chen Y. Circulating miR-21 serves as a serum biomarker for hepatocellular carcinoma and correlated with distant metastasis. Oncotarget. 2017;8:44050–44058.

39. Qi P, Cheng S, Wang H, Li N, Chen Y, Gao C. Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. PLoS One. 2011;6:e28486.

40. Koberle V, Kronenberger B, Pleli T, Trojan J, Imelmann E, Peveling-Oberhag J, et al. Serum microRNA-1 and microRNA-122 are prognostic markers in patients with hepatocellular carcinoma. Eur. J. Cancer. 2013;49:3442–3449.

41. Bihrer V, Friedrich-Rust M, Kronenberger B, Forestier N, Haupenthal J, Shi Y, et al. Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. Am. J. Gastroenterol. 2011;106:1663–1669.

42. Waidmann O, Bihrer V, Pleli T, Farnik H, Berger A, Zeuzem S, et al. Serum microRNA-122 levels in different groups of patients with chronic hepatitis B virus infection. J. Viral Hepat. 2012;19:e58-65.

43. Qiu L, Fan H, Jin W, Zhao B, Wang Y, Ju Y, et al. miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. Biochem. Biophys. Res. Commun. 2010;398:771–777.

44. Spaniel C, Honda M, Selitsky SR, Yamane D, Shimakami T, Kaneko S, et al. microRNA-122 abundance in hepatocellular carcinoma and non-tumor liver tissue from Japanese patients with persistent HCV versus HBV infection. PLoS One. 2013;8:e76867.

45. Xu Y, Bu X, Dai C, Shang C. High serum microRNA-122 level is independently associated with higher overall survival rate in hepatocellular carcinoma patients. Tumour Biol. 2015;36:4773–4776.

46. Cho HJ, Kim JK, Nam JS, Wang HJ, Lee JH, Kim BW, et al. High circulating microRNA-122 expression is a poor prognostic marker in patients with hepatitis B virus-related hepatocellular carcinoma who undergo radiofrequency ablation. Clin. Biochem. 2015;48:1073–1078.

47. Kim SS, Nam JS, Cho HJ, Won JH, Kim JW, Ji J-H, et al. Plasma micoRNA-122 as a predictive marker for treatment response following transarterial chemoembolization in patients with hepatocellular carcinoma. J. Gastroenterol. Hepatol. 2017;32:199–207.

48. Finn RS. Progression-free survival: Starting point or endpoint in advanced HCC trial design? J. Hepatol. 2019;70:1062–1064.

CHAPTER 8

Circulating microRNA-21 and HIF-1α in chronic liver diseases and hepatocellular carcinoma

Filippo Pelizzaro

ABSTRACT

Background. MicroRNA-21 (miR-21) has been reported to be elevated in hepatocellular carcinoma (HCC) patients, but the available studies reached discordant conclusions. Therefore, we aimed to evaluate miR-21 levels in patients with chronic liver disease and HCC, and to assess its correlation with the severity of liver fibrosis and liver function laboratory tests. Moreover, considering the correlation between HIF-1α and miR-21, we assessed in the same groups the level of HIF-1α.

Methods. In this study, 84 subjects were included (16 healthy volunteers, 11 patients with NAFLD/NASH, 19 patients with chronic hepatitis C [CHC], 20 cirrhotics and 18 HCC). Serum level of miR-21 was measured with quantitative real time (qRT)-PCR, while HIF-1α was measured with ELISA.

Results. Compared to healthy subjects, NAFLD/NASH and CHC patients, a significantly higher level of miR-21 was detected in cirrhotics (2.13-fold, IQR 1.15-2.93) and in HCC patients (1.67-fold, IQR 1.17-2.10), without statistically significant differences between these two groups. Similar trends were demonstrated for HIF-1α. Considering together patients from all groups, a positive correlation was found between miR-21 and HIF-1α (r = 0.39, 95% CI 0.16-0.59; p=0.001). miR-21 was positively correlated with liver stiffness measured at transient elastography (r = 0.44, 95% CI 0.10-0.68; p=0.01), and F0-F1 patients showed lower levels of the marker compared to F2-F3 (p=0.03) and F4 patients (p=0.0009). In addition, miR-21 proved to be an independent predictor of F4 fibrosis. In chronic hepatitis and cirrhotic patients, miR-21 was correlated with AST (r = 0.40; p=0.001), ALP (r = 0.36; p=0.005), albumin (r = -0.42; p=0.0008) and INR (r = 0.39; p=0.002).

Conclusions. miR-21 is a useful marker in identifying the progression of liver damage, but it seems not accurate in diagnosis of HCC considering the similar level in cirrhotics compared to patients with cancer. It correlates with liver fibrosis, hepatic necroinflammatory activity and liver function parameters, and it could be useful in providing information on different aspects of chronic liver diseases.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies worldwide, and it ranks third in mortality among cancers (1). The majority of HCCs develops in a fibrotic or already cirrhotic liver, which are the result of chronic inflammation caused by hepatitis B (HBV) or hepatitis C virus (HCV), as well as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (2).

MIcroRNAs (miRNAs) are small non-coding RNA molecules (19-24 nucleotides long), extensively involved in the regulation of gene expression. They bind to partially complementary recognition sequences of target mRNAs and play a role in regulating almost all main cellular pathways (3). Altered expression of miRNAs has been associated with liver metabolism dysregulation, liver injury, liver fibrosis and tumor development (4). In particular, upregulation of serum and hepatic microRNA-21 (miR-21) has been reported in several liver diseases, thus attracting intense interest (5). miR-21 was found to be upregulated in patients infected with HBV (6,7), and some studies suggest that HCV is able to increase expression of this miRNA in hepatocyte cell lines and primary human hepatocytes (8). Hepatic miR-21 expression was reported to be significantly increased in patients with NAFLD/NASH (9–11), as well as patients with alcoholic liver disease (12). Patients with acute liver failure have high serum level of miR-21, which is even higher in patients with spontaneous recovery compared to non-recovered patients (13). Moreover, miR-21 promotes HCC proliferation and is one of the most overexpressed miRNAs in liver cancer (14,15). The levels of this onco-miRNA detected in serum have been reported to be significantly elevated in patients with HCC (16,17), and therefore it has been suggested as diagnostic biomarker (18). Nevertheless, the results of different studies comparing miR-21 circulating level in HCC and chronic liver diseases are discordant. In addition, different controls groups were used and none of these studies reported the differential levels of this marker in various stages of liver disease (17,19–23).

In this study we aimed to comprehensively evaluate the differential miR-21 circulating level in HCC and chronic liver disease, and its correlation with the severity of liver fibrosis and liver function laboratory tests. Moreover, based on previous results showing a potential role of miR-21 in modulating angiogenesis in human cancers including HCC (24–27), we assessed in the same groups also the levels of HIF-1α.

PATIENTS AND METHODS

Study groups

In this study, blood samples from 16 healthy volunteers, 11 patients with NAFLD/NASH, 19 patients with chronic hepatitis C (CHC), 20 cirrhotics and 18 HCC patients collected between January 2019 and June 2021, were retrospectively evaluated.

Blood samples from patients with NAFLD/NASH, CHC and liver cirrhosis were obtained from patients managed at the outpatient clinic of the Gastroenterology Unit of the Padova University Hospital. In the NAFLD/NASH group, patients with metabolic liver disease diagnosed according to the recommendations of the European guidelines were included (28). The CHC group consisted of non-cirrhotic patients with chronic HCV infection waiting for treatment with Direct Acting Antivirals (DAAs). Patients were diagnosed with cirrhosis, and consequently included in the relative group, when fulfilling the following criteria: International Normalized Ratio (INR) >1.2, White Blood Cell count <4.4 x 10^9/L, Platelets <150 x 10^9/L (at least two of three) and abdomen ultrasonography (US) showing findings compatible with cirrhosis. All cirrhotic patients were regularly surveilled for the development of HCC with US every six months, and the presence of HCC was ruled out with dynamic computed tomography (CT) or magnetic resonance imaging (MRI) at the time of study entry. HCC patients included in the study were admitted to Gastroenterology Unit of Padova University Hospital for treatment (DEB-TACE). Diagnosis of HCC was achieved with the typical features at dynamic CT

or MRI (enhancement in the arterial phase and washout in delayed phases), according to European guidelines (29). In this subgroup, the blood samples were collected immediately before TACE.

The following clinical and tumor-related variables were recorded: sex, age, etiology, comorbidities, presence of clinically relevant portal hypertension (CRPH), main serological parameters (aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma-glutamyl transpeptidase [gGT], alkaline phosphatase [ALP], total bilirubin, international normalized ratio [INR], albumin and alpha-fetoprotein [AFP]), Child-Pugh class and Model for End-Stage Liver Disease (MELD). CRPH was defined as presence of splenomegaly, esophageal varices or ascites, and platelets count $<100 \times 10^9$/L (30). In NAFLD/NASH and in CHC groups also liver stiffness measured with transient elastography was collected, while this data was available only in 7 cirrhotic patients. Genotype and HCV-RNA were registered for patients with CHC. In HCC patients, number and size of liver nodules, presence of macrovascular invasion (MVI) and/or extrahepatic spread (EHS), evaluated with dynamic CT or MRI, were recorded. Patients were staged according to the Barcelona Clinic Liver Cancer (BCLC) system. The efficacy of TACE was evaluated with dynamic CT or MRI performed four weeks after the treatment and the Modified Response Evaluation Criteria In Solid Tumors (mRECIST) were used to classify the radiological response in complete (CR), partial (PR), stable disease (SD) or progressive disease (PD) (31).

RNA isolation and miRNAs analysis

From 10 milliliters of venous blood, 5 mL were used for serum and plasma separation. Samples were preserved at − 80 °C till the assays of biochemical markers.

Plasma samples were used for the determination of miRNAs. Total RNA was extracted from 200 µL of plasma using the miRneasy Serum/Plasm Advanced kit (Qiagen, GmbH, Hilden, Germany). Extraction efficiency was checked through adding synthetic oligonucleotides (UniSp2, UniSp4, UniSp5) at recommended concentrations. Reverse transcription for cDNA synthesis was performed

using the miRCURY LNA RT kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription efficiency was checked through adding synthetic oligonucleotides (UniSp6). The expression of miRNAs was evaluated by quantitative Real Time (qRT)-PCR analysis (miRCURY LNA miRNA PCR Assays and PCR Panels, Qiagen, GmbH, Hilden, Germany), according to the manufacturer's instructions, on a PRISM 7900HT system (Applied Biosystems, Foster City, California, USA) with miR-93, miR-103a, miR-425 as internal reference controls for normalization. To assess hemolysis, 2 miRNAs were used: miR-451, which is expressed in red blood cells, and miR-23a, which is relatively stable in serum and plasma but not affected by hemolysis. The ratio between these 2 miRNAs correlated with the degree of hemolysis. Samples with ratios above 7.0 have an increased risk of being affected by hemolysis, as opposed to samples with lower ratios. Therefore, samples with ratios above 7.0 were excluded from the analysis.

The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ (fold-change, fc) method, using healthy subjects as the reference group for the normalization.

HIF-1α assay

A commercial ELISA kit (Cloud-Clone Corp., Katy, Texas, USA) was used to determine HIF-1α in the serum samples from cirrhotics and HCC, according to manufacturer's instructions. The amount of HIF-1α (ng/mL) was derived by interpolation of samples absorbance on the calibration curves plotted with calibrators. Briefly, plates precoated with an antibody specific to HIF-1α were incubated with 100 μL of serum. HIF-1α was revealed by the addition of Detection Reagent A and B at 450 nm.

Statistical analysis

Quantitative variables were reported as median and interquartile range (IQR), while categorical variables as absolute frequency and percentage. Kruskal-Wallis and Mann-Whitney tests were used to compare quantitative variables. The comparison between categorical data was performed with

chi-square or Fischer's exact tests. The correlations between continuous variables were established calculating the non-parametric Spearman coefficient.

In order to identify independent predictors F4 fibrosis, a multivariate logistic regression was performed including in the model only variables significantly or borderline ($p \leq 0.1$) associated with fibrosis stage at the univariate analysis.

A p-value (two-tails) <0.05 was considered as significant in this study. IBM SPSS Statistics (Version 25.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 8.3.1 (GraphPad Software, La Jolla, California, USA) were used for all the calculations.

RESULTS

Baseline characteristics

Baseline characteristics of patients included in the study are shown in Table 1.

A significantly higher proportion of male patients was present in cirrhotics and HCC groups. Healthy volunteers were significantly younger compared to NAFLD/NASH, CHC, cirrhosis and HCC groups.

CHC had a significantly lower liver stiffness compared to NAFLD/NASH, while as expected cirrhotics had markedly higher stiffness (although this data was available only in 7/20 [35%] patients). Compared to NAFLD/NASH, cirrhotics and HCC patients demonstrated significantly lower levels of white blood cells (WBC) and platelets, but higher levels of bilirubin and INR. Cirrhotic patients showed significantly lower albumin compared to NAFLD/NASH, while HCC patients had higher AFP levels (despite the low clinical significance considering that the median AFP level in HCC patients was 4.9 ng/mL [IQR 2.8-11.6]).

No statistically significant differences in liver function (Child-Pugh class and MELD score) were registered between cirrhotics and HCC patients. These latter showed a median number of 3 nodules (IQR 1-4) with a median diameter of 2.0 cm (IQR 1.8-3.6). The majority of HCC patients had a BCLC

stage A or B tumor (7 patients in both groups). Seven patients (38.9%) had a CR to TACE, while 6 (33.3%) had a PR.

Table 1. Baseline characteristics of the study populations.

	Controls (n=16)	**NAFLD/NASH (n=11)**	**CHC (n=19)**	**Cirrhosis (n=20)**	**HCC (n=18)**
Clinical variables					
Sex- male	4 (25.0)	5 (45.5)	7 (33.3)	12 (66.7) *	14 (77.8) #
Age (years)	50 (39-59)	66 (56-73) #	61 (47-72) *	68 (58-72) #	66 (62-75) †
Liver biopsy	-	5 (45.5)	4 (19.0)	0 (0) #	2 (11.1)
Etiology					
HBV HCV NAFLD/NASH Alcohol	-	0 (0) 0 (0) 11 (100.0) 0 (0)	0 (0) 19 (100.0) ‡ 0 (0) ‡ 0 (0)	2 (10.0) 5 (25.0) 4 (20.0) ‡ 9 (45.0) #	0 (0) 7 (38.9) * 3 (16.7) ‡ 8 (44.4) *
HCV genotype 1b	-	-	11 (57.9)	-	-
Liver stiffness (kPa)	-	6.8 (5.3-8.4)	5.3 (4.7-6.7) *	26.4 (21.1-43.5) †,a	- b
WBC (x10^9/L)	-	7.88 (6.03-9.50)	5.36 (4.50-7.50) *	5.46 (3.51-7.13) *	5.49 (3.45-6.33) #
Hb (g/dL)	-	14.2 (12.8-14.9)	14.6 (13.9-15.6)	11.8 (8.7-13.3) *	13.3 (12.1-15.1)
PLT (x10^9/L)	-	226 (188-282)	214 (157-258)	126 (67-195) #	135 (73-177) †
AST (U/L)	-	27 (22-41)	39 (28-61) *	48 (24-76)	39 (28-49)
ALT (U/L)	-	32 (23-59)	45 (25-99)	19 (15-49)	29 (24-44)
gGT (U/L)	-	68 (27-172)	30 (15-84)	81 (32-162)	72 (52-156)
ALP (U/L)	-	97 (76-108)	78 (62-101)	107 (82-149)	93 (86-118)
Bilirubin (umol/L)	-	8.7 (7.3-12-3)	10.5 (7.6-16.7)	22.0 (8.7-37.6)	15.0 (11.3-32.8) *
INR	-	1.04 (1.00-1.07)	1.02 (0.98-1.07)	1.27 (1.13-1.43) ‡	1.12 (1.09-1.32) #
Albumin	-	4.1 (3.8-4.5)	4.3 (4.1-4.5)	3.5 (2.9-4.1) *	4.1 (3.2-4.5)
AFP (ng/mL)	-	2.2 (1.7-3.5)	3.6 (2.0-5.3)	3.7 (2.3-7.8)	4.9 (2.8-11.6) *
CRPH	-	-	-	17 (85.0)	10 (55.6) *
Child-Pugh class					
A B C	-	-	-	10 (50.0) 6 (30.0) 4 (20.0)	14 (77.8) 4 (22.2) 0 (0)
MELD	-	-	-	11 (10-19)	10 (8-14)
Tumor-related variables					
Number of nodules	-	-	-	-	3 (1-4)
Diameter (cm)					2.0 (1.8-3.6)
MVI and/or EHS	-	-	-	-	2 (11.1)
BCLC stage					
0 A B C	-	-	-	-	2 (11.1) 7 (38.9) 7 (38.9) 2 (11.1)
Radiological response (mRECIST)					
CR PR SD PD	-	-	-	-	7 (38.9) 6 (33.3) 2 (11.1) 3 (16.7)

a) Data on liver stiffness was available only in 7 cirrhotic patients
b) Data on liver stiffness was not available for patients with HCC
Reference groups for comparisons: controls for sex and age; NAFLD/NASH for liver biopsy, liver stiffness, etiology, WBC, Hb, PLT, AST, ALT, gGT, ALP, bilirubin, INR, albumin, and AFP; cirrhotics for CRPH, Child-Pugh and MELD.
* p<0.05 and ≥0.01; # p<0.01 and ≥0.001; † p<0.001 and ≥0.0001; ‡ p<0.0001
Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; CHC, chronic hepatitis C, HCC, hepatocellular carcinoma; HBV, hepatitis B virus, HCV, hepatitis C virus; WBC, white blood cells; Hb, hemoglobin; PLT, platelets; AST, aspartate aminotransferase; ALT, alanine aminotransferase; gGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; INR, international normalized ratio; AFP, alpha-fetoprotein; CRPH, clinically relevant portal hypertension; MELD, Model for End-Stage Liver Disease; MVI, macrovascular invasion; BCLC, Barcelona Clinic Liver Cancer; mRECIST, modified Response Evaluation Criteria In Solid Tumors; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Levels of circulating miR-21 and HIF-1α

miR-21 levels were significantly different between groups (Kruskall-Wallis p<0.0001). Median values of miR-21 were 1.07-fc (IQR 0.84-1.25) in controls, 0.77-fc (IQR 0.60-1.15) in NAFLD/NASH patients and 1.01-fc (IQR 0.78-1.50) in CHC patients. Cirrhotics (2.13-fold, IQR 1.15-2.93) had significantly higher levels of miR-21 compared to controls (p=0.002), NAFLD/NASH (p=0.001) and CHC (p=0.005). In HCC, the levels of miR-21 decreased to 1.67-fold (IQR 1.17-2.10) but remained significantly higher compared to controls (p=0.001), compared to NASH (p=0.001) and compared to CHC patients (p=0.03). There was no statistically significant difference in miR-21 level between cirrhotics and HCC patients (p=0.23) (Figure 1a).

Similar figures were found for variation of HIF-1α in different phases of chronic liver disease (Kruskall-Wallis p=0.0008). The median value in controls was 0.12 ng/mL (IQR 0.07-0.17). Compared to these latter, no significant differences were demonstrated in NAFLD/NASH group (median 0.07 ng/mL, IQR 0.05-0.19; p=0.56) and CHC group (median 0.14 ng/mL, IQR 0.06-0.38; p=0.73). Cirrhotics (0.29 ng/mL, IQR 0.18-0.55) had a statistically significant higher level of circulating HIF-1α compared to controls (p=0.007), NAFLD/NASH (p=0.001) and CHC (p=0.01). Compared to controls, HCC patients showed higher levels of the marker (0.28 ng/mL, IQR 0.18-0.50; p=0.009). A statistical significance difference was maintained when HCC patients were compared to NAFLD/NASH (p=0.001) and to CHC patients (p=0.04). There was no statistically significant difference in HIF-1α between cirrhotics and HCC patients (p=0.77) (Figure 1b).

In all the groups, no significant correlations between miR-21 and HIF-1α were demonstrated. Nevertheless, when patients from all groups were considered together, a statistically significant positive correlation was found (r = 0.39, 95% CI 0.16-0.59; p=0.001) (Figure 2).

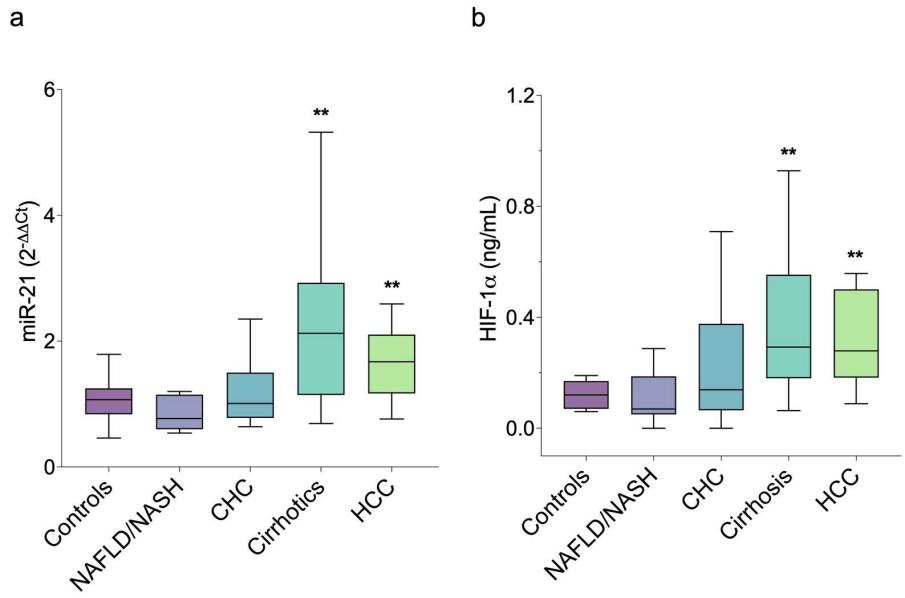


Figure 1. Box and whisker plots showing circulating levels of miR-21 (**a**) and HIF-1α (**b**) in controls, NAFLD/NASH, CHC patients, cirrhotics and HCC patients. Cirrhotics and HCC patients had significantly higher levels of miR21 and HIF-1α compared to controls. (** p<0.01 and ≥0.001)

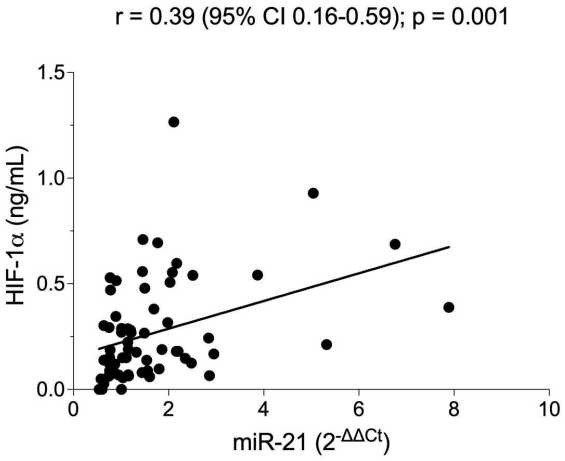


Figure 2. Correlations between miR-21 and HIF-1α levels.

Correlation between miR-21 and clinical parameters

Hypothesizing that miR-21 serum levels might be related to liver fibrosis, we investigated the correlation between levels of this miRNA and liver stiffness measured at transient elastography,

finding a positive correlation (r = 0.44, 95% CI 0.10-0.68; p=0.01) (Figure 3a). Moreover, this association was confirmed when patients were divided according to the fibrosis score (Figure 4a). Patients with F0-F1 fibrosis had a median value of miR-21 of 0.90-fc (IQR 0.73-1.26), which was significantly lower than that of F2-F3 patients (1.65-fc, IQR 1.23-2.07; p=0.03) and F4 patients (1.65-fc, IQR 1.23-2.07; p=0.0009). No significant differences were demonstrated between F2-F3 patients and F4 patients.

While no significant correlation was demonstrated between liver stiffness and HIF-1α levels (Figure 3b), a progressive increase of the marker was shown when patients were divided according to the fibrosis score (Figure 4b). HIF-1α levels were higher in F2-F3 compared to F0-F1 patients, although not reaching the statistical significance (median 0.19 ng/mL [IQR 0.02-0.43] vs. 0.12 ng/mL [IQR 0.06-0.29], respectively; p=0.85). F4 patients had a significantly higher level of HIF-1α compared to F0-F1 patients (0.28 ng/mL, IQR 0.18-0.55; p=0.002). Similarly to miR-21, no statistically significant difference was demonstrated between F2-F3 and F4 patients (p=0.24).

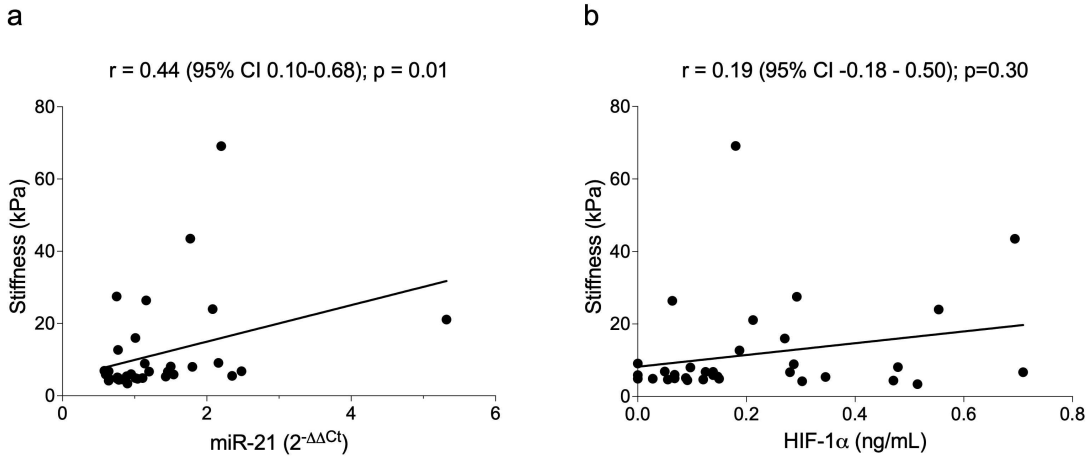


Figure 3. Correlation between miR-21 (**a**) and HIF-1α levels (**b**) and liver stiffness.

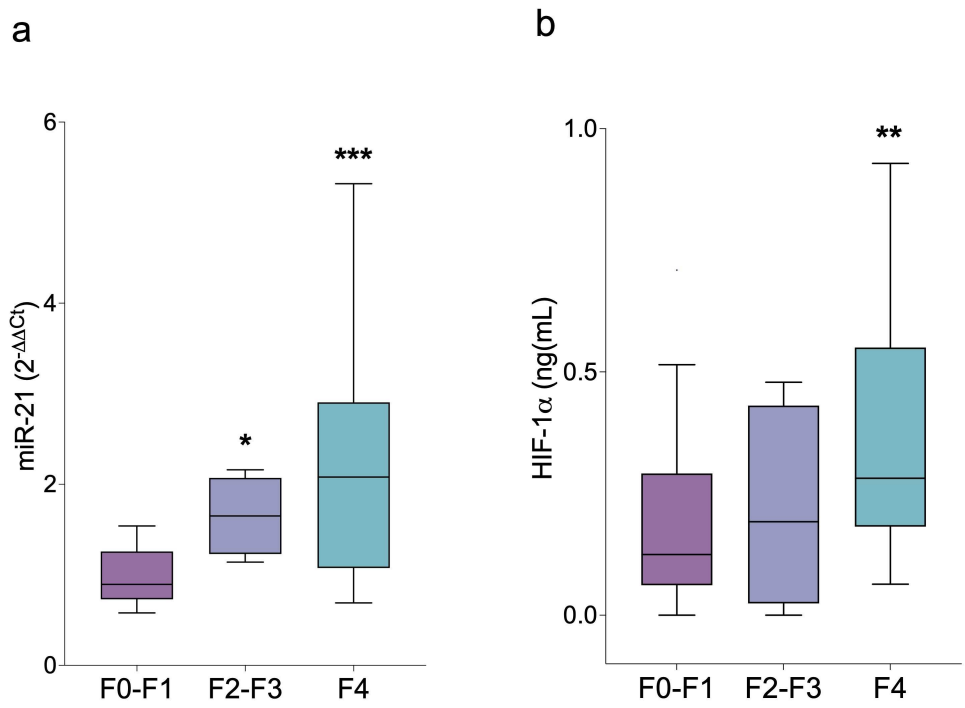


Figure 4. Levels of miR-21 (**a**) and HIF-1α (**b**) according to liver fibrosis measured at transient elastography. Compared to F0-F1 patients, miR-21 levels were significantly higher in F2-F3 and in F4 patients (**a**), while HIF-1α was higher in F4 patients (**b**). (* p<0.05 and ≥0.01; ** p<0.01 and ≥0.001; *** p<0.001 and ≥0.0001)

In patients with chronic hepatitis, cirrhosis and HCC, miR-21 levels correlated positively with AST (r = 0.40, 95% CI 0.16-0.60; p=0.001) and ALP (r = 0.36, 95% CI 0.11-0.57; p=0.005), but not with ALT (r = 0.14, 95% CI -0.12-0.38; p=0.29), gGT (r = 0.08, 95% CI -0.18-0.33; p=0.54) and bilirubin (r = 0.21, 95% CI -0.05-0.44; p=0.10). In addition, miR-21 levels were negatively correlated with albumin (r = -0.42, 95% CI -0.62 - -0.18; p=0.0008) and positively correlated with INR (r = 0.39, 95% CI 0.15-0.59; p=0.002) (Figure 5). A significantly higher level of miR-21 was demonstrated in patients with abnormal AST (1.89-fc, IQR 1.42-2.39) compared to patients with no abnormalities (1.03-fc, IQR 0.76-1.66; p=0.0004). On the contrary, no statistically significant differences were demonstrated between patients with abnormal vs. normal ALT (1.52-fc [IQR 1.01-2.19] vs. 1.18-fc [0.78-2.00], respectively; p=0.28) (Supplementary Figure 1). Similar results in correlations were observed in a sensitivity analysis with the exclusion of HCC patients (Supplementary Figure 2).

In the analysis restricted to patients with chronic hepatitis (either NAFLD/NASH or CHC), miR-21 levels positively correlated with both AST (r=0.52, 95% CI 0.17-0.75; p=0.005) and ALT (r=0.45, 95%

CI 0.08-0.71; p=0.02), while the correlation with ALP became non-significant even though a borderline p-value was obtained (r = 0.34, 95% CI -0.06 – 0.64; p=0.08) (Figure 6). Moreover, in the CHC group, miR-21 was positively correlated with HCV-RNA levels (r = 0.41, 95% CI 0.14-0.63; p=0.003).

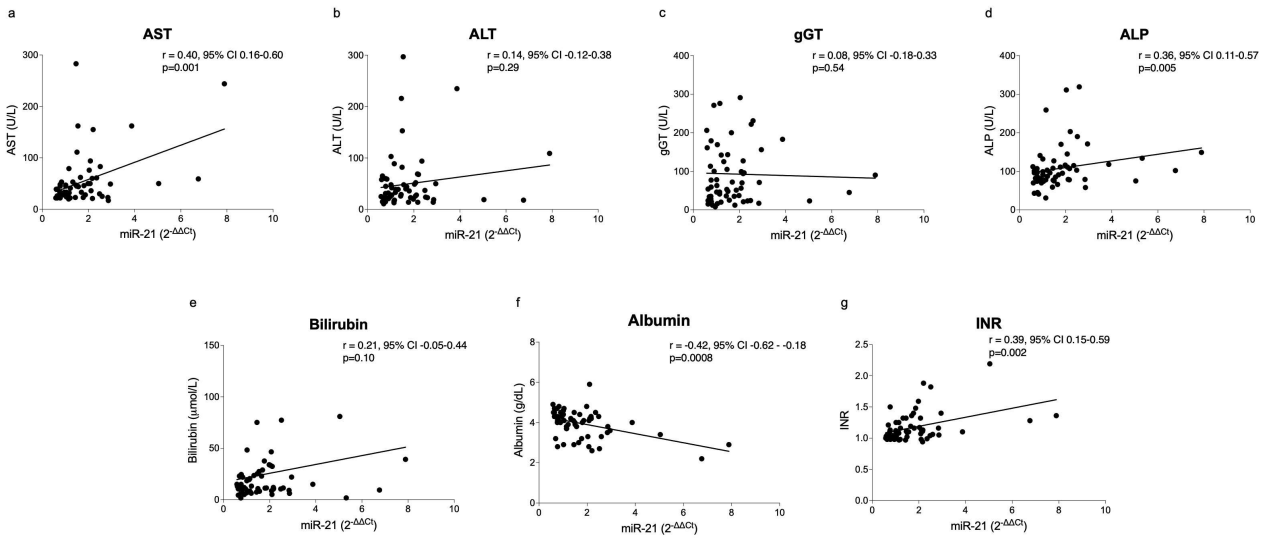


Figure 5. Correlations between miR-21 levels and AST (**a**), ALT (**b**), gGT (**c**), ALP (**d**), bilirubin (**e**), albumin (**f**) and INR (**g**) in chronic hepatitis, cirrhotics and HCC patients. A positive correlation was demonstrated between miR-21 levels and AST, ALP and INR, while a negative correlation was shown with albumin.

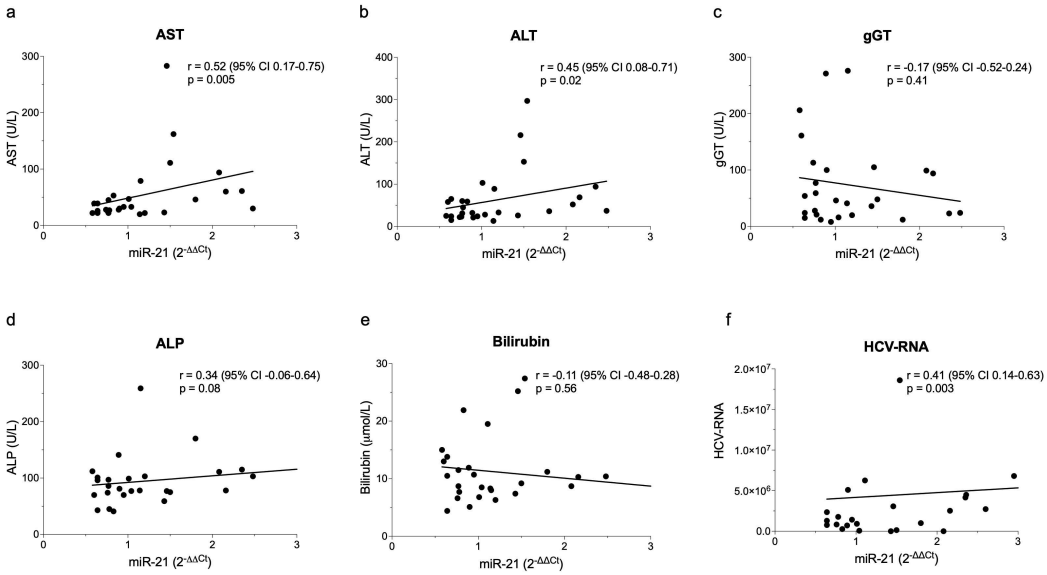


Figure 6. Correlations between miR-21 levels and AST (**a**), ALT (**b**), gGT (**c**), ALP (**d**), bilirubin (**e**) and HCV-RNA (**f**) in chronic hepatitis patients. A positive correlation was demonstrated between miR-21 levels and AST, ALT and HCV-RNA levels (in patients with CHC).

In order to evaluate if miR-21 levels could be a predictor of severe fibrosis (F4) we performed a multivariate logistic regression analysis, including variables associated with F4 fibrosis at the univariate analysis (Table 2). miR-21 turned out to be the only independent predictor of severe fibrosis (OR=5.77, 95% CI 1.04-32.03).

Table 2. Univariate and multivariate logistic regression analysis of factors associated with F4 fibrosis.

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p	OR (95% CI)	p
Sex	2.25 (0.71-7.14)	0.17		
Age	1.03 (0.98-1.08)	0.28		
Albumin	0.05 (0.01-0.33)	0.002	0.91 (0.05-16.21)	0.95
Bilirubin	1.08 (1.01-1.16)	0.04	1.16 (0.97-1.39)	0.09
logAST	2.15 (0.28-16.44)	0.46		
logALT	0.08 (0.01-0.71)	0.02	0.01 (0.00-1.27)	0.06
logGGT	3.13 (0.73-13.52)	0.13		
logALP	17.04 (0.57-506.86)	0.10		
miR-21	4.40 (1.62-11.95)	0.004	5.77 (1.04-32.03)	0.04

Abbreviations: logAST, logarithm of aspartate aminotransferase; logALT, logarithm of alanine aminotransferase; logGGT, logarithm of gamma-glutamyl transpeptidase; logALP, logarithm of alkaline phosphatase.

DISCUSSION

Several data demonstrated that miR-21, one of the most overexpressed miRNAs in liver cancer, promotes HCC development and progression (14,15). Some studies found a significant elevation of miR-21 serum levels in patients with HCC (17), therefore proposing its determination as a valuable tool for diagnosis (18). Nevertheless, available studies used different control groups (healthy subjects, patients with viral chronic hepatitis or cirrhotics) (17,19–23) and not conclusive data are available regarding the changes of this miRNA in different phases of liver disease. Bihrer et al. demonstrated that, compared to healthy subjects, miR-21 was elevated in HCC patients, but the latter had similar levels compared to cancer-free CHC patients (21). Tomimaru et al. showed significantly higher levels of miR-21 in HCC patients with chronic hepatitis, but in this study were not included patients with cirrhosis (17). Other more recent studies conducted in China, where HBV is the leading etiology of chronic liver disease and HCC, compared miR-21 levels between HCC

patients and chronic hepatitis B patients reaching opposite results: Guo et al. found higher miR-21 levels in HCC patients (22), while Pu et al. and Xu et al. reported the highest level in patients with chronic hepatitis (20,23). As far as miR-21 levels in NAFLD are concerned, the results in the comparison with controls varied depending on the studies (32,33).

We report here that miR-21 levels were higher in patients with HCC compared to healthy subjects and patients with chronic liver diseases (either NAFLD/NASH of CHC). However, the highest values of miR-21 were found in cirrhotics and no statistically significant differences were demonstrated between this group and patients with cancer. Similarly to our previous results (27), HCC patients had lower levels of miR-21 compared to cirrhotics, even though in this study the difference was not statistically significant, possibly due to the small number of patients included. Moreover, patients with NAFLD/NASH and CHC showed no differences in the levels of miR-21 compared to controls. According to our results, miR-21 is significantly overexpressed in advanced liver disease and in HCC. The overexpression of this onco-miRNA seems to be a very early event in liver carcinogenesis, with this marker fully expressed in cirrhosis which is the common pre-cancerous stage that eventually leads to the development of HCC. Consequently, miR-21 appears not to be a useful diagnostic biomarker for cancer detection, at least in Western countries where the great majority of HCCs arise from a cirrhotic background. More importantly, the adoption of miR-21 as a diagnostic tool appears to be hindered by the lack of standard operating procedures and uniform method for normalizing the miRNA level (5).

In this study, also HIF-1α was found at higher level in cirrhotic and HCC patients compared to controls, NAFLD/NASH and CHC patients. Even though HIF-1α was higher in cirrhotics compared to HCC, no statistically significant differences between these two groups were demonstrated. Similarly to what we found in our previous study (27), a statistically significant positive correlation was found between miR-21 and HIF-1α when all patients were considered together. This suggests a role of HIF-

1α in modulating angiogenesis, as already demonstrated in other conditions (24–26). However, when the groups of patients were considered separately, the existence of a correlation was not confirmed, probably as a result of the limited number of patients for each group.

A relevant finding of this study is the association of miR-21 with liver fibrosis measured at transient elastography. miR-21 has been linked to fibrosis in the lung (34), heart (35), and it has already been demonstrated that the level of this miRNA correlates with hepatic fibrosis (36). A close link between miR-21 and hepatic fibrosis is supported by the finding that transforming growth factor β (TGF-β), a critical mediator of hepatic fibrogenesis (37,38), promotes the expression of miR-21 (39), and that miR-21 decreases the expression of SMAD7 (36), a negative regulator of TGF-β signaling (38). In this study, we confirmed a significant correlation between levels of miR-21 and liver stiffness. Moreover, when patients were divided according to their fibrosis score (from F0 to F4), a progressive increase of miR-21 was demonstrated. miR-21 revealed also to be an independent predictor of F4 fibrosis at the multivariate logistic regression analysis, and this further confirms the central role of this miRNA in the progression of liver damage. Indeed, there is clear evidence for a close relation between miR-21 and hepatic fibrosis. The hepatic level of miR-21 appears to correlate with the stage of liver fibrosis (36) and it is strongly expressed not only in tumor cells, but also in tumors associated fibroblast (40). As far as circulating levels are concerned, a previous study showed that, although not reaching the statistical significance, F4 patients had higher levels of serum miR-21 compared to previous stages (41).

Several correlations between miR-21 and liver function laboratory tests were showed in this study. Similarly to the results obtained by Bihrer et al. (21), we found that miR-21 levels positively correlated with AST, ALP and albumin, and negatively correlated with INR in patients with chronic hepatitis, cirrhosis and HCC. A sensitivity analysis performed excluding patients with liver cancer, confirmed this result. Moreover, when only patients with chronic hepatitis were considered, miR-

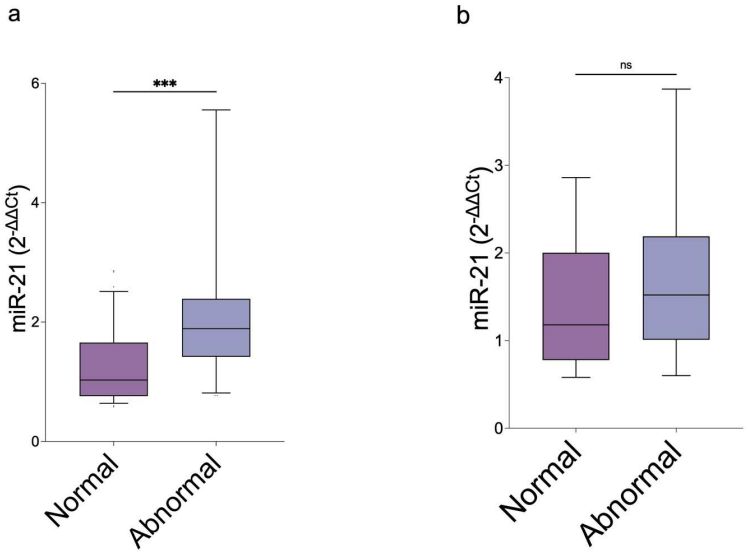
21 levels correlated with parameters of necroinflammatory activity (AST and ALT). In patients with CHC, miR-21 was positively correlated with HCV-RNA levels, and this is in agreement with previous data showing that miR-21 expression in liver tissue was associated with viral load (36).

The results of this study suggest that miR-21 concentration might be a useful parameter to differentiate patients with mild vs. moderate to severe liver fibrosis. In addition, also considering previous findings on the correlation between miR-21 serum levels and histologic activity index (HAI) score (21), this miRNA could discriminate patients with minimal vs. mils-severe necroinflammation in the liver, and with preserved vs. impaired liver function, which are of clinical relevance.

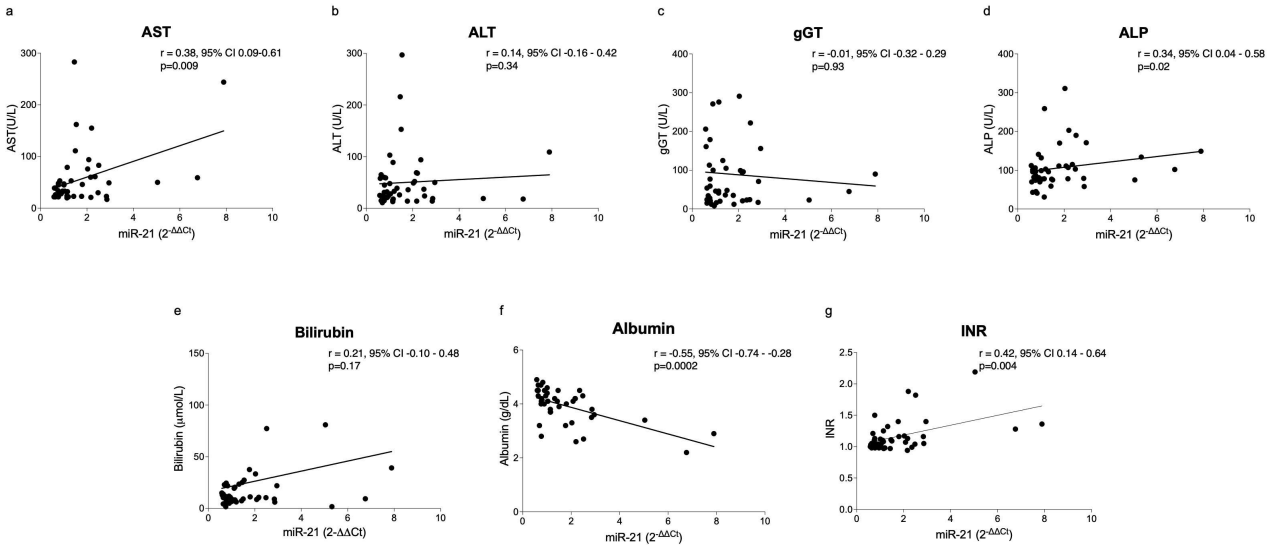
Despite its strengths (i.e., the analysis of groups of patients with different phases of liver damage), this study has also some limitations, the most important of which is its retrospective nature. In addition to potentially introducing unintended bias, this study design prevented us from collecting some data relevant for the analyses (for instance, liver stiffness at transient elastography was known only in 7 cirrhotic patients). Furthermore, the limited number of patients for each group likely prevented us from achieving more robust results.

In conclusion, we showed that miR-21 in a suitable marker in identifying the progression of liver damage, with highest levels found in cirrhosis and HCC patients compared to healthy subjects and patients with chronic liver disease. Since no differences in miR-21 levels were observed between cirrhotics and HCC patients, the evaluation of this biomarker seems not to be useful in diagnosing HCC (at least in Western countries, where the vast majority of cancers arises from a cirrhotic background). HIF-1α, when all patients were considered together, confirmed to be associated with miR-21, suggesting once more the role of this miRNA in modulating angiogenesis. Consistently with previous findings, miR-21 correlated with fibrosis, hepatic necroinflammatory activity and liver function parameters, and it could be a very useful marker in providing information on different aspects of chronic liver diseases.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Levels of miR-21 in patients with normal and abnormal AST (a) and ALT (b). Patients with elevated AST had significantly higher levels of miR-21 (a). (*** p<0.001 and ≥0.0001; ns, non-significant)



Supplementary Figure 2. Correlations between miR-21 levels and AST (**a**), ALT (**b**), gGT (**c**), ALP (**d**), bilirubin (**e**), albumin (**f**) and INR (**g**) in chronic hepatitis and cirrhotic patients. A positive correlation was demonstrated between miR-21 levels and AST, ALP and INR, while a negative correlation was shown with albumin.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA. Cancer J. Clin. 2021;71:209–249.
2. Arnold M, Abnet CC, Neale RE, Vignat J, Giovannucci EL, McGlynn KA, et al. Global Burden of 5 Major Types of Gastrointestinal Cancer. Gastroenterology. 2020;159:335-349.e15.
3. Ambros V. The functions of animal microRNAs. Nature. 2004;431:350–355.
4. Wang X, He Y, Mackowiak B, Gao B. MicroRNAs as regulators, biomarkers and therapeutic targets in liver diseases. Gut. 2021;70:784–795.
5. Zhang T, Yang Z, Kusumanchi P, Han S, Liangpunsakul S. Critical Role of microRNA-21 in the Pathogenesis of Liver Diseases. Front. Med. 2020;7:7.
6. Damania P, Sen B, Dar SB, Kumar S, Kumari A, Gupta E, et al. Hepatitis B virus induces cell proliferation via HBx-induced microRNA-21 in hepatocellular carcinoma by targeting programmed cell death protein4 (PDCD4) and phosphatase and tensin homologue (PTEN). PLoS One. 2014;9:e91745.
7. Wei Y-F, Cui G-Y, Ye P, Chen J-N, Diao H-Y. MicroRNAs may solve the mystery of chronic hepatitis B virus infection. World J. Gastroenterol. 2013;19:4867–4876.
8. Bandiera S, Pernot S, El Saghire H, Durand SC, Thumann C, Crouchet E, et al. Hepatitis C Virus-Induced Upregulation of MicroRNA miR-146a-5p in Hepatocytes Promotes Viral Infection and Deregulates Metabolic Pathways Associated with Liver Disease Pathogenesis. J. Virol. 2016;90:6387–6400.
9. Wu H, Ng R, Chen X, Steer CJ, Song G. MicroRNA-21 is a potential link between non-alcoholic fatty liver disease and hepatocellular carcinoma via modulation of the HBP1-p53-Srebp1c pathway. Gut. 2016;65:1850–1860.
10. Loyer X, Paradis V, Hénique C, Vion A-C, Colnot N, Guerin CL, et al. Liver microRNA-21 is overexpressed in non-alcoholic steatohepatitis and contributes to the disease in experimental models by inhibiting PPARα expression. Gut. 2016;65:1882–1894.
11. Vinciguerra M, Sgroi A, Veyrat-Durebex C, Rubbia-Brandt L, Buhler LH, Foti M. Unsaturated fatty acids inhibit the expression of tumor suppressor phosphatase and tensin homolog (PTEN) via microRNA-21 up-regulation in hepatocytes. Hepatology. 2009;49:1176–1184.
12. Wu N, McDaniel K, Zhou T, Ramos-Lorenzo S, Wu C, Huang L, et al. Knockout of microRNA-21 attenuates alcoholic hepatitis through the VHL/NF-κB signaling pathway in hepatic stellate cells. Am. J. Physiol. Gastrointest. Liver Physiol. 2018;315:G385–G398.
13. John K, Hadem J, Krech T, Wahl K, Manns MP, Dooley S, et al. MicroRNAs play a role in spontaneous recovery from acute liver failure. Hepatology. 2014;60:1346–1355.
14. Pan X, Wang Z-X, Wang R. MicroRNA-21: a novel therapeutic target in human cancer. Cancer Biol. Ther. 2010;10:1224–1232.
15. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology. 2007;133:647–658.
16. Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, et al. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J. Clin. Oncol. 2011;29:4781–4788.
17. Tomimaru Y, Eguchi H, Nagano H, Wada H, Kobayashi S, Marubashi S, et al. Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J. Hepatol. 2012;56:167–175.
18. Liao Q, Han P, Huang Y, Wu Z, Chen Q, Li S, et al. Potential Role of Circulating microRNA-21 for Hepatocellular Carcinoma Diagnosis: A Meta-Analysis. PLoS One. 2015;10:e0130677.
19. Bharali D, Banerjee BD, Bharadwaj M, Husain SA, Kar P. Expression Analysis of MicroRNA-21 and MicroRNA-122 in Hepatocellular Carcinoma. J. Clin. Exp. Hepatol. 2019;9:294–301.
20. Pu C, Huang H, Wang Z, Zou W, Lv Y, Zhou Z, et al. Extracellular Vesicle-Associated mir-21 and mir-144 Are Markedly Elevated in Serum of Patients With Hepatocellular Carcinoma. Front. Physiol. 2018;9:930.
21. Bihrer V, Waidmann O, Friedrich-Rust M, Forestier N, Susser S, Haupenthal J, et al. Serum microRNA-21 as

marker for necroinflammation in hepatitis C patients with and without hepatocellular carcinoma. PLoS One. 2011;6:e26971.

22. Guo X, Lv X, Lv X, Ma Y, Chen L, Chen Y. Circulating miR-21 serves as a serum biomarker for hepatocellular carcinoma and correlated with distant metastasis. Oncotarget. 2017;8:44050–44058.

23. Xu J, Wu C, Che X, Wang L, Yu D, Zhang T, et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol. Carcinog. 2011;50:136–142.

24. Liu L-Z, Li C, Chen Q, Jing Y, Carpenter R, Jiang Y, et al. MiR-21 induced angiogenesis through AKT and ERK activation and HIF-1alpha expression. PLoS One. 2011;6:e19139.

25. Donnem T, Fenton CG, Lonvik K, Berg T, Eklo K, Andersen S, et al. MicroRNA signatures in tumor tissue related to angiogenesis in non-small cell lung cancer. PLoS One. 2012;7:e29671.

26. Sabry D, El-Deek SEM, Maher M, El-Baz MAH, El-Bader HM, Amer E, et al. Role of miRNA-210, miRNA-21 and miRNA-126 as diagnostic biomarkers in colorectal carcinoma: impact of HIF-1alpha-VEGF signaling pathway. Mol. Cell. Biochem. 2019;454:177–189.

27. Pelizzaro F, Cardin R, Sartori A, Imondi A, Penzo B, Aliberti C, et al. Circulating MicroRNA-21 and MicroRNA-122 as Prognostic Biomarkers in Hepatocellular Carcinoma Patients Treated with Transarterial Chemoembolization. Biomedicines. 2021;9.

28. Marchesini G, Day CP, Dufour JF, Canbay A, Nobili V, Ratziu V, et al. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J. Hepatol. 2016;64:1388–1402.

29. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.

30. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology. 2015;62:440–451.

31. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. Semin. Liver Dis. 2010;30:52–60.

32. Sun C, Huang F, Liu X, Xiao X, Yang M, Hu G, et al. miR-21 regulates triglyceride and cholesterol metabolism in non-alcoholic fatty liver disease by targeting HMGCR. Int. J. Mol. Med. 2015;35:847–853.

33. Yamada H, Suzuki K, Ichino N, Ando Y, Sawada A, Osakabe K, et al. Associations between circulating microRNAs (miR-21, miR-34a, miR-122 and miR-451) and non-alcoholic fatty liver. Clin. Chim. Acta. 2013;424:99–103.

34. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, et al. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J. Exp. Med. 2010;207:1589–1597.

35. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008;456:980–984.

36. Marquez RT, Bandyopadhyay S, Wendlandt EB, Keck K, Hoffer BA, Icardi MS, et al. Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans. Lab. Invest. 2010;90:1727–1736.

37. Matsuzaki K, Murata M, Yoshida K, Sekimoto G, Uemura Y, Sakaida N, et al. Chronic inflammation associated with hepatitis C virus infection perturbs hepatic transforming growth factor beta signaling, promoting cirrhosis and hepatocellular carcinoma. Hepatology. 2007;46:48–57.

38. Dooley S, Hamzavi J, Ciuclan L, Godoy P, Ilkavets I, Ehnert S, et al. Hepatocyte-specific Smad7 expression attenuates TGF-beta-mediated fibrogenesis and protects against liver damage. Gastroenterology. 2008;135:642–659.

39. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature. 2008;454:56–61.

40. Yamamichi N, Shimomura R, Inada K, Sakurai K, Haraguchi T, Ozaki Y, et al. Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. Clin. cancer Res. an Off. J. Am. Assoc. Cancer Res. 2009;15:4009–4016.

41. Cermelli S, Ruggieri A, Marrero JA, Ioannou GN, Beretta L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. PLoS One. 2011;6:e23937.

CHAPTER 9

Circulating Prostaglandin E_2: a novel potential prognostic biomarker in patients with hepatocellular carcinoma

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ABSTRACT

Purpose. We aimed to explore the activation of Monoacylglycerol lipase (MAGL)/Cyclooxygenase-2 (COX-2)/Prostaglandin E_2 (PGE_2) axis in hepatocellular carcinoma (HCC), evaluating circulating PGE_2 as prognostic biomarker in HCC patients.

Methods. PGE_2 levels were measured in blood samples from 24 cirrhotics and 34 HCC patients consecutively collected between January 2016 and December 2017. In a subgroup of patients, tissue expression of MAGL mRNA and immunohistochemistry for MAGL and COX-2 were obtained.

Results. Despite tumor tissues showing overexpression of MAGL mRNA and higher levels of both MAGL and COX-2 at immunohistochemistry, PGE_2 levels were not significantly different in HCC and cirrhotics. HCC patients with circulating PGE_2 levels >14 pg/mL had a significantly shorter overall survival (19.4 vs. 49.9 months; p=0.03), the finding being confirmed by the multivariate analysis (HR 3.37 [95% CI 1.00 – 11.60]; p=0.05).

Conclusion. The MAGL/COX-2/PGE2 axis is activated in HCC, and circulating PGE_2 proved to be a potential prognostic biomarker.

INTRODUCTION

Hepatocellular carcinoma (HCC) develops almost invariably on a chronic liver disease characterized by persistent inflammation (1) and several evidences suggest that prostaglandins are involved in its pathogenesis (2). Monoacylglycerol lipase (MAGL) is the major source of pro-inflammatory prostaglandins in the liver (3). In addition to its role in lipid metabolism (hydrolysis of monoacylglycerols in glycerol and fatty acids (4)), MAGL acts as a pivotal enzyme in the endocannabinoid system converting 2-arachidonoylglycerol (2-AG) into arachidonic acid (AA). Although most of AA comes from MAGL activity, other enzymes such as fatty acids amide hydrolase (FAAH) are involved in its production (5). AA, in turn, is converted in prostaglandin E_2 (PGE_2), which is the most abundant prostaglandin in HCC, by cyclooxygenase-2 (COX-2) (6,7).

In different pre-clinical models, MAGL proved to have a role in the pathogenesis of different liver diseases, including Non-Alcoholic Fatty-Liver Disease and primary sclerosing cholangitis (PSC) (3,8–10). In mice models, MAGL deletion has been associated with reduced liver inflammation and resistance to fibrosis (3). Moreover, its inhibition slows down fibrosis progression and reduce liver injury caused by ischemia/reperfusion (3,8). MAGL knock-out prevent liver steatosis in mice fed with a high-fat diet promoting lipid storage in adipose tissue and malabsorption of fatty acids (9) and is involved in the development of cholestasis and cholangitis (10). The upregulation of MAGL in HCC cells promotes tumor growth, cell proliferation and invasiveness, enhancing epithelial-to-mesenchymal transition (11,12), and some evidences demonstrate that its overexpression is associated with worse prognosis (12). Similarly, the down-regulation of COX-2 expression or treatment with celecoxib, a selective COX-2 inhibitor, reduce tumor cell proliferation and induce cell cycle arrest (13,14). Also COX-2 overexpression could predict shorter survival (14,15). PGE_2, the main product of MAGL/COX-2 axis, is involved in cell proliferation, inhibition of apoptosis, with consequent tumor growth, and enhancement of invasion and migration (16–22). The central role of

MAGL, COX-2 and PGE_2 in the pathogenesis of HCC is an additional demonstration of the relevance of the inflammatory milieu in tumor development and progression.

In HCC, a tumor still characterized by a dismal prognosis, there is a continuous search for new reliable biomarkers, in particular in the predictive and prognostic setting. Several molecules have been investigated as potential HCC marker (23,24), but to the best of our knowledge, no data are currently available on the determination of circulating PGE_2 as a HCC biomarker. Therefore, in this study we aimed not only to confirm the activation of the MAGL/COX-2/PGE_2 pathway in HCC, but also to investigate serum PGE_2 as potential prognostic biomarker.

PATIENTS AND METHODS

Blood samples of 34 HCC patients consecutively admitted to the Gastroenterology Unit of Padova University Hospital for trans-arterial chemoembolization (TACE) and 24 cirrhotics were collected between January 2016 and December 2017 and retrospectively analyzed. In HCC patients the blood sample was collected immediately before TACE. Dynamic computed tomography (CT), magnetic resonance (MRI) or liver biopsy were used, at the time of the study entry, to diagnose the tumor in HCC patients (according to European guidelines (25)) or rule it out in cirrhotics.

Standard demographic, clinical and tumor variables (number/size of lesions, presence of macrovascular invasion and extra-hepatic spread) were recorded. The Barcelona Clinic Liver Cancer (BCLC) system was used for tumor staging.

Patients provided written informed consent for the participation to this study, which was conducted in accordance to the Declaration of Helsinki and approved by the local Ethics Committee (approval number 3312/AO/14).

PGE_2 quantification

A commercial ELISA kit (Cusabio, Texas, USA) was used to determine PGE_2 in serum samples, according to manufacturer's instructions. The amount of PGE_2 (pg/mL) was derived by interpolation of samples absorbance on the calibration curves. Plates precoated with a PGE_2 specific antibody were incubated with 50 µL of serum. PGE_2 was revealed by the Detection Reagent A/B at 450 nm.

MAGL mRNA quantification and immunohistochemistry for MAGL, COX-2 and FAAH

Formalin-fixed, paraffin-embedded tissue samples from 10 HCC patients (29.4%) and in 10 cirrhotics (41.7%), who subsequently underwent liver transplantation for end-stage liver disease, were analyzed.

RecoverAll™ Total Nucleic Acid Isolation Kit and *SuperScript™ VILO™ cDNA Synthesis Kit* (Invitrogen, California, USA) were used for total RNA extraction and reverse transcription for cDNA synthesis, respectively. The expression of MAGL mRNA was evaluated by quantitative Real Time PCR (*EXPRESS SYBR™ GreenER™ qPCR Supermix, Universal Kit;* Invitrogen, California, USA), according to the manufacturer's instructions, on a PRISM 7900HT system (Applied Biosystems, Foster City, California, USA) with β-actin as internal reference. The relative expression of MAGL mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (fold-change).

Immunohistochemistry for MAGL and FAAH was performed using *Anti-MGLL and Anti-FAAH antibodies* (ATLAS Antibodies, Stockholm, Sweden), while COX-2 expression was analyzed with the *COX-2/Cyclooxygenase 2/PTGS2 Polyclonal ANTIBODY Kit* (PROTEINTECH, Manchester, UK), according to manufacturer's instructions. The expression of MAGL and FAAH was reported as H-score, a semi-quantitative score based on positive percentage and intensity of staining, graded as weak (1+), moderate (2+) or strong (3+). The H-score was calculated as follows: *(1+)% x 1 + (2+)% x 2 + (3+)% x 3*. Due to the very low percentage of cells positive for COX-2, tissue slices were classified as negative, if no positivity for COX-2 was detected, or otherwise positive.

Statistical analysis

Median (interquartile range [IQR]) and absolute frequency (percentage) were used to report quantitative and categorical variables, respectively. Mann-Whitney, χ^2 and Fischer's exact tests were used in the comparisons, as appropriate. Correlations were established calculating the Spearman coefficient.

Overall survival (OS) (median and 95% confidence interval [CI]), was calculated from the date of TACE to death, drop-out or data censoring (30th April 2020). The value of PGE_2 with maximal sensitivity and specificity (Receiver Operating Characteristics curve) was selected as prognostic cut-off. Kaplan-Meier method and log-rank test were used to estimate and compare survival curves. The independent prognostic variables were identified with a Cox multivariate analysis, inserting in the model only survival predictors ($p \leq 0.1$) at univariate analysis.

A p-value <0.05 was considered as significant and IBM SPSS Statistics (Version 25.0. Armonk, NY: IBM Corp.) was used for all the calculations.

RESULTS

PGE_2 circulating levels

Baseline characteristics of included patients are shown in Table 1.

There were no statistically significant differences in PGE_2 levels between HCC and cirrhotics (30.3 [22.3-39.6] and 20.4 [12.0-47.8] pg/mL; p=0.73) (Figure 1a), possibly depending to the wide variability of the results in cirrhotics.

In HCC, significantly higher PGE_2 levels were found in males (32.9 [23.3-42.7] vs. 3.3 [1.2-5.5] pg/mL; p<0.0001), and in patients younger than 65 years (37.6 [22.9-51.3] vs. 26.5 [15.2-35.5] pg/mL; p=0.046), without differences according to etiology and severity of liver disease (Child-Pugh class and MELD). PGE_2 was positively correlated with number of liver lesions (r=0.50, 95% CI 0.19-0.72; p=0.002) but not with their diameter (r=0.08, 95% CI -0.28-0.41; p=0.66), and was higher in patients with multifocal tumors (35.8 [23.6-49.6] vs. 22.5 [4.8-27.85] pg/mL; p=0.002). Patients with BCLC 0-

A stage had significantly lower PGE_2 circulating levels than those with BCLC B-C stage (22.7 [6.9-30.3] vs. 36.2 [32.4-51.5] pg/mL; p=0.006. (Figure 1b-f)

In cirrhotics, there was a trend to higher levels of PGE_2 in males compared to females (39.6 [16.1-53.0] vs. 10.2 [4.2-33.3] pg/mL; p=0.07).

In the subgroup of 10 patients with available tumor histology, the two patients with Edmondson's grading G3 showed higher PGE_2 levels compared to G1-2 patients (51.1 pg /mL [49.6-52.6] vs. 30.2 pg/mL [22.5-40.0], respectively; no statistical comparison was performed since only 2 patients with grading G3 were included in our population). In the five patients with microvascular tumor invasion PGE_2 circulating levels were similar to those of patients without vascular invasion (35.3 pg/mL [19.9-52.6] and 37.8 pg/mL [23.6-49.4], respectively; p=0.84).

Table 1. Baseline characteristics of cirrhotics and HCC patients.

Variable		**Cirrhotics n=24**	**HCC n=34**	**p[a]**
Males/Females		19/5 (79.2/20.8)	30/4 (88.2/11.8)	0.47
Age (years)		63 (49-70)	67 (61-75)	0.04
Viral etiology		6 (25.0)	21 (61.8)	0.008
CRPH		21 (87.5)	17 (50.0)	0.005
Bilirubin (μmol/L)		32.8 (10.2-69.0)	13.7 (9.0-21.8)	0.02
INR		1.42 (1.13-1.79)	1.15 (1.08-1.25)	0.002
Albumin (mg/dL)		3.3 (2.9-3.8)	3.5 (3.2-3.8)	0.60
Creatinine (μmol/L)		88 (69-112)	78 (67-87)	0.07
CRP (mg/L)		10.4 (5.1-17.9)	2.9 (1.7-4-1)	<0.0001
AFP (ng/mL)		2.4 (1.9-4.0)	20.7 (4.7-70-4)	<0.0001
Platelets (x 10^9/L)		100 (69-149)	102 (69-139)	0.95
Child-Pugh	A B C	11 (45.8) 7 (29.2) 6 (25.0)	29 (85.3) 5 (14.7) -	0.001
MELD		13 (8-21)	9 (8-10)	0.002
Number of nodules		-	2 (1-4)	-
Diameter (cm)		-	2.0 (1.5-3.9)	-
MVI or EHS		-	2 (5.9)	-
ECOG-PS 0			34 (100)	
BCLC	0-A B-C	-	19 (55.9) 15 (44.1)	-

a) Mann-Whitney test, Fischer's exact test or χ^2 test, as appropriate.
Categorical variables are presents as absolute frequency and percentage, while continuous variables as median and interquartile range.
Abbreviations: CRPH, clinically relevant portal hypertension; INR, International Normalized Ratio; CRP, C-reactive protein; AFP, alpha-fetoprotein; MELD, Model for End-Stage Liver Disease; MVI, macrovascular invasion; EHS, extra-hepatic spread; BCLC, Barcelona Clinic Liver Cancer.

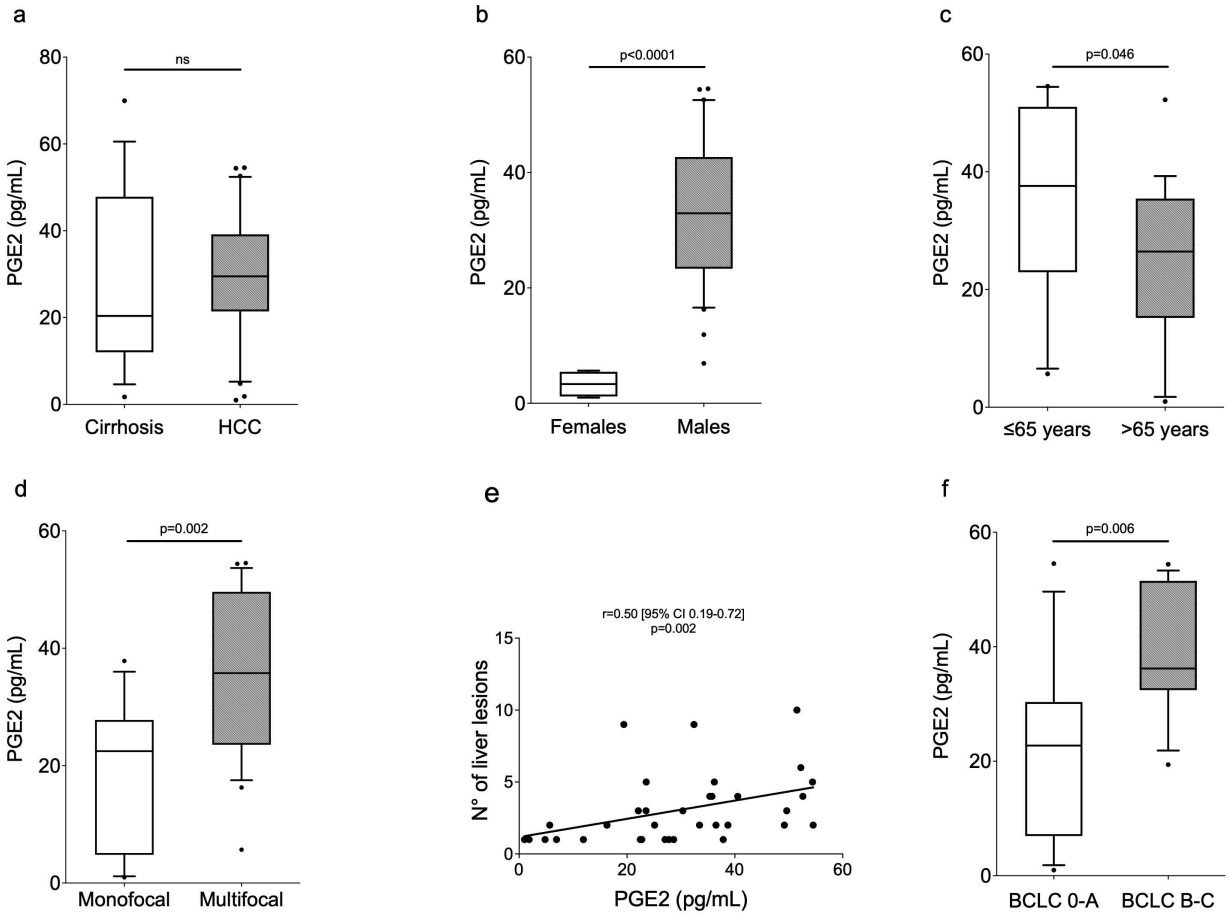


Figure 1. Circulating PGE_2 levels according to demographic, clinical and oncologic characteristics. Levels of circulating PGE_2 in cirrhotics and HCC patients are not significantly different (**a**). In patients with tumor, higher levels of PGE_2 were found in males (**b**) and in patients younger than 65 years (**c**). Patients with multifocal tumor had significantly higher levels of PGE_2 (**d**), and the marker was positively correlated with number of neoplastic liver lesions (**e**). Moreover, patients with BCLC B-C stages had higher levels of circulating PGE_2 compared to BCLC 0-A patients (**f**). (In the figure, boxes represent median and interquartile range, while whiskers represent 10^{th} and 90^{th} percentiles)

MAGL, COX-2 and FAAH tissue analysis (Figure 2)

HCC patients had a statistically significant higher relative expression of MAGL mRNA compared to cirrhotics (1.55-fold [0.23-6.05] vs. 0.0-fold [0.0-0.08]; p=0.002). Notably, in almost all cirrhotics (80%) no MAGL mRNA could be identified. At the immunohistochemistry, HCC patients had a significantly higher MAGL H-score compared to cirrhotics (275.0 [IQR, 227.5-290.0] vs. 185.0 [IQR, 150.0-202.5], respectively; p<0.0001), but a comparable expression of FAAH (100.0 [IQR 18.8-120.0] in HCC patients vs. 85.0 [IQR 32.5-112.5] in cirrhotics; p=0.87). COX-2 also was preferentially expressed in HCC patients: 7 were positive for COX-2 compared to only 3 cirrhotics (χ^2=3.2; p=0.07).

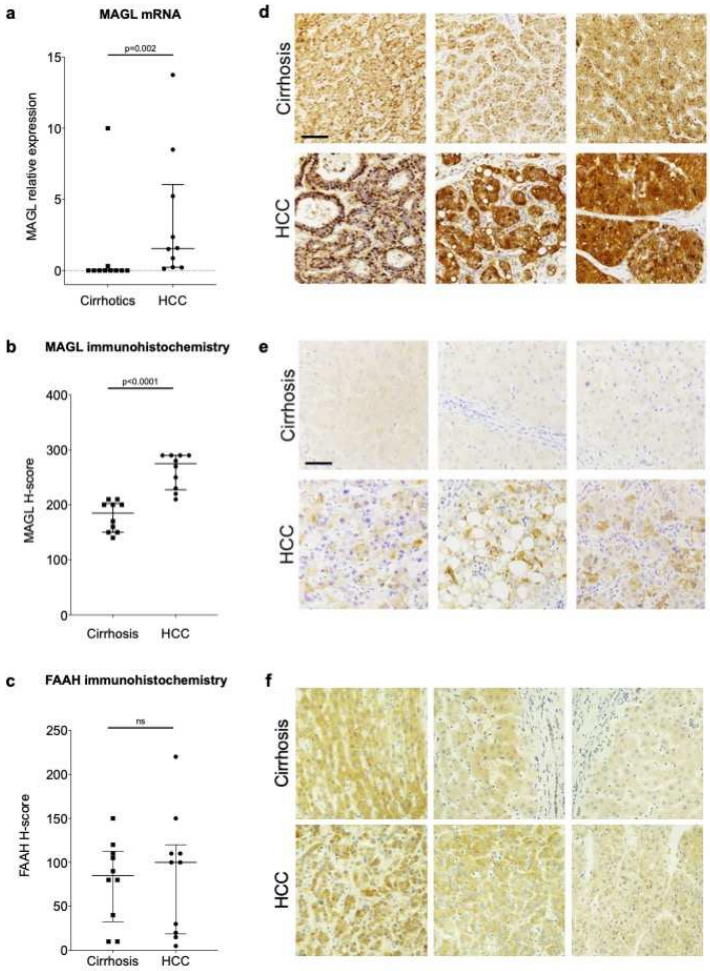


Figure 2. MAGL and COX-2 expression is increased in HCC patients compared to cirrhotics, while FAAH expression is similar in the two groups. MAGL mRNA relative expression (**a**) and MAGL H-score at immunohistochemistry (**b**) were significantly higher in HCC compared to cirrhotic patients; FAAH H-score was not significantly different between cirrhotics and HCC patients (**c**) (line at median and whiskers representing interquartile range). At the immunohistochemical evaluation, in HCC samples MAGL staining is stronger (**d**) and COX-2 expression is more frequent (**e**) compared to cirrhotic liver samples, while no differences between the two groups was demonstrated for FAAH staining (magnification 10x)

Survival analysis

The prognostic cut-off for circulating PGE_2 was identified at 14 pg/mL. Using this threshold, the marker stratified the patients according to survival. Median OS was 49.9 months (95% CI 32.1-59.7) in patients with PGE_2 ≤14 pg/mL and 19.4 months (95% CI 10.0-28.7) in the comparator group (p=0.03) (Figure 3). Moreover, PGE_2 proved to be an independent predictor of survival at the multivariate analysis (HR 3.37 [95% CI 1.00 – 11.60]; p=0.05) (Table 2).

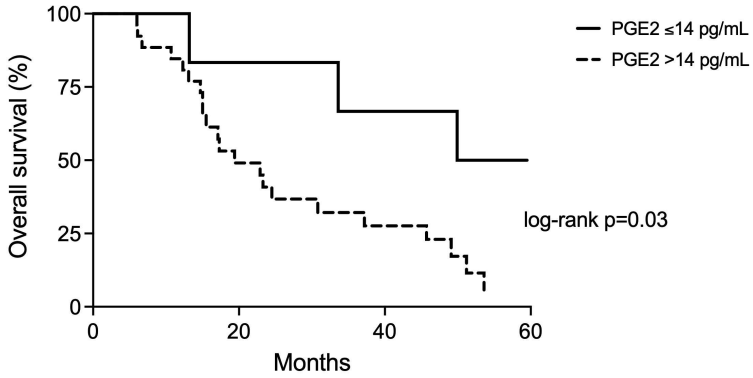


Figure 3. Kaplan-Meier survival curves according to the levels of PGE_2. Patients with PGE_2 ≤14 pg/mL had an overall survival significantly longer compared to patients with marker levels above the cut-off (p=0.03).

Table 2. Univariate and multivariate Cox regression analyses for independent predictors of survival.

Variable		Univariate analysis		Multivariate analysis	
		HR (95% CI)	p	HR (95% CI)	p
PGE_2 (pg/mL)	≤ 14	Ref	-	Ref	-
	> 14	3.57 (1.04-12.24)	0.04	3.37 (1.00-11.60)	0.05
Sex	Females	Ref	-	Ref	-
	Males	3.62 (0.84-15.58)	0.08	1.44 (0.13-15.98)	0.77
Age (years)	≤ 65	Ref	-		
	> 65	2.01 (0.85-4.78)	0.12		
Etiology	Not viral	Ref	-		
	Viral	0.87 (0.38-2.01)	0.75		
CRPH	No	Ref	-		
	Yes	1.45 (0.65-3.23)	0.36		
Child-Pugh	A	Ref	-	Ref	-
	B	3.05 (1.10-8.45)	0.03	2.11 (0.68-6.54)	0.19
MELD		1.28 (1.04-1.58)	0.02	1.26 (1.03-1.53)	0.02
Multifocality	No	Ref	-		
	Yes	1.86 (0.77-4.50)	0.17		
Diameter (cm)	≤ 3	Ref	-		
	> 3	1.01 (0.44-2.27)	0.97		
BCLC stage	0/A	Ref	-	Ref	-
	B/C	2.13 (0.95-4.78)	0.07	1.31 (0.51-3.33)	0.57
AFP (ng/mL)	≤ 200	Ref	-		
	> 200	1.10 (0.41-2.97)	0.85		
Radiological response	CR+PR+SD	Ref	-		
	PD	0.84 (0.33-2.12)	0.84		
Post-TACE treatments	No	Ref	-	Ref	-
	Yes	0.45 (0.19-1.09)	0.08	0.58 (0.22-1.56)	0.28

Abbreviations: HR, hazard ratio; Ref, reference group; PGE2, prostaglandin E_2; CRPH, clinically relevant portal hypertension; MELD, Model for End-Stage Liver Disease; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein; TACE, transarterial chemoembolization.

DISCUSSION

An amount of data has been produced on the involvement of MAGL and its metabolites in the development and progression of liver diseases. In pre-clinical studies, MAGL inhibition proved to be

beneficial in slowing down liver fibrosis progression (3), in reducing ischemia/reperfusion injury (8), in preventing liver steatosis (9) and in protecting against biliary fibrosis and inflammation in sclerosing cholangitis models (10). In addition, it has been recently demonstrated that MAGL and its metabolites are able to modulate gut-liver axis: the activation of nuclear receptors (PPARα, PPARγ and RXRα) by arachidonic acid leads to a decrease intestinal inflammation and PGE_2 levels, and triggers protective mechanisms against cholestatic liver disease progression (10). In addition, several studies demonstrated an involvement of the MAGL/COX-2/PGE_2 pathway in the pathogenesis of HCC (12–14,16–20), and high MAGL and COX-2 levels seem to predict poorer prognosis (12,14,15). However, to the best of our knowledge, there is a substantial lack of data in the literature on the role of PGE_2, the final product of this metabolic pathway, as circulating biomarker. In this study, we found that PGE_2 may represent a promising prognostic marker, since it is able to differentiate HCC patients according to their prognosis. Patients with higher circulating prostaglandin showed a worse prognosis compared to those with lower levels.

Zang S et al. evaluated tissue PGE_2 levels, demonstrating higher levels of prostaglandin in HCC compared to peritumoral and normal liver tissue (7), but in our experience circulating PGE_2 levels were not significantly different between HCC and cirrhotic patients. Prostaglandins level in serum reflect not only liver specific, but also systemic inflammation. Cirrhosis has been recognized as a systemic inflammatory multiorgan disease, with systemic inflammation playing a central role in the development of decompensation and organ dysfunction in liver disease (26). Indeed, in cirrhosis, inflammatory markers (such as white blood cell count, activated circulating neutrophils and monocytes, plasma C-reactive protein, pro-inflammatory cytokines, markers of macrophage activation) are often increased (27). This could contribute to explain our findings since, in our cohort, cirrhotics were found to be more frequently decompensated than HCC patients (with higher rates of CRPH, more frequent Child-Pugh class B-C and higher MELD score). Since systemic inflammation

is associated with decompensation of liver disease, the lack of statistical difference between cirrhotics and HCC patients could be attributed to the more pronounced systemic inflammation in the former. This is also confirmed by the significantly higher levels of CRP in cirrhotics. Nevertheless, we observed a higher expression of MAGL and COX-2 in neoplastic tissue, confirming the importance of the activation of this inflammatory pathway in HCC (12), and PGE_2 levels, despite not significantly different, were almost 50% higher, as a median, in HCC patients than in cirrhotics.

Since MAGL is not the only enzyme responsible for providing the precursors for prostaglandin synthesis with the degradation of 2-AG, we also evaluated the expression of FAAH in cirrhotics and HCC patients. We found comparable expression levels in the two groups, demonstrating that although the amount of circulating PGE_2 level is not only the result of MAGL activity, the overexpression of this enzyme in neoplastic tissue is responsible for the largest share of prostaglandins found in patients with HCC.

PGE_2 circulating levels were significantly higher in HCC males compared to females, and this was confirmed in cirrhotics as a trend. There are some data in literature proving that estradiol decreases PGE_2 production (28,29). Despite the altered sex hormones profiles in patients with chronic liver disease (30) and the limited number of females in our cohort, we can speculate on a role of sex hormones in modulating levels of prostaglandins.

Patients with higher levels of PGE_2 were characterized by more advanced tumors, with multinodular disease and advanced BCLC stages, with a positive correlation between the levels of the marker and number of nodules. These results confirm previous data (7) and underline the importance of inflammation and PGE_2 in promoting neoplastic progression (17–22).

In this study, we confirmed the activation of MAGL/COX-2/PGE_2 pathway in HCC and, to the best of our knowledge, reported for the first time a potential role of the determination of circulating PGE_2 in predicting prognosis of patients treated with TACE. Despite its limitations (in particular the

retrospective nature and the small number of patients included), this study should be interpreted as a proof of concept, aimed at providing the rationale for future investigations confirming the role of serum PGE_2 as a biomarker of HCC, also given the simple methodology of the determination.

REFERENCES

1. Elsharkawy AM, Mann DA. Nuclear factor-κB and the hepatic inflammation-fibrosis-cancer axis. Hepatology. 2007;46:590–597.
2. Wu T. Cyclooxygenase-2 in hepatocellular carcinoma. Cancer Treat. Rev. 2006;32:28–44.
3. Habib A, Chokr D, Wan JH, Hegde P, Mabire M, Siebert M, et al. Inhibition of monoacylglycerol lipase, an anti-inflammatory and antifibrogenic strategy in the liver. Gut. 2019;68:522–532.
4. Zechner R, Zimmermann R, Eichmann TO, Kohlwein SD, Haemmerle G, Lass A, et al. FAT SIGNALS - Lipases and lipolysis in lipid metabolism and signaling. Cell Metab. 2012;15:279–291.
5. Tripathi RKP. A perspective review on fatty acid amide hydrolase (FAAH) inhibitors as potential therapeutic agents. Eur. J. Med. Chem. 2020;188.
6. Tardelli M. Monoacylglycerol lipase reprograms lipid precursors signaling in liver disease. World J. Gastroenterol. 2020;26:3577–3585.
7. Zang S, Ma X, Wu Y, Liu W, Cheng H, Li J, et al. PGE2 synthesis and signaling in malignant transformation and progression of human hepatocellular carcinoma. Hum. Pathol. 2017;63:120–127.
8. Cao Z, Mulvihill MM, Mukhopadhyay P, Xu H, Erdélyi K, Hao E, et al. Monoacylglycerol lipase controls endocannabinoid and eicosanoid signaling and hepatic injury in mice. Gastroenterology. 2013;144:808–817.
9. Tardelli M, Bruschi F V., Claudel T, Fuchs CD, Auer N, Kunczer V, et al. Lack of monoacylglycerol lipase prevents hepatic steatosis by favoring lipid storage in adipose tissue and intestinal malabsorption. J. Lipid Res. 2019;60:1284–1292.
10. Tardelli M, Bruschi F V., Fuchs CD, Claudel T, Auer N, Kunczer V, et al. Monoacylglycerol Lipase Inhibition Protects From Liver Injury in Mouse Models of Sclerosing Cholangitis. Hepatology. 2020;71:1750–1765.
11. Zhu W, Zhao Y, Zhou J, Wang X, Pan Q, Zhang N, et al. Monoacylglycerol lipase promotes progression of hepatocellular carcinoma via NF-κB-mediated epithelial-mesenchymal transition. J. Hematol. Oncol. 2016;9:127.
12. Zhang J, Liu Z, Lian Z, Liao R, Chen Y, Qin Y, et al. Monoacylglycerol Lipase: A Novel Potential Therapeutic Target and Prognostic Indicator for Hepatocellular Carcinoma. Sci. Rep. 2016;6:35784.
13. Lv X, Chen Z, Li S, Xie H. Knockdown of cyclooxygenase-2 leads to growth inhibition and cell cycle arrest in hepatocellular carcinoma cells. Onco. Targets. Ther. 2019;12:4341–4349.
14. Tai Y, Zhang LH, Gao JH, Zhao C, Tong H, Ye C, et al. Suppressing growth and invasion of human hepatocellular carcinoma cells by celecoxib through inhibition of cyclooxygenase-2. Cancer Manag. Res. 2019;11:2831–2848.
15. Chen G, Li X, Yang J, Li J, Wang X, He J, et al. Prognostic signifcance of cyclooxygenase-2 expression in patients with hepatocellular carcinoma: A meta-analysis. Arch. Med. Sci. 2016;12:1110–1117.
16. Leng J, Han C, Demetris AJ, Michalopoulos GK, Wu T. Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: Evidence for Akt inhibition in celecoxib-induced apoptosis. Hepatology. 2003;38:756–768.
17. Li T, Zhong J, Dong X, Xiu P, Wang F, Wei H, et al. Meloxicam suppresses hepatocellular carcinoma cell proliferation and migration by targeting COX-2/PGE2-regulated activation of the β-catenin signaling pathway. Oncol. Rep. 2016;35:3614–3622.
18. Cheng SY, Zhang H, Zhang M, Xia SK, Bai XM, Zhang L, et al. Prostaglandin E2 receptor EP2 mediates Snail expression in hepatocellular carcinoma cells. Oncol. Rep. 2014;31:2099–2106.
19. Xia S, Ma J, Bai X, Zhang H, Cheng S, Zhang M, et al. Prostaglandin E2 promotes the cell growth and invasive ability of hepatocellular carcinoma cells by upregulating c-Myc expression via EP4 receptor and the PKA signaling pathway. Oncol. Rep. 2014;32:1521–1530.
20. Bai X, Wang J, Guo Y, Pan J, Yang Q, Zhang M, et al. Prostaglandin E2 stimulates β1-integrin expression in hepatocellular carcinoma through the EP1 receptor/PKC/NF-κB pathway. Sci. Rep. 2014;4:6538.
21. Mayoral R, Fernández-Martínez A, Boscá L, Martín-Sanz P. Prostaglandin E2 promotes migration and adhesion in hepatocellular carcinoma cells. Carcinogenesis. 2005;26:753–761.

22. Zhang H, Cheng S, Zhang M, Ma X, Zhang L, Wang Y, et al. Prostaglandin E2 promotes hepatocellular carcinoma cell invasion through upregulation of YB-1 protein expression. Int. J. Oncol. 2014;44:769–780.

23. Qu L, Cai X, Xu J, Wei X, Qu X, Sun L, et al. Six long noncoding RNAs as potentially biomarkers involved in competitive endogenous RNA of hepatocellular carcinoma. Clin. Exp. Med. 2020;20:437–447.

24. Weng J, Atyah M, Zhou C, Ren N. Prospects and challenges of circulating tumor DNA in precision medicine of hepatocellular carcinoma. Clin. Exp. Med. 2020;20:329–337.

25. Llovet JM, Ducreux M, Lencioni R, Di Bisceglie AM, Galle PR, Dufour JF, et al. EASL-EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2012;56:908–943.

26. Bernardi M, Moreau R, Angeli P, Schnabl B, Arroyo V. Mechanisms of decompensation and organ failure in cirrhosis: From peripheral arterial vasodilation to systemic inflammation hypothesis. J. Hepatol. 2015;63:1272–1284.

27. Waidmann O, Brunner F, Herrmann E, Zeuzem S, Piiper A, Kronenberger B. Macrophage activation is a prognostic parameter for variceal bleeding and overall survival in patients with liver cirrhosis. J. Hepatol. 2013;58:956–961.

28. Chaud M, Faletti A, de Estrada MB, Gimeno AL, Gimeno MAF. Synthesis and release of prostaglandins D2 and E2 by rat uterine tissue throughout the sex cycle. Effects of 17-β-estradiol and progesterone. Prostaglandins, Leukot. Essent. Fat. Acids. 1994;51:47–50.

29. Asselin E, Goff AK, Bergeron H, Fortier MA. Influence of sex steroids on the production of prostaglandins F2α and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium. Biol. Reprod. 1996;54:371–379.

30. Neong SF, Billington EO, Congly SE. Sexual Dysfunction and Sex Hormone Abnormalities in Patients With Cirrhosis: Review of Pathogenesis and Management. Hepatology. 2019;69:2683–2695.

CHAPTER 10

Prognostic role of platelets-to-lymphocytes ratio (PLR) and neutrophils-to-lymphocytes ratio (NLR) in hepatocellular carcinoma patients

Filippo Pelizzaro

ABSTRACT

Background. Platelets-to-lymphocytes ratio (PLR) and neutrophils-to-lymphocytes ratio (NLR) have been proposed as prognostic biomarkers in several cancers. In this study we aimed to evaluate their role as prognostic biomarkers in a large cohort of hepatocellular carcinoma (HCC) patients.

Methods. From the Italian Liver Cancer (ITA.LI.CA) database, data of 2,513 patients with available platelets, neutrophils and lymphocytes values were retrieved. PLR and NRL prognostic cut-offs were established with the ROC curve method. A subanalysis dividing patients according to treatment received was also performed.

Results. A significantly better prognosis was demonstrated in patients with PLR below the cut-off of 113.7 (38.0 [95% CI 34.3-41.7] vs. 29.0 [95% CI 24.3-33.7] months; p<0.0001) and NLR below the cut-off of 3.2 (40.1 [95% CI 35.6-44.6] vs. 25.3 [95% CI 21.7-28.9] months; p<0.0001), and both resulted independently associated with survival. The combination of PLR and NLR demonstrated a better prognostic stratification: the median survival was 40.5 months (95% CI 35.4-45.6) in patients with low levels of both biomarkers, 31.0 months (95% CI 25.9-36.1) in patients with one biomarker positive, and 24.6 months (95% CI 19.8-29.4) in patients with both NLR and PLR above their cut-off (p<0.0001). In the subanalysis according to treatment, PLR was an independent predictor in patients treated with ablation and systemic therapies, while NLR in patients treated with ablation and intra-arterial therapies.

Conclusions. PLR and NLR confirmed to be promising prognostic biomarkers, and particularly when combined they provide better stratify patients according to their survival. These scores appear to be more useful in specific treatment subgroups, but additional studies, possibly prospective, should be conducted in order to establish the precise setting of their applicability.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the most common primary liver cancer, and both its incidence and mortality are projected to increase in the near future (1). With a 5-year survival of 18% in United States (2) and 20% in Italy (3), HCC is one of the leading causes of cancer related mortality. Therefore, the identification of reliable prognostic parameters, including circulating biomarkers, is fundamental for an optimal clinical management of these patients. Alpha-fetoprotein (AFP) remains the most widely used and accepted serum biomarker in patients with HCC. Nevertheless, AFP performance as prognostic biomarker in HCC is not completely satisfying (4), making the identification of new markers urgently needed.

Non-resolving inflammation has a central role in carcinogenesis, contributing to the development of a malignant phenotype (5,6). Systemic inflammatory responses are involved in the promotion of angiogenesis, DNA damage and tumor invasion through up-regulation of cytokines (6). This is particularly relevant in HCC, that develops almost invariably on a chronic liver disease characterized by persistent inflammation (7). Recently, we demonstrated the role as prognostic biomarker of prostaglandin E_2, a key mediator of liver phlogosis (8). The association of inflammatory-based markers with the prognosis of HCC have been actively explored, and several inflammation and immune-based scores have been developed to predict survival and recurrence (9–14).

Among these scores, platelets-to-lymphocytes ratio (PLR) and neutrophils-to-lymphocytes ratio (NLR) seem to be very promising prognostic markers, also considering the easy determination. Inflammatory cells, such as platelets and neutrophils, can contribute to tumor cell invasion into the peripheral blood (15). Several studies demonstrated that platelets can protect circulating tumor cells from shear stresses during circulation, induce epithelial–mesenchymal transition, and promote tumor cell extravasation to metastatic sites (16–18). Neutrophils can promote adhesion and seeding of distant organ sites through secretion of circulating growth factors (12,13,19). By contrast,

lymphocytes are crucial in defense against tumors dictating the host immune response to malignancy by inducing cytotoxic cell death and inhibiting tumor cell proliferation and migration (20). Therefore, PLR and NLR reflect the potential balance between the platelets- and neutrophils-associated pro-tumor inflammation and lymphocytes-dependent anti-tumor immune-function (21). High levels of NLR and PLR may represent a trend toward increased pro-tumor inflammation and decreased anti-tumor immune function.

Several studies ad metanalysis showed that PLR and NLR could be useful in prognostic stratification (9,12,13,22–25). In this study, we aimed to confirm the potential prognostic role of PLR and NLR in a large population of HCC patients, regardless of the treatment received. In addition, a subanalysis according to treatment was performed.

PATIENTS AND METHODS

In this retrospective study, data were retrieved from the Italian Liver Cancer Database (ITA.LI.CA) database, a multicenter registry including 7,817 HCC patients prospectively collected from January 1988 to December 2018 in 24 participating Institutions. Data are updated every 2 years, and their accuracy is controlled by a data manager in the coordinating center (Bologna University). From the entire population of patients included in the database, for the purpose of the present study all the patients diagnosed with HCC between January 2000 and December 2018 and with available values of platelets, lymphocytes and neutrophils were selected (n=2,513). HCC diagnosis was histologically confirmed in 493 patients (19.6%), whereas in the remaining cases it was based on the radiological criteria (at computed tomography [CT] or magnetic resonance imaging [MRI]), according to guidelines (26).

In the ITA.LI.CA database, demographic and clinicopathological data, such as age, sex, comorbidities, etiology of the underlying liver disease, main serological parameters (albumin, bilirubin,

international normalized ratio [INR], creatinine, alpha-fetoprotein [AFP], blood count, including data about White Blood Cells [WBC], neutrophils, lymphocytes and platelets), Child-Pugh class, Model for End Stage Liver Disease (MELD) score, presence of ascites and hepatic encephalopathy and Eastern Cooperative Oncology Group performance status (ECOG-PS), are recorded. The presence of clinically significant portal hypertension (CSPH) is registered in the database and its diagnosis was based on unequivocal signs (presence of splenomegaly, varices, ascites) and platelet count <100 x 10^9/L (27). The database also reports main macroscopic tumor characteristics (location and size, number of nodules, macrovascular invasion [MVI] and extra-hepatic spread [EHS]) evaluated with dynamic CT or MRI. In this study, for staging purposes we used the BCLC staging system (26).

The complete sequence of treatments for every patient is also registered in the ITA.LI.CA database. The following treatment groups were considered in the present study: liver transplantation (LT), liver resection (LR), ablative procedures (ABL: percutaneous ethanol injection, percutaneous or laparoscopic thermal ablation), intra-arterial therapies (transarterial chemoembolization [TACE], trans-arterial embolization [TAE], selective internal radiation therapy [SIRT]), systemic therapy with sorafenib (SOR) and best supportive care (BSC).

Statistical analysis

Categorical variables were reported as absolute and relative frequency (percentages), while quantitative variables as median and interquartile range (IQR). Mann-Whitney test was used to compare quantitative variables, meanwhile χ^2 test and Fischer's exact test were used in the comparison of categorical variables as appropriate.

The value of PLR and NLR with maximal sensitivity and specificity (Receiver Operating Characteristics curve) was selected as prognostic cut-off in the whole population of patients and, specifically, in each treatment subgroup.

Overall survival (OS), expressed as median and 95% confidence interval (CI), was calculated from diagnosis to death from any cause or last follow-up. For patients alive at the end of the study, survival was censored at December 31st 2018. Survival curves were calculated with the Kaplan-Meier method and compared by the log-rank test. The independent variables predicting survival were identified by the multivariate Cox regression analysis, including in the analysis the variables associated with survival ($p \leq 0.1$) at the univariate analysis. In order to avoid collinearities between variables, three multivariate models were created, all containing clinical and oncologic features associated with survival, but different liver function variables: model 1 contained MELD score, Model 2 contained Child-Pugh class, and in Model 3 the variables forming these scores were included.

In all the analyses a two-tails p value <0.05 was considered as statistically significant. Data were analyzed by IBM SPSS Statistics (version 25.0. Armonk, NY: IBM Corp) and GraphPad Prism version 8.3.1 (GraphPad Software, La Jolla, California, USA).

RESULTS

Baseline characteristics

Baseline characteristics of included patients are shown in Table 1.

In the overall population of patients, the prognostic cut-off established with the ROC curve method for PLR was 113.7 (Supplementary Figure 1a). Patients with PLR above the cut-off were significantly older (71 [64-77] vs. 69 [60-76] years; p<0.0001), less frequently diagnosed with HCC under surveillance (52.6% vs. 62.7%; p<0.0001) and less frequently cirrhotics (82.1% vs. 91.1%; p<0.0001). Liver disease was virus-related in the majority of patients in both groups, but not-viral liver disease was more frequent in those with higher PLR levels (42.2% vs. 37%; p=0.02). The percentage of patients with CSPH was lower in patients with PLR above the cut-off (70.0% vs. 81.8%; p<0.0001).

As far as tumor-related variables are concerned, patients with higher PLR levels had slightly larger liver lesions (2.7 cm [1.8-4.5] vs. 2.4 cm [1.7-4.0]; p<0.0001), more frequently MVI (13.3% vs. 8.7%; p=0.001), more frequently EHS (11.4% vs. 5.2%; p<0.0001) and higher levels of AFP (≥200 ng/mL in 20.0% vs. 14.8%; p=0.001). BCLC B-C stage tumors were diagnosed more frequently in patients with PLR values above the cut-off, and these patients were managed less frequently with LT (2.9% vs. 3.7%) and ABL (24.0% vs. 33.0%), and more frequently with LR (13.4% vs. 9.8%), SOR (15.7% vs. 11.5%) and BSC (5.4% vs. 4.1%).

The prognostic cut-off for NLR identified with the ROC curve method was 3.2 (Supplementary Figure 1b). Patients in the high and low NLR group were comparable for gender and age, while a statistically significant higher percentage of patients was diagnosed under surveillance in the low NLR group (61.3% vs. 55.2%; p=0.004). Viral etiology was less frequent in patients with levels of NLR above the cut-off (43.9% vs. 51.3%; p=0.001). Patients with high NLR levels had more frequently CSPH (81.2% vs. 76.5% p=0.008) and less preserved liver function (significantly higher MELD values and significantly lower percentage of Child-Pugh class A). In addition, these patients showed a greater tumor burden, with higher number of liver lesions (2 [1-3] vs. 1 [1-3]; p<0.0001), larger tumor diameter (2.7 cm [1.8-4.5] vs. 2.4 cm [1.7-3.9]; p<0.0001), higher frequency of MVI (13.5% vs. 8.6%; p=0.0002) and EHS (10.3% vs. 5.8%; p<0.0001), and higher AFP levels (20.4% vs. 14.7%; p=0.0005). BCLC C and D stage tumors were more frequent in patients with NLR above the cut-off, and this group of patients was less frequently managed with ABL (23.9% vs. 32.8%) and more frequently treated with IAT (41.1% vs. 36.2%).

Table 1. Baseline characteristics of the study population.

Variables	PLR ≤113.7 (n=1699)	PLR >113.7 (n=814)	p	NLR ≤3.2 (n=1730)	NLR >3.2 (n=783)	p
Gender - Male	1331 (78.3)	636 (78.1)	0.88	1345 (77.7)	622 (79.4)	0.35
Age (years)	69 (60-76)	71 (64-77)	<0.0001	70 (61 -76)	70 (62-76)	0.70
Surveillance	1065 (62.7)	428 (52.6)	<0.0001	1061 (61.3)	432 (55.2)	0.004
Liver disease						
Cirrhosis Chronic hepatitis NAFLD/NASH Healthy liver	1547 (91.1) 107 (6.3) 33 (1.9) 12 (0.7)	668 (82.1) 82 (10.1) 35 (4.3) 29 (3.6)	<0.0001	1538 (88.9) 131 (7.6) 40 (2.3) 21 (1.2)	677 (86.5) 58 (7.4) 28 (3.6) 20 (2.6)	0.02
Etiology						
Viral Not viral Viral+other	849 (50.0) 629 (37.0) 221 (13.0)	383 (47.0) 345 (42.4) 86 (10.6)	0.02	888 (51.3) 630 (36.4) 212 (12.3)	344 (43.9) 344 (43.9) 95 (12.2)	0.001
CSPH	1389 (81.8)	570 (70.0)	<0.0001	1323 (76.5)	636 (81.2)	0.008
MELD	9 (8-12)	9 (8-11)	0.007	9 (8-11)	10 (8-12)	<0.0001
Child-Pugh class						
A B C	1152 (67.8) 500 (29.4) 47 (2.8)	552 (67.8) 242 (29.7) 20 (2.5)	0.90	1238 (71.6) 458 (26.5) 34 (2.0)	466 (59.5) 284 (36.3) 33 (4.2)	<0.0001
Albumin (g/dL)	3.6 (3.2 -4.0)	3.6 (3.2 -4.0)	0.90	3.7 (3.3 – 4.0)	3.6 (3.1 – 3.9)	<0.0001
Bilirubin (mg/dL)	1.0 (0.7 -1.6)	1.0 (0.7 -1.4)	0.002	1.00 (0.70 – 1.42	1.10 (0.80 – 1.80)	<0.0001
INR	1.18 (1.1 – 1.30)	1.15 (1.08 – 1.25)	<0.0001	1.17 (1.09 – 1.28)	1.19 (1.10 – 1.30	0.001
Creatinine (mg/dL)	0.83 (0.70 -1.00)	0.88 (0.74 – 1.04)	0.0003	0.83 (0.70 – 1.00)	0.88 (0.72 – 1.07)	0.003
Ascites	280 (16.5)	171 (21.0)	0.006	257 (14.9)	194 (24.8)	<0.0001
HE	86 (5.1)	40 (4.9)	0.92	71 (4.1)	55 (7.0)	0.003
ECOG-PS						
0 1-2 3-4	1234 (72.6) 436 (25.7) 29 (1.7)	561 (68.9) 235 (28.9) 18 (2.2)	0.14	1279 (73.9) 424 (24.5) 27 (1.6)	516 (65.9) 247 (31.5) 20 (2.6)	0.0001
Number	2 (1-3)	2 (1-3)	0.07	1 (1-3)	2 (1-3)	<0.0001
Diameter (cm)	2.4 (1.6 – 3.7)	2.7 (1.8 – 4.5)	<0.0001	2.4 (1.7 – 3.8)	2.7 (1.8 – 4.5)	<0.0001
MVI	147 (8.7)	108 (13.3)	0.001	149 (8.6)	106 (13.5)	0.0002
EHS	88 (5.2)	93 (11.4)	<0.0001	100 (5.8)	81 (10.3)	<0.0001
AFP >200 ng/mL	252 (14.8)	163 (20.0)	0.001	255 (14.7)	160 (20.4)	0.0005
BCLC stage						
0 A B C D	255 (15.0) 647 (38.1) 214 (12.6) 511 (30.1) 72 (4.2)	85 (10.4) 264 (32.4) 116 (14.3) 312 (38.3) 37 (4.5)	<0.0001	265 (15.8) 655 (37.9) 228 (13.2) 522 (30.2) 60 (3.5)	75 (9.6) 256 (32.7) 102 (13.0) 301 (38.4) 49 (6.3)	<0.0001
Treatment						
LT LR ABL IAT SOR BSC	63 (3.7) 167 (9.8) 560 (33.0) 643 (37.8) 196 (11.5) 70 (4.1)	24 (2.9) 109 (13.4) 195 (24.0) 305 (37.5) 128 (15.7) 53 (6.5)	<0.0001	62 (3.6) 199 (11.5) 568 (32.8) 626 (36.2) 212 (12.3) 63 (3.6)	25 (3.2) 77 (9.8) 187 (23.9) 322 (41.1) 112 (14.3) 60 (7.7)	<0.0001

Data are shown as median and interquartile range or absolute value and percentage.

Abbreviations: PLR, platelets-to-lymphocytes ratio; NLR, neutrophils-to-lymphocytes ratio; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; INR, international normalized ratio; HE, hepatic encephalopathy; ECOG-PS, Eastern Cooperative Oncology Group performance status; MVI, macrovascular invasion; EHS, extrahepatic spread; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; LT, liver transplantation; LR, liver resection; ABL, ablation, IAT, intra-arterial therapies; SOR, systemic therapies; BSC, best supportive care.

Survival analysis

In the whole population, the median follow-up was 24.8 months (95% CI 23.8-25.8) and the median OS was 35.2 months (95% CI 32.5-37.9) with a 5-years survival rate of 35.0%. At the end of the follow-up 1,135 patients were dead (45.2%), the majority from HCC progression (n=622, 54.8%). Liver decompensation and other causes were responsible for death in 281 patients (24.8%) and 232 patients (20.4%), respectively.

PLR proved to be able to stratify the survival of patients at the established cut-off, with a statistically significant better prognosis for patients with low PLR level. In fact, the median OS in patients with PLR ≤113.7 was 38.0 months (95% CI 34.3-41.7) vs. 29.0 months (95% CI 24.3-33.7) in patients with PLR >113.7 (p<0.0001) (Figure 1a). Also patients with higher levels of NLR showed worse prognosis compared to patients with lower NLR values. The median OS of NLR ≤3.2 group was 40.1 months (95% CI 35.6-44.6) compared to 25.3 months (95% CI 21.7-28.9) in patients with NLR >3.2 (p<0.0001) (Figure 1b).

Applying the ROC curve method, a prognostic cut-off was also established for platelets (110 x 10^9/L), neutrophils (5.35 x 10^9/L) and lymphocytes (1.44 x 10^9/L) alone. All these markers were associated with patient survival. Patients with high neutrophils level had shorter survival compared to patients with low neutrophils (31.5 months [95% CI 23.1-39.9] vs. 36.0 months [95% CI 33.0-39.1], p=0.02). By contrast, a poorer prognosis was demonstrated in patients with low levels of lymphocytes (29.1 months [95% CI 26.4-31.8] in patients with lymphocytes ≤1.44 x 10^9/L vs. 51.8 months [95% CI 43.5-60.1] in patients with higher levels; p<0.0001) and low level of platelets (30.4 months [95% CI 27.7-33-2] in patients with platelets ≤110 x 10^9/L vs. 43.2 months [95% CI 38.2-48.2] in those with higher levels; p=0.001).

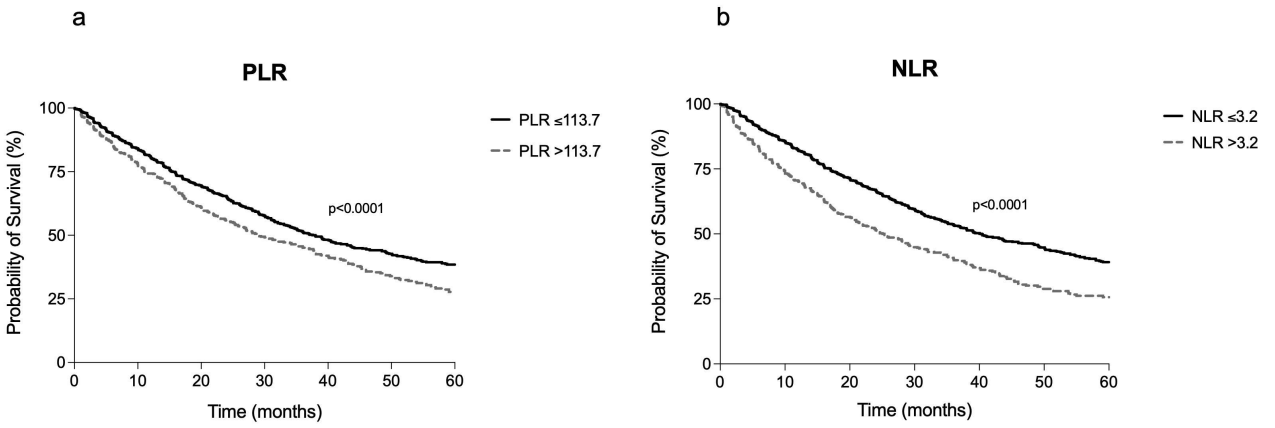


Figure 1. Survival of patients according to the level of PLR (a) and NLR (b). (a) Patients with PLR ≤113.7 had a statistically significant longer OS compared to patients with higher PLR values (p<0.0001). (b) Patients with NRL below the cut-off (3.2) had a statistically significant better prognosis compared to patients with NLR>3.2 (p<0.0001).

After adjustment for confounders (model 1), high PLR values remained independently associated with higher mortality risk (HR= 1.24, 95% CI 1.07-1.43). Similarly, also NLR was independently associated with survival (HR=1.40, 95% CI 1.21-1.62) (Table 2). The same results were obtained in model 2 (Supplementary Table 1) and 3 (Supplementary Table 2). Other relevant prognostic parameters were presence of CSPH, residual liver function, AFP levels, BCLC stage and treatment. When both PLR and NLR were included in multivariate model, the latter maintained its independent prognostic role (HR=1.34, 95% CI 1.15-1.57, in model 1), while PLR resulted not independently associated with survival (HR=1.09, 95% CI 0.93-1.28, in model 1) (see Supplementary Table 3 for model 2 and 3).

Table 2. Multivariate Cox analysis (model 1) for factors independently associated with survival.

	PLR model 1		**NLR model 1**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
PLR >113.7	1.24 (1.07-1.43)	0.004	-	-
NLR >3.2	-	-	1.40 (1.21-1.62)	<0.0001
Gender - males	1.17 (0.98-1.39)	0.09	1.17 (0.98-1.40)	0.08
Surveillance	0.96 (0.83-1.10)	0.54	0.96 (0.83-1.10)	0.54
Etiology				
Viral	Ref	-	Ref	-
Not viral	1.01 (0.87-1.18)	0.88	0.98 (0.84-1.14)	0.81
Viral + other	1.08 (0.87-1.34)	0.51	1.06 (0.85-1.31)	0.63
CSPH	1.46 (1.20-1.78)	0.0002	1.42 (1.17-1.73)	0.0004
MELD	1.06 (1.04-1.08)	<0.0001	1.06 (1.04-1.08)	<0.0001

logAFP	1.24 (1.26-1.32)	<0.0001	1.24 (1.17-1.32)	<0.0001
BCLC stage				
0	Ref	-	Ref	-
A	1.57 (1.16-2.12)	0.004	1.56 (1.15-2.12)	0.004
B	2.29 (1.63-3.20)	<0.0001	2.27 (1.62-3.18)	<0.0001
C	2.98 (2.19-4.06)	<0.0001	2.97 (2.18-4.04)	<0.0001
D	2.67 (1.71-4.15)	<0.0001	2.66 (1.71-4.14)	<0.0001
Treatment				
BSC	Ref	-	Ref	-
LT	0.03 (0.02-0.06)	<0.0001	0.03 (0.02-0.06)	<0.0001
LR	0.14 (0.10-0.21)	<0.0001	0.15 (0.10-0.22)	<0.0001
ABL	0.17 (0.12-0.23)	<0.0001	0.18 (0.13-0.24)	<0.0001
IAT	0.22 (0.16-0.29)	<0.0001	0.22 (0.17-0.30)	<0.0001
SOR	0.51 (0.38-0.69)	<0.0001	0.55 (0.41-0.74)	<0.0001

Abbreviations: PLR, platelets-to-lymphocytes ratio; NLR, neutrophils-to-lymphocytes ratio; HR, hazard ratio; CI, confidence interval; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; logAFP, logarithm of alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; LT, liver transplantation; LR, liver resection; ABL, ablation, IAT, intra-arterial therapies; SOR, systemic therapies; BSC, best supportive care.

The combination of NLR and PLR (defined as CNP) provided a better stratification of patient prognosis. We assigned to CNP a value of 0 with both PLR and NLR below their respective cut-offs, 1 with PLR or NLR above their respective cut-offs, and 2 with PLR and NLR above their respective cut-offs. The median OS was 40.5 months (95% CI 35.4-45.6) in patients with CNP 0, 31.0 months (95% CI 25.9-36.1) in patients with CNP 1, and 24.6 months (95% CI 19.8-29.4) in patients with CNP 2 (p<.0001) (Figure 2). The independent prognostic role of CNP, with survival progressively worsening from CNP 0 to CNP 2, was confirmed after adjustment for confoundings in multivariate analysis (Table 3).

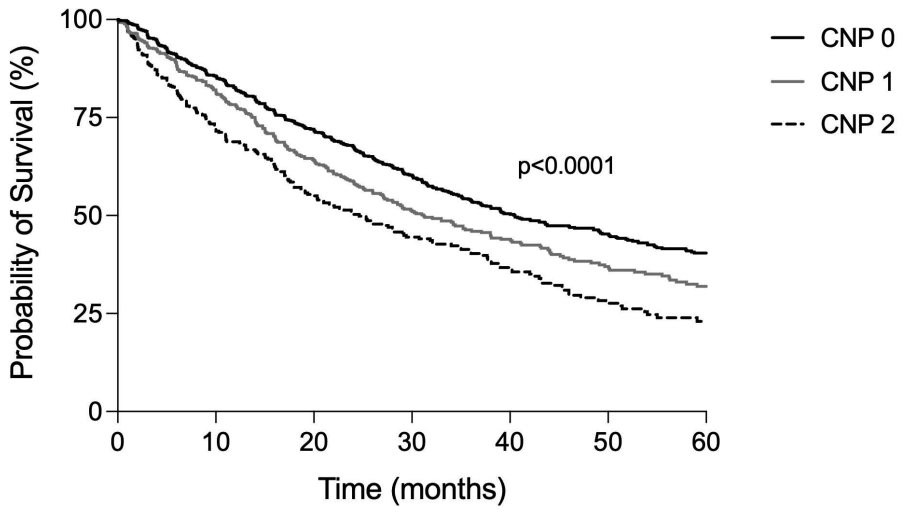


Figure 2. Survival of patients according to the combination of PLR and NLR values (CNP).

Table 3. Multivariate Cox models with adjustment of prognostic role of CNP for confounders.

	Model 1		Model 2		Model 3	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
CNP						
0	Ref	-	Ref	-	Ref	-
1	1.32 (1.12-1.55)	0.001	1.33 (1.13-1.56)	0.0005	1.29 (1.10-1.52)	0.001
2	1.43 (1.20-1.71)	<0.0001	1.41 (1.18-1.69)	0.0001	1.38 (1.16-1.64)	0.0003
Gender - males	1.16 (0.97-1.38)	0.11	1.12 (1.01-1.45)	0.04	1.20 (1.00-1.43)	0.05
Surveillance	1.04 (0.90-1.20)	0.57	0.96 (0.83-1.11)	0.60	0.97 (0.84-1.12)	0.70
Etiology						
Viral	Ref	-	Ref	-	Ref	-
Not viral	0.99 (0.85-1.15)	0.87	0.99 (0.85-0.16)	0.93	0.97 (0.83-1.13)	0.72
Viral + other	1.09 (0.87-1.35)	0.46	1.05 (0.84-1.31)	0.68	1.07 (0.86-1.33)	0.54
CSPH	1.48 (1.21-1.80)	0.0001	1.41 (1.15-1.73)	0.001	-[a]	-[a]
MELD	1.06 (1.04-1.08)	<0.0001	-	-	-	-
Child-Pugh class						
A			Ref	-		
B	-	-	1.49 (1.28-1.73)	<0.0001	-	-
C			2.76 (1.57-4.85)	0.0004		
Albumin	-	-	-	-	0.69 (0.60-0.79)	<0.0001
Bilirubin	-	-	-	-	1.07 (1.04-1.10)	<0.0001
INR	-	-	-	-	1.28 (0.98-1.69)	0.07
Creatinine (mg/dL)	-	-	-	-	1.05 (0.91-1.21)	0.51
Ascites	-	-	-	-	0.85 (0.71-1.01)	0.06
HE	-	-	-	-	1.09 (0.82-1.45)	0.54
logAFP	1.24 (1.16-1.32)	<0.0001	1.23 (1.16-1.31)	<0.0001	1.19 (1.12-1.27)	<0.0001
BCLC stage						
0	Ref	-	Ref	-	Ref	-
A	1.57 (1.16-2.12)	0.004	1.49 (1.09-2.02)	0.01	1.54 (1.15-2.08)	0.004
B	2.25 (1.61-3.16)	<0.0001	2.16 (1.54-3.04)	<0.0001	2.15 (1.54-3.01)	<0.0001
C	2.94 (2.16-4.00)	<0.0001	2.80 (2.05-3.84)	<0.0001	2.93 (2.16-3.97)	<0.0001
D	2.61 (1.67-4.06)	<0.0001	2.03 (1.15-3.58)	0.01	2.27 (1.43-3.60)	0.001
Treatment						
BSC	Ref	-	Ref	-	Ref	-
LT	0.03 (0.02-0.06)	<0.0001	0.03 (0.02-0.06)	<0.0001	0.04 (0.02-0.07)	<0.0001
LR	0.14 (0.10-0.21)	<0.0001	0.13 (0.09-0.19)	<0.0001	0.15 (0.10-0.22)	<0.0001
ABL	0.17 (0.13-0.24)	<0.0001	0.16 (0.12-0.22)	<0.0001	0.19 (0.14-0.26)	<0.0001
IAT	0.22 (0.16-0.30)	<0.0001	0.20 (0.15-0.27)	<0.0001	0.25 (0.18-0.33)	<0.0001
SOR	0.53 (0.39-0.71)	<0.0001	0.49 (0.37-0.66)	<0.0001	0.57 (0.42-0.77)	0.0003

a) not included to avoid collinearity with ascites.

CNP is defined as combination of PLR and NLR: 0 = PLR and NLR below their respective cut-offs; 1 = PLR or NLR above their respective cut-offs; 2 = PLR and NLR above their respective cut-offs.

Abbreviations: CNP, combination of NLR and PLR; HR, hazard ratio; CI, confidence interval; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; INR, international normalized ratio; HE, hepatic encephalopathy; logAFP, logarithm of alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; LT, liver transplantation; LR, liver resection; ABL, ablation, IAT, intra-arterial therapies; SOR, systemic therapies; BSC, best supportive care.

Subanalysis according to treatment

A prognostic cut-off for PLR and NLR was calculated with the ROC curve method in each treatment subgroup. Both PLR and NLR confirmed their prognostic role at the unadjusted analysis in all treatment subgroups, except for NLR in LT patients and both biomarkers in LR patients (the difference in survival did not reach the statistical significance, even though borderline p-values were demonstrated) (Table 3). After adjustment for confounders, an independent prognostic role was maintained for PLR in ABL, SOR and BSC subgroups. NLR demonstrated to be able to independently predict prognosis in ABL and IAT groups. PLR and NLR resulted to be significantly associated with prognosis in LT group after adjustment for confounders, but the estimated HR for both biomarkers seem not to be accurate, probably due to the low number of patients included in this group.

Table 3. Survival analysis according to treatment.

PLR	Median OS (months) (95% CI)	p	Adjusted HR (95% CI) [a]	NLR	Median OS (months) (95% CI)	p	Adjusted HR (95% CI) [a]
Liver transplantation							
≤108.7 >108.7	NE (NE-NE) 61.1 (NE-NE)	0.049	Ref 18.14 (2.08-158.02)	≤2.5 >2.5	NE (NE-NE) 119.0 (NE-NE)	0.25	Ref 8.39 (1.17-60.15)
Liver resection							
≤120.3 >120.3	78.2 (44.6-111.8) 68.2 (53.9-82.5)	0.08	Ref 1.24 (0.73-2.11)	≤3.6 >3.6	68.9 (45.7-92.2) 54.0 (28.5-79.5)	0.07	Ref 1.41 (0.70-2.85)
Ablation							
≤77.4 >77.4	62.0 (46.3-77.7) 44.1 (34.5-53.6)	0.007	Ref 1.47 (1.08-1.99)	≤2.9 >2.9	55.5 (47.4-63.6) 41.2 (33.0-49.4)	0.003	Ref 1.36 (1.01-1.84)
Intra-arterial therapies							
≤111.4 >111.4	32.1 (28.5-35.6) 27.2 (24.0-30.4)	0.02	Ref 1.24 (0.98-1.55)	≤2.3 >2.3	35.1 (30.3-39.8) 26.5 (23.5-29.6)	0.0002	Ref 1.32 (1.06-1.64)
Systemic therapies							
≤62.5 >62.5	17.7 (8.0-27.4) 10.2 (7.6-12.8)	0.01	Ref 2.03 (1.34-3.09)	≤3.3 >3.3	14.1 (10.4-17.8) 6.7 (5.3-8.1)	0.009	Ref 1.36 (0.96-1.94)
Best supportive care							
≤88.8 >88.8	6.0 (1.8-10.2) 3.0 (2.1-3.9)	0.048	Ref 2.12 (1.22-3.70)	≤2.9 >2.9	6.1 (3.2-8.9) 2.4 (1.5-3.4)	0.009	Ref 1.32 (0.77-2.25)

[a] adjusted for gender, etiology of the underlying liver disease, surveillance, presence of CSPH, BCLC stage, AFP levels and MELD score. Similar results were obtained after correction with Child-Pugh class instead of MELD score at the multivariate analysis.
Abbreviations: PLR, platelets-to-lymphocytes ratio; NLR, neutrophils-to-lymphocytes ratio; OS, overall survival; HR, hazard ratio; CI, confidence interval; NE, not estimable.

DISCUSSION

In neoplastic process, inflammatory cells are important tumor promoters. They produce an attractive environment for tumor growth, facilitating genomic instability and promoting angiogenesis (28). Several studies demonstrated pro-tumor function of platelets and neutrophils. Platelets induce circulating tumor cell epithelial-to-mesenchymal transition and promote extravasation to metastatic sites (16–18). Neutrophils enhance cancer cell invasion, proliferation, and assist cancer cells with evading immune surveillance. Moreover, they promote adhesion and seeding of distant organ sites through secretion of circulating growth factors such as Vascular Endothelial Growth Factor (VEGF) and proteases (12,13,19). By contrast, lymphocytes play a central role in host anti-tumor immune responses by inducing cytotoxic cell death and inhibiting tumor cell proliferation and migration (20). Recently, the efficacy of immune checkpoint inhibitors, that boost the anti-tumor activity of lymphocytes, is starting to emerge (29–34). Therefore, the predictive power of PLR and NLR for cancer outcome might be due to the function of these three types of cells: the more the balance is shifted towards a high value of platelets and neutrophils and a low value of lymphocytes, the worse the prognosis.

Confirming previous results (22–25), in this study we demonstrated that the inflammatory-based biomarkers PLR and NLR are valuable prognostic parameters in patients with HCC. Even though both markers were shown to be independent predictors of prognosis, NLR maintained its independent prognostic role and demonstrated a higher prognostic power over PLR when the two scores were included in the same multivariate model. Accordingly, it has been recently demonstrated that NLR may be superior to PLR in predicting prognosis of HCC patients treated with TACE (35). The lower accuracy of PLR as prognostic biomarker is reasonable, considering that the level of platelets is not only influenced by tumor-associated inflammation, but also by the severity of portal hypertension. In fact, low PLR may reflect the presence of CSPH, that is associated with poor prognosis, also due

to the reduced possibility of applying curative treatments (i.e., liver resection). This is confirmed by the fact that, when platelet count was considered alone as a prognostic parameter, the longer survival was demonstrated for patients with higher platelet count.

Although both PLR and NLR revealed to be useful prognostic parameters, a better stratification of patient survival could be obtained when these two biomarkers are considered together. The longer survival was demonstrated for patients with both biomarkers negative, whereas the worse prognosis was shown for patients with both markers above their respective cut-off. These results are in agreement with some recent studies that demonstrated that an accurate prognostic stratification in patients with HCC managed with different therapies could be obtained combining PLR and NLR (36–42).

Available evidence demonstrates that PLR and NLR are able to stratify patient survival in different therapeutic setting, from LT to systemic treatments (9,12,13,40,43–46). In this study, we confirmed that PLR and NLR maintained the ability to stratify patients according to prognosis in groups of patients homogeneous for treatment (except for NLR in LT patients). In particular, after adjustment for confounders, PLR maintained its association with survival in patients treated with ABL and SOR, while NLR was independently associated with prognosis in patients treated with ABL and IAT. Compared to previous literature, the prognostic predictive power of both PLR and NLR appeared to be lower for patients managed with surgical treatments (12,40,43,44,46). In addition, our results on the role of NRL in patients treated with systemic therapy slightly differ from what has been already reported (9). Nevertheless, we obtained an HR approaching significance after adjustment for confounders, and we can confidently conclude that NRL may be a useful prognostic biomarker also in this setting. The evaluation of PLR and NRL in patients treated with systemic therapy will became even more important with the advent of immune checkpoint inhibitors, that rely their activity in boosting anti-cancer immune response (39). Recent data show that integrating parameters

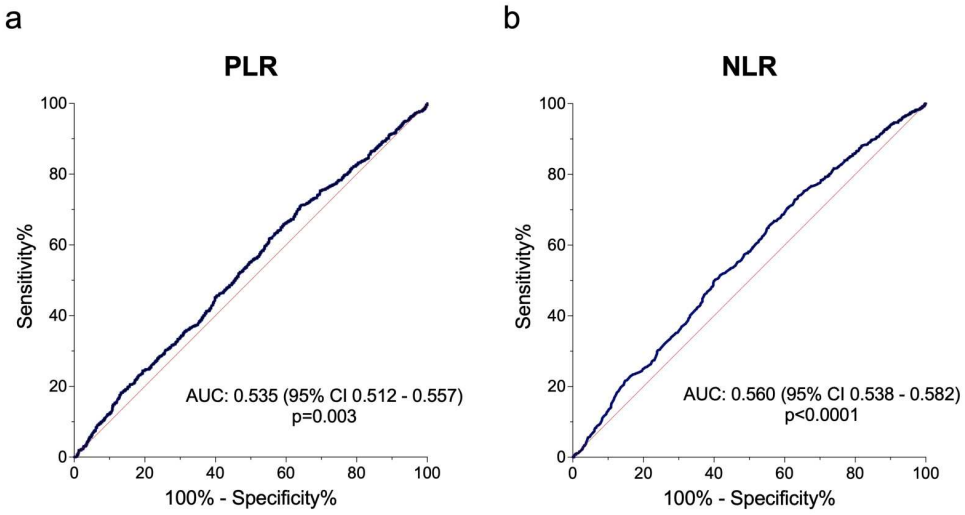
evaluating systemic inflammation in a prognostic score with AFP can accurately predict the prognosis of HCC patients treated with immunotherapy (47).

Despite its strength, in particular the great number of patients included, this study has some limitations, the most important of which is its retrospective nature that could have introduced unintended biases. A further limitation was that, in the subanalysis according to treatment, other therapies after the first-line treatment were not considered. This could be a potential source of bias, influencing the analysis of prognostic accuracy of PLR and NLR, since the survival of HCC patients is function of all the treatments performed during patient clinical history (48).

Even though several studies have been published on the prognostic role of PLR and NLR, the wide variability in the adopted cut-off values among different studies is a major limitation in clinical practice applicability. In systematic reviews and metanalyses, a great variability of cut-offs has been reported for both NLR (from 1.77 to 6 (25)) and PLR (from 87.87 to 290 (22)). This discordance mainly depends on the relative frequency of early-stage or advances patients included in the analysis, and could be best resolved with a prospective study.

In conclusion, in this study PLR and NLR confirmed to be useful prognostic parameters in patients with HCC. The stratification of patient survival is improved when these biomarkers are considered together. In addition, PLR and NLR revealed to be potentially useful in patients managed with different treatments, although additional studies, possibly prospective, should be conducted in order to establish the precise setting of applicability of these biomarkers and the possible combination with markers of cancer aggressiveness (e.g., AFP). The low cost, the easy determination and reproducibility of a blood count make PLR and NLR a promising tool for assessing HCC prognosis in future clinical practice.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. ROC curves used to identify the optimal prognostic cut-off for PLR (**a**) and NLR (**b**). (**a**) The identified cut-off for PLR was 113.7, with a sensitivity of 71.1% and a specificity of 35.8%. (**b**) The identified prognostic cut-off for NRL was 3.2, with a sensitivity of 73.8% and a specificity of 36.2%.

Supplementary Table 1. Multivariate Cox analysis (model 2) for factors independently associated with survival.

	PLR model 2		**NLR model 2**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
PLR >113.7	1.22 (1.06-1.41)	0.007	-	-
NLR >3.2	-	-	1.40 (1.21-1.61)	<0.0001
Gender - males	1.23 (1.02-1.47)	0.03	1.23 (1.03-1.47)	0.02
Surveillance	0.96 (0.83-1.11)	0.55	0.96 (0.83-1.11)	0.58
Etiology				
Viral	Ref	-	Ref	-
Not viral	1.02 (0.87-1.18)	0.83	0.99 (0.85-1.15)	0.89
Viral + other	1.04 (0.83-1.29)	0.75	1.02 (0.82-1.27)	0.87
CSPH	1.39 (1.13-1.71)	0.002	1.36 (1.11-1.66)	0.003
Child-Pugh class				
A	Ref	-	Ref	-
B	1.51 (1.30-1.76)	<0.0001	1.48 (1.27-1.72)	<0.0001
C	2.75 (1.56-4.85)	0.0005	2.52 (1.44-4.38)	0.001
logAFP	1.23 (1.15-1.31)	<0.0001	1.24 (1.16-1.32)	<0.0001
BCLC stage				
0	Ref	-	Ref	-
A	1.49 (1.09-2.02)	0.01	1.49 (1.10-2.03)	0.01
B	2.19 (1.56-3.08)	<0.0001	2.19 (1.56-3.08)	<0.0001
C	2.84 (2.08-3.89)	<0.0001	2.84 (2.08-3.89)	<0.0001
D	2.10 (1.19-3.71)	0.01	2.19 (1.26-3.82)	0.006
Treatment				
BSC	Ref	-	Ref	-
LT	0.03 (0.02-0.06)	<0.0001	0.03 (0.02-0.06)	<0.0001
LR	0.13 (0.09-0.19)	<0.0001	0.14 (0.10-0.20)	<0.0001
ABL	0.16 (0.12-0.22)	<0.0001	0.17 (0.12-0.23)	<0.0001
IAT	0.20 (0.15-0.26)	<0.0001	0.20 (0.15-0.27)	<0.0001
SOR	0.48 (0.36-0.64)	<0.0001	0.50 (0.38-0.67)	<0.0001

Abbreviations: PLR, platelets-to-lymphocytes ratio; NLR, neutrophils-to-lymphocytes ratio; HR, hazard ratio; CI, confidence interval; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; logAFP, logarithm of alpha-fetoprotein;

BCLC, Barcelona Clinic Liver Cancer; LT, liver transplantation; LR, liver resection; ABL, ablation, IAT, intra-arterial therapies; SOR, systemic therapies; BSC, best supportive care.

Supplementary Table 2. Multivariate Cox analysis (model 3) for factors independently associated with survival.

	PLR model 3		**NLR model 3**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
PLR >113.7	1.25 (1.08-1.45)	0.003	-	
NLR >3.2	-		1.38 (1.20-1.60)	<0.0001
Gender - males	1.21 (1.01-1.46)	0.04	1.21 (1.01-1.45)	0.04
Surveillance	0.97 (0.84-1.12)	0.67	0.97 (0.84-1.12)	0.68
Etiology				
Viral	Ref	-	Ref	-
Not viral	0.98 (0.84-1.15)	0.83	0.97 (0.83-1.14)	0.71
Viral + other	1.04 (0.84-1.30)	0.71	1.02 (0.82-1.27)	0.85
CSPH	- [a]	- [a]	- [a]	- [a]
Albumin (g/dL)	0.72 (0.63-0.83)	<0.0001	0.74 (0.64-0.84)	<0.0001
Bilirubin (mg/dL)	1.08 (1.05-1.11)	<0.0001	1.07 (1.04-1.10)	<0.0001
INR	1.18 (0.88-1.60)	0.27	1.17 (0.86-1.58)	0.31
Creatinine (mg/dL)	1.07 (0.93-1.23)	0.35	1.10 (0.96-1.27)	0.18
Ascites	0.88 (0.74-1.06)	0.17	0.89 (0.75-1.07)	0.22
HE	1.06 (0.80-1.40)	0.71	1.12 (0.84-1.49)	0.45
logAFP	1.20 (1.12-1.28)	<0.0001	1.21 (1.13-1.29)	<0.0001
BCLC stage				
0	Ref	-	Ref	-
A	1.51 (1.11-2.05)	0.008	1.52 (1.12-2.07)	0.007
B	2.18 (1.55-3.07)	<0.0001	2.20 (1.56-3.09)	<0.0001
C	2.89 (2.12-3.95)	<0.0001	2.92 (2.14-3.98)	<0.0001
D	2.29 (1.43-3.66)	0.001	2.38 (1.50-3.79)	0.0003
Treatment				
BSC	Ref	-	Ref	-
LT	0.03 (0.02-0.06)	<0.0001	0.03 (0.02-0.06)	<0.0001
LR	0.16 (0.11-0.23)	<0.0001	0.16 (0.11-0.24)	<0.0001
ABL	0.18 (0.13-0.25)	<0.0001	0.19 (0.13-0.26)	<0.0001
IAT	0.23 (0.17-0.31)	<0.0001	0.23 (0.17-0.32)	<0.0001
SOR	0.56 (0.41-0.75)	0.0002	0.58 (0.43-0.79)	0.0004

a) not included to avoid collinearity with ascites.

Abbreviations: PLR, platelets-to-lymphocytes ratio; NLR, neutrophils-to-lymphocytes ratio; HR, hazard ratio; CI, confidence interval; CSPH, clinically significant portal hypertension; INR, international normalized ratio; HE, hepatic encephalopathy; logAFP, logarithm of alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; LT, liver transplantation; LR, liver resection; ABL, ablation, IAT, intra-arterial therapies; SOR, systemic therapies; BSC, best supportive care.

Supplementary Table 3. Multivariate Cox models including both PLR and NLR.

	Model 1		**Model 2**		**Model 3**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
PLR >113.7	1.09 (0.93-1.28)	0.28	1.08 (0.92-1.26)	0.38	1.12 (0.95-1.31)	0.19
NLR >3.2	1.34 (1.15-1.57)	0.0003	1.35 (1.15-1.58)	0.0002	1.31 (1.12-1.54)	0.001
Gender - males	1.17 (0.98-1.39)	0.09	1.22 (1.02-1.46)	0.03	1.21 (1.01-1.45)	0.04
Surveillance	0.96 (0.83-1.11)	0.55	0.96 (0.83-1.11)	0.58	0.97 (0.84-1.12)	0.70
Etiology						
Viral	Ref	-	Ref	-	Ref	-
Not viral	0.98 (0.84-1.14)	0.79	0.99 (0.85-1.15)	0.87	0.96 (0.82-1.13)	0.65
Viral + other	1.07 (0.86-1.33)	0.55	1.03 (0.83-1.28)	0.80	1.04 (0.83-1.29)	0.75
CSPH	1.44 (1.18-1.76)	0.0003	1.37 (1.12-1.69)	0.003	- [a]	- [a]
MELD	1.06 (1.04-1.08)	<0.0001	-	-	-	-

Child-Pugh class						
A B C	-	-	Ref 1.49 (1.28-1.73) 2.66 (1.51-4.67)	- <0.0001 0.001	-	-
Albumin (g/dL)	-	-	-	-	0.73 (0.63-0.83)	<0.0001
Bilirubin (mg/dL)	-	-	-	-	1.07 (1.04-1.10)	<0.0001
INR	-	-	-	-	1.18 (0.87-1.59)	0.28
Creatinine (mg/dL)	-	-	-	-	1.09 (0.94-1.26)	0.26
Ascites	-	-	-	-	0.89 (0.75-1.07)	0.22
HE	-	-	-	-	1.10 (0.83-1.47)	0.51
logAFP	1.24 (1.16-1.32)	<0.0001	1.23 (1.16-1.31)	<0.0001	1.20 (1.12-1.28)	<0.0001
BCLC stage						
0	Ref	-	Ref	-	Ref	-
A	1.56 (1.15-2.12)	0.004	1.49 (1.09-2.03)	0.01	1.52 (1.12-2.06)	0.008
B	2.26 (1.62-3.17)	<0.0001	2.18 (1.56-3.07)	<0.0001	2.18 (1.55-3.06)	<0.0001
C	2.94 (2.16-4.01)	<0.0001	2.82 (2.06-3.86)	<0.0001	2.88 (2.11-3.93)	<0.0001
D	2.61 (1.68-4.07)	<0.0001	2.08 (1.18-3.67)	0.01	2.30 (1.44-3.67)	0.0005
Treatment						
BSC	Ref	-	Ref	-	Ref	-
LT	0.03 (0.02-.06)	<0.0001	0.03 (0.02-0.06)	<0.0001	0.03 (0.02-0.06)	<0.0001
LR	0.15 (0.10-0.21)	<0.0001	0.14 (0.09-0.20)	<0.0001	0.16 (0.11-2.39)	<0.0001
ABL	0.18 (0.13-0.24)	<0.0001	0.17 (0.12-0.23)	<0.0001	0.18 (0.13-0.25)	<0.0001
IAT	0.22 (0.16-0.30)	<0.0001	0.20 (0.15-0.26)	<0.0001	0.23 (0.17-0.31)	<0.0001
SOR	0.54 (0.40-0.72)	<0.0001	0.49 (0.37-0.66)	0.0002	0.57 (0.42-0.77)	0.0003

a) not included to avoid collinearity with ascites.

Abbreviations: PLR, platelets-to-lymphocytes ratio; NLR, neutrophils-to-lymphocytes ratio; HR, hazard ratio; Ci, confidence interval; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; INR, international normalized ratio; HE, hepatic encephalopathy; logAFP, logarithm of alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; LT, liver transplantation; LR, liver resection; ABL, ablation, IAT, intra-arterial therapies; SOR, systemic therapies; BSC, best supportive care.

REFERENCES

1. Villanueva A. Hepatocellular Carcinoma. N. Engl. J. Med. 2019;380:1450–1462.
2. Jemal A, Ward EM, Johnson CJ, Cronin KA, Ma J, Ryerson B, et al. Annual Report to the Nation on the Status of Cancer, 1975-2014, Featuring Survival. J. Natl. Cancer Inst. 2017;109.
3. Italian Association of Cancer Registries (AIRTUM) - available at https://www.registri-tumori.it/cms/pubblicazioni/i-numeri-del-cancro-italia-2020.
4. Galle PR, Foerster F, Kudo M, Chan SL, Llovet JM, Qin S, et al. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. Liver Int. 2019;39:2214–2229.
5. Yu L-X, Ling Y, Wang H-Y. Role of nonresolving inflammation in hepatocellular carcinoma development and progression. NPJ Precis. Oncol. 2018;2:6.
6. Ferrucci PF, Gandini S, Battaglia A, Alfieri S, Di Giacomo AM, Giannarelli D, et al. Baseline neutrophil-to-lymphocyte ratio is associated with outcome of ipilimumab-treated metastatic melanoma patients. Br. J. Cancer. 2015;112:1904–1910.
7. Elsharkawy AM, Mann DA. Nuclear factor-κB and the hepatic inflammation-fibrosis-cancer axis. Hepatology. 2007;46:590–597.
8. Pelizzaro F, Kitenge MP, Cardin R, Ponzoni A, Cillo U, Vitale A, et al. Circulating prostaglandin E(2): a novel potential prognostic biomarker in patients with hepatocellular carcinoma. Clin. Exp. Med. 2021;21:675–682.
9. Casadei Gardini A, Scarpi E, Faloppi L, Scartozzi M, Silvestris N, Santini D, et al. Immune inflammation indicators and implication for immune modulation strategies in advanced hepatocellular carcinoma patients receiving sorafenib. Oncotarget. 2016;7:67142–67149.
10. Conroy G, Salleron J, Belle A, Bensenane M, Nani A, Ayav A, et al. The prognostic value of inflammation-based scores in advanced hepatocellular carcinoma patients prior to treatment with sorafenib. Oncotarget. 2017;8:95853–95864.
11. Diaz-Beveridge R, Bruixola G, Lorente D, Caballero J, Rodrigo E, Segura Á, et al. An internally validated new clinical and inflammation-based prognostic score for patients with advanced hepatocellular carcinoma treated with sorafenib. Clin. Transl. Oncol. Off. Publ. Fed. Spanish Oncol. Soc. Natl. Cancer Inst. Mex. 2018;20:322–329.
12. Halazun KJ, Hardy MA, Rana AA, Woodland DC 4th, Luyten EJ, Mahadev S, et al. Negative impact of neutrophil-lymphocyte ratio on outcome after liver transplantation for hepatocellular carcinoma. Ann. Surg. 2009;250:141–151.
13. Dan J, Zhang Y, Peng Z, Huang J, Gao H, Xu L, et al. Postoperative neutrophil-to-lymphocyte ratio change predicts survival of patients with small hepatocellular carcinoma undergoing radiofrequency ablation. PLoS One. 2013;8:e58184.
14. Hu B, Yang X-R, Xu Y, Sun Y-F, Sun C, Guo W, et al. Systemic immune-inflammation index predicts prognosis of patients after curative resection for hepatocellular carcinoma. Clin. cancer Res. an Off. J. Am. Assoc. Cancer Res. 2014;20:6212–6222.
15. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–674.
16. Placke T, Salih HR, Kopp H-G. GITR ligand provided by thrombopoietic cells inhibits NK cell antitumor activity. J. Immunol. 2012;189:154–160.
17. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell. 2011;20:576–590.
18. Schumacher D, Strilic B, Sivaraj KK, Wettschureck N, Offermanns S. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. Cancer Cell. 2013;24:130–137.
19. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J. Clin. Invest. 2013;123:3446–3458.
20. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454:436–444.
21. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140:883–899.

22. Song W, Wang K, Zhong F-P, Fan Y-W, Peng L, Zou S-B. Clinicopathological and prognostic significance of platelet-to-lymphocyte ratio in patients with hepatocellular carcinoma. Oncotarget. 2016;7:81830–81838.

23. Xiao W-K, Chen D, Li S-Q, Fu S-J, Peng B-G, Liang L-J. Prognostic significance of neutrophil-lymphocyte ratio in hepatocellular carcinoma: a meta-analysis. BMC Cancer. 2014;14:117.

24. Qi X, Li J, Deng H, Li H, Su C, Guo X. Neutrophil-to-lymphocyte ratio for the prognostic assessment of hepatocellular carcinoma: A systematic review and meta-analysis of observational studies. Oncotarget. 2016;7:45283–45301.

25. Min G-T, Li Y-M, Yao N, Wang J, Wang H-P, Chen W. The pretreatment neutrophil-lymphocyte ratio may predict prognosis of patients with liver cancer: A systematic review and meta-analysis. Clin. Transplant. 2018;32.

26. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.

27. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology. 2015;62:440–451.

28. Llovet JM, Pena CEA, Lathia CD, Shan M, Meinhardt G, Bruix J. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. Clin. Cancer Res. 2012;18:2290–2300.

29. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. Lancet. 2017;389:2492–2502.

30. Zhu AX, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, et al. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. Lancet Oncol. 2018;19:940–952.

31. Finn RS, Ryoo BY, Merle P, Kudo M, Bouattour M, Lim HY, et al. Pembrolizumab As Second-Line Therapy in Patients With Advanced Hepatocellular Carcinoma in KEYNOTE-240: A Randomized, Double-Blind, Phase III Trial. J. Clin. Oncol. 2020;38:193–202.

32. Yau T, Park J-W, Finn RS, Cheng A-L, Mathurin P, Edeline J, et al. Nivolumab versus sorafenib in advanced hepatocellular carcinoma (CheckMate 459): a randomised, multicentre, open-label, phase 3 trial. Lancet. Oncol. 2021;

33. Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim T-Y, et al. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. N. Engl. J. Med. 2020;382:1894–1905.

34. https://www.astrazeneca.com/content/astraz/media-centre/press-releases/2021/imfinzi-and-tremelimumab-improved-os-in-liver-cancer.html.

35. Young S, Cam I, Gencturk M, Rubin N, D'souza D, Flanagan S, et al. Inflammatory Scores: Comparison and Utility in HCC Patients Undergoing Transarterial Chemoembolization in a North American Cohort. J. Hepatocell. carcinoma. 2021;8:1513–1524.

36. Zhang L, Yan Z-P, Hou Z-H, Huang P, Yang M-J, Zhang S, et al. Neutrophil-to-Lymphocyte and Platelet-to-Lymphocyte Ratios as Predictors of Outcomes in Patients With Unresectable Hepatocellular Carcinoma Undergoing Transarterial Chemoembolization Plus Sorafenib. Front. Mol. Biosci. 2021;8:624366.

37. Kong W, Qu E, Sheng N, Zhang J, Li X, Zheng J, et al. Prognostic significance of inflammation-based score in patients with hepatocellular carcinoma after liver transplantation. Eur. J. Gastroenterol. Hepatol. 2021;Publish Ah.

38. Suner A, Carr BI. Platelet-to-lymphocyte and neutrophil-to-lymphocyte ratios predict tumor size and survival in HCC patients: Retrospective study. Ann. Med. Surg. 2020;58:167–171.

39. Dharmapuri S, Özbek U, Lin J-Y, Sung M, Schwartz M, Branch AD, et al. Predictive value of neutrophil to lymphocyte ratio and platelet to lymphocyte ratio in advanced hepatocellular carcinoma patients treated with anti-PD-1 therapy. Cancer Med. 2020;9:4962–4970.

40. Kabir T, Ye M, Mohd Noor NA, Woon W, Junnarkar SP, Shelat VG. Preoperative Neutrophil-to-Lymphocyte Ratio Plus Platelet-to-Lymphocyte Ratio Predicts the Outcomes after Curative Resection for Hepatocellular Carcinoma. Int. J. Hepatol. 2019;2019:4239463.

41. He C, Zhang Y, Cai Z, Lin X. The prognostic and predictive value of the combination of the neutrophil-to-

lymphocyte ratio and the platelet-to-lymphocyte ratio in patients with hepatocellular carcinoma who receive transarterial chemoembolization therapy. Cancer Manag. Res. 2019;11:1391–1400.

42. Chen K, Zhan M-X, Hu B-S, Li Y, He X, Fu S-R, et al. Combination of the neutrophil to lymphocyte ratio and the platelet to lymphocyte ratio as a useful predictor for recurrence following radiofrequency ablation of hepatocellular carcinoma. Oncol. Lett. 2018;15:315–323.

43. Ismael MN, Forde J, Milla E, Khan W, Cabrera R. Utility of Inflammatory Markers in Predicting Hepatocellular Carcinoma Survival after Liver Transplantation. Biomed Res. Int. 2019;2019:7284040.

44. Mano Y, Shirabe K, Yamashita Y-I, Harimoto N, Tsujita E, Takeishi K, et al. Preoperative neutrophil-to-lymphocyte ratio is a predictor of survival after hepatectomy for hepatocellular carcinoma: a retrospective analysis. Ann. Surg. 2013;258:301–305.

45. McNally ME, Martinez A, Khabiri H, Guy G, Michaels AJ, Hanje J, et al. Inflammatory markers are associated with outcome in patients with unresectable hepatocellular carcinoma undergoing transarterial chemoembolization. Ann. Surg. Oncol. 2013;20:923–928.

46. Fu S-J, Shen S-L, Li S-Q, Hua Y-P, Hu W-J, Liang L-J, et al. Prognostic value of preoperative peripheral neutrophil-to-lymphocyte ratio in patients with HBV-associated hepatocellular carcinoma after radical hepatectomy. Med. Oncol. 2013;30:721.

47. Scheiner B, Pomej K, Kirstein MM, Hucke F, Finkelmeier F, Waidmann O, et al. Prognosis of patients with hepatocellular carcinoma treated with immunotherapy - development and validation of the CRAFITY score. J. Hepatol. 2021;

48. Vitale A, Farinati F, Pawlik TM, Frigo AC, Giannini EG, Napoli L, et al. The concept of therapeutic hierarchy for patients with hepatocellular carcinoma: A multicenter cohort study. Liver Int. 2019;39:1478–1489.

CHAPTER 11

Surveillance as determinant of long-term survival in non-transplanted hepatocellular carcinoma patients

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ABSTRACT

Purpose: We aimed at assessing the impact of surveillance on long-term survival in HCC patients. **Methods**: From the ITA.LI.CA database, we selected 1,028 cases with long (≥5 years, LS group) and 2,721 controls with short-term survival (<5 years, SS group). The association between surveillance and LS was adjusted for confounders by multivariable logistic regression analysis. Survival of surveilled patients was presented both as observed and corrected for the lead-time bias, and the comparison of survival between surveillance and no surveillance groups was also performed after balancing the baseline characteristics with inverse probability weights (IPW). **Results**: LS patients were more frequently diagnosed under surveillance (p<0.0001), and had more favorable baseline characteristics. Surveillance was an independent predictor of LS (OR=1.413, 95% CI 1.195–1.671; p<0.0001). The observed and the lead-time corrected survival of surveilled patients were significantly longer compared to the survival of not surveilled patients (p<0.0001 and p=0.0008, respectively). In IPW adjusted populations, no survival differences were demonstrated between the two groups (p=0.30). **Conclusions**: Surveillance, increasing early-stage diagnosis and applicability of curative treatments, is a fundamental determinant of long-term survival in HCC patients. A wide implementation of surveillance programs should be pursued in order to improve HCC patients' prognosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide (1). According to the International Agency for Research on Cancer, incidence and mortality in 2018 involved 841,080 and 781,631 patients, respectively, with an age-standardized incidence rate of 9.3/100,000 and an age-standardized mortality of 8.5/100,000 (1). This small difference could be explained by the low five-year survival rate of HCC patients (currently 12–14% in the United States) (2). In Italy, despite the improvement of prognosis recently observed (3), the long-term survival rate remains around 20% (4).

The individual prognosis of HCC patients is however highly unpredictable and not always dismal. The great variability in survival is justified by the peculiar features of these patients in whom prognosis depends on several parameters, not only including tumor burden, liver functional reserve, and general conditions (characteristics incorporated in the most commonly used staging approach, the Barcelona Clinic Liver Cancer [BCLC] system (5)), but also tumor biology (6–9), gender (10), immunological response of the host (11), and therapeutic choices (12). As a result, HCC patients may survive from a few months to many years. Studies looking for the predictors of long-term survival showed that early stage at diagnosis, preserved liver function and type of treatment performed are pivotal parameters in predicting a good prognosis (13–15). With the aim of improving patients' prognosis, by increasing early diagnosis and applicability of curative treatments, international guidelines recommend periodic surveillance in patients at risk of developing HCC (5,16). These indications are supported by data deriving from two Chinese randomized controlled trials conducted in HBV-infected patients (17,18), several cohort studies (19–24), and meta-analysis (25,26). Although a previous report indicate that the benefit of surveillance over no surveillance strategies is evident from the third year of follow-up (27), only limited data are currently available

about the role of periodic screening in achieving a long-term survival. In this study, we aimed at evaluating the impact of surveillance on long-term survival in non-transplanted HCC patients.

MATERIALS AND METHODS

In the Italian Liver Cancer (ITA.LI.CA) database, including 7,816 HCC patients consecutively evaluated and managed from January 1987 to December 2018 in 24 participating Institutions, data are prospectively collected, updated every 2 years, and periodically revised by the ITA.LI.CA coordinator center (Semeiotics Unit, Alma Mater Studiorum-Bologna University).

The study was approved by the Institutional Review Board of the ITA.LI.CA coordinating center, Alma Mater Studiorum University of Bologna (approval number 99/2012/O/Oss), and it was conducted in accordance to the ethical guidelines of the 1975 Declaration of Helsinki.

From the ITA.LI.CA database, we selected the patients diagnosed with HCC from January 2000 to December 2013 (n = 4,194). After the removal of 199 patients treated with liver transplantation (since transplant opens a peculiar scenario in terms of long-term survival), 210 Child-Pugh C patients (excluded from surveillance because advanced liver failure prevents effective HCC therapies), and 36 patients without survival data, in this study, 3,749 patients were considered. Patients were divided in two groups according to their survival: 1,028 patients (27.4%) showing a survival ≥5 years entered in the case group (long-term survivors, LS), while the remaining with a survival shorter than 5 years (n = 2,721; 72.6%) were selected as controls (short-term survivors, SS) (Figure 1).

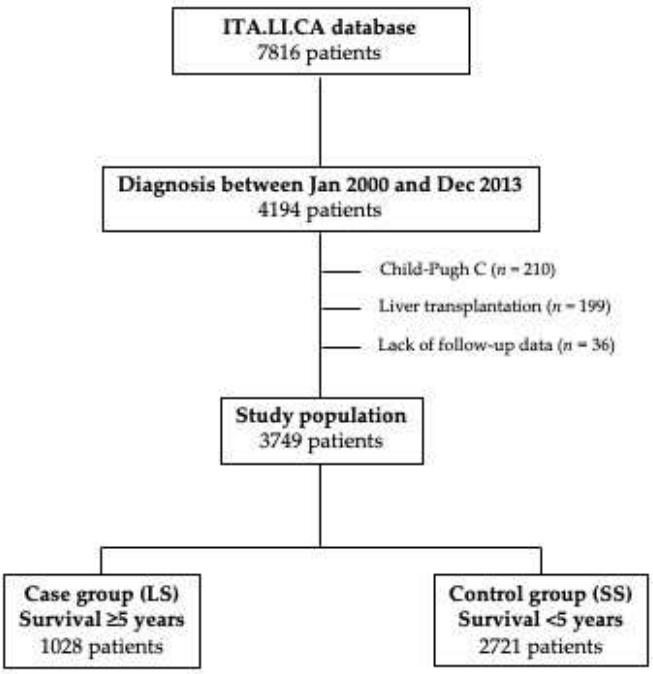


Figure 1. Study flow chart. Selection of patients finally included in the case (long-term survivors—LS) and control (short-term survivors—SS) groups.

All patients included in this study fitted the criteria for entering in a surveillance program according to guidelines (cirrhotic patients in Child-Pugh classes A and B; non-cirrhotic HBV patients at intermediate or high risk of HCC; non-cirrhotic F3 patients perceived at high risk of tumor development) (5). In the ITA.LI.CA database, the modality of HCC diagnosis (casual, achieved under surveillance, or as a consequence of the development of cancer-related symptoms) is recorded. In patients diagnosed under surveillance, data about the interval and the surveillance tests are also collected. Considering the nature of ITA.LI.CA database, surveillance protocols were not standardized across different Institutions. The interval of surveillance was established by the referring physician of each patient who was not necessarily one of the ITA.LI.CA clinicians, since a number of patients included in the database are referred to ITA.LI.CA Institutions after diagnosis for treatment purposes. Nevertheless, the six-months interval was the most frequently adopted among the patients included in the ITA.LI.CA database. As far as surveillance tests are considered, in all patients diagnosed under surveillance included in this study, the periodic repetition of liver

ultrasonography was performed, with or without the adjunctive determination of alpha-fetoprotein (AFP) (left as a complementary choice of the clinician).

HCC diagnosis was histologically confirmed in 215 LS patients (20.9%) and in 468 SS patients (17.2%), whereas in the remaining cases, it was based on the typical features at imaging (i.e., at dynamic computed tomography or magnetic resonance), according to guidelines (5).

In the ITA.LI.CA database, the following standard demographic and clinical data are collected: age, sex, comorbidities, body mass index (BMI), Eastern Cooperative Oncology Group performance status (ECOG-PS), general symptoms, modality of HCC diagnosis (unequivocal and radiological findings or biopsy/surgical specimens), etiology, serological parameters (albumin, bilirubin, INR, creatinine, sodium, platelet count, AFP), Child-Pugh score, Model for End-Stage Liver Disease (MELD) score, and clinically significant portal hypertension (CSPH). Tumor characteristics (location, size and number of nodules, macrovascular invasion [MVI], and extrahepatic spread [EHS]) and cause of death are also collected. CSPH diagnosis was based on unequivocal clinical signs (presence of esophageal varices, ascites, splenomegaly) or platelet count <100,000/mL, since hepatic venous gradients are not generally assessed (28).

Recently, the ITA.LI.CA staging system, externally validated (29,30), demonstrated the highest prognostic power compared to the other prognostic systems and was therefore considered in the present study.

Moreover, for the purpose of this paper, each ITA.LI.CA Institution was categorized, considering the volume of patients managed, in “low-” or “high-volume” centers, according to the average annual HCC case volume (below vs. above the median of the 24 centers, respectively).

From the therapeutic point of view, five groups were created: liver resection (LR), ablation (ABL, including percutaneous ethanol injection, radiofrequency, and microwave ablation, either percutaneous or laparoscopic); intra-arterial therapies (IAT), systemic therapy with sorafenib (SOR),

and “other” therapies (including best supportive care [BSC]). In patients managed with more than one treatment, only the more radical one (main treatment) was considered, according to the following hierarchy: LR, ABL, IAT, SOR, and OTHERS (12).

Statistical Analysis

Categorical variables were expressed as absolute frequency and percentage, while continuous variables as medians and interquartile range (IQR). Quantitative data were compared with Student’s t test, while categorical variables with χ^2 test and Fischer’s exact test, as appropriate.

A multiple logistic regression analysis was performed to identify independent predictors of LS, considering only the variables significantly or borderline ($p \leq 0.10$) associated with survival in the univariate analysis. Since the aim of this study was to evaluate the impact of surveillance on long-term survival, multicollinearity analysis was performed. To exclude multicollinearity between surveillance and other variables, we analyzed tolerance (an indicator of how much collinearity that a regression analysis can tolerate) and variance inflation factor (an indicator of how much of the inflation of the standard error could be caused by collinearity) using a specific “collinearity diagnostics package” for STATA (31). We also evaluated the calibration of the final model using the Calibration belt and test (32). Finally, 1000 bootstrap replications of the final model were performed (reporting bootstrap standard errors and confidence intervals) to correct for optimism.

Survivals were expressed as median and 95% confidence interval (CI). Overall survival was calculated from HCC diagnosis to death, drop-out, or last follow-up visit, with data censored on 31st December 2018. The Kaplan–Meier method and the log-rank test were used to estimate and compare survival curves. Survival analyses were performed both before and after correction for the lead time bias in patients with HCC diagnosed under surveillance, as previously reported (27). Moreover, in order to correct for all biases in the comparison between surveillance and no surveillance groups, propensity score values and inverse probability weights (IPW) were then calculated using generalized boosted

models as described by McCaffrey et al. (33). This is a machine learning technique using a flexible estimation method that can adjust for a large number of covariates. All potential confounders were included in boosted models: sex, age, etiology, liver function, tumor related variables, radical treatment, and center volume. In order to reduce the type I error rate (because of the inflated sample size in the pseudo data), we used stabilized weights (SW) according the formula:

$$SW = p/PS$$

for the study group,

$$SW = (1-p)/(1-PS)$$

for the control group, where p is the probability of etiology without considering covariates and PS is the propensity score.

Finally, weighted survival curves were calculated using the Kaplan–Meier method and compared using the Log Rank test.

Missing data of study covariates always involved less than 10% of patients. Thus, they were estimated using the Maximum Likelihood Estimation method (34).

In all analyses, a two-tailed p-value < 0.05 was considered statistically significant. All analyses were performed in JMP® 9.0.1 package (1989–2010 SAS Institute Inc., Cary, NC, USA), STATA13.0 (Copyright 1985–2013 StataCorp LP, College Station, TX, USA), and R. app 4.0.0 GUI 1.71 (S. Urbanek & H.-J. Bibiko, © R Foundation for Statistical Computing, 2016).

RESULTS

Patients' characteristics

The median follow-up was 92.3 months (95% CI 89.2–94.0) in LS group and 19.0 months (18.0–20.0) in SS group. During the follow-up, 470 patients (45.7%) in LS group died, 166 (35.3%) from tumor progression, 73 (15.5%) from liver failure, 160 (34.1%) from other causes, and 71 (15.1%) from

unspecified causes. All SS patients were dead at the end of the follow-up, with tumor progression being the most frequent cause (n = 1,118 patients, 41.1%), followed by liver failure (n = 331, 12.1%), other causes (n = 1,006, 37.0%), and not reported causes (n = 266, 9.8%).

The median overall survival (OS) was 120.0 months (95% CI 109.7–130.3) in LS patients and 19.0 months (95% CI 18.1–19.9) in SS patients ($p < 0.0001$).

Baseline characteristics of LS and SS patients are shown in Table 1. Cases and controls were comparable for gender, presence of type 2 diabetes mellitus and viral etiology. LS patients were slightly younger than SS patients (p=0.04) and showed a significantly higher prevalence of overweight (35.7% vs. 27.3%), but the two groups were comparable in the prevalence of metabolic disfunction-associated fatty liver disease (MAFLD) (14.2% vs. 12.0%, respectively; p=0.08). LS patients showed a higher prevalence of HCC developed on a non-cirrhotic liver (8.3% vs. 5.0%; p=0.0002) and a lower prevalence of CSPH (72.0% vs. 83.2%, p<0.0001). Liver function was better preserved in LS than in SS patients (Child-Pugh class A in 86.9% vs. 68.0%, and median MELD score of 9 [7–10] vs. 10 [8–12], respectively; p<0.0001 in both cases).

LS and SS patients significantly differed in terms of diagnosis under surveillance (67.9% vs. 55.7%; p<0.0001). The median duration of surveillance was 48.0 months (IQR, 16.0–120.0) in SS and 60.0 months (IQR, 24.0–120.0) in LS patients (p=0.06). Of the patients included in this study, 1,539 (69.5%) underwent semiannual surveillance (76.2% in LS and 65.7% in SS group), 266 (12.0%) annual surveillance (9.8% in LS and 12.9% in SS groups), and 330 (14.9%) were followed-up with a three-month schedule (12.0% in LS and 16.2% in SS group). Other surveillance intervals were less frequently adopted.

Table 1. Baseline characteristics of cases (long-term survivors – LS) and controls (short-term survivors – SS).

Variable	**Cases – LS n = 1028**	**Controls – SS n = 2721**	**p †**
Gender - males	791 (76.9)	2067 (76.0)	0.55
Age (years)	69 (62–74)	69 (62–75)	0.04
BMI (kg/m^2)			
≤25 25-30 >30	661 (64.3) 264 (25.7) 103 (10.0)	1977 (72.7) 532 (19.5) 212 (7.8)	<0.0001
T2DM	339 (33.0)	891 (32.7)	0.91
Cirrhosis	943 (91.7)	2586 (95.0)	0.0002
Viral etiology	712 (69.3)	1899 (69.8)	0.75
MAFLD	146 (14.2)	327 (12.0)	0.08
CSPH	740 (72.0)	2263 (83.2)	<0.0001
Child-Pugh class			
A B	893 (86.9) 135 (13.1)	1850 (68.0) 871 (32.0)	<0.0001
MELD	9 (7–10)	10 (8–12)	<0.0001
Surveillance	698 (67.9)	1516 (55.7)	<0.0001
ECOG-PS 0	893 (86.9)	1880 (69.1)	<0.0001
Multifocality	257 (25.0)	1476 (54.2)	<0.0001
Number of nodules	1 (1–2)	2 (1–4)	<0.0001
Diameter (cm)	2.7 (2.0–3.7)	3.5 (2.3–5.3)	<0.0001
MVI	35 (3.4)	432 (15.9)	<0.0001
EHS	8 (0.8)	128 (4.7)	<0.0001
AFP ≤ 200 ng/mL	816 (79.4)	1802 (66.2)	<0.0001
ITA.LI.CA staging system			
0 A B1 B2 B3 C D	265 (25.8) 431 (42.0) 198 (19.3) 59 (5.7) 29 (2.8) 27 (2.6) 19 (1.8)	332 (12.2) 582 (21.4) 701 (25.8) 290 (10.6) 233 (8.6) 310 (11.4) 273 (10.0)	<0.0001
Main treatment			
LR ABL IAT SOR Other	301 (29.3) 487 (47.4) 138 (13.4) 11 (1.1) 91 (8.8)	310 (11.4) 757 (27.8) 743 (27.3) 166 (6.1) 745 (27.4)	<0.0001
Management in "Low-volume" Institutions	338 (32.9)	550 (20.2)	<0.0001

Continuous data are presented as median and interquartile range, while categorical variables are expressed as absolute frequency and percentage. † Student's t test, χ^2 test or Fischer's exact test, as appropriate.

Abbreviations: LS, long-term survivors; SS, short-term survivors; BMI, body mass index; T2DM, type 2 diabetes mellitus; MAFLD, metabolic defunction associated fatty liver disease; CSPH, clinically significant portal hypertension; MELD, Model for End Stage Liver Disease; ECOG-PS, Eastern Cooperative Oncology Group performance status; MVI, macrovascular invasion; EHS, extra-hepatic spread; AFP, alpha-fetoprotein; ITA.LI.CA, Italian Liver Cancer; LR, liver resection; ABL, ablation; IAT, intra-arterial therapy; SOR, sorafenib.

As far as oncological variables are concerned, LS patients showed better preserved clinical conditions (ECOG-PS 0 in 86.9% vs. 69.1%; p < 0.0001), lower number (p<0.0001) and size (p<0.0001)

of nodules, lower prevalence of MVI (3.4% vs. 15.9%; p<0.0001), EHS (0.8% vs. 4.7%; p<0.0001), and AFP levels (≤200 ng/mL in 79.4% vs. 66.2%; p<0.0001). Early-stage tumor, according to ITA.LI.CA classification, were more frequently diagnosed in LS patients (stages 0–A in 67.8% of LS and in 33.6% of SS patients).

Lastly, considering the main treatment, LS patients more frequently underwent LR (29.3% vs. 11.4%) and ABL (47.4% vs. 27.8%), and less frequently IAT (13.4% vs. 27.3%), SOR (1.1% vs. 6.1%) and BSC or other treatments (8.8% vs. 27.4%). Eight hundred and eighty-eight patients (23.7%) were managed in "low-volume" centers and 2861 patients (76.3%) in "high-volume" Institutions, with a significantly higher prevalence of LS compared to SS patients managed in "low-volume" hospitals (32.9% vs. 20.2%; p<0.0001).

Multivariable logistic regression analysis

In addition to diagnosis under surveillance (odds ratio [OR] = 1.681, 95% CI 1.445–1.956; p<0.0001), several other variables resulted associated (p≤0.10) with the survival group at the univariate logistic regression analysis: age, overweight, cirrhosis, presence of MAFLD, ECOG-PS, CSPH, MELD score, Child-Pugh class, multifocality, tumor size, MVI, EHS, AFP, ITA.LI.CA stage, "volume" of the ITA.LI.CA Institution, and main treatment. Considering that the aim of this study was to determine the impact of surveillance on long-term survival, we performed a multicollinearity analysis in order to exclude from the multivariable model variables collinear with surveillance. The final model obtained is described in Table 2. Diagnosis under surveillance remained independently associated with long-term survival (adjusted OR = 1.413, 95% CI 1.195–1.671; p<0.0001). Other variables significantly associated with LS were lower age, presence of MAFLD, absence of CSPH, lower MELD score, and being managed in low-volume centers. As expected, the variable with the strongest independent impact on long-term survival was main treatment: curative therapies (LR + ABL) were associated with an OR of long-term survival of 3.924 (95% CI 3.312–4.650; p < 0.0001).

Table 2. Univariate and multivariate logistic regression analysis for independent predictors of long-term survivors (LS) group membership.

Variable	Univariable Analysis		Multivariable Analysis	
	OR (95% CI)	p	aOR (95% CI)	p
Surveillance	1.681 (1.445–1.956)	<0.0001	1.413 (1.195–1.671)	<0.0001
Gender - male	0.947 (0.799–1.122)	0.53		
Age †	0.993 (0.986–0.999)	0.04	0.989 (0.982–0.997)	0.008
BMI (kg/m^2) >25	1.475 (1.266-1.719)	<0.0001		
T2DM	1.011 (0.867–1.177)	0.89		
Cirrhosis	0.579 (0.437–0.767)	<0.0001		
Viral etiology	0.975 (0.835–1.140)	0.75		
MAFLD	1.212 (0.983–1.495)	0.07	1.299 (1.032–1.636)	0.03
CSPH	0.520 (0.439–0.616)	<0.0001	0.705 (0.582–0.854)	0.0003
Child-Pugh B	0.321 (0.263–0.391)	<0.0001		
MELD †	0.840 (0.816–0865)	<0.0001	0.877 (0.850–0.905)	<0.0001
ECOG-PS ≥1	0.338 (0.277–0.412)	<0.0001		
Multifocality	0.281 (0.240–0.330)	<0.0001		
Diameter (cm) >5	0.325 (0.261–0.405)	<0.0001		
MVI	0.187 (0.131–0.266)	<0.0001		
EHS	0.159 (0.077–0.326)	<0.0001		
AFP (ng/mL) >200	0.509 (0.429–0.604)	<0.0001		
ITA.LI.CA stage B-D	0.241 (0.207–0.281)	<0.0001		
Curative treatment (LR and ABL)	4.810 (4.083–5.667)	<0.0001	3.924 (3.312–4.650)	<0.0001
ITA.LI.CA Institution - LV	1.934 (1.647–2.270)	<0.0001	1.741 (1.463–2.070)	<0.0001

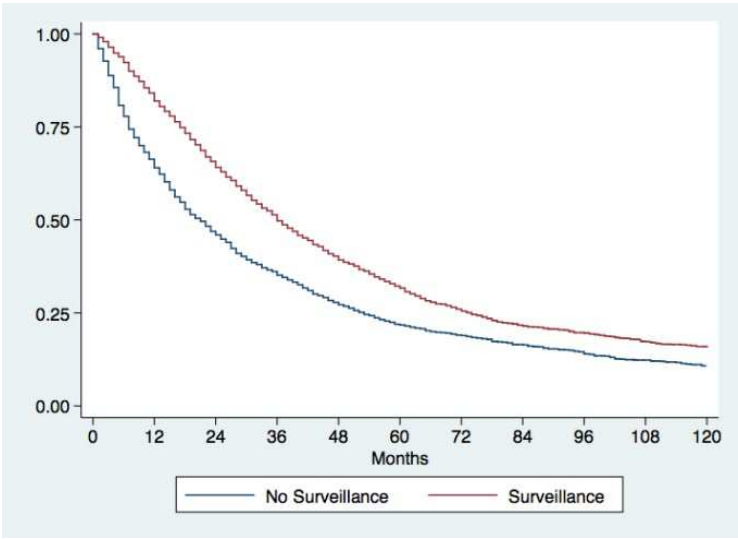
† In univariate and multivariate analysis age and MELD were considered as continuous variables.
Abbreviations: OR, Odds Ratio; CI, confidence interval; aOR, adjusted Odds Ratio; BMI, body mass index; T2DM, type 2 diabetes mellitus; MAFLD, metabolic disfunction associated fatty liver disease; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; ECOG-PS, Eastern Cooperative Oncology Group performance status; MVI, macrovascular invasion; EHS, extrahepatic spread; AFP, alpha-fetoprotein; ITA.LI.CA, Italian Liver Cancer; LV, low-volume institutions.

The results of the calibration test (Supplementary Figure 1; statistic = 0.09, p = 0.76) suggest that the hypothesis of good calibration of the final model is not rejected (at the classically adopted 0.05 level). Similar conclusions can be drawn from the interpretation of the produced plot (calibration belt), reported in the Supplementary Figure 1. We note that both the 80% and 95% calibration belts encompass the bisector over the whole range of the predicted probabilities. This suggests that the predictions of the model do not significantly deviate from the observed rate in the training sample (which means that the model's internal calibration is acceptable). Moreover, bootstrap standard

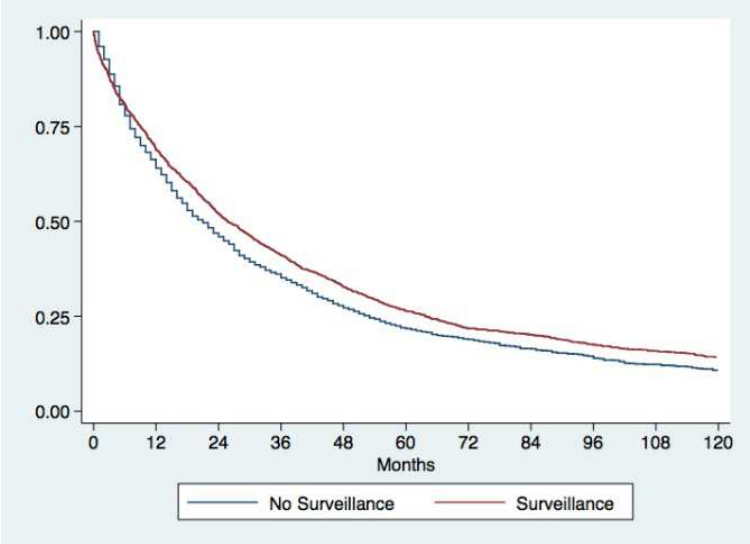
errors and confidence intervals (Supplementary Table 1) overlapped with that of the final model in Table 2, suggesting that the final model doesn't suffer of optimistic bias.

Survival Analysis

The unadjusted Kaplan–Meier analysis demonstrated a considerable survival advantage in patients diagnosed under surveillance compared to patients diagnosed incidentally or because the development of symptoms. Surveilled patients had a median OS of 36.0 months (95% CI 33.9–38.1) compared to 20.0 months (95% CI 18.0–22.0) in not-surveilled patients, with five-year survival rates of 31.5% and 21.5%, respectively ($p<0.0001$) (Figure 2a). Even after correction for lead-time bias, surveillance remained associated with a better prognosis. The median survival of surveilled patients corrected for the lead-time bias was 25.6 months (95% CI 23.6–27.5), with a five-year corrected survival rate of 26.3%. These figures were again significantly higher than those observed in not-surveilled patients ($p=0.0008$) (Figure 2b).



(**a**)



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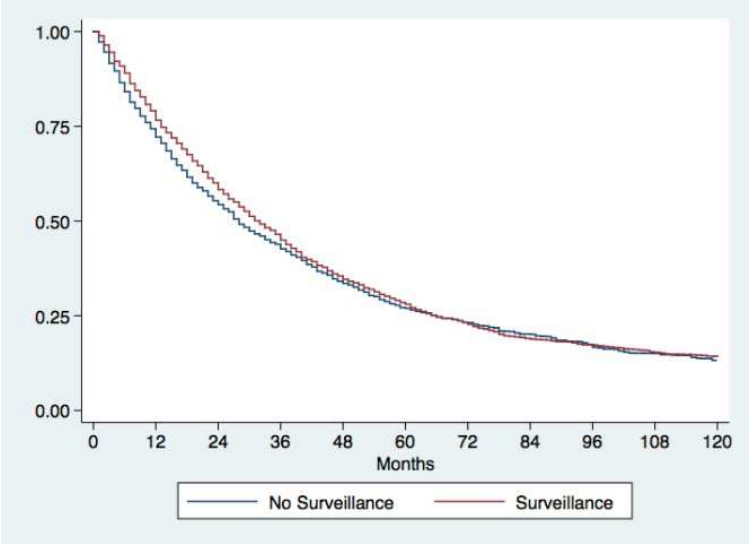


(**c**)

Figure 2. Kaplan–Meier survival curves comparing surveillance and no surveillance groups. (**a**) Observed survival of patients diagnosed under surveillance or with a casual/symptomatic diagnosis. Patients diagnosed under surveillance demonstrated a significantly longer survival (p<0.0001). (**b**) Observed survival of patients with casual/symptomatic diagnosis compared to corrected survival in surveilled patients. Surveillance significantly improves prognosis of patients even after correction for the lead-time bias (p=0.0008). (**c**) Comparison of survival between surveilled and not surveilled patients after adjustment for adjustment for confounders with IPW. The two groups of patients showed similar survival (p=0.30).

In order to correct for all biases in the comparison between surveillance and no surveillance groups, an IPW analysis was performed. Baseline characteristics of surveillance and no surveillance groups before and after IPW are showed in Table 3. Before IPW, in the surveillance group, there was a significant lower percentage of males, of patients with BMI >25 kg/m^2, with type 2 diabetes mellitus and MAFLD, and a significantly higher percentage of cirrhotics, with a virus-related liver disease and

CSPH. Surveilled patients had a better-preserved liver function (Child-Pugh A in 76.6% vs. 68.0%; p<0.0001) and better clinical conditions (ECOG-PS 0 in 81.3% vs. 63.5%; p<0.0001). As far as oncological variables were concerned, surveilled patients presented an overall lower tumor burden and significantly lower levels of AFP. Finally, a significant higher proportion of patients diagnosed during surveillance underwent to LR or ABL. After IPW, two populations absolutely comparable in all the baseline characteristics were obtained (Table 3). The survival analysis performed in the two IPW adjusted populations demonstrated no differences in prognosis between surveilled and not surveilled groups (median OS in surveilled group 31.0 months [95% CI 30.0–33.0] vs. 28.0 months [95% CI 26.0–30.0] in not surveilled patients; five-year survival rates 28.0% and 27.0% respectively; p = 0.30) (Figure 2c).

Table 3. Baseline characteristics of surveillance and no surveillance groups before and after inverse probability weights.

Variable	Before IPW			After IPW		
	Surveillance (n = 2214)	**No Surveillance (n = 1535)**	**p †**	**Surveillance (n = 2215)**	**No Surveillance (n = 1531)**	**p †**
Gender—males	1621 (73.2)	1237 (80.6)	<0.0001	1676 (75.7)	1158 (75.7)	0.97
Age—≤70 years	1250 (56.5)	859 (56.0)	0.76	1228 (55.5)	853 (55.7)	0.95
BMI >25 kg/m^2	621 (28.0)	490 (31.9)	0.01	627 (28.3)	451 (29.4)	0.46
T2DM	681 (30.8)	549 (35.8)	0.002	708 (32.0)	505 (33.0)	0.52
Cirrhosis	2140 (96.7)	1389 (90.5)	<0.0001	2089 (94.3)	1443 (94.2)	0.94
Viral etiology	1723 (77.8)	888 (57.8)	<0.0001	1544 (69.7)	1062 (69.4)	0.83
MAFLD	199 (9.0)	274 (17.8)	<0.0001	260 (11.7)	194 (12.6)	0.39
CSPH	1833 (82.8)	1170 (76.2)	<0.0001	1792 (80.9)	1235 (80.7)	0.90
Child-Pugh A	1699 (76.7)	1044 (68.0)	<0.0001	1606 (72.5)	1106 (72.2)	0.82
MELD >10	845 (38.2)	591 (38.5)	0.84	848 (38.3)	584 (38.1)	0.97
ECOG-PS 0	1799 (81.3)	974 (63.5)	<0.0001	1641 (74.1)	1129 (73.8)	0.82
Multifocality	852 (38.5)	881 (57.4)	<0.0001	1025 (46.3)	708 (46.2)	1.00
Diameter >5 cm	207 (9.4)	597 (38.9)	<0.0001	475 (21.4)	331 (21.6)	0.90
MVI	155 (7.0)	312 (20.3)	<0.0001	290 (13.1)	197 (12.9)	0.88
EHS	35 (1.6)	101 (6.6)	<0.0001	86 (3.9)	56 (3.7)	0.93
AFP ≤ 200 ng/mL	1628 (73.5)	990 (64.5)	<0.0001	1559 (70.4)	1073 (70.1)	0.83
ITA.LI.CA stage						
0–A B–D	1184 (53.5) 1030 (46.5)	426 (27.8) 1109 (72.2)	<0.0001	944 (42.6) 1271 (57.4)	652 (42.5) 880 (57.5)	0.95
Treatment						
LR + ABL IAT + SOR + Other	1299 (58.7) 915 (41.3)	602 (39.2) 933 (60.8)	<0.0001	1120 (50.6) 1094 (49.4)	771 (50.4) 760 (49.6)	0.89
LV Institutions	543 (24.5)	345 (22.5)	0.15	537 (24.2)	374 (24.4)	0.88

† Student's t test, χ^2 test or Fischer's exact test, as appropriate.

Abbreviations: IPW, inverse probability weights; BMI, body mass index; T2DM, type 2 diabetes mellitus; MAFLD, metabolic associated fatty liver disease; CSPH, clinically significant portal hypertension; MELD, Model for End Stage Liver Disease; ECOG-PS, Eastern Cooperative Oncology Group performance status; MVI, macrovascular invasion; EHS, extra-hepatic spread; AFP, alpha-fetoprotein; ITA.LI.CA, Italian Liver Cancer; LR, liver resection; ABL, ablation; IAT, intra-arterial therapy; SOR, sorafenib; LV, low volume.

DISCUSSION

Several attempts to establish the HCC prognosis, in both untreated and treated patients have been made so far, also with the aim of determining the actual survival benefit of each treatment in each cancer stage (7,10,12,29,35–38). In untreated patients, for instance, median OS has been reported to range from 25–38 months in BCLC stages 0–A and to be of 6 months in BCLC D (10). The amenability to the most effective treatment, defined on an individualized basis, is an additional relevant factor that increase the prognostic variability among patients (12). In this respect, it is worth noting that LR achieves a net survival benefit over loco-regional treatments across different BCLC stages (39). Nevertheless, the indicated treatment may be not always prescribed or available, even in wealthy countries (40). Beyond that, the survival of HCC patients can be unexpectedly long, or short, irrespective of what can be foreseen considering baseline clinical characteristics and treatment received, since the biologic aggressiveness of the tumor and the immunologic defenses of the host play a crucial role in determining the treatment outcome (6–9,11).

Some studies tried to clarify the factors associated with long-term survival in different therapeutic settings. Following LR, tumor diameter, presence of single node, and absence of microvascular invasion (13,14), as well as absence of cirrhosis (15), independently predict a very long survival. Other studies focused on the prediction of the outcome after ABL (41,42), IAT (43,44), or systemic therapies (45,46). However, for unselected HCC patients, models based on routinely available clinical characteristics capable to predict long-term survival without liver transplant are still lacking. Beyond that, in the prognostic stratification of HCC patients, surveillance is an important parameter that has to be considered. Although only two randomized controlled trials have ever been conducted on this topic (17,18), several cohort studies (19–24) and meta-analyses (25,26) showed that surveillance is associated with a better prognosis. As a matter of fact, all the major international

guidelines recommend surveillance in patients at risk of developing HCC, with the aim of maximizing survival probabilities, achieving an early diagnosis which allows the applicability of potentially curative treatments (5,16). In the literature, some data demonstrate that surveillance strategies exert their benefit on survival depending on the length of follow-up. The survival benefit provided by surveillance over casual/symptomatic diagnosis become factual for long follow-up (i.e., after the third year), with the short-term survival advantage being largely attributable to lead-time bias (27). However, the actual role of surveillance in achieving a long-term survival is still not defined.

Bearing this in mind, we aimed to evaluate the impact of surveillance on long-term survival comparing a group of non-transplanted HCC patients showing a survival ≥5 years with a group of contemporaneous patients with shorter survival. As expected, LS patients showed favorable baseline characteristics in terms of severity of liver disease (lower rates of CSPH, better Child-Pugh class and lower MELD score levels), clinical conditions (better ECOG-PS), and tumor burden (fewer and smaller nodules, less frequent MVI and EHS presence, lower levels of AFP). Overall, cancer stage at diagnosis was significantly earlier in LS patients and this, in addition to better preserved liver function and clinical conditions, allowed a higher applicability of curative treatments (LR and ABL). Concerning death causes, despite that a higher proportion of death for HCC progression could be expected in SS group, about 35% of LS patients eventually died from late tumor recurrence, without differences between cases and controls. Only less than half of LS patients (45.7%) were dead at the end of follow-up and this could have influenced this result. However, even patients with long survival after curative therapies persist at risk of recurrence and progression, with the five-year recurrence rates after LR being around 70% (5).

Although in both LS and SS groups a relatively high percentage of patients (more than 50%) was diagnosed under surveillance, in the former group, surveilled patients were significantly more represented (67.9% vs. 55.7%; $p<0.0001$). Despite the fact that these figures substantially differ

from other experiences published in the literature, which reported <20% of cirrhotics undergoing surveillance (47,48), the percentage of surveilled patients in this study is in line with previous works of the ITA.LI.CA group (3,49).

After correction for confounders, excluding from the multivariable model collinear variables, surveillance maintained an independent association with long-term survival. Other variables independently associated with long-term survival were younger age, absence of CSPH, and preserved liver function (lower MELD score). In addition, MAFLD, compared to other etiologies, proved to be associated with better prognosis. An intriguing result, that may seem counterintuitive, is the lower probability of long-term survival for patients managed in "high-volume" centers. We can speculate that this reflects the referral of patients more complex and "difficult to treat" to high-volume tertiary centers, as already demonstrated in other liver diseases, such as in primary sclerosing cholangitis (50).

Treatment emerged as a fundamental prognostic variable in the multivariable logistic regression analysis, with radical therapies (LR and ABL) being the strongest predictors of a better prognosis. Our data fuel the debated issue of HCC treatment. The BCLC system, endorsed by the European and American Guidelines (5,51), relies on a "stage hierarchy" philosophy, which recommends a specific treatment for each stage (52). However, numerous studies report a poor adherence to its therapeutic indications (53–55), and several data show that curative therapies are superior to the standard of care in selected intermediate or advanced patients (52). The so-called "therapeutic hierarchy" approach, which indicates a sequence of HCC treatments hierarchically organized according to their proven effectiveness (survival benefit), is now gaining ground as a strategy well in line with the evolving concept of "precision medicine", i.e., a patient-tailored rather than a stage-dictated management (52).

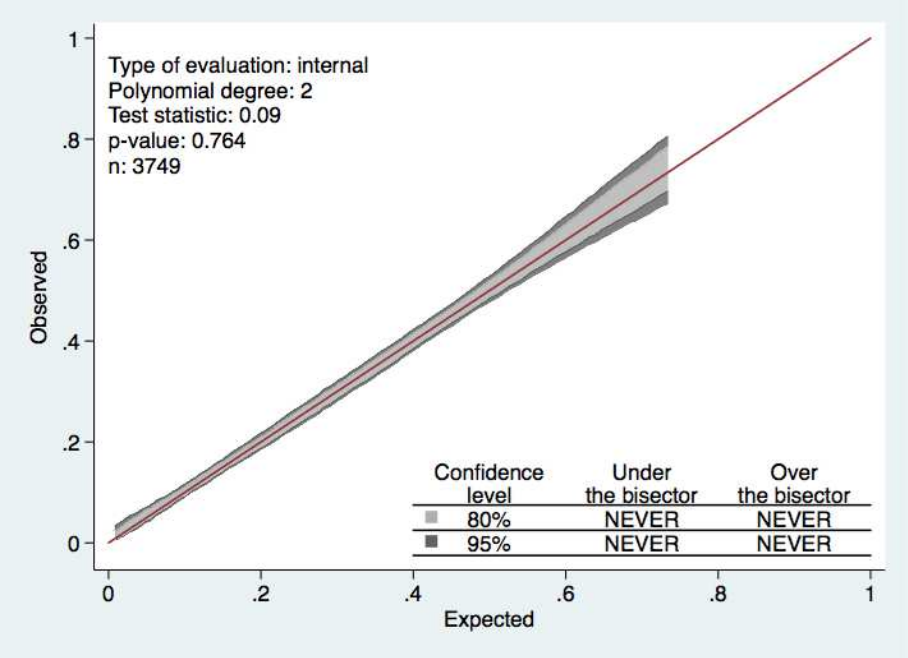
In this study, as already demonstrated (19,20), diagnosis under surveillance proved to be associated with a better prognosis compared to casual/symptomatic diagnosis in the unadjusted survival analysis. Our study, as all cohort studies on surveillance performance, may suffer from length-time and lead-time biases (27,56). Surveillance preferentially detects tumors with slow growth (length-time bias), and it may be possible that a higher percentage of aggressive HCC is present in SS group. However, in this study, the confounding effect of length-time bias was minimized by keeping in the surveillance group the patients in whom HCC diagnosis was anticipated (with respect to the scheduled surveillance test) due to the development of symptoms (56). Although the lead-time bias loses most of its importance in long-surviving patients (27), we also accounted for its confounding effect in this study, correcting the survival of surveilled patients for the calculated lead-time. Surveillance maintained its prognostic benefit over casual/symptomatic diagnosis even after this correction. It can be speculated that patients who adhere to a regular surveillance schedule have also a higher compliance to the entire diagnostic and therapeutic process, thus improving their prognosis. Nevertheless, in order to account for all potential confounders, survival of surveillance and no surveillance groups were compared after adjustment for baseline characteristics with IPW. In these populations, the survival benefit of surveillance disappeared. This is reasonable because the benefit of surveillance relies not on an intrinsic property of the modality of diagnosis, but derives from the ability of periodic screening to detect HCC at an early stage and, in turn, increase the proportion of patients amenable to effective treatments. Therefore, in groups adjusted for baseline oncologic and therapeutic variables, surveillance lost its association with better prognosis. In any case, our findings support once more the recommendation of a widespread use of surveillance in all patients at risk for HCC, despite the lack of randomized controlled trials in cirrhotics and HCV patients with advanced fibrosis (5,57).

Despite the attempt made to minimize all confounding factors, the retrospective nature of our study makes it vulnerable to several unintended biases. However, we feel that the limitations of this study are overweighted by its strengths, among which the adjustment only for factors not collinear with surveillance in the multivariable logistic regression model to evaluate its independent prognostic role. Moreover, the survival benefit of surveillance was firstly adjusted for the lead-time bias and subsequently tested in populations balanced with IPW. We believe that our results strengthen the pivotal role of surveillance as prognostic predictor and further underlines the need to develop extensive screening programs and to foster a high adherence, in order to improve HCC patients' prognosis through early diagnosis and delivery of curative treatments.

CONCLUSIONS

In addition to well-known predictors of survival, regular surveillance of patients at risk is a fundamental parameter that must be considered in the aim of achieving a long-term survival. Surveillance benefit are driven by an increase in early-stage tumor detection and amenability to potentially curative treatments. Our results further and strongly underline the importance of implementing surveillance programs in all patients at risk of developing HCC.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Calibration belt and test of the final multivariable logistic regression model.

Supplementary Table 1. Bootstrap standard errors and confidence intervals of our multivariable logistic model. One thousand bootstrap replications have been performed.

Variable		Bootstrap OR	Bootstrap 95% CI	Bootstrap Standard Error	p
Surveillance	No	Ref	Ref	-	-
	Yes	1.413	1.200–1.664	0.178	<0.0001
Age †		0.989	0.982–0.997	0.004	0.007
MAFLD	No	Ref	Ref	-	-
	Yes	1.299	1.039–1.624	0.148	0.02
CSPH	No	Ref	Ref	-	-
	Yes	0.705	0.579–0.858	0.071	0.001
MELD †		0.877	0.848-0.906	0.015	<0.0001
Treatment	Palliative	Ref	Ref	-	-
	Curative	3.924	3.316–4.645	0.337	<0.0001
Volume	HV	Ref	Ref	-	-
	LV	1.741	1.457–2.079	0.158	<0.0001

† Age and MELD were considered as continuous variables. Palliative treatment: IAT, SOR and other; curative treatments: LR and ABL. Abbreviations: OR, Odds Ratio; CI, confidence interval; aOR, adjusted Odds Ratio; Ref, reference group; MAFLD, metabolic associated fatty liver disease; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; ITA.LI.CA, Italian Liver Cancer; HV, high-volume institutions; LV, low-volume institutions.

REFERENCES

1. Fact Sheets by Population - Globocan - IARC n.d. [Internet]. [cited 2020 Oct 16];Available from: https://gco.iarc.fr/today/fact-sheets-populations
2. Mittal S, El-Serag HB. Epidemiology of hepatocellular carcinoma: consider the population. J. Clin. Gastroenterol. 2013;47 Suppl:S2–S6.
3. Bucci L, Garuti F, Lenzi B, Pecorelli A, Farinati F, Giannini EG, et al. The evolutionary scenario of hepatocellular carcinoma in Italy: an update. Liver Int. 2017;37:259–270.
4. Italian Association of Cancer Registries (AIRTUM) - available at https://www.registri-tumori.it/cms/pubblicazioni/i-numeri-del-cancro-italia-2020.
5. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.
6. Villa E, Critelli R, Lei B, Marzocchi G, Camma C, Giannelli G, et al. Neoangiogenesis-related genes are hallmarks of fast-growing hepatocellular carcinomas and worst survival. Results from a prospective study. Gut. 2016;65:861–869.
7. Villa E, Moles A, Ferretti I, Buttafoco P, Grottola A, Del Buono M, et al. Natural history of inoperable hepatocellular carcinoma: estrogen receptors' status in the tumor is the strongest prognostic factor for survival. Hepatology. 2000;32:233–238.
8. Iizuka N, Oka M, Yamada-okabe H, Nishida M, Maeda Y, Mori N, et al. Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. Lancet. 2003;361:923–929.
9. Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, et al. Gene Expression in Fixed Tissues and Outcome in Hepatocellular Carcinoma. N. Engl. J. Med. 2008;359:1995–2004.
10. Giannini EG, Farinati F, Ciccarese F, Pecorelli A, Rapaccini GL, Di Marco M, et al. Prognosis of untreated hepatocellular carcinoma. Hepatology. 2015;61:184–190.
11. Garnelo M, Tan A, Her Z, Yeong J, Lim CJ, Chen J, et al. Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. Gut. 2017;66:342–351.
12. Vitale A, Farinati F, Pawlik TM, Frigo AC, Giannini EG, Napoli L, et al. The concept of therapeutic hierarchy for patients with hepatocellular carcinoma: A multicenter cohort study. Liver Int. 2019;39:1478–1489.
13. Zheng J, Kuk D, Gönen M, Balachandran VP, Kingham TP, Allen PJ, et al. Actual Ten-Year Survivors after Resection of Hepatocellular Carcinoma. Ann. Surg. Oncol. 2017;24:1358–1366.
14. Kim KH, Choi Y-K. Long-term survival after resection of hepatocellular carcinoma. Korean J. Hepato-Biliary-Pancreatic Surg. 2012;16:98–104.
15. Wu K-T, Wang C-C, Lu L-G, Zhang W-D, Zhang F-J, Shi F, et al. Hepatocellular carcinoma: Clinical study of long-term survival and choice of treatment modalities. World J. Gastroenterol. 2013;19:3649–3657.
16. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68:723–750.
17. Yang B, Zhang B, Xu Y, Wang W, Shen Y, Zhang A, et al. Prospective study of early detection for primary liver cancer. J. Cancer Res. Clin. Oncol. 1997;123:357–360.
18. Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. J. Cancer Res. Clin. Oncol. 2004;130:417–422.
19. Trevisani F, De Notariis S, Rapaccini G, Farinati F, Benvegnù L, Zoli M, et al. Semiannual and annual surveillance of cirrhotic patients for hepatocellular carcinoma: Effects on cancer stage and patient survival (Italian experience). Am. J. Gastroenterol. 2002;97:734–744.
20. Trevisani F, Santi V, Gramenzi A, Di Nolfo MA, Del Poggio P, Benvegnu L, et al. Surveillance for early diagnosis of hepatocellular carcinoma: is it effective in intermediate/advanced cirrhosis? Am. J. Gastroenterol. 2007;102:2448–57.

21. Santi V, Trevisani F, Gramenzi A, Grignaschi A, Mirici-Cappa F, Del Poggio P, et al. Semiannual surveillance is superior to annual surveillance for the detection of early hepatocellular carcinoma and patient survival. J. Hepatol. 2010;53:291–297.

22. Thompson Coon J, Rogers G, Hewson P, Wright D, Anderson R, Jackson S, et al. Surveillance of cirrhosis for hepatocellular carcinoma: A cost-utility analysis. Br. J. Cancer. 2008;98:1166–1175.

23. Santagostino E, Colombo M, Rivi M, Rumi MG, Rocino A, Linari S, et al. A 6-month versus a 12-month surveillance for hepatocellular carcinoma in 559 hemophiliacs infected with the hepatitis C virus. Blood. 2003;102:78–82.

24. Giannini EG, Cucchetti A, Erroi V, Garuti F, Odaldi F, Trevisani F. Surveillance for early diagnosis of hepatocellular carcinoma : How best to do it ? World J. Gastroenterol. 2013;19:8808–8821.

25. Singal A, Volk ML, Waljee A, Salgia R, Higgins P, Rogers MAM, et al. Meta-analysis: Surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment. Pharmacol. Ther. 2009;30:37–47.

26. Singal AG, Pillai A, Tiro J. Early Detection, Curative Treatment, and Survival Rates for Hepatocellular Carcinoma Surveillance in Patients with Cirrhosis: A Meta-analysis. PLoS Med. 2014;11:e1001624.

27. Cucchetti A, Trevisani F, Pecorelli A, Erroi V, Farinati F, Ciccarese F, et al. Estimation of lead-time bias and its impact on the outcome of surveillance for the early diagnosis of hepatocellular carcinoma. J. Hepatol. 2014;61:333–341.

28. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology. 2015;62:440–451.

29. Farinati F, Vitale A, Spolverato G, Pawlik TM, Huo T, Lee Y-H, et al. Development and Validation of a New Prognostic System for Patients with Hepatocellular Carcinoma. PLoS Med. 2016;13:e1002006.

30. Borzio M, Dionigi E, Rossini A, Marignani M, Sacco R, De Sio I, et al. External validation of the ITA.LI.CA prognostic system for patients with hepatocellular carcinoma: A multicenter cohort study. Hepatology. 2018;67:2215–2225.

31. https://stats.idre.ucla.edu/stata/webbooks/logistic/chapter3/lesson-3-logistic-regression-diagnostics/.

32. Nattino G, Lemeshow S, Phillips G, Finazzi S, Bertolini G. Assessing the Calibration of Dichotomous Outcome Models with the Calibration Belt. Stata J. Promot. Commun. Stat. Stata. 2017;17:1003–1014.

33. Mccaffrey DF, Griffin BA, Almirall D, Slaughter ME, Ramchand R, Burgette LF. A tutorial on propensity score estimation for multiple treatments using generalized boosted models. Stat. Med. 2013;32:3388–3414.

34. Baraldi AN, Enders CK. An introduction to modern missing data analyses. J. Sch. Psychol. 2010;48:5–37.

35. Llovet JM, Bustamante J, Castells A, Vilana R, Ayuso M del C, Sala M, et al. Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. Hepatology. 1999;29:62–67.

36. Cabibbo G, Enea M, Attanasio M, Bruix J, Craxì A, Cammà C. A meta-analysis of survival rates of untreated patients in randomized clinical trials of hepatocellular carcinoma. Hepatology. 2010;51:1274–1283.

37. Farinati F, Vanin V, Giacomin A, Pozzan C, Cillo U, Vitale A, et al. BCLC stage B hepatocellular carcinoma and transcatheter arterial chemoembolization: A 20-year survey by the Italian Liver Cancer group. Liver Int. 2015;35:223–231.

38. Cillo U, Vitale A, Volk ML, Frigo AC, Grigoletto F, Brolese A, et al. The survival benefit of liver transplantation in hepatocellular carcinoma patients. Dig. Liver Dis. 2010;42:642–649.

39. Vitale A, Burra P, Frigo AC, Trevisani F, Farinati F, Spolverato G, et al. Survival benefit of liver resection for patients with hepatocellular carcinoma across different Barcelona Clinic Liver Cancer stages: a multicentre study. J. Hepatol. 2015;62:617–624.

40. Tan D, Yopp A, Beg MS, Gopal P, Singal AG. Meta analysis: underutilization and disparities of treatment among patients with hepatocellular carcinoma in the united states. Aliment. Pharmacol. Ther. 2013;38:703–712.

41. Sparchez Z, Mocan T, Radu P, Mocan LP, Sparchez M, Leucuta DC, et al. Prognostic factors after percutaneous radiofrequency ablation in the treatment of hepatocellular carcinoma. Impact of incomplete ablation on recurrence and overall survival rates. J. Gastrointest. Liver Dis. 2018;27:399–407.

42. Ryu T, Takami Y, Wada Y, Hara T, Sasaki S, Saitsu H. Actual 10-Year Survival After Surgical Microwave Ablation

for Hepatocellular Carcinoma: A Single-Center Experience in Japan. Ann. Surg. Oncol. 2019;26:4126–4133.

43. Kadalayil L, Benini R, Pallan L, O'Beirne J, Marelli L, Yu D, et al. A Simple Prognostic Scoring System for Patients Receiving Transarterial Embolisation for Hepatocellular Cancer. Ann. Oncol. 2013;24:2565–2570.

44. Kim BK, Kim SU, Kim KA, Chung YE, Kim MJ, Park MS, et al. Complete response at first chemoembolization is still the most robust predictor for favorable outcome in hepatocellular carcinoma. J. Hepatol. 2015;62:1304–1310.

45. Lu LC, Shao YY, Chan SY, Hsu CH, Cheng AL. Clinical characteristics of advanced hepatocellular carcinoma patients with prolonged survival in the era of anti-angiogenic targeted-therapy. Anticancer Res. 2014;34:1047–1052.

46. Lombardi G, Zustovich F, Farinati F, Cillo U, Vitale A, Zanus G, et al. Pegylated liposomal doxorubicin and gemcitabine in patients with advanced hepatocellular carcinoma: Results of a phase 2 study. Cancer. 2011;117:125–133.

47. Davila JA, Henderson L, Kramer JR, Kanwal F, Richardson PA, Duan Z, et al. Utilization of surveillance for hepatocellular carcinoma among hepatitis C virus-infected veterans in the United States. Ann. Intern. Med. 2011;154:85–93.

48. Singal AG, Yopp A, S. Skinner C, Packer M, Lee WM, Tiro JA. Utilization of hepatocellular carcinoma surveillance among American patients: A systematic review. J. Gen. Intern. Med. 2012;27:861–867.

49. Garuti F, Neri A, Avanzato F, Gramenzi A, Rampoldi D, Rucci P, et al. The changing scenario of hepatocellular carcinoma in Italy: an update. Liver Int. 2020;

50. Boonstra K, Weersma RK, van Erpecum KJ, Rauws EA, Spanier BWM, Poen AC, et al. Population-based epidemiology, malignancy risk, and outcome of primary sclerosing cholangitis. Hepatology. 2013;58:2045–2055.

51. Heimbach JK, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, et al. AASLD guidelines for the treatment of hepatocellular carcinoma. Hepatology. 2018;67:358–380.

52. Vitale A, Trevisani F, Farinati F, Cillo U. Treatment of hepatocellular carcinoma in the Precision Medicine era: from treatment stage migration to therapeutic hierarchy. Hepatology. 2020;72:2206–2218.

53. Park JW, Chen M, Colombo M, Roberts LR, Schwartz M, Chen PJ, et al. Global patterns of hepatocellular carcinoma management from diagnosis to death: The BRIDGE Study. Liver Int. 2015;35:2155–2166.

54. Giannini EG, Bucci L, Garuti F, Brunacci M, Lenzi B, Valente M, et al. Patients with advanced hepatocellular carcinoma need a personalized management: A lesson from clinical practice. Hepatology. 2018;67:1784–1796.

55. Sangiovanni A, Triolo M, Iavarone M, Forzenigo L V., Nicolini A, Rossi G, et al. Multimodality treatment of hepatocellular carcinoma: How field practice complies with international recommendations. Liver Int. 2018;38:1624–1634.

56. Cucchetti A, Garuti F, Pinna AD, Trevisani F. Length time bias in surveillance for hepatocellular carcinoma and how to avoid it. Hepatol. Res. 2016;46:1275–1280.

57. Kanwal F, Singal AG. Surveillance for Hepatocellular Carcinoma: Current Best Practice and Future Direction. Gastroenterology. 2019;157:54–64.

CHAPTER 12

Surveillance for hepatocellular carcinoma with a 3-months interval in “extremely high-risk” patients does not further improve survival

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ABSTRACT

Background. An enhanced surveillance schedule has been proposed for cirrhotics with viral etiology, who are considered at extremely high-risk of hepatocellular carcinoma (HCC).

Aims. We compared the 3- and 6-months surveillance interval, evaluating cancer stage at diagnosis and patient survival.

Methods. Data of 777 HBV and HCV cirrhotic patients with HCC diagnosed under a 3-months (n=109, 3MS group) or a 6-months (n=668, 6MS group) surveillance were retrieved from the Italian Liver Cancer database. Survival in the 3MS group was considered as observed and adjusted for lead-time bias, and survival analysis was repeated after a propensity score matching.

Results. The 3-months surveillance interval neither reduced the share of patients diagnosed outside the Milano criteria, nor increased their probability to receive curative treatments. The median survival of 6MS patients (55.0 months [95% CI 45.9-64.0]) was not significantly different from the observed (47.0 months [95% CI 35.0-58.9]; p=0.43) and adjusted (44.9 months [95% CI 33.4-56.4]; p=0.30) survival of 3MS patients. A propensity score analysis confirmed the absence of a survival advantage for 3MS patients.

Conclusions. A tightening of surveillance schedule does not increase the diagnosis of early-stage tumors, the feasibility of curative treatments and the survival. Therefore, we should maintain the 6-months interval in the surveillance of viral cirrhotics.

INTRODUCTION

Primary liver cancer is the seventh most common cancer and the second most common cause of cancer-related death worldwide (1). Despite the expected decline in the coming years of their role, hepatitis B virus (HBV) and hepatitis C virus (HCV) are still the most important global risk factors for hepatocellular carcinoma (HCC) (2).

In order to reduce disease-specific mortality, by increasing early diagnosis and the delivery of curative treatments, international guidelines recommend surveillance in individuals at risk of developing HCC (3–7). These indications are supported by data deriving from two Chinese randomized controlled trials conducted in HBV-infected patients (8,9), several cohort studies (10–14) and by meta-analyses (15,16). Guidelines uniformly recommend liver ultrasonography (US) for surveillance, with some disagreement about the use of tumor markers (4–7). Concerning the surveillance schedule, the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Diseases (AASLD) and the Asian Pacific Association for the Study of the Liver (APASL) guidelines indicate a 6-months interval (4,5,7). A longer interval is associated with lower probability of diagnosing early stage HCC (10) and shorter survival (12), while no additional clinical benefit is obtained by reducing the interval to 3-months in unselected patients at risk (17). Japanese guidelines, by contrast, identify patients with HBV and HCV-related cirrhosis as an extremely high-risk group for HCC development considering their higher risk compared to non-viral cirrhotics (18), and recommend to perform in these patients US and tumor markers evaluation (alpha-fetoprotein [AFP], des-gamma-carboxy prothrombin and fucosylated fraction of AFP) every 3-4 months, plus optional dynamic computed tomography (CT) or magnetic resonance imaging (MRI) every 6-12 months (6). However, there is no clear evidence that this tighter surveillance interval improves patient survival in viral cirrhosis. Therefore, we aimed at comparing, in patients

with HBV- and HCV-related cirrhosis, the 3- and the 6-months surveillance intervals in terms of HCC stage at diagnosis and survival.

PATIENTS AND METHODS

Study groups

In this retrospective study, data were retrieved from the Italian Liver Cancer (ITA.LI.CA) database, a multicenter registry including 6,991 HCC patients consecutively managed from January 1987 to December 2017 at any of the 24 participating Institutions. We selected all the patients with HBV or HCV-related liver cirrhosis and HCC diagnosed under regular US surveillance (n=2,829). The liver disease was classified as HBV- or HCV-related if patients were hepatitis B surface antigen (HBsAg) carriers or were positive for serum anti-HCV antibodies, respectively. The diagnosis of cirrhosis was either confirmed histologically or made unequivocal by clinical, radiological and biochemical findings. Only patients diagnosed with HCC between January 2009 and December 2013 (n=1,169) were included, thus obtaining a homogeneous group in terms of HCC treatment and antiviral therapy availability. After the exclusion of HIV co-infected patients (n=16) and of patients surveilled with different intervals than those selected (n=376), 109 patients (14%) surveilled with a 3±1 months schedule (3MS group) and 668 patients (86%) surveilled with a 6±1 months schedule (6MS group) were finally included in this study (Supplementary Figure 1).

The management of the ITA.LI.CA database conforms to the Italian legislation on privacy. According to the Italian laws, no specific patient approval is needed for any retrospective analysis, but patients provided written informed consent for every diagnostic and therapeutic procedure, as well as for having their clinical data recorded anonymously in the ITA.LI.CA database. The study was conducted in accordance to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008).

Details about HCC diagnosis (symptomatic, casual or during surveillance) are regularly registered in the database. The surveillance interval and tests are established by the referring physician of each patient. US was used as surveillance test in all the patients included in the study, while the adjunctive determination of AFP was left to the attending clinician's choice.

HCC diagnosis was histological in 108 patients (13.9%), whereas in the majority of the cases it was based on the typical features at imaging (CT or MRI) according to guidelines (4,5).

As described elsewhere (19), patient demographic, clinicopathological and laboratory data , as well as tumor characteristics (tumor location and size, number of nodules, macrovascular invasion [MVI] and extra-hepatic spread [EHS]) were collected. Clinically significant portal hypertension (CSPH) was defined as the presence of unequivocal signs (splenomegaly, varices, ascites) or platelet count <100 × 10^9/L (20). At diagnosis, tumor burden was evaluated with dynamic CT or MRI (plus additional investigations to detect metastases when extra-hepatic involvement was suspected) in all cases. For the purpose of the present study, HCC was staged as: solitary tumor ≤2 cm without MVI and/or EHS (very early); solitary tumor 2.1-3.0 cm without MVI and/or EHS; solitary tumor 3.1-5.0 cm without MVI and/or EHS; 2-3 lesions each ≤3 cm without MVI and/or EHS; advanced tumor (outside the Milano criteria (21)).

Six therapeutic subgroups were considered: liver transplantation (LT), liver resection (LR), ablation (ABL), intra-arterial therapies (IAT), sorafenib (SOR) and best supportive care (BSC). In patients managed along their clinical history with more than one treatment modality, only the main therapy was considered, defined as the most beneficial according to the following hierarchy: LT, LR, ABL, IAT, SOR and BSC (22).

Statistical analysis

Categorical variables were reported with absolute and relative frequencies, while quantitative data were summarized with median and interquartile range. Mann-Whitney test, χ^2 test and Fischer's exact test were used to compare variables, as appropriate.

Variables independently associated with cancer stage at diagnosis were identified by univariate and multivariate logistic regression analysis. Surveillance interval, sex, age, year of diagnosis, etiology, CRPH, body mass index (BMI), Model for End-Stage Liver Disease (MELD) score, Child-Pugh class, and log-transformed AFP were tested at univariate analysis, and only variables predictive of cancer stage ($p \leq 0.1$) were included in the multivariate models. Similarly, multivariate logistic regression analysis was used to identify factors independently associated with increased likelihood of receiving curative treatment (LT, LR or ABL).

Overall survival (OS), expressed as median and 95% confidence interval (CI), was calculated from the date of HCC diagnosis to the date of death from any cause, last follow-up evaluation or data censoring (December 31st, 2017). Survival curves were estimated and compared using Kaplan-Meier method and log-rank test. The confounding effect of the lead-time bias was minimized adjusting the observed survival of 3MS patients for the calculated lead-time. This was obtained using the Schwartz's formula (23): $t = DT \times 3 \times \log(d_1/d_0)/\log(2)$, in which t is the lead-time in days, DT is the mean tumor volume doubling time reported in a recent metanalysis (4.6 months) (24), d_0 and d_1 are the median HCC sizes in 3MS and 6MS groups, respectively. If after the subtraction of the lead-time the adjusted survival became negative, a survival (in deceased patients) or a follow-up (in living patients) of 1 day was attributed. The 3MS group survival was analyzed as both observed and adjusted. Multivariate Cox proportional hazard models were used to identify the independent prognostic factors among variables significantly or borderline ($p \leq 0.1$) associated with survival at univariate analysis. To avoid collinearity between residual liver function variables, in multivariate logistic regression and Cox analyses, two models were created including either MELD score or Child-

Pugh class. In addition, we explored whether the surveillance schedule had an impact on cause-specific survival. HCC progression, cirrhosis decompensation and not liver-related causes of death were treated as competing risks and the association of the surveillance interval with each outcome was evaluated with a competing risk survival analysis.

Considered the non-randomized nature of this study, in order to correct for potential biases in the allocation to 3- or 6-months surveillance interval, we selected two homogeneous groups by propensity score matching. Variables that might affect the selection of surveillance schedule (sex, age, etiology, presence of CSPH, residual liver function) were included in the propensity score model. Binary logistic regression was used to calculate a continuous propensity score from 0 to 1. A nearest-neighbor match without replacement in a 1:2 ratio, with a pre-defined caliper width (0.2 of the standard deviation of the logit of the propensity score), was used to match 3MS and 6MS patients.

In order to estimate the increase in direct costs of surveillance adopting a 3-months schedule, the time interval between the diagnosis of cirrhosis and HCC (monitoring time lapse) was calculated and then divided by the interval of surveillance in months (three in 3MS patients and six in 6MS patients), obtaining the number of US examinations performed in each patient. These figures were multiplied by 80 €, which is the cost for an US examination calculated by the Italian National Health System, thus providing the direct costs of surveillance per patient. The sum of these costs in each group was then divided by the number of patients belonging to one or the other group, obtaining the average cost of surveillance per patient in 3MS and 6MS groups. In summary, the formula used for this calculation was: *Average cost = ∑ [(Time under surveillance in months/surveillance interval in months) x US cost] / number of patients*.

In all the analysis, a 2-tailed p value <0.05 was considered statistically significant. IBM SPSS Statistics (Version 25.0. Armonk, NY: IBM Corp.), STATA 13.0 (1985-2013 StataCorp LP) and GraphPad Prism

version 8.3.1 (GraphPad Software, La Jolla, California, USA) were used for all the calculations in this study.

RESULTS

Patient characteristics, tumor stage and treatment

The median duration of surveillance was 6 years (2-17) in 3MS and 9 years (3.5-17) in S6M group. Baseline characteristics of the patients are depicted in Table 1. 3MS patients were more frequently males (p=0.04), younger (p=0.004) and affected by HBV-related cirrhosis (p=0.01). No significant difference in tumor burden, AFP levels and presence of MVI and EHS was recorded between the 2 groups. The majority of patients were diagnosed with solitary tumors ≤5 cm or 2-3 nodules ≤3 cm in size, but 35.8% patients in 3MS group and 31.7% patients in 6MS group were diagnosed outside the Milano criteria (p=0.89). The different types of treatment were equally distributed in the two groups: the majority of patients underwent LT, LR or ABL, the latter treatment being the most frequently adopted (45.0% in 3MS and 48.8% in 6MS patients).

Table 1. Baseline demographic and clinical characteristics of patients.

Variable	**3MS (n=109, 14%)**	**6MS (n=668, 86%)**	**p[a]**
Males – n (%)	82 (75.2)	435 (65.1)	0.04
Age (years)	70 (62 – 76)	72 (64 – 77)	0.004
Etiology – n (%)			
HBV HCV HBV+HCV	24 (22.0) 80 (73.4) 5 (4.6)	84 (12.6) 568 (85.0) 16 (2.4)	0.01
BMI (Kg/m^2)	24.9 (22.9 – 27.0)	25.3 (23.2 – 27.6)	0.33
CSPH – n (%)	69 (63.3)	475 (71.1)	0.11
MELD score	10 (8 – 12)	9 (7 – 11)	0.02
Child-Pugh class – n (%)			
A B C	76 (69.8) 31 (28.4) 2 (1.8)	476 (71.3) 173 (25.9) 19 (2.8)	0.74
ECOG-PS – n (%)			
0 1 2	92 (84.4) 17 (15.6) 0 (0)	474 (71.0) 172 (25.7) 22 (3.3)	0.007
Number of nodules	1 (1 – 3)	1 (1 – 3)	0.72

Diameter (cm)	2.7 (2.0 – 4.8)	3.0 (2.0 – 4.0)	0.11
MVI – n (%)	10 (9.2)	43 (6.4)	0.31
EHS – n (%)	2 (1.8)	10 (1.5)	0.68
AFP (ng/mL)	20.0 (4.5 – 323.0)	21.0 (5.0 – 249.3)	0.63
Cancer stage – n (%)			
Solitary ≤2 cm, no MVI, no EHS	33 (30.3)	203 (30.4)	0.89
Solitary 2.1-3 cm, no MVI, no EHS	13 (11.9)	96 (14.4)	
Solitary 3.1-5 cm, no MVI, no EHS	10 (9.2)	59 (8.8)	
2-3 nodules, ≤ 3 cm, no MVI, no EHS	14 (12.8)	98 (14.7)	
Outside Milano criteria	39 (35.8)	212 (31.7)	
Main treatment – n (%)			
LT	11 (10.1)	32 (4.8)	0.28
LR	16 (14.7)	98 (14.7)	
ABL	49 (45.0)	326 (48.8)	
IAT	25 (22.9)	146 (21.9)	
SOR	5 (4.6)	31 (4.6)	
BSC	3 (2.7)	35 (5.2)	

[a] Mann-Whitney test, χ^2 test and Fischer's exact test, as appropriate.
Categorical variables are presented ad absolute frequency and percentage, while continuous variables are presented as median and interquartile range.
Abbreviations: 3MS, 3-months surveillance group; 6MS, 6-months surveillance group; BMI, body mass index; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; ECOG-PS, Eastern Cooperative Oncology Group performance status; MVI, macrovascular invasion; EHS, extra-hepatic spread; AFP, alpha-fetoprotein; LT, liver transplantation; LR, liver resection; ABL, ablation; IAT, intra-arterial therapies; SOR, sorafenib; BSC, best supportive care.

By univariate logistic regression analysis, the 6-months surveillance interval was not associated with an increased risk of HCC diagnosis outside the Milano criteria compared to the 3-months interval (odds ratio [OR] = 0.83, 95% CI 0.55-1.28; p=0.40), as instead were sex, CSPH, MELD score, Child-Pugh class and AFP levels. Independent predictors of diagnosis in advanced stage at the multivariate logistic regression analysis were male sex, Child-Pugh B and high AFP levels (Table 2). Only high levels of AFP and BMI (although this latter borderline) resulted independent predictors of diagnosis beyond the very early stage (Supplementary Table 1).

Table 2. Independent risk factors for the detection of HCC outside the Milano criteria.

Variable	**Univariate analysis**		**Multivariate analysis Model 1[a]**		**Multivariate analysis Model 2[a]**	
	OR (95% CI)	**p**	**aOR (95% CI)**	**p**	**aOR (95% CI)**	**p**
Surveillance group						
3MS	Ref	-	-	-	-	-
6MS	0.83 (0.55-1.28)	0.40				
Sex						
Female	Ref	-	Ref	-	Ref	-
Male	1.35 (0.97-1.86)	0.07	1.48 (1.04-2.13)	0.03	1.51 (1.05-2.16)	0.03
Age (per 10-years increase)	0.96 (0.82-1.11)	0.55	-	-	-	-
Year of diagnosis	0.95 (0.86-1.06)	0.38	-	-	-	-

Etiology						
HBV	Ref	-	-	-	-	-
HCV	0.99 (0.64-1.52)	0.95				
HBV+HCV	1.28 (0.49-3.38)	0.61				
BMI (Kg/m^2)	1.03 (0.97-1.08)	0.36	-	-	-	-
CSPH						
No	Ref	-	Ref	-	Ref	-
Yes	1.51 (1.08-2.13)	0.27	1.12 (0.76-1.67)	0.57	1.02 (0.68-1.53)	0.92
MELD score	1.05 (1.01-1.10)	0.02	1.02 (0.97-1.08)	0.43	-	-
Child-Pugh class						
A	Ref	-	-	-	Ref	-
B	1.73 (1.24-2.41)	0.001			1.50 (1.01-2.23)	0.04
C	2.77 (1.15-6.65)	0.02			1.26 (0.46-3.45)	0.65
logAFP	2.41 (2.05-2.83)	<0.0001	2.40 (2.03-2.82)	<0.0001	2.39 (2.03-2.82)	<0.0001

[a] In order to avoid collinearity between liver function variables, MELD score and Child-Pugh class were included as covariates separately in model 1 and model 2, respectively.

Abbreviations: OR, Odds ratio; aOR, adjusted OR; CI, confidence interval; Ref, reference; 3MS, 3-months surveillance group; 6MS, 6-months surveillance group; HBV, hepatitis B virus; HCV, hepatitis C virus; BMI, Body Mass Index; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; logAFP, logarithm of alpha-fetoprotein.

The likelihood of receiving curative treatment was similar between patients surveilled every 3- and 6-months (OR=0.93, 95% CI 0.60-1.45; p=0.76). By contrast, high ECOG-PS, poor residual liver function (Child-Pugh class B), high AFP levels and larger tumor burden were independently associated with a decreased probability to be treated with LT, LR or ABL (Table 3).

Table 3. Factors associated with the probability of receiving a curative treatment (liver transplantation, liver resection or ablation) for HCC.

Variable	**Univariate analysis**		**Multivariate analysis Model 1[a]**		**Multivariate analysis Model 2[a]**	
	OR (95% CI)	**p**	**aOR (95% CI)**	**p**	**aOR (95% CI)**	**p**
Surveillance group						
3MS	Ref	-	-	-	-	-
6MS	0.93 (0.60-1.45)	0.76				
Sex						
Female	Ref	-	-	-	-	-
Male	1.00 (0.73-1.38)	0.99				
Age (per 10-years increase)	0.95 (0.81-1.10)	0.48	-	-	-	-
Year of diagnosis	0.99 (0.90-1.11)	0.96	-	-	-	-
Etiology						
HBV	Ref	-	-	-	-	-
HCV	0.77 (0.49-1.22)	0.26				
HBV+HCV	0.73 (0.27-2.00)	0.55				
BMI (Kg/m^2)	0.97 (0.91-1.02)	0.25	-	-	-	-
CSPH						
No	Ref	-	Ref	-	Ref	-
Yes	0.44 (0.31-0.64)	<0.0001	0.67 (0.44-1.03)	0.07	0.77 (0.50-1.18)	0.23
MELD score	0.92 (0.88-0.96)	0.0002	0.99 (0.93-1.04)	0.62	-	-
Child-Pugh class						
A	Ref	-	-	-	Ref	-

B	0.39 (0.28-0.55)	<0.0001			0.65 (0.43-0.98)	0.04
C	0.13 (0.05-0.35)	<0.0001			0.43 (0.13-1.43)	0.17
EGOG-PS						
0	Ref	-	Ref	-	Ref	-
1	0.23 (0.16-0.32)	<0.0001	0.28 (0.19-0.41)	<0.0001	0.30 (0.20-0.44)	<0.0001
2	0.04 (0.01-0.15)	<0.0001	0.09 (0.02-0.32)	0.0002	0.12 (0.03-0.44)	0.002
logAFP	0.50 (0.43-0.58)	<0.0001	0.70 (0.58-0.84)	0.0002	0.70 (0.58-0.84)	0.0002
Cancer stage						
Solitary ≤2 cm, no MVI, no EHS	Ref	-	Ref	-	Ref	-
Solitary 2.1-3 cm, no MVI, no EHS	0.48 (0.27-0.86)	0.01	0.52 (0.28-0.96)	0.03	0.52 (0.28-0.96)	0.03
Solitary 3.1-5 cm, no MVI, no EHS	0.24 (0.13-0.43)	<0.0001	0.49 (0.24-0.98)	0.04	0.48 (0.24-0.98)	0.04
2-3 nodules, ≤ 3 cm, no MVI, no EHS	0.45 (0.26-0.80)	0.007	0.48 (0.26- 0.87)	0.01	0.46 (0.25-0.85)	0.01
Outside Milano criteria	0.13 (0.09-0.21)	<0.0001	0.22 (0.11-0.37)	<0.0001	0.22 (0.13-0.38)	<0.0001

[a] In order to avoid collinearity between liver function variables, MELD score and Child-Pugh class were included as covariates separately in model 1 and model 2, respectively.

Abbreviations: OR, Odds ratio; aOR, adjusted OR; CI, confidence interval; Ref, reference; 3MS, 3-months surveillance group; 6MS, 6-months surveillance group; HBV, hepatitis B virus; HCV, hepatitis C virus; BMI, Body Mass Index; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; ECOG-PS, Eastern Cooperative Oncology Group performance status; logAFP, logarithm of alpha-fetoprotein; MVI, macrovascular invasion; EHS, extrahepatic spread.

Survival analysis

The median duration of follow-up in the whole study population was 50.0 months (95% CI 21.0-67.5) and during this period 69 patients (63.3%) in 3MS group and 373 patients (55.8%) in 6MS group died. 3MS and 6MS groups were similar in the proportion of death from HCC progression (66.7% vs. 57.4%, respectively; p=0.18), cirrhosis decompensation (21.7% vs. 25.7%, respectively; p=0.55) and not liver-related causes of death (11.6% vs. 16.9%, respectively; p=0.37).

The median OS in the whole population of patients was 52.0 months (95% CI 44.2-59.8), with a 5-years survival rate of 46.3%. The observed survival of 3MS patients (47.0 months, 95% CI 35.0-58.9), albeit slightly shorter, was not significantly different compared to that of 6MS patients (55.0 months, 95% CI 45.9-64.0; hazard ratio (HR)=0.90 [95% CI 0.70-1.17]; p=0.43). The 5-years survival rates were 40.7% and 47.2%, respectively (Figure 1a).

According to the Schwartz's formula, the calculated lead-time was 63 days. The median adjusted survival of 3MS group was 44.9 months (95% CI 33.4-56.4), with a 5-years survival rate of 40.0%. These figures did not significantly differ than those observed in 6MS group (HR=0.87, 95% CI 0.67-1.13; p=0.30; Figure 1b).

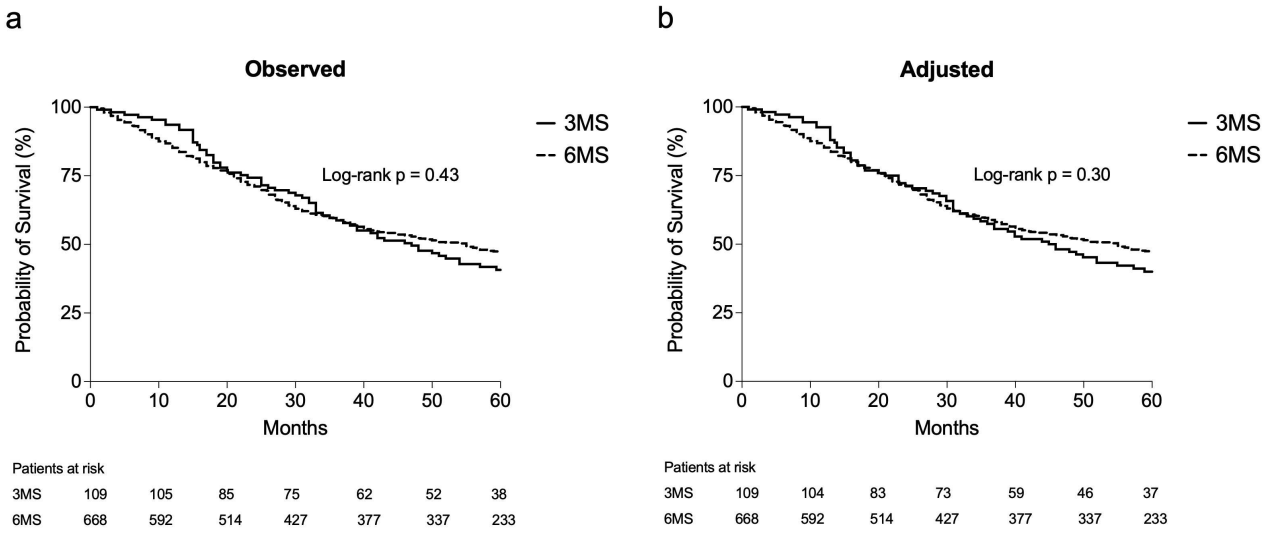


Figure 1. Kaplan-Meier curves for overall survival in the 3MS and 6MS groups. 6MS survival was not statistically significant different from that of 3MS group, the latter considered both as observed (**a**) and adjusted for the lead-time bias (**b**).

By multivariate analyses, year of diagnosis, residual liver function (MELD score and Child-Pugh class in model 1 and 2, respectively), advanced ECOG-PS, tumor burden and main treatment were independently associated with an increased risk of death (Table 4). Among these, treatment was the major predictor of prognosis, with a clear hierarchy in survival benefit (LT>LR>ABL>IAT>SOR>BSC). After correction for other independent predictors of prognosis, the 6MS interval was again not associated with an increased risk of mortality (adjusted HR=0.92 [95% CI 0.70-1.21], p=0.54 in model 1 including MELD score and 0.89 [95% CI 0.68-1.18], p=0.42 in model 2 including Child-Pugh class). At the univariate competing risk survival analysis, 3MS was not associated with a decreased mortality risk due to HCC progression. By contrast, a slightly and non-statistically significant lower risk of cancer-related death was evident for semiannual surveillance (HR=0.75, 95% CI 0.55-1.02; p=0.07) (Supplementary Figure 2a). As expected, the surveillance schedule was not associated with mortality risk due to cirrhosis decompensation (HR=1.03, 95% CI 0.59-1.78; p=0.10) and not liver-related causes of death (HR=1.30, 95% CI 0.63-2.70; p=0.71) (Supplementary Figure 2b and 2c).

Table 4. Variables independently associated with mortality (survival adjusted for the lead-time was used in 3MS patients).

Variable	Univariate analysis		Multivariate analysis Model 1[a]		Multivariate analysis Model 2[a]	
	HR (95% CI)	p	aHR (95% CI)	p	aHR (95% CI)	p
Surveillance group						
3MS	Ref	-	-	-	-	-
6MS	0.87 (0.67-1.13)	0.30				
Sex						
Female	Ref	-	-	-	-	-
Male	1.01 (0.83-1.23)	0.92				
Age (per 10-years increase)	1.08 (0.98-1.19)	0.14	-	-	-	-
Year of diagnosis	0.85 (0.80-0.91)	<0.0001	0.84 (0.79-0.90)	<0.0001	0.83 (0.78-0.89)	<0.0001
Etiology						
HBV	Ref	-	-	-	-	-
HCV	1.04 (0.79-1.37)	0.78				
HBV+HCV	1.17 (0.64-2.13)	0.62				
BMI (Kg/m^2)	0.98 (0.95-1.02)	0.27	-	-	-	-
CSPH						
No	Ref	-	Ref	-	Ref	-
Yes	1.60 (1.29-1.99)	<0.0001	1.20 (0.94-1.52)	0.14	1.19 (0.93-1.51)	0.16
MELD score	1.07 (1.05-1.10)	<0.0001	1.06 (1.03-1.09)	<0.0001	-	-
Child-Pugh class						
A	Ref	-	-	-	Ref	-
B	1.74 (1.42-2.13)	<0.0001			1.60 (1.28-2.02)	<0.0001
C	2.08 (1.22-3.56)	0.007			1.95 (1.07-3.53)	0.03
ECOG-PS						
0	Ref	-	Ref	-	Ref	-
1	1.80 (1.47-2.22)	<0.0001	1.23 (0.98-1.53)	0.08	1.18 (0.94-1.47)	0.16
2	2.83 (1.71-4.69)	<0.0001	0.46 (0.25-0.85)	0.01	0.38 (0.20-0.72)	0.003
logAFP	1.36 (1.24-1.48)	<0.0001	1.06 (0.96-1.18)	0.26	1.06 (0.95-1.17)	0.29
Cancer stage						
Solitary ≤2 cm, no MVI, no EHS	Ref	-	Ref	-	Ref	-
Solitary 2.1-3 cm, no MVI, no EHS	1.33 (0.95-1.86)	0.09	1.15 (0.82-1.62)	0.42	1.19 (0.85-1.67)	0.32
Solitary 3.1-5 cm, no MVI, no EHS	2.60 (1.83-3.68)	<0.0001	2.10 (1.44-3.05)	0.0001	2.22 (1.53-3.22)	<0.0001
2-3 nodules, ≤ 3 cm, no MVI, no EHS	1.92 (1.41-2.62)	<0.0001	1.69 (1.24-2.31)	0.001	1.72 (1.26-2.35)	0.001
Outside Milano criteria	2.59 (2.01-3.32)	<0.0001	1.64 (1.22-2.20)	0.001	1.66 (1.24-2.22)	0.001
Treatment						
BSC	Ref	-	Ref	-	Ref	-
LT	0.03 (0.02-0.07)	<0.0001	0.03 (0.01-0.07)	<0.0001	0.03 (0.01-0.07)	<0.0001
LR	0.09 (0.06-0.14)	<0.0001	0.12 (0.07-0.20)	<0.0001	0.11 (0.06-0.18)	<0.0001
ABL	0.12 (0.08-0.17)	<0.0001	0.15 (0.10-0.24)	<0.0001	0.15 (0.09-0.23)	<0.0001
IAT	0.24 (0.17-0.35)	<0.0001	0.26 (0.16-0.41)	<0.0001	0.24 (0.15-0.37)	<0.0001
SOR	0.49 (0.30-0.80)	0.004	0.55 (0.32-0.92)	0.02	0.50 (0.30-0.83)	0.008

[a] In order to avoid collinearity between liver function variables, MELD score and Child-Pugh class were included as covariates separately in model 1 and model 2, respectively.

Abbreviations: HR, hazard ratio; aHR, adjusted HR; CI, confidence interval; Ref, reference; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; ECOG-PS, Eastern Cooperative Group performance status; MVI, macrovascular invasion, EHS, extrahepatic spread; logAFP, logarithm of alpha-fetoprotein; BSC, best supportive care; LT, liver transplantation; LR, liver resection; ABL, ablation; IAT, intra-arterial therapies; SOR, sorafenib.

Propensity score matching survival analysis

After propensity score matching, 74 3MS patients and 148 6MS patients were selected, obtaining two subgroups absolutely comparable in baseline characteristics (Table 5).

The median OS in this sub-population was 46.0 months (95% CI 35.3-56.7), with a 5-years survival rate of 43.1%. As in the whole study population, there was no significant difference between the observed survival of 3MS and 6MS patients (48.0 months [95% CI 37.6-58.4] vs. 40.0 months [95% CI 19.5-60.5]; HR=0.97, 95% CI 0.68-1.38; p=0.86). The 5-years survival rates were 41.5% and 43.9%, respectively (Figure 2a). Despite the slightly higher median OS recorded for 3MS patients, 5-years survival rates were higher in 6MS group and this is explained by the crossing over of the survival curves in the proximity of the median OS. The median survival of the 3MS patients adjusted for the estimated lead-time (85 days) was 45.2 months (95% CI 34.8-55.5), with a 5-years corrected survival rate of 39.7%, again not different from that of 6MS patients (HR=0.93, 95% CI 0.65-1.32; p=0.67; Figure 2b).

Table 5. Baseline demographic and clinical characteristics of patients after propensity score matching.

Variable	**3MS (n=74)**	**6MS (n=148)**	**p[a]**
Males – n (%)	57 (77.0)	111 (75.0)	0.74
Age (years)	71 [62 - 76]	69 [61 - 76]	0.87
Etiology – n (%)			
HBV HCV HBV+HCV	12 (16.2) 59 (79.7) 3 (4.1)	25 (16.9) 118 (79.7) 5 (3.4)	0.96
BMI (Kg/m^2)	25.2 [23.0 – 28.2]	25.4 [22.9 – 27.6]	0.82
CSPH – n (%)	46 (62.2)	89 (60.1)	0.77
MELD score	9 [8 - 11]	9 [7 - 11]	0.23
Child-Pugh class – n (%)			
A B C	54 (73.0) 18 (24.3) 2 (2.7)	110 (74.3) 36 (24.3) 2 (1.4)	0.77
ECOG-PS – n (%)			
0 1 2	65 (87.8) 9 (12.2) 0 (0)	114 (77.0) 33 (22.3) 1 (0.7)	0.14
Number of nodules	1 [1 - 3]	1 [1 - 3]	0.96
Diameter (cm)	2.7 [2.0 – 4.8]	3.0 [2.0 – 4.0]	0.11
MVI – n (%)	5 (6.8)	9 (6.1)	1.00
EHS – n (%)	2 (2.7)	3 (2.0)	1.00
AFP (ng/mL)	37.5 [4.8 – 682.0]	17.5 [5.3 – 203.3]	0.33
Cancer stage – n (%)			
Solitary ≤2 cm, no MVI, no EHS	18 (24.3)	44 (29.7)	0.87

Solitary 2.1-3 cm, no MVI, no EHS	9 (12.2)	19 (12.8)	
Solitary 3.1-5 cm, no MVI, no EHS	6 (8.1)	8 (5.4)	
2-3 nodules, ≤ 3 cm, no MVI, no EHS	12 (16.2)	21 (14.2)	
Outside Milano criteria	29 (39.2)	56 (37.8)	
Main treatment – n (%)			
LT	8 (10.8)	10 (6.8)	0.89
LR	11 (14.9)	25 (16.9)	
ABL	32 (43.2)	71 (48.0)	
IAT	18 (24.3)	32 (21.6)	
SOR	3 (4.1)	5 (3.4)	
BSC	2 (2.7)	5 (3.4)	

[a] Mann-Whitney test, χ^2 test and Fischer's exact test, as appropriate.

Categorical variables are presented ad absolute frequency and percentage, while continuous variables are presented as median and interquartile range.

Abbreviations: 3MS, 3-months surveillance group; 6MS, 6-months surveillance group; BMI, body mass index; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; ECOG-PS, Eastern Cooperative Oncology Group performance status; MVI, macrovascular invasion; EHS, extra-hepatic spread; AFP, alpha-fetoprotein; LT, liver transplantation; LR, liver resection; ABL, ablation; IAT, intra-arterial therapies; SOR, sorafenib; BSC, best supportive care.

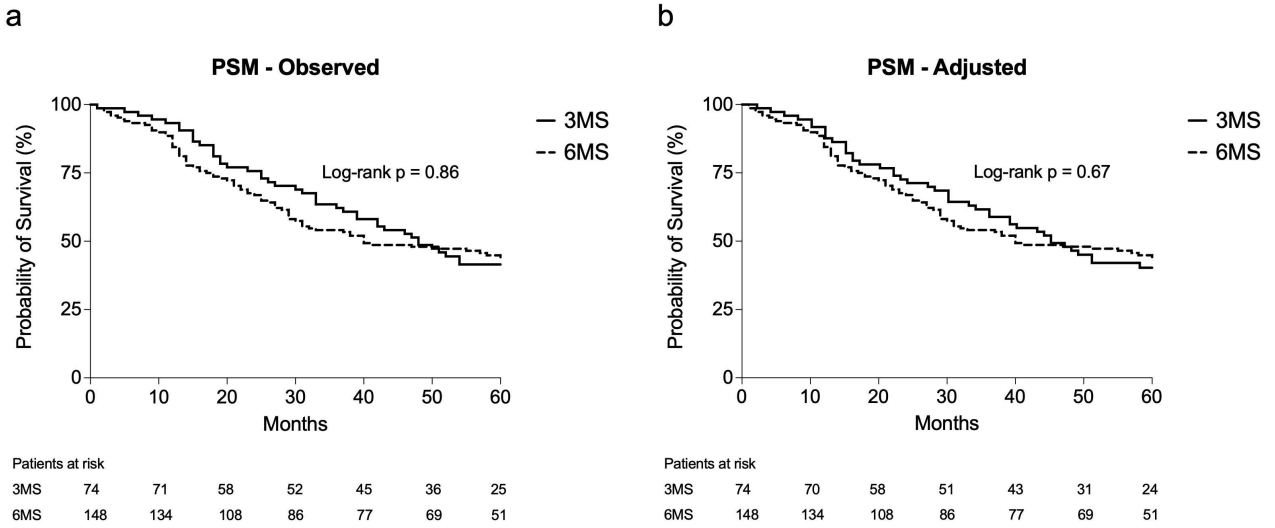


Figure 2. Kaplan-Meier curves for overall survival in the 3MS and 6MS groups after propensity score matching analysis. The survival of 6MS patients was not different from the observed (**a**) and adjusted (**b**) survival of 3MS patients.

Cost analysis

The overall cost of surveillance was 316,645 € in 3MS group and 1,217,764 € in 6MS group. These figures were then divided by the number of patients included in each group, obtaining the average cost per patient: 2,905 € for a patient surveilled quarterly and 1,823 € for a patient tested twice a year, with a difference of 1,082 € between the two surveillance schedules. Only the direct costs of US surveillance were estimated, not including the expenses for the determination of biomarkers, which are frequently used in clinical practice, as well as the indirect costs of recall procedures. In

the evaluation of the economic impact of the surveillance schedule, also the cost of the procedures adopted to achieve HCC diagnosis, including liver biopsy, should be considered. Although our study was not designed to compare the cost of diagnosing HCC in the two groups, a greater expense could be postulated in 3MS group since the diagnosis was histological in a significantly higher proportion of 3MS (n=24, 22.0%) compared to 6MS patients (n=84, 12.6%) (p=0.01).

DISCUSSION

Despite some improvement in survival, HCC should be still considered as a highly lethal cancer and, with the aim of reducing disease-specific mortality, international guidelines recommend regular surveillance in patients at risk (4–7). While semiannual surveillance is suggested by EASL, AASLD and APASL guidelines (4,5,7), the Japanese ones recommend an enhanced schedule (shorter interval between US, periodic assessment of tumor markers and optional CT/MRI every 6-12 months) in HBV and HCV-related cirrhotics, who are considered at “extremely high-risk” of developing HCC (6). By contrast, Western guidelines does not even suggest the determination of AFP (4,5). Experimental data proving an advantage in shortening the surveillance interval are still lacking. A single trial compared the 3- and the 6-months schedules and reported no difference in terms of early diagnosis and survival, but it was not specifically addressed at evaluating viral cirrhotics (17).

In this study we assessed whether a 3-months US surveillance schedule is associated with an improvement of prognosis in Caucasian patients with a virus-related cirrhosis, compared to the routine 6-months interval. 3MS was not superior to 6MS schedule in terms of number of tumors detected and median size of nodules, as also demonstrated by the comparable cancer stage at diagnosis. At the logistic regression analysis, 6MS schedule was not associated with an increased risk of missing patients with an early-stage tumor (within the Milano criteria). Moreover, as far as therapeutic allocation was concerned, between the two groups there were no significant

differences: the majority of patients were managed with curative therapies (in particular ABL) in both groups and the tighter surveillance schedule was not associated with an increased likelihood to receive a radical treatment.

The prognostic benefit of regular surveillance is a well-known concept, since the survival of periodically monitored patients is significantly longer compared to casual/symptomatic diagnosis (10,11,25,26). This benefit is indirectly confirmed also in this study considering that the median survival demonstrated for all the included surveilled patients was remarkably high (52.0 months). Surveillance exerts its benefit on prognosis through an early diagnosis, which allows the delivery of curative treatments. Since in this study there was no difference in cancer stage at diagnosis and treatments between the two groups, the 3MS did not demonstrate to improve the survival as compared to the 6MS surveillance schedule. This was further confirmed when cause-specific survival was analyzed, with 3MS not proving to be associated with a decrease of cancer-related mortality risk.

Studies on surveillance for HCC are potentially limited by the existence of “lead-time” and “length-time” biases (25,27). We here minimized the former by adjusting the observed survival of 3MS patients for the calculated lead-time, and again no statistically significant difference in survival between 3MS and 6MS patients was observed after correction. The length-time bias (i.e., the relative excess of slowly growing tumors detected in surveillance as compared to symptomatic diagnosis) cannot be avoided, but it was minimized considering that in the ITA.LI.CA database patients in whom the diagnosis is anticipated between the scheduled appointments due to the development of symptoms are considered as surveilled.

In addition to being useless in reducing mortality, the 3MS schedule accounted for an increased direct cost of surveillance. The 3-months interval was associated with an approximately 1,000 € higher expenses per patient. Moreover, the enhanced surveillance program cost is likely to be

higher since we did not consider the cost of biomarkers (e.g., AFP, widely used despite not recommended by EASL guidelines) and, more importantly, the indirect cost of surveillance. Indeed, as already demonstrated by Trinchet et al. (17), a shorter surveillance interval increases the detection of focal lesions, leading to a higher number of recall procedures to achieve a diagnosis.

The present study underlines some limitations of the current surveillance policy. A high proportion of patients (about 30% in the entire cohort) was diagnosed with a tumor burden outside the Milano criteria, similarly to what has been previously found in other studies on surveillance (12,17). The relatively high prevalence of tumors deriving from a multicentric carcinogenesis may explain this result, but these data also emphasize the need for improving surveillance methods.

This study has some limitations and may suffer from unintended biases, due to its retrospective nature. The first, and most important limitation, is the selection bias which derives from the subjective and not standardized choice of the surveillance schedule. Clinicians tend to shorten the surveillance interval in patients considered at very high risk to develop HCC (such as HBV patients), and this could have increased the number of high-risk patients assigned to a 3-months surveillance schedule (as suggested by the higher prevalence of young males with HBV-related chronic liver disease in this group). Therefore, in order to support our findings, we created two groups comparable for the variables that may influence the choice of surveillance interval with a propensity score matching. While in the unadjusted survival analysis 3MS patients demonstrated a slightly shorter median OS compared to 6MS group, these figures were reversed after matching (although 5-years survival rates were still slightly higher in 6MS group). Moreover, the median OS of 6MS patients decreased from 55.0 months in the unadjusted analysis to 40.0 months in the propensity score analysis, while the median OS of 3MS patients remained approximately the same (47.0 months and 48.0 months, respectively). This seems to confirm the existence of a selection bias, whereby patients at higher risk and probably those who will develop more aggressive tumors were

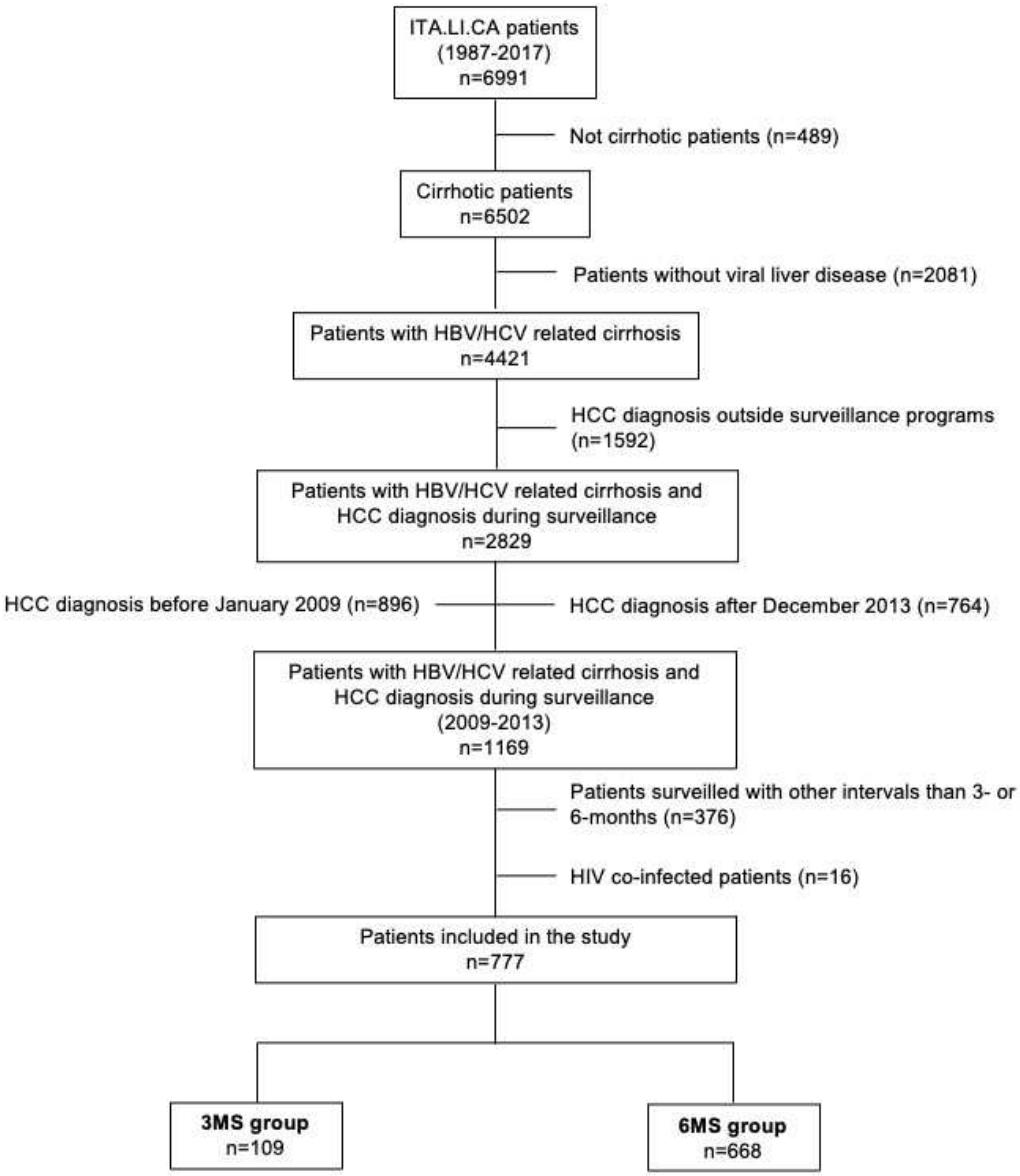
predominantly allocated in the 3MS group. The median OS of semiannually surveilled patients singled out with propensity score matching drops to 40.0 months, this being probably due to their baseline characteristics and risk profile similar to 3MS patients. Nevertheless, even after correction for this selection bias, the 3-months surveillance schedule proved not to be superior to the routine 6-months interval, both considering the observed and the lead-time adjusted survival. This lends additional support to the conclusion that a more stringent surveillance schedule does not improve the prognosis of HBV and HCV-related cirrhotic patients.

Even if after the propensity score matching we obtained two comparable groups, it was not possible to take into account all the confounders that can influence the choice of the surveillance interval. In the ITA.LI.CA database information about the presence of precancerous lesion or undefined nodules, which may have influenced the adoption of a tighter surveillance interval, are not collected and therefore cannot be included in the analysis. The ITA.LI.CA database collects patients managed over a 30-years period of time, during which there have been several improvements in diagnostic and therapeutic techniques (recruitment time bias). Trying to minimize the influence of the period of HCC diagnosis on surveillance effectiveness and survival outcomes, we included in our analysis only patients diagnosed in a narrow temporal window (2009-2013). As a drawback, HCV-cirrhotics treated with direct-acting antiviral agents and the impact of this effective therapy on surveillance for HCC were not considered in this study. Although it is well recognized that a sustained virological response (SVR) reduces the HCC risk in HCV cirrhotics (28), this study was limited by the fact that we were not able to consider the role of SVR because HCV-RNA levels were known only in 254 HCV patients (38.0%). However, we can reasonably assume that an enhanced surveillance, which did not improve survival of patients before the availability of an effective HCV eradication therapy, might be even less useful after viral clearance, thus further reinforcing our conclusions. In addition, we did not consider the status of the patients regarding HBV-antiviral therapy. It is known that high level

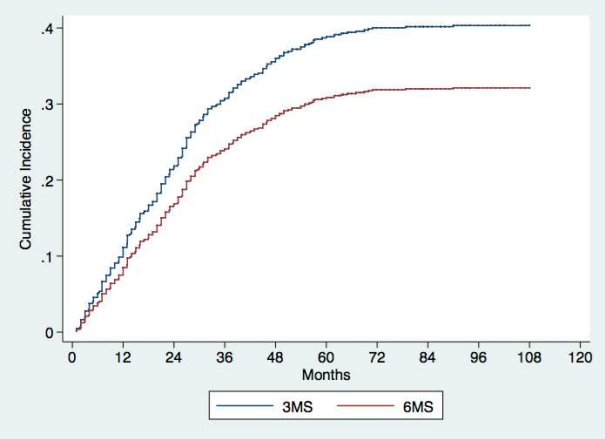
of serum HBV-DNA are associated with higher risk of HCC (29), while the suppression of viral replication could reduce the risk and improve survival (30,31). Unfortunately, data regarding HBV-antiviral therapy and HBV-DNA levels were not available for the majority of patients, preventing us from correcting even for these confounders.

In conclusion, the 3-months surveillance interval in HBV and HCV cirrhotic patients does not increase the detection of early-stage cancers, the probability to receive curative treatments and, most importantly, the overall survival compared to the 6-months schedule. Moreover, the 3-months schedule increases the direct cost of surveillance, and could likely increase also the indirect ones. According to our results, the surveillance of HBV and HCV cirrhotic patients should be performed on a 6-months basis, as recommended by EASL, AASLD and APASL guidelines (4,5,7).

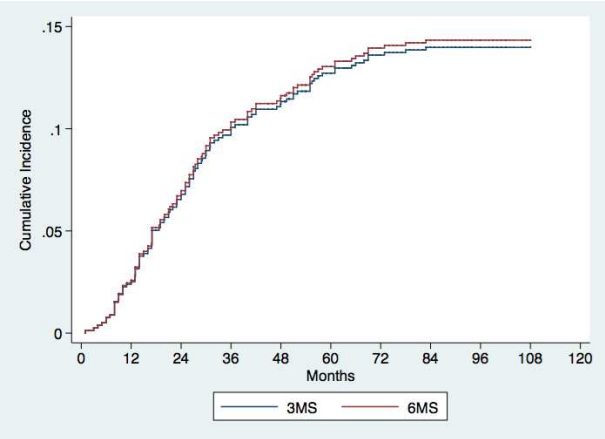
SUPPLEMENTARY MATERIAL



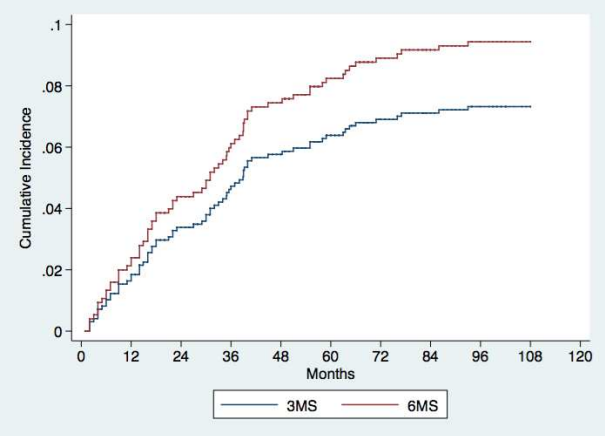
Supplementary Figure 1. Flow-chart of inclusion. (Abbreviations: ITA.LI.CA, Italian Liver Cancer; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HIV, Human Immunodeficiency Virus; 3MS, 3-months surveillance group; 6MS, 6-months surveillance group)



(**a**)



(**b**)



(**c**)

Supplementary Figure 2. Cumulative incidence plots comparing the risk of death from HCC progression (**a**), cirrhosis decompensation (**b**) and not liver-related causes (**c**) in 3MS and 6MS groups. Despite the slightly lower risk of mortality from cancer progression in 6MS group, no statistically significant difference was shown between the two groups (HR=0.75, 95% CI 0.55-1.02; p=0.07) (**a**). Similarly, no differences in the risk of death from cirrhosis decompensation (HR=1.03, 95% CI 0.59-1.78; p=0.10) (**b**) and not liver-related causes (HR=1.30, 95% CI 0.63-2.70; p=0.71) (**c**) were demonstrated between 3MS and 6MS groups. (Abbreviations: 3MS, 3-months surveillance schedule; 6MS, 6-months surveillance schedule; HR, hazard ratio).

Supplementary Table 1. Independent risk factors for the detection of HCC beyond the very early stage (i.e., solitary lesion ≤2 cm, without MVI and/or EHS).

Variable	Univariate analysis		Multivariate analysis Model 1		Multivariate analysis Model 2	
	OR (95% CI)	p	aOR (95% CI)	p	aOR (95% CI)	p
Surveillance group						
3MS	Ref	-	-	-	-	-
6MS	0.99 (0.64-1.55)	0.98				
Sex						
Female	Ref	-	-	-	-	-
Male	1.15 (0.83-1.58)	0.41				
Age (per 10-years increase)	1.01 (0.86-1.17)	0.95	-	-	-	-
Year of diagnosis	0.96 (0.86-1.07)	0.46	-	-	-	-
Etiology						
HBV	Ref	-	-	-	-	-
HCV	1.05 (0.68-1.63)	0.82				
HBV+HCV	1.47 (0.50-4.34)	0.49				
CSPH						
No	Ref	-	-	-	-	-
Yes	1.23 (0.89-1.71)	0.22				
BMI (Kg/m^2)	1.05 (1.00-1.11)	0.05	1.05 (0.99-1.11)	0.08	1.05 (0.99-1.11)	0.09
MELD score	1.08 (1.02-1.13)	0.005	1.04 (0.97-1.10)	0.29	-	-
Child-Pugh class						
A	Ref	-	-	-	Ref	-
B/C	1.61 (1.13-2.30)	0.009			1.19 (0.74-1.89)	0.48
logAFP	2.86 (2.32-3.54)	<0.0001	2.40 (1.79-3.22)	<0.0001	2.41 (1.80-3.23)	<0.0001

Note: in order to avoid collinearity between residual liver function variables, two model were fit, the first with MELD score and the second with Child-Pugh class.

Abbreviations: OR, Odds ratio; aOR, adjusted OR; CI, confidence interval; Ref, reference; 3MS, 3-months surveillance group; 6MS, 6-months surveillance group; HBV, hepatitis B virus; HCV, hepatitis C virus; CSPH, clinically significant portal hypertension; BMI, Body Mass Index; MELD, Model for End-Stage Liver Disease; logAFP, logarithm of alpha-fetoprotein.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. 2018;68:394–424.
2. McGlynn KA, Petrick JL, El-Serag HB. Epidemiology of Hepatocellular Carcinoma. Hepatology. 2020;hep.31288.
3. Kanwal F, Singal AG. Surveillance for Hepatocellular Carcinoma: Current Best Practice and Future Direction. Gastroenterology. 2019;157:54–64.
4. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.
5. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68:723–750.
6. Kokudo N, Takemura N, Hasegawa K, Takayama T, Kubo S, Shimada M, et al. Clinical practice guidelines for hepatocellular carcinoma: The Japan Society of Hepatology 2017 (4th JSH-HCC guidelines) 2019 update. Hepatol. Res. 2019;49:1109–1113.
7. Omata M, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, et al. Asia–Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. Hepatol. Int. 2017;11:317–370.
8. Yang B, Zhang B, Xu Y, Wang W, Shen Y, Zhang A, et al. Prospective study of early detection for primary liver cancer. J. Cancer Res. Clin. Oncol. 1997;123:357–360.
9. Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. J. Cancer Res. Clin. Oncol. 2004;130:417–422.
10. Trevisani F, De Notariis S, Rapaccini G, Farinati F, Benvegnù L, Zoli M, et al. Semiannual and annual surveillance of cirrhotic patients for hepatocellular carcinoma: Effects on cancer stage and patient survival (Italian experience). Am. J. Gastroenterol. 2002;97:734–744.
11. Trevisani F, Santi V, Gramenzi A, Di Nolfo MA, Del Poggio P, Benvegnu L, et al. Surveillance for early diagnosis of hepatocellular carcinoma: is it effective in intermediate/advanced cirrhosis? Am. J. Gastroenterol. 2007;102:2448–57.
12. Santi V, Trevisani F, Gramenzi A, Grignaschi A, Mirici-Cappa F, Del Poggio P, et al. Semiannual surveillance is superior to annual surveillance for the detection of early hepatocellular carcinoma and patient survival. J. Hepatol. 2010;53:291–297.
13. Thompson Coon J, Rogers G, Hewson P, Wright D, Anderson R, Jackson S, et al. Surveillance of cirrhosis for hepatocellular carcinoma: A cost-utility analysis. Br. J. Cancer. 2008;98:1166–1175.
14. Santagostino E, Colombo M, Rivi M, Rumi MG, Rocino A, Linari S, et al. A 6-month versus a 12-month surveillance for hepatocellular carcinoma in 559 hemophiliacs infected with the hepatitis C virus. Blood. 2003;102:78–82.
15. Singal A, Volk ML, Waljee A, Salgia R, Higgins P, Rogers MAM, et al. Meta-analysis: Surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment. Pharmacol. Ther. 2009;30:37–47.
16. Singal AG, Pillai A, Tiro J. Early Detection, Curative Treatment, and Survival Rates for Hepatocellular Carcinoma Surveillance in Patients with Cirrhosis: A Meta-analysis. PLoS Med. 2014;11:e1001624.
17. Trinchet JC, Chaffaut C, Bourcier V, Degos F, Henrion J, Fontaine H, et al. Ultrasonographic surveillance of hepatocellular carcinoma in cirrhosis: A randomized trial comparing 3- and 6-month periodicities. Hepatology. 2011;54:1987–1997.
18. Ioannou GN, Splan MF, Weiss NS, McDonald GB, Beretta L, Lee SP. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. 2007;5:938–45, 945.e1–4.
19. Pelizzaro F, Penzo B, Peserico G, Imondi A, Sartori A, Vitale A, et al. Monofocal hepatocellular carcinoma: How much does size matter? Liver Int. 2021;41:396–407.
20. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of

hepatocellular cancer. Hepatology. 2015;62:440–451.

21. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N. Engl. J. Med. 1996;334:693–699.

22. Vitale A, Farinati F, Pawlik TM, Frigo AC, Giannini EG, Napoli L, et al. The concept of therapeutic hierarchy for patients with hepatocellular carcinoma: A multicenter cohort study. Liver Int. 2019;39:1478–1489.

23. Schwartz M. A Biomathematical Approach to Clinical Tumor Growth. Cancer. 1961;14:1272–1294.

24. Nathani P, Gopal P, Rich N, Yopp A, Yokoo T, John B, et al. Hepatocellular carcinoma tumour volume doubling time: a systematic review and meta-analysis. Gut. 2021;70:401–407.

25. Cucchetti A, Trevisani F, Pecorelli A, Erroi V, Farinati F, Ciccarese F, et al. Estimation of lead-time bias and its impact on the outcome of surveillance for the early diagnosis of hepatocellular carcinoma. J. Hepatol. 2014;61:333–341.

26. Pelizzaro F, Vitale A, Sartori A, Vieno A, Penzo B, Russo FP, et al. Surveillance as determinant of long-term survival in non-transplanted hepatocellular carcinoma patients. Cancers (Basel). 2021;13:1–16.

27. Cucchetti A, Garuti F, Pinna AD, Trevisani F. Length time bias in surveillance for hepatocellular carcinoma and how to avoid it. Hepatol. Res. 2016;46:1275–1280.

28. Kanwal F, Kramer J, Asch SM, Chayanupatkul M, Cao Y, El-Serag HB. Risk of Hepatocellular Cancer in HCV Patients Treated With Direct-Acting Antiviral Agents. Gastroenterology. 2017;153:996-1005.e1.

29. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA Level. JAMA. 2006;295:65–73.

30. Wu CY, Lin JT, Ho HJ, Su CW, Lee TY, Wang SY, et al. Association of nucleos(T)ide analogue therapy with reduced risk of hepatocellular carcinoma in patients with chronic hepatitis B - A nationwide cohort study. Gastroenterology. 2014;147:143–151.

31. Lim YS, Han S, Heo NY, Shim JH, Lee HC, Suh DJ. Mortality, liver transplantation, and hepatocellular carcinoma among patients with chronic hepatitis B treated with entecavir vs lamivudine. Gastroenterology. 2014;147:152–161.

CHAPTER 13

Monofocal hepatocellular carcinoma: how much does size matter?

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ABSTRACT

Background & Aims: According to the Barcelona Clinic Liver Cancer (BCLC) staging system, monofocal hepatocellular carcinoma (HCC) is classified as early (BCLC A) irrespective of its size, even though controversies still exist regarding staging and treatment of large tumors. We aimed at evaluating the appropriate staging and treatment for large (>5 cm) monofocal (HCC).

Methods: From the Italian Liver Cancer database, we selected 924 patients with small early monofocal HCC (2-5 cm; SEM-HCC), 163 patients with larger tumors (>5 cm; LEM-HCC) and 1,048 intermediate stage patients (BCLC B).

Results: LEM-HCC patients had a worse overall survival (OS) than SEM-HCC (31.0 vs. 49.0 months; p<0.0001), and this was confirmed at multivariate analysis (HR 1.63, 95% CI 1.29–2.05; p<0.0001). The small difference in OS between LEM-HCC and BCLC B patients (31.0 vs. 27.0 months; p=0.03) disappeared in the multivariate model (HR 0.98, 95% CI 0.77–1.25; p=0.89). In all monofocal tumors, treatment was the strongest independent predictor of survival, with a progressively decreasing survival benefit moving from “curative” to “palliative” therapies. The survival of resected patients with LEM-HCC was significantly shorter than that of SEM-HCC (44.0 vs. 78.0 months; p=0.002), but liver resection provided the highest survival benefit in both groups compared to other treatments.

Conclusions: Monofocal HCC larger than 5 cm should not be staged as BCLC A and either a different staging system or a different subgrouping of patients (e.g., BCLC AB) should be used. Liver resection, if feasible, remains the recommended treatment for all these patients.

INTRODUCTION

Prognostic assessment in patients with hepatocellular carcinoma (HCC) is complex, being survival determined not only by tumor burden, but also by liver function and general health status (1). Over the last 30 years, several prognostic systems have been proposed for HCC in the attempt to capture the complex interrelationship between the prognostic factors (2,3,12–14,4–11). Among them, the Barcelona Clinic Liver Cancer (BCLC) system, endorsed by the European and American guidelines (1,15), is the most widely used. In its original version (4), the early stage (BCLC A) included solitary HCC <5 cm or up to 3 lesions each <3 cm; the classification of single large (>5 cm) tumors was ambiguous, as resectability, rather than tumor size, was considered to be the indicator for the allocation in the early or intermediate stage. In the 2011 update (16) and in the last version of BCLC (1), all monofocal HCCs without macrovascular invasion and/or extrahepatic spread are classified in the early stage, irrespective of the tumor diameter. Despite some proposals to classify solitary HCC larger than 5 cm in the intermediate stage (17–19), current Western guidelines recommend the allocation of these patients in the BCLC A stage (1,15), because of the higher survival when treated with liver resection (LR) compared to alternative treatments (20,21). Nevertheless, the post-resection outcome worsens with increasing tumor size: the greater the diameter, the higher the risk of early tumor recurrence (22), vascular invasion, intra-/extra-hepatic spread (23) and mortality (17). Liver transplantation (LT) is not indicated for patients with single HCC >5 cm according to Milan criteria (24). In large HCC, thermal ablation with radiofrequency is unable to achieve response rates and outcomes comparable to those observed in smaller tumors (1), and the efficacy of transarterial chemoembolization is debatable (25,26). Recently, a therapeutic hierarchy (determined by the decreasing survival benefit starting from LT, through progressively less radical treatments, to best supportive care [BSC]) has been demonstrated, irrespective of stage (27).

In this study, we performed a survival analysis aimed to evaluate the most appropriate stage allocation for large (>5 cm) monofocal HCC, the influence of tumor size on the therapeutic choices made in clinical practice and their outcomes. Considering that the 5 cm cut-off initially included in the BCLC staging system was based on Milan criteria (24), and that nowadays the "up-to-7" (28) criteria are widely used in the selection of patients for LT, we also conducted a sub-analysis taking into account the 7 cm threshold.

METHODS

Study groups

In this retrospective study, data were retrieved from the Italian Liver Cancer (ITA.LI.CA) database, a multicenter registry including 6,669 HCC patients consecutively managed at any of the 24 participating Institutions from January 1987 to March 2015. Among the patients diagnosed after January 2002 (n=4,867), we selected all the patients (n=1,087) with a monofocal HCC classifiable as early according to the latest version of the BCLC staging system (>2 cm in size, no macrovascular invasion or metastasis, preserved liver function and good general clinical conditions as assessed with Eastern Cooperative Oncology Group performance status [ECOG-PS]) (1). These patients were divided in two groups according to tumor size: the Small Early Monofocal (SEM)-HCC group (diameter ≤5 cm, n=924) and the Large Early Monofocal (LEM)-HCC group (>5 cm, n=163). For comparison, all the patients diagnosed with an intermediate stage tumor in the same time period (n=1,048) were also considered (BCLC B group). Moreover, in order to conduct the sub-analysis considering the 7 cm cut-off value, early monofocal HCC patients were subsequently regrouped as follows: HCC ≤7 cm (n=1,035; 95.2%) and tumors >7 cm (n=52; 4.8%).

The management of the ITA.LI.CA database conforms to the Italian legislation on privacy. According to Italian laws, no specific request and patient approval are needed for retrospective studies, but

patients provided written informed consent for every diagnostic and therapeutic procedure, as well as for having their data recorded anonymously in the ITA.LI.CA database. This study was conducted in accordance to the ethical guidelines of the Declaration of Helsinki and it was approved by the Institutional Review Board of the participating Institutions.

HCC diagnosis was histological in 162 (14.9%) patients with monofocal HCC and in 169 (16.1%) patients in the BCLC B group, whereas in the remaining cases it was based on the typical features at imaging (dynamic computed tomography [CT] or magnetic resonance imaging [MRI]), according to guidelines (1,15).

Standard demographic and clinicopathological data were recorded, such as age, sex, etiology of the underlying liver disease, main serological parameters (albumin, bilirubin, INR, creatinine, sodium, platelet count, alpha-fetoprotein [AFP]), Child-Pugh class, Model for End Stage Liver Disease (MELD) score, presence of ascites, clinically significant portal hypertension (CSPH), ECOG-PS, tumor radiological characteristics (location and size, number of nodules, macrovascular invasion and extra-hepatic spread) and BCLC stage. Tumor burden was evaluated with dynamic CT or MRI. CSPH diagnosis was based on unequivocal signs (presence of splenomegaly, varices, ascites) or platelet count $<100 \times 10^9$/L (29).

In total, six therapeutic subgroups were considered: LT, LR, ablation (ABL: percutaneous ethanol injection, radiofrequency and microwave ablation), intra-arterial therapies (IAT: transarterial chemoembolization, simple embolization), sorafenib (SOR) and BSC. For patients managed along their clinical history with more than one treatment modality, only the main therapy was considered, defined as the more radical according to the following hierarchy: LT, LR, ABL, IAT, SOR and BSC (27).

Statistical analysis

Categorical variables were reported as absolute and relative frequency, while quantitative data as median and interquartile range. Mann-Whitney test was used to compare quantitative data, meanwhile χ^2 test and Fischer's exact test were used for categorical variables, as appropriate.

Survivals were expressed as medians and 95% confidence interval (CI). Overall survival (OS) was calculated from the date of HCC diagnosis to the date of death from any cause, last follow-up evaluation or data censoring (December 31st, 2016). Survival curves were estimated using the Kaplan-Meier method and the difference between curves was assessed by the log-rank test.

Multivariate Cox proportional hazard models were used to identify the independent prognostic factors. Firstly, multivariate analyses were conducted in all patients with monofocal HCC, and separately in SEM-HCC and LEM-HCC, in order to identify independent predictors of survival in each group. Subsequently, another multivariate model was developed including all patients, aimed at estimating the survival differences between SEM-HCC, LEM-HCC and BCLC B groups adjusted for confoundings. In Cox regression models, continuous variables were categorized according to the following selected cut-offs: age 65 years, MELD score 9 (median value), platelet count 100 x 10^9/L and AFP 200 ng/mL. Only variables significantly or borderline ($p \leq 0.1$) associated with survival at univariate analysis were included in multivariate models.

In all the analysis, a 2-tailed p-value <0.05 was considered statistically significant. IBM SPSS Statistics (Version 25.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 8.3.1 (GraphPad Software, La Jolla, California, USA) were used for all the calculations in this study.

RESULTS

Patient characteristics

The baseline characteristics of included patients with monofocal HCC are described in Table 1. Compared to LEM-HCC, female sex (29.5% vs. 19.0%, p=0.006), viral etiology (73.8% vs. 58.9%,

p=0.0002) and CSPH (64.4% vs. 55.2%, p=0.03) were more frequent in SEM-HCC group. Moreover, SEM-HCC patients had lower platelet count (p<0.0001) and AFP levels (p<0.0001). Regarding the main treatment, LEM-HCC patients were more frequently treated with LR (41.7% vs. 25.8%) and IAT (27.0% vs. 18.7%), while ABL was less frequently adopted (14.1% vs. 44.9%).

There was a statistically significant difference among the causes of death between SEM-HCC and LEM-HCC groups (p=0.0004). At the end of the follow-up 474 SEM-HCC patients (51.8%) were dead, 212 (44.7%) from tumor progression, 106 (22.4%) from liver failure and 156 (32.9%) from sepsis, bleeding or other causes. During the follow-up, 95 LEM-HCC patients (58.3%) died, because of tumor progression (n=54, 56.8%), liver decompensation (n=29, 30.5%), and infections, bleeding or other causes (n=12, 12.7%).

Table 1. Baseline demographic and clinical characteristics of Early Monofocal HCC patients, with the comparison between SEM-HCC and LEM-HCC groups.

Variable		**Early monofocal HCC (n = 1087, 100%)**	**SEM-HCC (n = 924, 85%)**	**LEM-HCC (n = 163, 15%)**	**p†**
Males		783 (72.0)	651 (70.5)	132 (81.0)	0.006
Age (years)		70 (63-75)	70 (63-75)	71 (63-77)	0.22
Viral etiology		778 (71.6)	682 (73.8)	96 (58.9)	0.0002
CSPH		685 (63.0)	595 (64.4)	90 (55.2)	0.03
Ascites		254 (23.4)	211 (22.8)	43 (26.4)	0.32
Platelets (x 10^9/L)		126 (93-157)	126 (91-152)	130 (107-187)	<0.0001
MELD score		9 (7-11)	9 (7-11)	9 (7-11)	0.66
Child-Pugh class A		869 (79.9)	743 (80.4)	126 (77.3)	0.39
AFP (ng/mL)		24.0 (6.0-315.0)	21.0 (6.0-315.0)	68.0 (7.0-1810.0)	<0.0001
Main treatment	LT	33 (3.0)	28 (3.0)	5 (3.1)	<0.0001
	LR	306 (28.1)	238 (25.8)	68 (41.7)	
	ABL	438 (40.3)	415 (44.9)	23 (14.1)	
	IAT	217 (20.0)	173 (18.7)	44 (27.0)	
	SOR	27 (2.5)	18 (2.0)	9 (5.5)	
	BSC	66 (6.1)	52 (5.6)	14 (8.6)	

† Mann-Whitney test, χ^2 test and Fischer's exact test, as appropriate

Categorical variables are shown as absolute frequency and percentage, while continuous data are shown as median and range.

There were no statistically significant differences between SEM-HCC and LEM-HCC group in the following serological parameters: albumin, bilirubin, INR, creatinine and sodium levels (Data not shown).

Abbreviations: HCC, hepatocellular carcinoma; SEM-HCC, small early monofocal hepatocellular carcinoma; LEM-HCC, large early monofocal hepatocellular carcinoma; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; AFP, alpha-fetoprotein; INR, international normalized ratio; LT, liver transplantation; LR, liver resection; ABL, ablation; IAT, intra-arterial therapies; SOR, sorafenib; BSC, best supportive care.

Early monofocal HCC survival analysis

The median OS of all patients with solitary HCC was 47.0 months (95% CI 43.1–50.9), with a 5-year survival of 40.9%. The LEM-HCC group had a statistically significant shorter OS compared to the SEM-HCC group [31.0 months (95% CI 22.1–39.9) vs. 49.0 months (95% CI 45.2–52.8); hazard ratio (HR) 1.50 (95% CI 1.20–1.87); p<0.0001] (Figure 1). The 5-year survival rates were 33.3% and 42.2%, respectively.

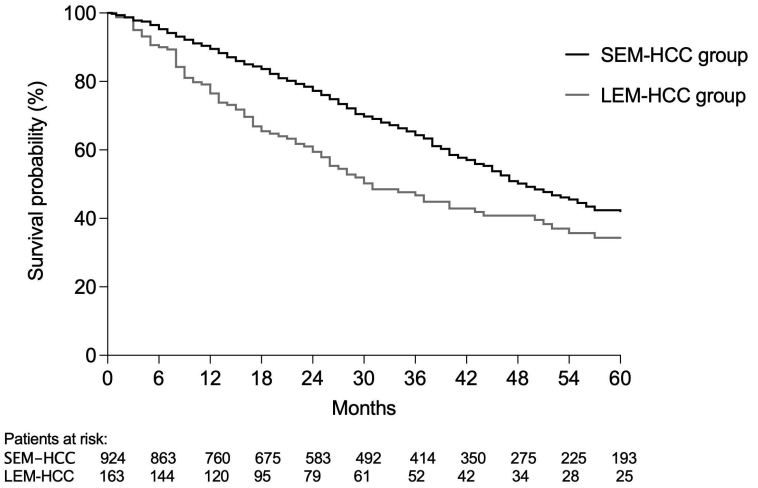

Figure 1. Kaplan-Meier survival curves of patients with a monofocal HCC subdivided according to the 5 cm diameter cut-off. Small Early Monofocal (SEM)-HCC patients had a statistically significant longer survival compared to Large Early Monofocal (LEM)-HCC patients (p<0.0001).

The main treatment strategy had a strong impact on OS with a clear hierarchical order of survival benefit. As shown in Table 2, there was a progressive decrease in survival rates moving from “curative” to “not-curative” therapies (5-year survival rates of 63.6% in LT, 55.3% in LR, 39.8% in ABL, 28.7% in IAT, 10.2% in SOR and 9.5% in BSC). This declining benefit of different treatments modalities was maintained in both SEM-HCC and LEM-HCC groups (Table 2). When SEM-HCC and LEM-HCC patients were compared according to the treatment subgroups, SEM-HCC patients had better 5-years survival rates and longer median OS compared to LEM-HCC patients in every treatment subset.

Table 2. Five-year survival rates (%) and median OS of the whole population of early monofocal HCC, SEM-HCC group and LEM-HCC group according to the main treatment.

	Early monofocal HCC			SEM-HCC group			LEM-HCC group		
	5-year survival rate (%)	**Median OS (months)**	**p**	**5-year survival rate (%)**	**Median OS (months)**	**p**	**5-year survival rate (%)**	**Median OS (months)**	**p**
LT	63.6	87.0 (NE – NE)	<0.0001	67.6	87.0 (NE – NE)	<0.0001	45.2	66.0 (16.6 – 115.4)	<0.0001
LR	55.3	72.0 (60.5 – 83.5)		59.5	78.0 (64.2 – 91.8)		37.7	44.0 (27.1 – 60.9)	
ABL	39.8	48.0 (43.6 – 52.4)		40.0	49.0 (44.3 – 53.7)		31.1	37.0 (15.0 – 59.0)	
IAT	28.7	37.0 (31.5 – 42.5)		28.1	38.0 (33.5 – 42.5)		22.6	28.0 (22.9 – 33.1)	
SOR	10.2	25.0 (12.0 – 38.0)		14.1	31.0 (21.3 – 40.7)		0.0	8.0 (5.2 – 10.8)	
BSC	9.5	13.0 (7.5 – 18.5)		8.9	15.0 (6.4 – 23.6)		0.0	8.0 (0.7 – 15.3)	

OS is presented as median and 95% confidence interval.

Abbreviations: HCC, hepatocellular carcinoma; SEM-HCC, small early monofocal hepatocellular carcinoma; LEM-HCC, large early monofocal hepatocellular carcinoma; OS, overall survival; NE, not estimable; LT, liver transplantation; LR, liver resection; ABL, ablation; IAT, intra-arterial therapies; SOR, Sorafenib; BSC, best supportive care; NE, not estimable.

Table 3. Multivariate analysis for factors independently associated with survival in the whole Early Stage Monofocal HCC population, SEM-HCC group and LEM-HCC group.

		Early Monofocal HCC		SEM-HCC		LEM-HCC	
Variable		**Adjusted HR (95% CI)**	**p**	**Adjusted HR (95% CI)**	**p**	**Adjusted HR (95% CI)**	**p**
Gender	Female	-	-	Ref	-	-	-
	Male			0.88 (0.72 – 1.08)	0.22		
Ascites	No	-	-	Ref	-	Ref	-
	Yes			0.98 (0.76 – 1.26)	0.88	2.26 (1.32 – 3.88)	0.003
Platelets ($x10^9/L$) †	> 100	Ref	-	Ref	-	-	-
	≤ 100	1.41 (1.17 – 1.70)	0.0003	1.36 (1.11– 1.67)	0.003		
MELD score	≤ 9	Ref	-	Ref	-	-	-
	> 9	1.23 (1.02 – 1.48)	0.03	1.31 (1.06 – 1.62)	0.01		
Child-Pugh class	A	Ref	-	Ref	-	-	-
	B	1.16 (0.93 – 1.45)	0.19	1.15 (0.89 – 1.50)	0.29		
Diameter (cm)	≤ 5	Ref	-	-	-	-	-
	> 5	1.63 (1.29 – 2.05)	<0.0001				
Treatment	BSC	Ref	-	Ref	-	Ref	-
	LT	0.11 (0.06 – 0.22)	< 0.0001	0.12 (0.05 – 0.25)	< 0.0001	0.12 (0.03 – 0.39)	0.001
	LR	0.20 (0.14 – 0.28)	< 0.0001	0.20 (0.14 – 0.29)	< 0.0001	0.15 (0.07 – 0.32)	< 0.0001
	ABL	0.26 (0.19 – 0.36)	< 0.0001	0.27 (0.19 – 0.39)	< 0.0001	0.18 (0.07 – 0.32)	< 0.0001
	IAT	0.33 (0.23 – 0.46)	< 0.0001	0.36 (0.24 – 0.52)	< 0.0001	0.18 (0.08 – 0.39)	< 0.0001
	SOR	0.74 (0.45 – 1.20)	0.22	0.64 (0.36 – 1.15)	0.14	0.82 (0.32 – 2.07)	0.82

† Both platelet count and CSPH were associated with survival at univariate analysis [HR 1.56 (95% CI 1.31-1.85) and HR 1.47 (95% CI 1.21-1.78), respectively], but in multivariate models only platelets were included to avoid collinearity between these two co-variates.

Abbreviations: HCC, hepatocellular carcinoma; SEM-HCC, small early monofocal hepatocellular carcinoma; LEM-HCC, large early monofocal hepatocellular carcinoma; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; BSC, best supportive care; LT, liver transplantation; LR, liver resection; ABL, ablation; IAT, intra-arterial therapies; SOR, sorafenib.

In patients with solitary HCC, CSPH, platelet count, MELD score, Child-Pugh class and treatment, in addition to tumor diameter, were associated with survival at univariate analysis. AFP did not predict patients' survival, whatever the cut-off (20, 200 or 400 ng/mL) chosen. At the Cox multivariate analysis, platelet count ≤100 x 10^9/L [adjusted HR 1.41 (95% CI 1.17–1.70); p=0.0003], MELD >9 [adjusted HR 1.23 (95% CI 1.02–1.48); p=0.03], diameter >5 cm [adjusted HR 1.63 (95% CI 1.29–2.05); p<0.0001] and treatment, with a decreasing survival benefit following the sequence LT, LR, ABL and IAT (SOR was not statistically significant superior to BSC) were identified as independent prognostic factors for patients with monofocal HCC (Table 3). A multivariate Cox model was separately developed in SEM-HCC and LEM-HCC groups, including the variables significantly associated with survival at the univariate analysis in each group. In both, the main independent prognostic factor was the treatment, with a decreasing risk of mortality compared to BSC in a hierarchical sequence (Table 3).

Comparison with the intermediate stage (BCLC B)

In the unadjusted survival analysis, compared to BCLC B patients [median OS 27.0 months (95% CI 24.6–29.4); 5-year survival rate 20.6%], an advantage was found for the SEM-HCC group [median OS 49.0 months; HR 0.53 (95% CI 0.48–0.60); p<0.0001] and, although much smaller, for the LEM-HCC group [median OS 31.0 months; HR 0.79 (95% CI 0.64–0.98); p=0.03] (Figure 2a). However, when the comparison was adjusted for the variables affecting prognosis at the univariate analysis (platelet count, MELD, Child-Pugh class, AFP levels and treatment), the better prognosis of SEM-HCC patients compared to that of BCLC B stage patients was confirmed [adjusted HR 0.63 (95% CI 0.53–0.74); p<0.0001], while the difference in survival between LEM-HCC and BCLC B disappeared [adjusted HR 0.98 (95% CI 0.77–1.25); p=0.89] (Table 4).

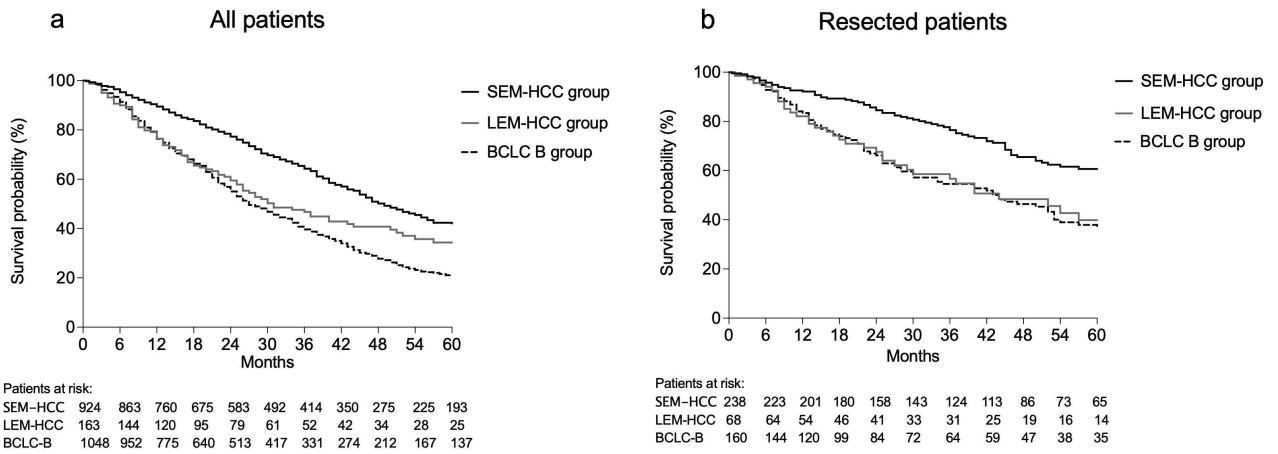

Figure 2. Kaplan-Meier survival curves for Small Early Monofocal (SEM)-HCC, Large Early Monofocal (LEM)-HCC and BCLC B patients. (**a**) SEM-HCC patients had a statistically significant longer survival compared to both LEM-HCC (p<0.0001) and BCLC B patients (p<0.0001), with a relatively small difference between the latter two (p=0.03). (**b**) Considering only the subgroups of patients treated with liver resection, SEM-HCC patients had a statistically significant longer survival compared to both LEM-HCC (p<0.0001) and BCLC B patients (p<0.0001), with no differences between the latter two (p=0.55).

Table 4. Multivariate Cox regression model for survival in the whole population of patients enrolled in the study.

Variable		**Multivariate model**		
		Adjusted HR	**95% CI**	**p**
Group	BCLC B	Ref	Ref	-
	SEM-HCC	0.63	0.53 – 0.74	< 0.0001
	LEM-HCC	0.98	0.77 – 1.25	0.89
Platelets (x 10^9/L) †	> 100	Ref	Ref	-
	≤ 100	1.26	1.08 – 1.46	0.003
MELD score	≤ 9	Ref	Ref	-
	> 9	1.25	1.07 – 1.45	0.004
Child-Pugh class	A	Ref	Ref	-
	B	1.13	0.95 – 1.35	0.18
AFP (ng/mL)	≤ 200	Ref	Ref	-
	> 200	1.20	1.03 – 1.39	0.02
Main treatment	BSC	Ref	Ref	-
	LT	0.08	0.04 – 0.14	< 0.0001
	LR	0.18	0.14 – 0.24	< 0.0001
	ABL	0.25	0.19 – 0.33	< 0.0001
	IAT	0.32	0.24 – 0.42	<0.0001
	SOR	0.56	0.38 – 0.83	0.004

† Both platelet count and CSPH were associated with survival at univariate analysis [HR 1.39 (95% CI 1.21-1.60) and HR 1.27 (95% CI 1.16-1.64), respectively], but in multivariate models only platelets were included to avoid collinearity between these two co-variates. Abbreviations: SEM-HCC, small early monofocal hepatocellular carcinoma; LEM-HCC, large early monofocal hepatocellular carcinoma; BCLC-B, Barcelona Clinic Liver Cancer stage B; CSPH, Clinically Significant Portal Hypertension; MELD, Model for End stage Liver Disease; AFP, alpha-fetoprotein; BSC, best supportive care; LT, liver transplant; LR, liver resection; ABL, ablation; IAT, intra-arterial therapies; SOR, sorafenib.

Excluding LT (the number of transplanted patients in LEM-HCC group was very small), LR was the treatment associated with the highest survival in both groups of monofocal tumors. Two hundred and thirty-eight SEM-HCC patients (25.8%), 68 LEM-HCC patients (41.7%) and 160 BCLC B patients

(15.3%) were treated with LR. The median OS of resected patients was 78.0 months (95% CI 64.2-91.8) in the SEM-HCC group, 44.0 months (95% CI 27.1–60.9) in LEM-HCC group, and 44.0 months (95% CI 31.1–56.9) in BCLC B group. According to these figures, SEM-HCC patients had a significantly longer OS than LEM-HCC [HR 0.55 (95% CI 0.38–0.80); p=0.002] and BCLC B patients [HR 0.49 (95% CI 0.37–0.65); p<0.0001], while no difference was shown between these latter two [HR 0.89 (95% CI 0.62– 1.30); p=0.55] (Figure 2b). Compared to BCLC B patients undergoing IAT (median OS 25.0 months, 95% CI 22.4-27.6), LEM-HCC managed with the same treatment had a similar survival [28.0 months (95% CI 22.9-33.1); HR 1.39 (95% CI 0.92-2.11); p=0.12], while they achieved a significantly better prognosis when treated with LR [44.0 months (95% CI 27.1-60.9); HR 0.56 (95% CI 0.40-0.77); p=0.0005].

Sub-analysis according to the 7 cm cut-off

Patients with solitary HCC >7 cm had a significantly shorter median OS compared to patients with smaller tumors [30.0 months (95% CI 8.1-51.9) vs. 47.0 months (95% CI 43.1-50.9); HR 1.48 (95% CI 1.02-2.15); p=0.04]. The 5-years survival rates were 32.8% and 41.2%, respectively. Diameter at the cut-off of 7 cm confirmed to be an independent predictor at the Cox multivariate analysis with worse survival in patients with larger monofocal tumors [adjusted HR 1.55 (95% CI 1.06-2.28); p=0.03]. The survival of patients with monofocal HCC ≤7 cm was significantly longer compared to that of BCLC B patients [HR 0.56 (95% CI 0.50-0.62); p<0.0001], while no differences were detected between these latter patients and those with solitary tumors >7 cm [HR 0.82 (95% CI 0.56-1.18); p=0.28] (Figure 3a).

The above reported therapeutic hierarchy was confirmed in patients with an HCC ≤7 cm (5-year survival rates of 63.6% in LT, 56.3% in LR, 39.8% in ABL, 28.7% in IAT, 12.1% in SOR and 10% in BSC; p<0.0001). Excluding LT (due to the relatively small sample size), LR confirmed to be the treatment with the highest survival in patients with monofocal tumors ≤7 cm (median OS 73.0 months, 95% CI

61.3-84.7). Two hundred and seventy-nine patients (27.0%) in the ≤7 cm group and 27 patients (51.9%) in the >7 cm group were treated with LR. Despite the longer median OS of the former group, a statistically significant difference was not achieved [73.0 months (95% CI 61.3-84.7) vs. 44.0 months (95% CI 8.5-79.5); HR 0.59 (95% CI 0.34-1.01); p=0.055]. Probably the limited number of patients with HCC >7 cm undergoing surgery prevented to have enough statistical power to detect a difference in survival. Compared to BCLC B resected patients [median OS 44.0 months (95% CI 31.1–56.9)], those with a tumor ≤7 cm had a significantly longer survival [HR 0.54 (95% CI 0.41-0,70); p<0.0001], while patients with larger monofocal tumors had the same prognosis [HR 0.91 (95% CI 0.53-1.57); p=0.74] (Figure 3b).

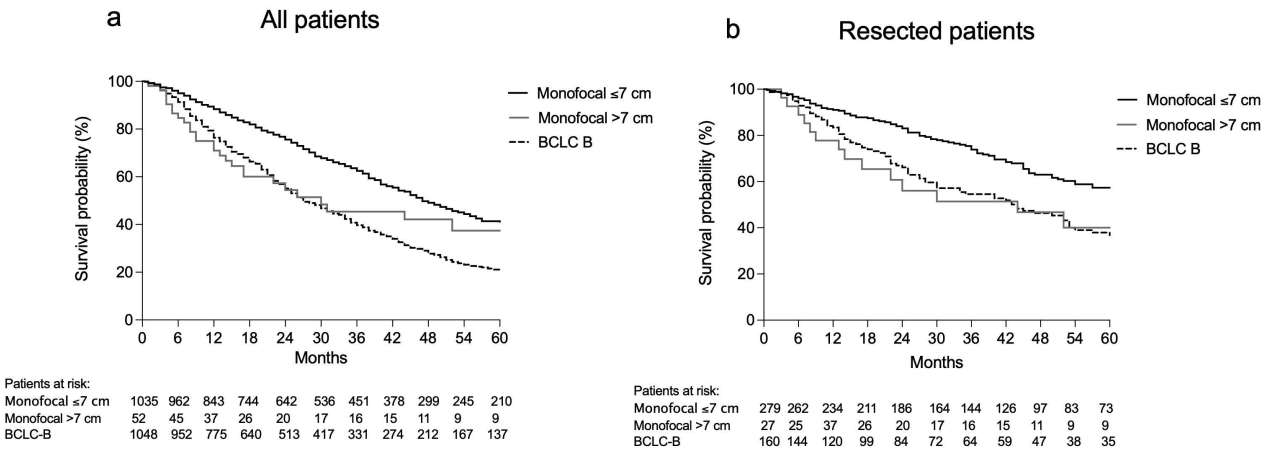

Figure 3. Kaplan-Meier survival curves for monofocal HCC ≤7 cm, monofocal HCC >7 cm and BCLC B patients. (**a**) Patients with monofocal HCC ≤7 cm had a statistically significant longer survival compared to patients with larger monofocal tumors (p=0.04) and BCLC B patients (p<0.0001); there was no difference in survival between patients with monofocal tumors >7 cm and BCLC B patients (p=0.28). (**b**) In patients treated with liver resection, the median survival of patients with a monofocal HCC ≤7 cm was longer compared to BCLC B patients (p<0.0001) and almost statistically significant longer compared to monofocal tumors >7 cm (p=0.055); no differences in prognosis were found between the latter two groups (p=0.74).

DISCUSSION

In the BCLC staging system, monofocal tumors without macrovascular invasion and extra-hepatic metastasis, along with preserved liver function and good clinical conditions, are included in the early stage irrespective of their size (1,15). Some authors, however, suggested that large (>5 cm) monofocal HCC should be staged as intermediate (BCLC B), because of the significantly worse

survival with respect to smaller tumors (17–19). Nevertheless, guidelines continue to support the classification of large tumors as BCLC A since these patients have the best survival benefit from LR, a treatment typically proposed for early tumors (20,21). However, as resected large HCCs have a worse prognosis than tumors ≤5 cm, it was proposed to designate this subgroup as BCLC AB stage (21). In line with this view, in the recently developed ITA.LI.CA tumor staging system, single tumors of 2-5 cm in size (SEM-HCC) are classified in stage A, while those >5 cm are classified as stage B1 (30).

In our study, patients with SEM-HCC had a statistically significant better prognosis than LEM-HCC patients, confirming previous results (31). Moreover, the prognostic importance of tumor diameter was definitely established by the multivariate analysis, showing that exceeding the 5 cm threshold independently predicted an increased mortality risk.

Therefore, the pertinent unmet need is to know if, from a prognostic standpoint, LEM-HCC should be allocated to BCLC B stage or to a new stage in between BCLC A and B stages. In previous papers (17–19), authors came to the conclusion that these HCCs should be classified as intermediate stage. In particular, Cho et al. (17) revealed a superior prognostic ability of the classification system when single large tumors were allocated in the BCLC B stage. Liu et al. (18) and Jung et al. (19) also concluded that the prognosis of monofocal HCC >5 cm and multifocal intermediate patients were similar. In our study, patients with LEM-HCC had a statistically significant longer survival compared to BCLC B patients at the unadjusted univariate analysis, with a 4.0 months difference in terms of median OS, but the difference disappeared after adjusting for the other variables affecting prognosis. Also, focusing the attention to resected patients, LEM-HCC and BCLC B cases had similar median OS. Therefore, LEM-HCC patients should not be grouped in the same stage of smaller solitary tumors (BCLC A), given their significantly worse survival, that is instead similar to that of BCLC B patients.

The BCLC system links stage with therapy and proposes only one treatment option for each stage or, for BCLC A, for each sub-stage (1,32). In the last version of European guidelines, the "treatment stage migration" strategy has been introduced in an attempt to attenuate the rigidity of the stage-dictated approach of this system which greatly limits the adherence to BCLC recommendations in clinical practice (1,33). Recently, it has been proposed the alternative concept of "therapeutic hierarchy", which postulates that, in each stage, the therapy with the highest survival benefit should be proposed and, when it is not feasible due to specific contraindications, alternative options should be considered in an order dictated by the declining survival benefit (27). For early stage tumors, outside the LT setting, LR is identified as the therapy with the highest survival benefit, followed by ABL, IAT and systemic therapies (27). Accordingly, in our population of early stage monofocal tumors we confirmed the highest survival rates with LT (that was however rarely adopted), followed by LR, ABL, IAT, SOR and BSC. Furthermore, treatment was the most important independent predictor of survival, with a confirmed decreasing benefit following the above reported hierarchy in the whole population and in both SEM-HCC and LEM-HCC. Excluding LT (in the LEM-HCC group only 5 patients were treated with LT, making impossible every comparison), LR was the best treatment option regardless of HCC size, although the median OS of the resected patients was remarkably influenced by this parameter. Indeed, in patients bearing small cancer, it exceeded by 34 months that of cases with large lesions. This is an expected result, as the post-surgical risk of early tumor recurrence (22), vascular invasion and intra-/extra-hepatic spread (23) is higher in large tumors. As a matter of fact, after resection, patients with large HCCs had an outcome similar to that found in those with surgically treated intermediate stage tumors, but this result does not stand against the preferential use of LR in large solitary tumors, considering its survival benefit over the other therapeutic options. Moreover, the survival of LEM-HCC compared to that of BCLC B patients undergoing IAT was significantly longer if they were treated with LR, while no differences existed if they were managed

with transarterial palliative treatments. Our data lend support to the belief that, in well selected candidates, LR is superior to non-surgical treatments, irrespective of the BCLC stage (21,27).

The sub-analysis adopting the 7 cm cut-off, despite being limited by the number of patients in the large volume group, confirmed the results obtained whit the 5 cm threshold. The prognosis of patients with large monofocal tumor (>7 cm) was significantly worse compared to patients with smaller lesions and similar to that of BCLC B. The same was true in patients treated with LR (despite a statistically significant difference in survival was not demonstrated between small and large HCC). In monofocal tumors up to 7 cm in size, we confirmed that curative therapies offer a survival advantage compared to palliative approaches, according to the established therapeutic hierarchy, and surgery remains the therapy of choice even when this threshold is considered. A direct comparison is not possible in this study, because of the overlap between the two subgroups, but in patients with tumors ≤7 cm we found an outcome after LR similar to that obtained in SEM-HCC group (median OS of 73.0 and 78.0 months, respectively). Due to the very limited number of patients in each therapy subgroup, it was not possible to compare LR with other treatment options in patients with HCC >7 cm. However, when resected these patients achieved the same median OS obtained in the LEM-HCC group (44.0 months in both cases).

The main limitation of our study is its retrospective design that makes selection and confounding biases unavoidable. Moreover, the number of patients with LEM-HCC was relatively small, probably affecting the results of sub-analyses on survival by treatment. A further limitation relies on the fact that, in patients managed with different therapies, we considered only one treatment strategy for each patient, whereas the survival is a function of all the treatments received. However, we think that these biases may have been mitigated by the fact that we considered not the first line therapy, but the main hierarchical therapy (according to the above reported hierarchy) that the patients had received in his/her history. Moreover, our results support the concept that the main treatment, as

indicated by the suggested therapeutic hierarchy, represents a prognostic corner stone for HCC patients, regardless of the therapeutic sequence adopted.

In conclusion, the prognosis of patients with monofocal HCC >5 cm is significantly worse than that of those with smaller tumors and it is similar to that of BCLC B patients. Hence, from the prognostic point of view, BCLC A should not be the designation stage for these patients. Nevertheless, as far as therapeutic allocation is concerned, LR is the recommended therapy for these tumors, considering its higher survival benefit in comparison to alternative treatments. The same is true if a higher cut-off (7 cm) is adopted. The approach proposed by the ITA.LI.CA study group, that classify this tumors as B1, could be useful in solving the dimensional issue regarding monofocal HCC, since it differentiates in the prognostic evaluation small tumors from larger lesions, thus capturing their diverse outcomes (30). Alternatively, the inclusion in the BCLC system of an additional stage (i.e., BCLC AB stage) could be considered (21).

REFERENCES

1. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.
2. Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J. Hepatol. 2001;35:421–430.
3. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005;42:1208–1236.
4. Llovet JM, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin. Liver Dis. 1999;19:329–338.
5. Yau T, Tang VYF, Yao T-J, Fan S-T, Lo C-M, Poon RTP. Development of Hong Kong Liver Cancer staging system with treatment stratification for patients with hepatocellular carcinoma. Gastroenterology. 2014;146:1691–700.e3.
6. Edge S, Byrd D, Compton C, Fritz A, Greene F, Trotti A. AJCC cancer staging manual. 7th edi- tion. Springer US; 2010.
7. Kim BH, Park J-W, Nam B-H, Kwak HW, Kim WR. Validation of a model to estimate survival in ambulatory patients with hepatocellular carcinoma: a single-centre cohort study. Liver Int. 2014;34:e317–e323.
8. Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer. 1985;56:918–928.
9. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. Hepatology. 1998;28:751–755.
10. Chevret S, Trinchet JC, Mathieu D, Rached AA, Beaugrand M, Chastang C. A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. J. Hepatol. 1999;31:133–141.
11. Leung TWT, Tang AMY, Zee B, Lau WY, Lai PBS, Leung KL, et al. Construction of the Chinese University Prognostic Index for hepatocellular carcinoma and comparison with the TNM staging system, the Okuda staging system, and the Cancer of the Liver Italian Program staging system: a study based on 926 patients. Cancer. 2002;94:1760–1769.
12. Kudo M, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). J. Gastroenterol. 2003;38:207–215.
13. Tateishi R, Yoshida H, Shiina S, Imamura H, Hasegawa K, Teratani T, et al. Proposal of a new prognostic model for hepatocellular carcinoma: an analysis of 403 patients. Gut. 2005;54:419–425.
14. Yang JD, Kim WR, Park KW, Chaiteerakij R, Kim B, Sanderson SO, et al. Model to estimate survival in ambulatory patients with hepatocellular carcinoma. Hepatology. 2012;56:614–621.
15. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68:723–750.
16. Llovet JM, Ducreux M, Lencioni R, Di Bisceglie AM, Galle PR, Dufour JF, et al. EASL-EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2012;56:908–943.
17. Cho Y, Sinn DH, Yu SJ, Gwak GY, Kim JH, Yoo YJ, et al. Survival Analysis of Single Large (>5 cm) Hepatocellular Carcinoma Patients: BCLC A versus B. PLoS One. 2016;11:e0165722–e0165722.
18. Liu P-H, Su C-W, Hsu C-Y, Hsia C-Y, Lee Y-H, Huang Y-H, et al. Solitary Large Hepatocellular Carcinoma: Staging and Treatment Strategy. PLoS One. 2016;11:e0155588–e0155588.
19. Jung YK, Jung CH, Seo YS, Kim JH, Kim TH, Yoo YJ, et al. BCLC stage B is a better designation for single large hepatocellular carcinoma than BCLC stage A. J. Gastroenterol. Hepatol. 2016;31:467–474.
20. Bruix J, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. Gut. 2014;63:844–855.

21. Vitale A, Burra P, Frigo AC, Trevisani F, Farinati F, Spolverato G, et al. Survival benefit of liver resection for patients with hepatocellular carcinoma across different Barcelona Clinic Liver Cancer stages: a multicentre study. J. Hepatol. 2015;62:617–624.

22. Bruix J, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. Gastroenterology. 2016;150:835–853.

23. Pawlik TM, Delman KA, Vauthey J-N, Nagorney DM, Ng IO-L, Ikai I, et al. Tumor size predicts vascular invasion and histologic grade: Implications for selection of surgical treatment for hepatocellular carcinoma. Liver Transpl. 2005;11:1086–1092.

24. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N. Engl. J. Med. 1996;334:693–699.

25. Hu HT, Kim JH, Lee L-S, Kim K-A, Ko G-Y, Yoon H-K, et al. Chemoembolization for hepatocellular carcinoma: multivariate analysis of predicting factors for tumor response and survival in a 362-patient cohort. J. Vasc. Interv. Radiol. 2011;22:917–923.

26. Yoon HM, Kim JH, Kim E-J, Gwon D Il, Ko G-Y, Ko HK. Modified cisplatin-based transcatheter arterial chemoembolization for large hepatocellular carcinoma: multivariate analysis of predictive factors for tumor response and survival in a 163-patient cohort. J. Vasc. Interv. Radiol. 2013;24:1639–1646.

27. Vitale A, Farinati F, Pawlik TM, Frigo AC, Giannini EG, Napoli L, et al. The concept of therapeutic hierarchy for patients with hepatocellular carcinoma: A multicenter cohort study. Liver Int. 2019;39:1478–1489.

28. Mazzaferro V, Llovet JM, Miceli R, Bhoori S, Schiavo M, Mariani L, et al. Predicting survival after liver transplantation in patients with hepatocellular carcinoma beyond the Milan criteria: a retrospective, exploratory analysis. Lancet Oncol. 2009;10:35–43.

29. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology. 2015;62:440–451.

30. Farinati F, Vitale A, Spolverato G, Pawlik TM, Huo T, Lee Y-H, et al. Development and Validation of a New Prognostic System for Patients with Hepatocellular Carcinoma. PLoS Med. 2016;13:e1002006.

31. Guo Z, Zhong J-H, Jiang J-H, Zhang J, Xiang B-D, Li L-Q. Comparison of survival of patients with BCLC stage A hepatocellular carcinoma after hepatic resection or transarterial chemoembolization: a propensity score-based analysis. Ann. Surg. Oncol. 2014;21:3069–3076.

32. Chapiro J, Geschwind J-F. Hepatocellular carcinoma: have we finally found the ultimate staging system for HCC? Nat. Rev. Gastroenterol. Hepatol. 2014;11:334–336.

33. Vitale A, Trevisani F, Farinati F, Cillo U. Treatment of hepatocellular carcinoma in the Precision Medicine era: from treatment stage migration to therapeutic hierarchy. Hepatology. 2020;72:2206–2218.

CHAPTER 14

Transarterial chemoembolization for hepatocellular carcinoma in clinical practice: temporal trends and survival outcomes of an iterative treatment

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ABSTRACT

Background. Transarterial chemoembolization (TACE) is one of the most frequently applied treatments for hepatocellular carcinoma (HCC) worldwide. In this study, we aimed at evaluating whether and how TACE application and repetition, as well as the related outcome, have changed over the last three decades in Italy.

Methods. Data of 7,184 patients with HCC were retrieved from the Italian Liver Cancer (ITA.LI.CA) database. Patients were divided according to the period of diagnosis in six cohorts: P1 (1988-1993), P2 (1994-1998), P3 (1999-2004), P4 (2005-2009), P5 (2010-2014), and P6 (2015-2019). All the analysis were repeated in the overall patient population and in Barcelona Clinic Liver Cancer (BCLC) B patients, who are the subgroup of HCC patients originally supposed to receive TACE according to guidelines. TACE was either defined as the first or the main (more effective) treatment.

Results. The proportion of patients receiving TACE as first or main therapy declined over time, and less than 50% of BCLC B patients were treated with chemoembolization from P3 onwards. Conversely, TACE was widely used even outside intermediate stage. Survival of TACE-treated patients progressively increased from P1 to P6. Although TACE was performed only once in the majority of patients, there was an increasing proportion of those receiving 2 or ≥3 treatments sessions over time. The overall survival (OS) of patients undergoing repeated treatments was significantly higher compared to those managed with a single TACE (median OS 40.0 vs. 65.0 vs. 71.8 months in 1, 2 and ≥3 TACE groups, respectively; $p<0.0001$). However, after a first-line TACE, the adoption of curative therapies provided longer survival than repeating TACE (83.0 vs. 42.0 months; $p<0.0001$), which in turn was associated with better outcomes compared to systemic therapies or best supportive care (BSC).

Conclusions. Despite a decline in the percentage of treated patients over time, TACE has still an important role in the management of HCC patients. The survival of TACE-treated patients gradually

improved over time, probably due to a better patients' selection. Iterative TACE is effective, but an up-ward shift to curative therapies provides better outcomes while transition to systemic therapies and BSC leads to a worse prognosis.

INTRODUCTION

Liver cancer ranked as the sixth most common cancer and the third leading cause of cancer-related death worldwide in 2020, with approximately 906,000 incident cases and about 830,000 deaths (1). Hepatocellular carcinoma (HCC), which represents about 90% of primary liver cancers, is a leading cause of mortality among cirrhotic patients (2,3). In most geographical areas the annual HCC mortality almost equals its incidence, confirming the high mortality rate of this tumor (5-year survival rate of 12-14% in the United States and 20% in Italy (4,5)). Despite efforts to foster surveillance programs, which could allow an earlier diagnosis and increase the percentage of patients amenable to curative treatments (6–8), HCC is frequently detected at an advanced stage, thus precluding the possibility to deliver curative treatments such as liver transplantation (LT), liver resection (LR) or ablation (ABL) (9).

According to the Barcelona Clinic Liver Cancer (BCLC) algorithm, transarterial chemoembolization (TACE) is the standard of care treatment in patients with intermediate stage HCC (9). However, it is also widely used outside the BCLC B stage and this makes TACE one of the most frequently used treatment for HCC in daily clinical practice worldwide (10,11). TACE is by definition a palliative and an iterative treatment, considered the low rates of complete response and the high risk of disease recurrence (12–14). There is no definitive evidence that scheduled TACE at regular intervals (e.g., every 2 months), irrespective of tumor response, has different effects on patient survival than on demand TACE. Nevertheless, the adoption of an aggressive schedule might lead to the development of liver failure in a high proportion of patients, most of whom are also affected by cirrhosis (15). Therefore, this approach has been substantially abandoned, following the recommendation of the guidelines to retreat with TACE only when residual viable tumor is detected at imaging, and to stop performing TACE when 2 subsequent attempts fail to obtain a significant oncologic response (9). Nevertheless, in clinical practice TACE is often repeated several times, particularly in patients with

partial response or after recurrence following an initial successful treatment. However, the benefit of retreating with TACE is uncertain, also because survival prediction in these patients is a difficult issue that only complicated recalibration (16) or time varying models (i.e., mHAP-III) (17) seem to accurately solve. This uncertainty has been increased by the growing availability of several lines of effective systemic therapy based on tyrosine kinase inhibitors, ramucirumab and immunotherapy (18–23). Indeed, systemic therapy may be a valid (and possibly better) alternative to iterative TACE. In order to support the decision to retreat patients, several algorithms, such as ART score (24,25) and ABCR score (26), have been proposed.

Although TACE is frequently used as treatment of HCC, few studies investigated whether its use has changed over time. Furthermore, little evidence is available regarding the percentage of patients retreated with TACE in real-life clinical practice, the changing trends of this percentage over time and the outcome of patients retreated with transarterial therapies compared to other therapeutic options. Considering the availability in the Italian Liver Cancer (ITA.LI.CA) database of a large series of patients managed along a period of thirty years, our study aimed to evaluate whether in real-life clinical practice the use of TACE and its outcome have changed over time, as well as the oncologic and clinical characteristics that guide the choice of this treatment. Moreover, we evaluated temporal trends in the attitude to repeat TACE and outcomes of patients managed with iterative treatment sessions.

PATIENTS AND METHODS

Study groups

In this retrospective study, data were retrieved from the ITA.LI.CA database, a multicenter registry including 7,817 HCC patients consecutively managed from January 1988 to December 2018 in 24

participating Institutions. Data are collected prospectively and updated every 2 years, and their accuracy is controlled by a data manager in the coordinating center (Bologna University).

The management of the ITA.LI.CA database conforms to the Italian legislation on privacy. According to Italian laws, specific patient's consent is not mandatory for any retrospective analysis, but patients provided written informed consent for every diagnostic and therapeutic procedure, as well as for having their clinical data anonymously recorded in the database. This study was conducted in accordance to the ethical guidelines of the Declaration of Helsinki and the study protocol was approved by the Institutional Review Board of the ITA.LI.CA coordinator center (Bologna University).

From the entire population of patients included in the database, 633 patients (8.1%) with missing data were excluded (in 153 patients informations on tumor burden or stage were missing, while treatment modality was not recorded in 480 cases), leaving 7,184 patients for the final analysis. These patients were divided in six 5-years cohorts on the basis of the year of diagnosis: P1 (1988-1993), P2 (1994-1998), P3 (1999-2004), P4 (2005-2009), P5 (2010-2014) and P6 (2015-2019). A flow chart of patient selection is provided in Supplementary Figure 1.

HCC diagnosis was histologically confirmed in 2,371 patients (33%), whereas in the remaining cases it was based on the radiological criteria (at computed tomography [CT] or magnetic resonance imaging [MRI]), according to guidelines available at the time of diagnosis (9,27).

In the ITA.LI.CA database, demographic and clinicopathological data, such as age, sex, comorbidities, etiology of the underlying liver disease, main serological parameters (albumin, bilirubin, international normalized ratio [INR], creatinine, platelet count, alpha-fetoprotein [AFP]), Child-Pugh class, Model for End Stage Liver Disease (MELD) score, presence of ascites and hepatic encephalopathy, clinically significant portal hypertension (CSPH), and Eastern Cooperative Oncology Group performance status (ECOG-PS), are recorded. CSPH diagnosis was based either on unequivocal signs (presence of splenomegaly, varices, ascites) or platelet count $<100 \times 10^9$/L (28).

The database also reports main macroscopic tumor characteristics (location and size, number of nodules, macrovascular invasion [MVI] and extra-hepatic spread [EHS]) evaluated with dynamic CT or MRI. In this study, also in order to evaluated the adherence to its therapeutic recommendation, for staging purposes we used the BCLC staging system (9).

The complete sequence of treatments for every patient is also registered in the ITA.LI.CA database. The following treatment groups were considered in the present study: liver transplantation (LT), liver resection (LR), ablative procedures (ABL: percutaneous ethanol injection, percutaneous or laparoscopic thermal ablation), TACE, trans-arterial embolization (TAE), selective internal radiation therapy (SIRT), systemic therapy with sorafenib or other tyrosine kinase inhibitors (SOR), best supportive care (BSC) and other treatments. In all the analysis, we evaluated the first therapeutic choice and the main (i.e., more effective) treatment according to the following hierarchy: LT, LR, ABL, TACE, TAE and SIRT, SOR, and BSC (29). The ITA.LI.CA database reports the treatment modality at each recurrence. In the present study, when different rounds of TACE were necessary to achieve a complete treatment (e.g., treatment of lesions in the left lobe and subsequent treatment of nodules in the right lobe), TACE was considered as a single procedure. On the contrary, when repeated at tumor recurrence, TACEs were considered as separate treatments. Regarding technical details, in the ITA.LI.CA database, chemotherapeutic drugs administered as well as the type of TACE (conventional vs. drug-eluting beads) are rarely registered and were not considered in this study. Response to TACE was evaluated using the modified Response Evaluation Criteria in Solid Tumors (mRECIST) and was categorized in complete response (CR), partial response (PR), stable disease (SD) and progressive disease (SD) (30).

Statistical analysis

Categorical variables were reported as absolute and relative frequency (percentages), while quantitative variables as median and interquartile range (IQR). Mann-Whitney test was used to

compare quantitative variables, meanwhile χ^2 test and Fischer's exact test were used in the comparison of categorical variables as appropriate.

In order to evaluate predictors of TACE treatment compared to potentially radical (LT, LR and ABL) and palliative (SOR and BSC) treatments, a multinomial logistic regression was performed. Variables significantly or borderline ($p \leq 0.1$) associated with treatment category at univariate analysis were included in multivariate models. The multinomial logistic regression analysis was used to establish the variables predicting TACE as first and main treatment in the overall population of patients and in the subgroup of BCLC B patients.

Overall survival (OS), expressed as median and 95% confidence interval (CI), was calculated from diagnosis to death from any cause or last follow-up. For patients alive at the end of the study, survival was censored at December 31st 2018. Survival curves were calculated with the Kaplan-Meier method and compared with the log-rank test. The independent predictors of survival were identified by the multivariate Cox regression analysis, including in the analysis the variables associated with survival ($p \leq 0.1$) at the univariate analysis.

In all the analyses a two-tails p value <0.05 was considered as significant. Data were analyzed by IBM SPSS Statistics (version 25.0. Armonk, NY: IBM Corp) and GraphPad Prism version 8.3.1 (GraphPad Software, La Jolla, California, USA).

RESULTS

TACE treatment in the whole population

Baseline demographic and clinical characteristics of patients in the six time periods are described in Table 1. Compared to P1 patients, those diagnosed in more recent periods were slightly older, more frequently diagnosed under surveillance and had less frequently a viral etiology and cirrhosis. More than half of patients in all time periods had CRPH, with slightly lower percentages in P5 and P6. Liver

function and AFP levels at diagnosis were similar among subgroups (except for a slightly lower MELD score in P5 and P6, and a lower median AFP level in P6). While the majority of patients presented with a single liver lesion at diagnosis in each time period, tumor size was significantly smaller in P2-P6 as compared to P1. As far as tumor stage at diagnosis is concerned, BCLC B patients progressively decreased, while the proportion of BCLC C patients increased over time.

The choice of prescribing TACE as the first therapeutic approach decreased across P2 and P3, remaining thereafter substantially stable. Namely, 45.7% of patients in P1, 45.9% in P2, 28.3% in P3, 28.9% in P4, 29.9% in P5 and 28.5% in P6 underwent TACE as first treatment (Table 1, Figure 1A). A very similar trend was demonstrated from for TACE used as the main treatment (45.7%, 44.6%, 25.3%, 24.0%, 23.7% and 22.9%, respectively) (Table 1, Figure 1B). In parallel to the decrease in TACE use, there was an increase of ABL and systemic therapies as both first and main treatment. The rate of LT and LR remained approximately stable across the six time periods considered.

Table 1. Baseline characteristics of the overall population of patients divided according to the period of diagnosis.

Variable	P1 (1988-1993) n=256	P2 (1994-1998) n=370	P3 (1999-2004) n=867	P4 (2005-2009) n=1323	P5 (2010-2014) n=2515	P6 (2015-2019) n=1853
Sex – males	197 (77.0)	279 (75.4)	657 (75.8)	1003 (75.8)	1932 (76.8)	1464 (79.0)
Age (years)	64 (58-68)	64 (57-70)	67 (61-74) ‡	68 (60-74) ‡	69 (60-75) ‡	69 (60-76) ‡
Surveillance	126 (49.2)	209 (56.5)	508 (58.6) #	831 (62.8) ‡	1637 (65.1) ‡	1073 (57.9) *
Etiology						
Viral	150 (58.6)	288 (77.8) ‡	613 (70.7) †	832 (62.9)	1443 (57.4)	941 (50.8) *
Not viral	54 (21.1)	45 (12.2) #	188 (21.7)	372 (28.1) *	815 (32.4) †	712 (38.4) ‡
Viral + other	52 (20.3)	37 (10.0) †	66 (7.6) ‡	119 (9.0) ‡	257 (10.2) ‡	200 (10.8) ‡
Liver disease						
Healthy liver	0 (0)	4 (1.1)	8 (0.9)	13 (1.0)	40 (1.6) *	41 (2.2) #
NAFLD	0 (0)	0 (0)	3 (0.3)	12 (0.9)	68 (2.7) #	50 (2.7) #
Fibrosis	6 (2.3)	7 (1.9)	49 (5.7) *	48 (3.6)	148 (5.9) *	139 (7.5) #
Cirrhosis	250 (97.7)	359 (97.0)	807 (93.1) #	1250 (94.5) *	2259 (89.8) ‡	1623 (87.6) ‡
ECOG-PS						
0	194 (75.8)	216 (58.4) ‡	698 (80.5)	923 (69.8)	1740 (69.2) *	1359 (73.3)
1-2	54 (21.1)	154 (41.6) ‡	166 (19.1) ‡	344 (26.0) ‡	672 (26.7) ‡	450 (24.3) ‡
3-4	8 (3.1)	0 (0) †	3 (0.3) †	56 (4.2)	103 (4.1)	44 (2.4)
CSPH	176 (68.7)	266 (71.9)	567 (65.4)	844 (63.8)	1514 (60.2) #	1128 (60.9) *
Child-Pugh						
A	170 (66.4)	234 (63.2)	552 (63.7)	889 (67.2)	1655 (65.8)	1305 (70.4)
B	75 (29.3)	105 (28.4)	256 (29.5)	340 (25.7)	757 (30.1)	465 (25.1)
C	11 (4.3)	31 (8.4)	59 (6.8)	94 (7.1)	103 (4.1)	83 (4.5)
MELD	10 (8-13)	10 (9-13)	10 (8-13)	10 (8-12)	10 (8-12) #	9 (8-11) †
AFP (ng/mL)	30.5 (9.0-201.5)	34.0 (9.0-172.8)	23.0 (7.0-210.0)	31.0 (6.0-330.0)	40.0 (5.0-567.0)	12.5 (4.0-239.3) ‡
Tumor morphology						
Monofocal	120 (46.9)	182 (49.2)	432 (49.8)	633 (47.8)	1267 (50.4)	990 (53.4)
Multifocal	112 (43.8)	169 (45.7)	375 (43.3)	587 (44.4)	1044 (41.5)	743 (40.1)
Infiltrative	15 (5.8)	15 (4.1)	33 (3.8)	69 (5.2)	141 (5.6)	61 (3.3) *
Massive	9 (3.5)	4 (1.1) *	27 (3.1)	34 (2.6)	63 (2.5)	59 (3.2)
Number	1 (1-4)	1 (1-4)	1 (1-3)	1 (1-3)	1 (1-2)	1 (1-2)
Diameter (cm)	3.5 (2.4-5.1)	3.0 (2.2-4.0) *	3.0 (2.2-4.5) *	3.0 (2.0-4.5) #	3.0 (2.0-5.0) *	3.0 (2.0-4.8) †
MVI	27 (10.5)	31 (8.4)	110 (12.7)	158 (11.9)	284 (11.3)	206 (11.1)
EHS	0 (0)	2 (0.5)	68 (7.8) ‡	139 (10.5) ‡	257 (10.2) ‡	189 (10.2) ‡
BCLC stage						
0	25 (9.8)	29 (7.8)	68 (7.8)	126 (9.5)	261 (10.4)	261 (14.1)
A	107 (41.8)	175 (47.3)	339 (39.1)	459 (34.7) *	934 (37.1)	685 (37.0)
B	78 (30.5)	101 (27.3)	216 (24.9)	235 (17.8) ‡	376 (15.0) ‡	264 (14.2) ‡
C	34 (13.3)	38 (10.3)	217 (25.0) ‡	439 (33.2) ‡	856 (34.0) ‡	594 (32.1) ‡
D	12 (4.7)	27 (7.3)	27 (3.1)	64 (4.8)	88 (3.5)	49 (2.6)
First treatment						

LT	5 (2.0)	16 (4.4)	28 (3.2)	34 (2.6)	49 (2.0)	33 (1.8)
LR	38 (14.8)	40 (10.8)	125 (14.4)	202 (15.3)	418 (16.6)	280 (15.1)
ABL	62 (24.3)	91 (24.6)	306 (35.3) †	430 (32.5) #	787 (31.3) *	608 (32.8) #
TACE	117 (45.7)	170 (45.9)	245 (28.3) ‡	383 (28.9) ‡	752 (29.9) ‡	528 (28.5) ‡
TAE/SIRT	0 (0)	0 (0)	1 (0.1)	3 (0.2)	21 (0.8)	75 (4.0) ‡
SOR	0 (0)	0 (0)	0 (0)	53 (4.0) †	229 (9.1) ‡	178 (9.6) ‡
BSC	6 (2.3)	7 (1.9)	78 (9.0) †	146 (11.0) ‡	218 (8.7) ‡	116 (6.3) #
Other	28 (10.9)	46 (12.4)	84 (9.7)	72 (5.5) #	41 (1.6) ‡	35 (1.9) ‡
Main treatment						
LT	5 (2.0)	22 (6.0) *	43 (5.0) *	83 (6.2) #	121 (4.8) *	86 (4.6) *
LR	38 (14.8)	40 (10.8)	127 (14.7)	214 (16.2)	432 (17.2)	306 (16.5)
ABL	62 (24.2)	90 (24.3)	315 (36.3) †	436 (33.0) *	862 (34.3) #	637 (34.4) #
TACE	117 (45.7)	165 (44.6)	219 (25.3) ‡	317 (24.0) ‡	597 (23.7) #	424 (22.9) ‡
TAE/SIRT	0 (0)	0 (0)	1 (0.1)	2 (0.2)	18 (0.7)	72 (3.9) #
SOR	0 (0)	0 (0)	0 (0)	53 (4.0) †	226 (9.0) ‡	176 (9.5) ‡
BSC	6 (2.4)	7 (1.9)	78 (9.0) †	146 (11.0) ‡	218 (8.7) ‡	116 (6.3) #
Other	28 (10.9)	46 (12.4)	84 (9.7)	72 (5.4) #	41 (1.6) ‡	36 (1.9) ‡

Continuous variables are reported as median and interquartile range (IQR), while categorical variables as absolute and relative frequencies.
The first cohort (1988-1993) is taken as reference in the comparison with other time periods.
* p<0.05 and ≥0.01
# p<0.01 and ≥0.001
† p<0.001 and ≥0.0001
‡ p<0.0001
Abbreviations: NAFLD, non-alcoholic fatty liver disease; ECOG-PS, Eastern Cooperative Oncology Group performance status; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; AFP, alpha-fetoprotein; MVI, macrovascular invasion; EHS, extrahepatic spread; BCLC, Barcelona Clinic Liver Cancer; LT, liver transplantation; LR, liver resection; ABL, ablation; TACE, transarterial chemoembolization; TAE, transarterial embolization; SIRT, selective internal radiation therapy; SOR, systemic therapy; BSC, best supportive care.

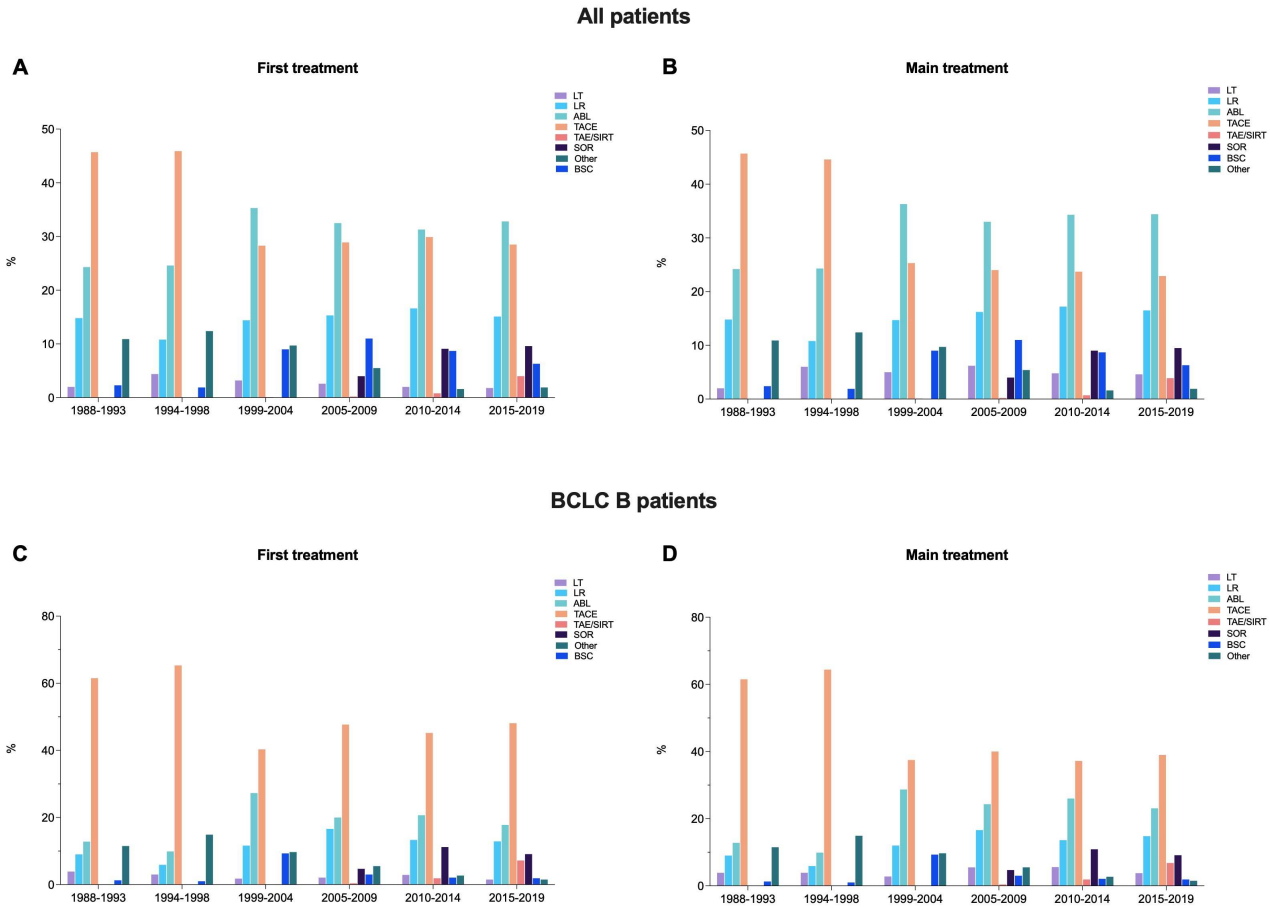


Figure 1. Distribution of the first and main treatment adopted in the overall population of patients (**A** and **B**) and in BCLC B patients (**C** and **D**) in the six time periods considered.

TACE treatment in BCLC B patients

Of the entire population of patients included in the study, 1,270 (17.7%) were classified as BCLC B at the time of diagnosis. Baseline demographic and clinical characteristics of these patients in the six time periods considered are shown in Table 2. As in the whole population, patients diagnosed in recent time cohorts were slightly older and more frequently diagnosed with HCC under surveillance. Non-viral etiologies increased over time. No statistically significant differences in the percentage of patients with CRPH was demonstrated between different groups. A better residual liver function (as evaluated with Child-Pugh score and MELD) was documented in patients more recently diagnosed. As far as tumor burden is considered, the number of liver lesions was significantly lower in the more recent cohorts while the size of the largest nodule remained stable across the different calendar periods.

As in the whole population, even in BCLC B patients there was a decrease in the use of TACE as the first therapeutic approach between P2 and P3. In fact, 61.5% of patients in P1 and 65.3% in P2 were treated with chemoembolization, while these figures were 40.3% in P3, 47.7% in P4, 45.2% in P5 and 48.1% in P6 (Table 2, Figure 1C). Despite TACE being the standard of care according to BCLC guidelines, patients with intermediate stage HCC diagnosed in more recent temporal cohorts underwent TACE as main treatment only in about one third of cases. Indeed, TACE was used as main treatment in 61.5% of P1, 64.4% of P2, 37.5% of P3, 40.0% of P4, 37.2% of P5 and 39.0% of P6 patients (Table 2, Figure 1D). Notably, recently diagnosed BCLC B patients more frequently underwent to curative treatments (LR and ABL) as main therapies.

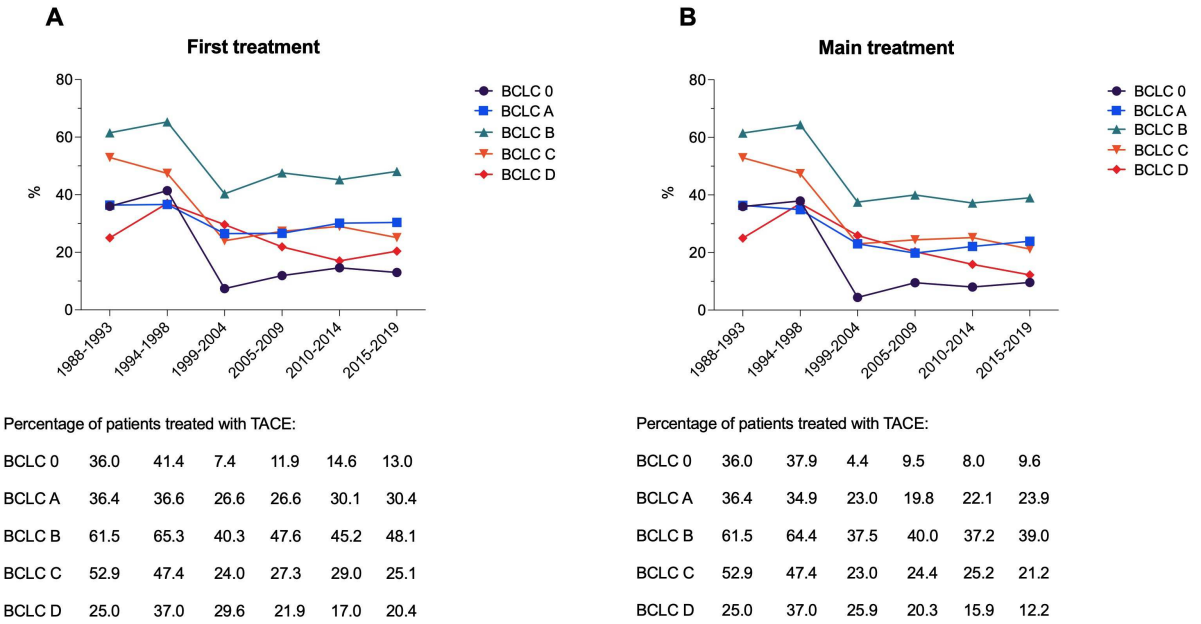
Beyond BCLC B patients, TACE was also widely used across all the other HCC stages (Figure 2). A substantial subgroup of BCLC 0 and A patients underwent TACE, as both first and main treatment, but even in these cases the use of such treatment dropped over time (from 36.0% in P1 to 9.6% in P6 as main treatment in BCLC 0; from 36.4% in P1 to 23.9% in P6 as main treatment in BCLC A). More than half of BCLC C patients were treated with TACE in P1 (52.9%), while this treatment was used in a lower proportion of patients both as first or main choice (25.1% and 21.2%, respectively) in P6.

Table 2. Baseline characteristics of the BCLC B patients divided according to the period of diagnosis.

Variable	P1 (1988-1993) n=78	P2 (1994-1998) n=101	P3 (1999-2004) n=216	P4 (2005-2009) n=235	P5 (2010-2014) n=376	P6 (2015-2019) n=264
Sex – males	68 (87.2)	76 (75.2)	164 (75.9) *	200 (85.1)	321 (85.4)	231 (87.5)
Age (years)	63 (58-68)	63 (57-70)	67 (60-73) #	66 (59-72) #	67 (59-74) †	68 (59-76) †
Surveillance	34 (43.6)	54 (53.5)	111 (51.4)	140 (59.6) *	221 (58.8) *	124 (47.0)
Etiology						
Viral	44 (56.5)	77 (76.2) #	151 (69.9) *	142 (60.4)	206 (54.8)	113 (42.8) *
Not viral	14 (17.9)	15 (14.9)	44 (20.4)	70 (29.8)	136 (36.2) #	111 (42.0) ‡
Viral + other	20 (25.6)	9 (8.9) #	21 (9.7) #	23 (9.8) †	34 (9.0) †	40 (15.2) *
Liver disease						
Healthy liver	0 (0)	3 (3.0)	1 (0.1)	1 (0.4)	13 (3.5)	3 (1.1)
NAFLD	0 (0)	0 (0)	2 (0.9)	3 (1.3)	9 (2.4)	11 (4.2)
Fibrosis	3 (3.8)	1 (1.0)	13 (6.0)	8 (3.4)	25 (6.6)	21 (8.0)
Cirrhosis	75 (96.2)	97 (96.0)	200 (92.6)	223 (94.9)	329 (87.5) *	229 (86.7) *
CSPH	51 (65.4)	72 (71.3)	130 (60.2)	131 (55.7)	202 (53.7)	158 (59.8)
Child-Pugh						
A	47 (60.3)	70 (69.3)	144 (66.7)	181 (77.0) #	283 (75.3) *	201 (76.1) #
B	31 (39.7)	31 (30.7)	72 (33.3)	54 (33.0)	93 (24.7)	63 (23.9)
MELD	11 (9-13)	11 (8-13)	10 (9-12)	10 (8-11) *	10 (8-11) †	9 (8-11) †
AFP (ng/mL)	40.0 (13.0-417.0)	30.5 (8.8-272.0)	50.0 (10.5-654.5)	39.5 (8.0-892.5)	92.0 (12.0-1158.0)	47.5 (7.0-1019.0)
Morphology						
2-3 lesions	2 (2.5)	4 (4.0)	35 (16.2) #	94 (40.0) ‡	224 (59.6) ‡	166 (62.9) ‡
>3 lesions	63 (80.8)	88 (87.1)	158 (73.1)	102 (43.4) ‡	111 (29.5) ‡	77 (29.2) ‡
Infiltrative/massive	13 (16.7)	9 (8.9)	23 (10.6)	39 (16.6)	41 (10.9)	21 (7.9) *
Number	4 (4-4)	4 (4-4)	4 (4-4) *	4 (2-4) ‡	3 (2-4) ‡	3 (2-4) ‡
Diameter (cm)	4.5 (3.5-6.7)	4.0 (2.9-5.0) *	4.0 (3.2-5.9)	4.0 (3.5-5.5)	4.0 (3.6-5.5)	4.0 (3.5-5.8)
First treatment						
LT	3 (3.9)	3 (3.0)	4 (1.8)	5 (2.1)	11 (2.9)	4 (1.5)
LR	7 (9.0)	6 (5.9)	25 (11.6)	39 (16.6)	50 (13.3)	34 (12.9)
ABL	10 (12.8)	10 (9.9)	59 (27.3) *	47 (20.0)	78 (20.7)	47 (17.8)
TACE	48 (61.5)	66 (65.3)	87 (40.3) #	112 (47.7) *	170 (45.2) #	127 (48.1) *
TAE/SIRT	0 (0)	0 (0)	0 (0)	1 (0.4)	7 (1.9)	19 (7.2) #
SOR	0 (0)	0 (0)	0 (0)	11 (4.7)	42 (11.2) †	24 (9.1) #
BSC	1 (1.3)	1 (1.0)	20 (9.3) *	7 (3.0)	8 (2.1)	5 (1.9)
Other	9 (11.5)	15 (14.9)	21 (9.7)	13 (5.5)	10 (2.7) #	4 (1.5) †
Main treatment						
LT	3 (3.9)	4 (3.9)	6 (2.8)	13 (5.5)	21 (5.6)	10 (3.8)
LR	7 (9.0)	6 (5.9)	26 (12.0)	39 (16.6)	51 (13.6)	39 (14.8)
ABL	10 (12.8)	10 (9.9)	62 (28.7) #	57 (24.3) *	98 (26.0) *	61 (23.1)
TACE	48 (61.5)	65 (64.4)	81 (37.5) †	94 (40.0) #	140 (37.2) †	103 (39.0) †
TAE/SIRT	0 (0)	0 (0)	0 (0)	1 (0.4)	7 (1.9)	18 (6.8) *

SOR	0 (0)	0 (0)	0 (0)	11 (4.7)	41 (10.9) †	24 (9.1) #
BSC	1 (1.3)	1 (1.0)	20 (9.3) *	7 (3.0)	8 (2.1)	5 (1.9)
Other	9 (11.5)	15 (14.9)	21 (9.7)	13 (5.5)	10 (2.7) #	4 (1.5) †

Continuous variables are reported as median and interquartile range (IQR), while categorical variables as absolute and relative frequencies.
The first cohort (1988-1993) is taken as reference in the comparison with other time periods.
* p<0.05 and ≥0.01
# p<0.01 and ≥0.001
† p<0.001 and ≥0.0001
‡ p<0.0001
Abbreviations: NAFLD, non-alcoholic fatty liver disease; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; AFP, alpha-fetoprotein; LT, liver transplantation; LR, liver resection; ABL, ablation; TACE, transarterial chemoembolization; TAE, transarterial embolization; SIRT, selective internal radiation therapy; SOR, systemic therapy; BSC, best supportive care.



Percentage of patients treated with TACE:

BCLC 0	36.0	41.4	7.4	11.9	14.6	13.0
BCLC A	36.4	36.6	26.6	26.6	30.1	30.4
BCLC B	61.5	65.3	40.3	47.6	45.2	48.1
BCLC C	52.9	47.4	24.0	27.3	29.0	25.1
BCLC D	25.0	37.0	29.6	21.9	17.0	20.4

Percentage of patients treated with TACE:

BCLC 0	36.0	37.9	4.4	9.5	8.0	9.6
BCLC A	36.4	34.9	23.0	19.8	22.1	23.9
BCLC B	61.5	64.4	37.5	40.0	37.2	39.0
BCLC C	52.9	47.4	23.0	24.4	25.2	21.2
BCLC D	25.0	37.0	25.9	20.3	15.9	12.2

Figure 2. Proportion of patients treated with TACE as first (**A**) and main (**B**) treatment in the six time periods considered, according to the BCLC stage.

Predictive factors of treatment with TACE

The multinomial logistic regression (Table 3) showed that, compared to potentially curative options (LT, LR, ABL), TACE was selected preferentially in older patients (aOR=0.88 per 10-year increase, 95% CI 0.82-0.94), in those with non-viral etiology (adjusted odds ratio [aOR]=0.82, 95% CI 0.69-0.97), with deteriorated clinical conditions (ECOG-PS ≥1), with CSPH (aOR=0.51, 95% CI 0.43-0.60) and with poor residual liver function (aOR=0.96, 95% CI 0.94-0.99, for MELD score). Moreover, patients with high tumor burden (number and size of liver lesions, and AFP levels) were less likely to receive LT/LR/ABL as the first therapeutic option. The same variables, with the addition of EHS (aOR=0.54, 95% CI 0.36-0.83), were also negatively associated with LT/LR/ABL compared to TACE as main treatment. By contrast, patients with deteriorated clinical conditions (ECOG-PS ≥1), poor liver function, and high tumor burden (number and size of liver tumors, presence of MVI and EHS) were more likely to receive systemic or palliative treatment as compared to TACE, as both first and main therapy. Diagnosis under regular surveillance was significantly associated with higher odds to receive TACE rather than SOR or BSC.

The role of residual liver function in the choice of treatment requires further clarification. Compared to TACE, while poor residual liver function was negatively associated with LT, LR and ABL considered together, this was not the case of patients treated specifically with transplantation. Indeed, higher MELD was a negative predictor of treatment with TACE when compared to LT: with the increase of MELD score, decreased the probability of being treated with TACE as first (aOR=0.92, 95% CI 0.87-0.97; p=0.003) and main treatment (aOR=0.95, 95% CI 0.91-0.99; p=0.03). Poor residual liver function favors LT compared to TACE, but at the same time it might contraindicate LR (particularly when large resections are needed). Therefore, considered the low number of patients managed with LT, it is not surprising that grouping together all curative treatments, the detrimental effect of

poor residual liver function on the possibility to treat patients with LR prevailed, and we found that higher MELD was associated with greater probability to receive TACE.

In BCLC B patients, negative independent predictors of potentially curative therapies as first treatment compared to TACE were older age (aOR=0.78 per 10-year increase, 95% CI 0.65-0.93), presence of CRPH (aOR=0.44, 95% CI 0.30-0.66) and higher number of liver lesions (aOR=0.87, 95% CI 0.76-0.99) (Supplementary Table 1). As far as the main treatment is concerned, in addition to these variables (age, residual liver function, number of liver nodules), also MELD score (aOR=0.91, 95% CI 0.85-0.98), size of liver lesions (aOR=0.91, 95% CI 0.83-0.99) and the period of diagnosis were associated with the probability to receive LT/LR/ABL rather than TACE. Compared to patients diagnosed in P1, those diagnosed from P3 to P6 were more likely to receive potentially curative treatments. Only MELD score (aOR=1.10, 95% CI 1.01-1.20) and tumor size (aOR=1.13, 95% CI 1.03-1.23) were independently associated with higher odds of receiving SOR or BSC as first treatment instead of TACE in BCLC B patients. In this subpopulation, tumor diameter was also the only predictive variable independently associated with increased probability of being treated with SOR or BSC as main treatment (aOR=1.10, 95% CI 1.01-1.21).

Table 3. Multinomial logistic regression showing independent factors associated with probability of receive TACE compared to potentially curative treatment (LT, LR and ABL) and palliative therapies (SOR and BSC).

Variables		Curative treatment (LT, LR and ABL)		Palliative treatment (SOR and BSC)		Curative treatment (LT, LR and ABL)		Palliative treatment (SOR and BSC)	
		First treatment				Main treatment			
		aOR (95% CI)	p	aOR (95% CI)	p	aOR (95% CI)	p	aOR (95% CI)	p
Sex	Females	Ref	-	Ref	-	Ref	-	Ref	-
	Males	0.95 (0.79-1.13)	0.54	0.81 (0.61-1.09)	0.15	1.00 (0.83-1.21)	0.98	0.82 (0.61-1.11)	0.19
Age (per 10-year increase)		0.88 (0.82-0.94)	0.0001	1.08 (0.97-1.21)	0.16	0.81 (0.75-0.87)	<0.0001	1.04 (0.92-1.16)	0.55
Period of diagnosis	P1	Ref	-	Ref	-	Ref	-	Ref	-
	P2	0.11 (0.01-0.88)	0.04	0.57 (0.03-10.75)	0.71	0.20 (0.03-1.37)	0.10	0.62 (0.03-114)	0.75
	P3	0.68 (0.20-2.35)	0.54	2.73 (0.42-17.98)	0.30	0.80 (0.23-2.79)	0.73	2.89 (0.45-18.75)	0.27
	P4	0.61 (0.18-2.08)	0.43	1.96 (0.30-12.69)	0.48	0.81 (0.23-2.79)	0.74	2.21 (0.35-14.13)	0.40
	P5	0.51 (0.15-1.75)	0.29	1.41 (0.22-9.12)	0.72	0.74 (0.22-2.53)	0.63	1.61 (0.25-10.20)	0.62
	P6	0.49 (0.14-1.67)	0.26	1.16 (0.18-7.49)	0.88	0.66 (0.19-2.26)	0.51	1.27 (0.20-8.14)	0.80
Etiology	Viral	Ref	-	Ref	-	Ref	-	Ref	-
	Not viral	0.82 (0.69-0.97)	0.02	1.02 (0.78-1.33)	0.90	0.82 (0.69-0.98)	0.03	1.01 (0.77-1.33)	0.93
	Viral+other	0.90 (0.69-1.17)	0.42	1.31 (0.89-1.94)	0.18	0.87 (0.66-1.14)	0.30	1.31 (0.88-1.96)	0.19
Surveillance	No	Ref	-	Ref	-	Ref	-	Ref	-
	Yes	1.08 (0.91-1.27)	0.38	0.62 (0.48-0.79)	0.0001	1.05 (0.88-1.25)	0.57	0.62 (0.48-0.80)	0.0002
ECOG-PS	0	Ref	-	Ref	-	Ref	-	Ref	-
	1-2	0.65 (0.54-0.78)	<0.0001	2.54 (1.99-3.24)	<0.0001	0.63 (0.53-0.76)	<0.0001	2.46 (1.92-3.16)	<0.0001
	3-4	0.39 (0.17-0.87)	0.02	11.85 (6.25-22.46)	<0.0001	0.35 (0.15-0.77)	0.01	10.71 (5.59-20.55)	<0.0001
CSPH	No	Ref	-	Ref	-	Ref	-	Ref	-
	Yes	0.51 (0.43-0.60)	<0.0001	1.05 (0.80-1.37)	0.75	0.60 (0.51-0.71)	<0.0001	1.11 (0.84-1.46)	0.48
MELD		0.96 (0.94-0.99)	0.001	1.09 (1.06-1.12)	<0.0001	0.96 (0.94-0.98)	0.0002	1.08 (1.05-1.11)	<0.0001
Number		0.66 (0.61-0.70)	<0.0001	1.16 (1.10-1.22)	<0.0001	0.70 (0.65-0.74)	<0.0001	1.14 (1.08-1.21)	<0.0001
Diameter (cm)		0.89 (0.86-0.93)	<0.0001	1.15 (1.10-1.21)	<0.0001	0.88 (0.84-0.92)	<0.0001	1.14 (1.08-1.19)	<0.0001
MVI	No	Ref	-	Ref	-	Ref	-	Ref	-
	Yes	0.80 (0.58-1.11)	0.18	1.75 (1.22-2.49)	0.002	0.73 (0.53-1.01)	0.06	1.61 (1.12-2.31)	0.01
EHS	No	Ref	-	Ref	-	Ref	-	Ref	-
	Yes	0.71 (0.47-1.09)	0.12	4.01 (2.71-5.93)	<0.0001	0.54 (0.36-0.83)	0.004	3.55 (2.40-5.26)	<0.0001
AFP (ng/mL)	≤20	Ref	-	Ref	-	Ref	-	Ref	-
	20-200	0.79 (0.66-0.95)	0.01	0.87 (0.64-1.18)	0.38	0.81 (0.66-0.98)	0.03	0.86 (0.63-1.18)	0.36
	>200	0.61 (0.51-0.74)	<0.0001	1.18 (0.90-1.55)	0.23	0.59 (0.49-0.72)	<0.0001	1.15 (0.87-1.51)	0.34

TACE treatment is the reference category of the multinomial logistic regression. OR<1 indicates that the variable is associated with higher probability of being treated with TACE rather than the comparison category (potentially curative treatments or palliative treatments). OR>1 indicates that the variable is associated with higher probability to be treated with potentially curative treatments (or palliative treatments) rather than TACE. In the multivariate models, BCLC stage was not included in in favor of the other constituent variables of the stage (number of liver tumors, size MVI, EHS, ECOG-PS and residual liver function). MELD was selected as the variable expressing residual liver function.

Abbreviations: LT, liver transplantation; LR, liver resection; ABL, ablation; SOR, systemic therapy; BSC, best supportive care; aOR, adjusted odds ratio; CI, confidence interval; ECOG-PS, Eastern Oncology Group performance status; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; MVI, macrovascular invasion; EHS, extrahepatic spread; AFP, alpha-fetoprotein.

Survival analysis

In the whole patient population, the median follow-up was 27.0 months (95% CI 12-54.4), and the median survival was 40.0 months (95% CI 38.4-41.6). The median survival of patients gradually improved from 28.0 months (95% CI 23.2-32.8) in P1 to 40.0 months (95% CI 36.9-43.1) in P5 and it was not evaluable in P6 ($p<0.0001$) (Figure 3A).

Similar trends were observed in patients treated with TACE as initial treatment (median OS 21.0 months [95% CI 16.2-25.8] in P1, 42.0 months [95% CI 37.7-46.3] in P5 and not estimable in P6; $p<0.0001$) (Figure 3B). Median OS was generally lower in patients treated with TACE as main therapy, but the improvement of prognosis over time was confirmed in this subgroup (Figure 3C). After adjustment for confounders (age, etiology, surveillance, CSPH, MELD, AFP level, BCLC stage and treatment, this latter only in the whole patient population), the improvement of survival over time was confirmed in all patients and in those treated with TACE as both first and main treatment (Table 4).

In BCLC B patients, the median follow-up was 24.0 months (95% CI 23.0-26.0) and the median OS was 32.0 months (95% CI 29.5-34.5). The median OS improved over time, from 16.0 months (95% CI 12.2-19.8) in P1 to 35.0 months (95% CI 30.0-40.0) in P5 and not estimable in P6 ($p<0.0001$) (Supplementary Figure 2A). This gradual OS improvement was confirmed in intermediate stage patients treated with TACE as both first (Supplementary Figure 2B) and main therapy (Supplementary Figure 2C). Similar to the results achieved in the whole patient population, the over time improvement of survival was confirmed after correction for confounders (Supplementary Table 2). Interestingly, in BCLC B patients a therapeutic hierarchy in terms of survival benefit (LT, LR, ABL, TACE, SOR, BSC) was demonstrated. Longer survival was shown in patients managed with potentially curative treatments compared to TACE which, in turn, was able to improve OS compared to systemic therapies (Figure 4). The independent prognostic role of treatment, with an established

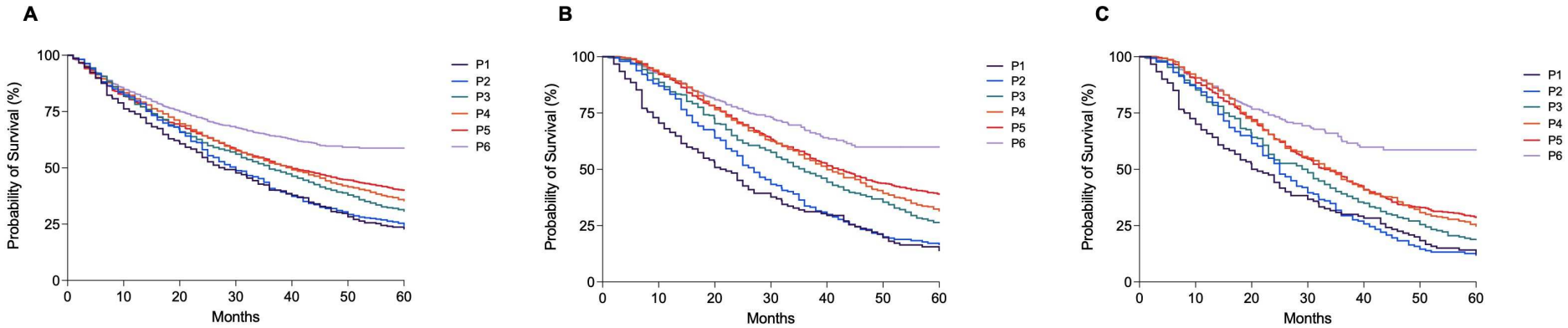


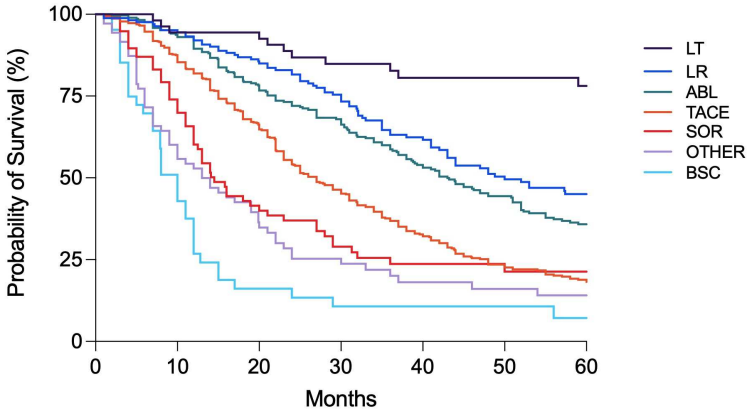
Figure 3. Kaplan-Meier curves showing overall survival according to the period of diagnosis in the overall population of patients (**A**), in patients treated with TACE as first treatment (**B**) and in those treated with TACE as main treatment (**C**) (all p<0.0001).

Table 4. Survival analysis according to the period of diagnosis in the overall population of patients.

Period of diagnosis	Median OS (months)	5-year survival (%)	aHR (95% CI) [a]	p
All patients				
P1	28.0 (23.2-32.8)	22.9	Ref	-
P2	28.0 (23.2-32.8)	24.2	1.00 (0.83-1.21)	0.99
P3	36.0 (32.6-39.4)	30.8	0.83 (0.71-0.98)	0.03
P4	39.9 (36.5-43.4)	35.4	0.67 (0.57-0.79)	<0.0001
P5	40.0 (36.9-43.1)	39.9	0.61 (0.52-0.71)	<0.0001
P6	NE (NE-NE)	58.5	0.49 (0.41-0.58)	<0.0001
Patients treated with TACE as first therapy				
P1	21.0 (16.2-25.8)	13.9	Ref	-
P2	27.0 (23.6-30.4)	16.6	0.96 (0.74-1.24)	0.74
P3	36.0 (31.4-40.6)	26.4	0.60 (0.46-0.77)	<0.0001
P4	40.0 (35.6-44.4)	31.6	0.51 (0.40-0.65)	<0.0001
P5	42.0 (37.7-46.3)	38.9	0.45 (0.36-0.57)	<0.0001
P6	NE (NE-NE)	59.7	0.31 (0.24-0.40)	<0.0001
Patients treated with TACE as main therapy				

P1	20.0 (15.0-25.0)	12.5	Ref	-
P2	25.0 (21.7-28.3)	11.9	0.97 (0.75-1.26)	0.84
P3	29.0 (23.8-34.1)	18.8	0.70 (0.54-0.90)	0.006
P4	34.0 (29.7-38.3)	24.8	0.61 (0.47-0.77)	<0.0001
P5	33.0 (29.3-36.6)	28.6	0.57 (0.45-0.73)	<0.0001
P6	NE (NE-NE)	58.6	0.38 (0.29-0.50)	<0.0001

a) Adjusted for: age, etiology, surveillance, CSPH, MELD, AFP level, BCLC stage and main treatment (this latter only in the group including all patients).
Abbreviations: OS, overall survival; aHR, adjusted hazard ratio; NE, not estimable; TACE, transarterial chemoembolization.



Treatment	Median OS (months)	5-year survival (%)
LT	NE (NE-NE)	78.1
LR	50.0 (38.4-61.6)	45.0
ABL	43.0 (37.6-48.4)	35.7
TACE	27.0 (23.8-30.2)	18.2
SOR	14.5 (11.7-17.4)	16.0
OTHER	13.0 (7.2-18.8)	13.9
BSC	10.0 (7.6-12.4)	7.2

Figure 4. Kaplan-Meier curves showing survival according to main treatment modality in BCLC B patients (p<0.0001). Median overall survival and 5-year survival rate are also shown for each treatment modality.

therapeutic hierarchy, was confirmed in BCLC B patients after adjustment for confounders (results of the Cox multivariate analysis are shown in Supplementary Table 3).

Temporal trends and survival of patients repeatedly treated with TACE

Three thousand and seven patients (41.9%) underwent at least a TACE in their clinical history, irrespective of the treatment sequence adopted. The percentage of these patients remained substantially stable across the calendar periods considered, except for P6 in which a lower proportion of patients who received this treatment was registered (35.4%). In BCLC B patients, these percentages were higher compared to the overall population in all the time periods; P3 was the cohort with the lower number of TACE-treated patients (65.3%), while in P4 the highest proportion was registered (91.1%) (Table 5). Both in the whole patient population and in BCLC B group, a forward shift of TACE treatment in the therapeutic sequence was observed over time. Indeed, the proportion of TACE applied as first-line treatment decreased, and consequently its adoption in second and subsequent lines increased (Table 5, Supplementary Figure 3). Treatment with TACE at recurrence (in second or subsequent lines), after the adoption of hierarchically superior treatments, was associated with better prognosis (Figure 5).

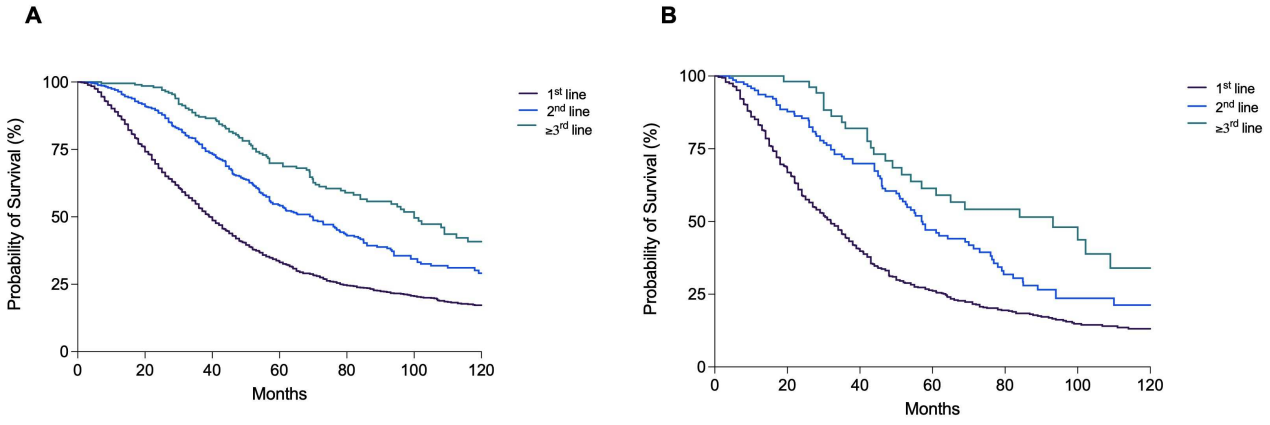


Figure 5. Kaplan Meier curves showing overall survival according to the line (1st, 2nd, ≥3rd) of TACE treatment during the patient clinical history in the overall patient population (**A**) and in BCLC B patients (**B**) (both p<0.0001).

Table 5. Characteristics of TACE treatment in the different calendar periods. All patients receiving at least a TACE, irrespective of the treatment sequence adopted, were considered.

	P1	P2	P3	P4	P5	P6
All patients						
Patients with at least a TACE	123/256 (48.0)	195/370 (52.7)	354/867 (40.8) *	601/1323 (45.4)	1078/2515 (42.9)	656/1853 (35.4) †
Line of TACE treatment						
1st line	117 (95.1)	170 (87.2) *	245 (69.2) ‡	383 (63.7) ‡	752 (69.7) ‡	528 (80.5) ‡
2nd line	6 (4.9)	17 (8.7)	61 (17.2) †	143 (23.8) ‡	237 (22.0) ‡	102 (15.5) †
≥3rd line	0 (0)	8 (4.1) *	48 (13.6) ‡	75 (12.5) ‡	89 (8.3) ‡	26 (4.0) *
Rounds of TACE per patient						
1	123 (100.0)	194 (99.9)	325 (91.8) †	431 (71.7) ‡	631 (58.6) ‡	446 (68.0) ‡
2	0 (0)	0 (0)	9 (2.5)	102 (17.0) ‡	257 (23.8) ‡	141 (21.5) ‡
≥3	0 (0)	1 (0.1)	20 (5.7) #	68 (11.3) ‡	190 (17.6) ‡	69 (10.5) ‡
Response to first TACE						
CR + PR	96 (78.1)	164 (84.1)	274 (77.4)	475 (79.0)	863 (80.1)	529 (80.7)
SD + PD	27 (21.9)	31 (15.9)	80 (22.6)	126 (21.0)	215 (19.9)	127 (19.3)
TACE as main treatment	117/123 (95.1)	165/195 (84.6) #	219/354 (61.9) ‡	317/601 (52.7) ‡	597/1078 (55.4) ‡	424/656 (64.6) ‡
BCLC B patients						
Patients with at least a TACE	61/78 (78.2)	82/101 (81.2)	141/216 (65.3) *	214/235 (91.1) #	329/376 (87.5) *	204/264 (77.3)
Line of TACE treatment						
1st line	48 (78.7)	66 (80.5)	87 (61.7) *	112 (52.3) †	170 (51.7) ‡	127 (62.3) *
2nd line	13 (21.3)	16 (19.5)	32 (22.7)	70 (32.7)	123 (37.4) *	56 (27.4)
≥3rd line	0	0	22 (15.6) †	32 (15.0) †	36 (10.9) #	21 (10.3) #
Rounds of TACE per patient						
1	61 (100.0)	82 (100.0)	134 (95.0)	156 (72.9) ‡	195 (59.3) ‡	131 (64.2) ‡
2	0 (0)	0 (0)	2 (1.4)	32 (15.0) †	75 (22.8) ‡	51 (25.0) ‡
≥3	0 (0)	0 (0)	5 (3.6)	26 (12.1) #	59 (17.9) ‡	22 (10.8) #
Response to first TACE						
CR + PR	45 (73.8)	65 (79.3)	108 (76.6)	163 (76.2)	234 (71.1)	157 (77.0)
SD + PD	16 (26.2)	17 (20.7)	33 (23.4)	51 (23.8)	95 (29.9)	47 (23.0)
TACE as main treatment	48 /61 (70.7)	65/82 (79.3)	81/141 (57.4) #	94/214 (43.9) ‡	140/329 (42.6) ‡	103/204 (50.5) †

The first cohort (P1, 1988-1993) is taken as reference for the comparison of the variable distribution.
Continuous variables are reported as median and interquartile range (IQR), while categorical variables as absolute and relative frequencies.
* p<0.05 and ≥0.01
# p<0.01 and ≥0.001
† p<0.001 and ≥0.0001
‡ p<0.0001
Abbreviations: TACE, trans-arterial chemoembolization; CR, complete response; OR, partial response; SD, stable disease; PD, progressive disease; BCLC, Barcelona Clinic Liver Cancer.

The objective response (CR+PR) to the first TACE was 79.8% in the whole population and 74.9% in BCLC B patients. No significant differences were demonstrated in radiological response, both overall and in BCLC B patients. In the whole population, patients with objective response had a longer median OS compared to non-responders (61.0 months [95% CI 56.0-66.0] vs. 41.0 months [95% CI 34.3-47.7]; p<0.0001) (Supplementary Figure 4A). A statistically significant difference in survival between responders (46.2 months [95% CI 40.9-51.5]) and non-responders (32.1 months [95% CI 21.2-43.0]) was also demonstrated in BCLC B patients (p=0.004) (Supplementary Figure 4B).

While in P1-P3 periods the vast majority of patients received only one session of TACE (91.8-100.0%), in P4-P6 periods a significantly higher percentage of patients received ≥2 TACEs. An increase over time of the percentage of patients treated with several TACE sessions was also observed in BCLC B patients (Table 5). Nevertheless, in all calendar periods, both overall and in intermediate stage, the percentage of patients treated with only 1 TACE was above 50%. The median OS of patients receiving only one TACE (40.0 months [95% CI 37.7-42.3]) was significantly lower compared to patients receiving 2 (65.0 months [95% CI 57.1-72.9]) and 3 or more TACE sessions (71.8 months [95% CI 61.1-82.4]) (p<0.0001) (Figure 6A). In BCLC B patients comparable results were obtained (30.4 months [95% CI 27.4-33.4] vs. 61.0 [95% CI 49.3-72.7] vs. 66.0 [95% CI 47.0-85.0], respectively; p<0.0001) (Figure 6B). Among the patients who received at least one TACE, 1,805 (60.0%) were dead at the end of the follow-up, mainly because of tumor progression (66.2%) and less frequently from liver decompensation (20.1%) or other causes (13.7%). The proportion of deaths from liver decompensation in patients treated with two (20.4%) and three or more TACEs (18.4%) was similar to that of patients receiving only one course of TACE (20.3%). The majority of patients in the three groups died from tumor progression (67.3% in the 1 TACE group, 61.3% in the 2 TACE group and 65.8% in the ≥3 TACE group).

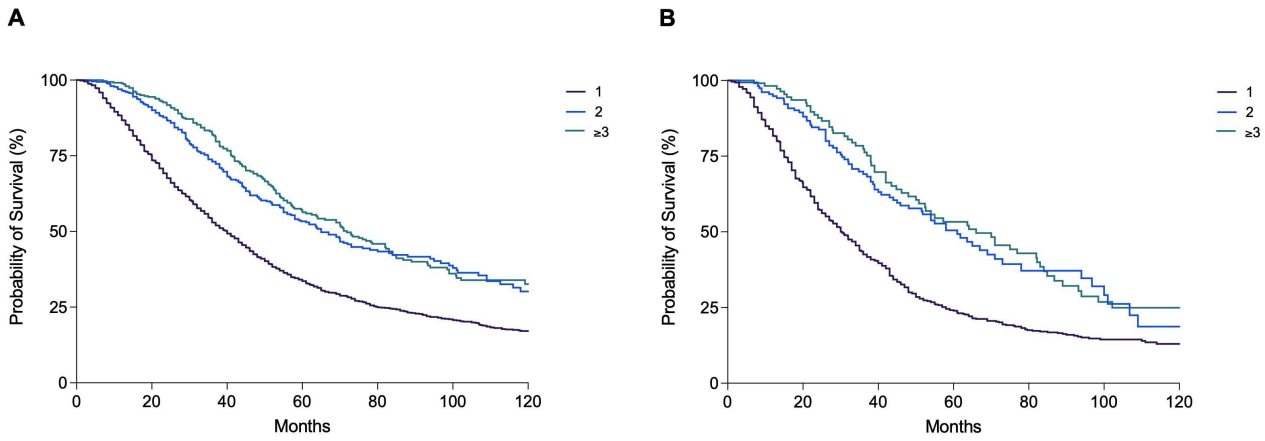


Figure 6. Kaplan-Meier curves showing overall survival according to the number of TACE performed in the overall patient population (**A**) and in BCLC B patients (**B**) (both p<0.0001).

In assessing whether TACE repetition can be considered as a positive or negative approach to the HCC treatment, the OS of patients who underwent an additional TACE in case of non-response or at the time of recurrence was compared to that of patients subsequently treated by curative treatments (LT, LR or ABL), with an upward shift, or by systemic treatments and BSC, with a downward transition. The upward shift after a TACE was associated with a significantly better survival compared to TACE repetition (83.0 months [95% CI 64.3-101.8] vs. 42.0 months [95% CI 38.4-45.7]; p<0.0001). This latter, in turn, provided a survival advantage compared to systemic therapies (27.0 months [95% CI 22.3-31.7]; p<0.0001) or BSC (29.0 months [95% CI 26.6-31.4]; p<0.0001) (Figure 7A). Similarly, in BCLC B patients, the upward shift after TACE led to a longer survival compared to a second TACE session (69.0 months [95% CI 29.7-108.3] vs. 35.0 months [95% CI 29.6-40.4]; p=0.002). Instead, the prognosis was similar in patients repeating TACE and in those receiving systemic therapies (27.4 months [95% CI 22.3-32.5]; p=0.44), while patients allocated to BSC had a significantly poorer prognosis (24.0 months [95% CI 21.9-26.1]; p=0.001) (Figure 7B).

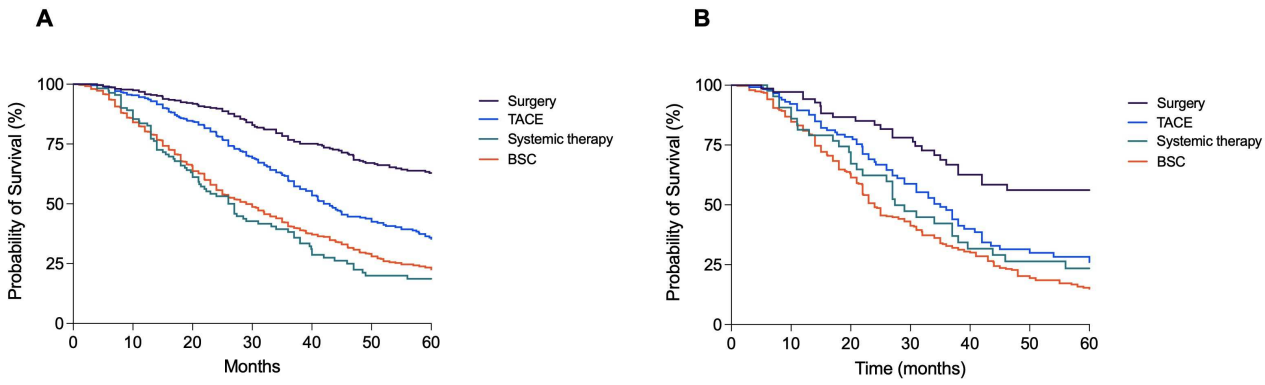


Figure 7. Kaplan-Meier curves showing the survival of patients treated with TACE in first-line according to the subsequent treatment. (**A**) In the overall patient population, those allocated to surgery had a significantly longer OS compared to those receiving another TACE (p<0.0001); these latter patients had in turn a better prognosis compared to those allocated to systemic therapies (p<0.0001) or BSC (p<0.0001). (**B**) In BCLC B patients, those treated with surgery had a better prognosis compared to patients repeating a second course of TACE (p=0.002); these latter had a similar survival compared to patients treated with systemic therapies (p=0.44), but maintained a significantly longer survival compared to those allocated to BSC (p=0.001).

DISCUSSION

With the single exception of LT, in most instances a single treatment, all therapies used in patients with HCC can be considered as iterative. In fact, the risk of tumor recurrence is high even after curative treatments (31), and both LR and ABL have been demonstrated to be safe and effective when repeated (32–37). Also systemic therapy can be seen as iterative, since drugs for first-, second- and even third-line therapy are now available (18–23). TACE, one of the most frequently used therapeutic strategies worldwide (10), could be considered by definition an iterative treatment, based on the low rates of complete response achievable and the high recurrence risk with this approach (12–14). Local tumor progression can generally benefit from repeated TACE sessions, but subsequent intra-arterial treatments have been indicated as responsible for an impairment of liver function (15). Although the evidence of TACE effectiveness for HCC treatment dates back of about 20 years (12,38), there is a lack of studies exploring whether and how the application of TACE and its relative survival benefit changed over time in real life clinical scenarios. Moreover, even less is known on TACE when considered as an iterative treatment, with few data available regarding the proportion of patients undergoing repetitive sessions. In order to give an answer to these questions,

we analyzed the ITA.LI.CA database, one of the largest registries in Europe collecting data of HCC patients managed in many referral Italian centers over more than three decades.

The results of this study indicate that, although declining over time, the percentage of patients treated with TACE remained rather elevated in all the calendar periods considered. TACE was indeed selected as first-line therapeutic choice in 45.7% of patients diagnosed in P1, and the percentage of these cases decreased from P3 onwards, until a figure of 28.5% in the last cohort (P6). The same trend was demonstrated when TACE was considered as the main (most radical) treatment applied, and less than a quarter of patients underwent TACE in P4-P6. Similar trends were detected in BCLC B patients, for whom TACE is considered as the standard of care treatment according to BCLC algorithm (9). Although a decline in its application as both first and main therapy was shown, the proportion of patients treated with TACE has stabilized in the last temporal cohorts and it is unlikely to decline further, as it remains a well-established option in the therapeutic algorithm of patients with HCC.

A not negligible proportion of patients in BCLC A, C and D stages was treated with TACE, and also some very-early stage patients received this treatment. Similarly to the trend demonstrated in the overall patient population and in BCLC B, in the other stages the percentage of patients receiving TACE was higher in P1 and P2, and gradually decreased thereafter. These results show that, in our country, the real-life therapeutic management of HCC frequently deviates from the therapeutic recommendations of the BCLC algorithm. A study investigating the management of HCC in the Campania region of Italy (39), as well as numerous studies worldwide (10,40–45), obtained comparable results regarding the poor adherence to guidelines, especially in intermediate and advanced stages. Indeed, adhering to BCLC therapeutic recommendations has been questioned by the vast amount of evidence demonstrating the better outcomes of patients undergoing treatments with potentially higher efficiency compared to the BCLC standard of care, and showing that the

treatment is an independent predictor of survival within each BCLC stage (28,42–48). Pertinently, a hierarchy of treatments in terms of survival benefit has been recently demonstrated in each tumor stage (29,49). Treatment selection in patients with HCC is a difficult issue, and several variables have to be considered. They include not only tumor burden, residual liver function and clinical conditions, but also location of the tumor in the liver, presence of significant portal hypertension, comorbidities, patient preference and, most importantly, the expected survival benefit of different treatments modalities. All of these are pivotal parameters that must be considered in order to tailor the treatment to the patient, with the aim of maximizing survival outcomes (49).

Despite being TACE the prototype of iterative treatments, our results demonstrated that in the "real-life" of the ITA.LI.CA centers most patients (both overall and in BCLC B stage) are treated with TACE only once during their clinical history. In the most recent cohorts compared to the previous ones, a greater proportion of patients were treated with 2 or ≥3 sessions of TACE, but patients who repeated the treatment remained a minority. Considered the attitude to repeat the treatment according to response, presumably patients undergoing several sessions of TACE were those with good tumor responses and a delayed recurrence or a slow progression of the treated lesion(s). Indeed, the survival of patients managed with 2 and ≥3 TACE during their clinical history was significantly longer than that of patients treated with a single TACE course. Moreover, although immortal-time bias may have played a role, this result probably reflects also the better prognosis of those patients who can be retreated at recurrence thank to favorable oncologic and clinical characteristics. Interestingly, repeating TACE did not seem to be associated with an increased risk of death from liver decompensation, since the proportion of patients who died from liver failure was similar in those receiving 1, 2 or ≥3 treatment sessions. However, this comforting finding could not be reproduced if HCC patients are managed outside expert centers.

Although repeating TACE in clinical practice was effective and safe, we also demonstrated that, whenever possible, potentially curative treatments should be preferred to TACE repetition in case of non-response or at the time of cancer recurrence after the first transarterial treatment. In fact, regardless of the tumor stage as well as in BCLC B patients, the up-ward shift toward curative therapies (LT, LR and ABL) made possible by TACE provided a longer survival compared to TACE repetition. The latter, in turn, was associated to better prognosis compared to systemic treatment or BSC. Since the survival of HCC patients is largely determined by the more effective treatment received, irrespective of the therapeutic sequence adopted (29), it was not surprising that, after a first-line TACE, the adoption of treatment that can provide a higher survival benefit was associated with better prognosis. Moreover, it has already been demonstrated that surgical treatment of HCC recurrence is a favorable prognostic factor (41,50,51). Therefore, the principle of firstly consider the therapy with the highest survival benefit is also valid in the second-line setting, in case of non-response or recurrence after the frontline therapy (49).

As expected, the variables impacting in treatment selection pertained to clinical conditions, residual liver function and tumor burden. TACE was preferred to curative approaches in older patients, in those with ECOG-PS≥1, CSPH, higher MELD (except for LT specifically) and greater tumor burden (in terms of number and size of nodules, MVI, EHS and high AFP levels). The opposite was found comparing TACE vs. more palliative treatments: patients who had compromised clinical conditions, higher MELD, increasing number and size of liver nodules and presence of MVI or EHS were more likely to receive SOR or BSC. In BCLC B patients age, CSPH, residual liver function and number and size of liver nodules influenced the selection of treatment. However, the probability of being treated with potentially curative therapies instead of TACE as main treatment increased from P3 onwards, suggesting that the attitude of treating intermediate stage patients with curative intent, whenever feasible, has progressively gained field in recent years.

Another key finding of this study is the progressive improvement of survival over time, not only irrespective of treatment, but also in patients treated with TACE as first-line or main therapy. This improvement occurred also in BCLC B patients, even if the median OS registered were lower in this group. In general, the progressive prolongation of survival may be the result of an earlier HCC diagnosis, a better management and the availability of effective therapies for the underlying liver disease (52) and a better HCC management. In patients treated with TACE a better selection of patients and technical advancements (e.g., superselective embolization to minimize ischemic injury to non-tumor tissue (53)) are probably the key determinants. In support to these considerations, it has already been demonstrated that refinements in the selection criteria, made possible by the publication of studies demonstrating TACE efficacy in selected patients, provided better survival outcomes despite the more advanced tumor stage of treated patients (54).

Despite this improvement, in intermediate stage patients, TACE remained less effective in terms of survival benefit than curative treatments. As already reported (48), TACE provided worse outcomes compared to LT, LR and ABL. Moreover, as the existence of a therapeutic hierarchy in BCLC B patients (LT>LR>ABL>TACE>SOR>BSC) was confirmed by our study, such evidence reinforces the concept that, whenever possible and once excluded specific contraindications, the treatment potentially offering the best survival should be chosen irrespective of the stage (29,49).

Despite its many strengths, our study also has some limitations, the most important of which is its retrospective nature which may have introduced unintended biases. Nevertheless, the aim of the study itself, which was to evaluate if and how the application of TACE and the attitude to repeat this treatment in clinical practice has changed in the last decades, required the analysis of a large dataset collecting real-life data. The ITA.LI.CA database offered us this opportunity, having collected data of HCC patients managed in clinical practice for more than three decades and being nowadays one of the largest European databases. However, the retrospective design of the study made it impossible

to determine the exact reasons behind the choice of TACE as the first-line or main HCC treatment. Moreover, the reasons that prompted clinicians to prescribe additional TACE after a first session or to switch to other treatments were not pre-defined and standardized among centers. We tried to evaluate which factors were associated with higher likelihood of receiving TACE compared to other treatments, but we could not consider all the variables implicated, including patient's unwillingness to accept the treatment, comorbidities, technical contraindications. Another major limitation of this study is that we could not provide technical details about TACE treatment. This therapy, which can be grossly divided in conventional TACE (cTACE) and TACE with drug-eluting beads (DEB-TACE), lack in standardization and is a rather heterogenous treatment (11). Unfortunately, in the ITA.LI.CA database a detailed description of the type of TACE is seldom available and therefore we could not assess the technical evolution of the procedure over time (which may partly explain the progressively better survival seen in recent years) and whether the attitude to treat patients with cTACE or TACE-DEB has changed. Technical skills and experience are fundamental for the effectiveness of TACE. Even though we did not measure these variables, all the Institutions collaborating to the ITA.LI.CA project are expert centers in the management of HCC patients that routinely performs TACE.

In conclusion, in this study we provided a comprehensive analysis of the changes in TACE treatment occurred in real-life clinical practice over the last three decades. The proportion of patients treated with TACE, also when BCLC B patients were specifically considered, declined over time but remained stable over the last calendar periods considered. In real-world clinical management of HCC, a substantial proportion of BCLC B patients are managed deviating from treatment recommendations of Western guidelines, and a relevant percentage of patients belonging to other stages are treated with TACE, confirming that expert centers have a poor adherence to BCLC indications. The better selection of patients, as well as the procedural improvements, may explain the progressive better

survival observed over time in patients undergoing TACE. Nevertheless, although this treatment could be safely and effectively repeated in expert centers, in this setting the majority of patients are treated with TACE only once during their clinical history. After a first-line TACE, a shift toward curative therapies (LT, LR and ABL) to refine the achieved result provides a higher survival benefit compared to TACE repetition and, therefore, it should be preferred whenever feasible.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Multinomial logistic regression showing independent factors associated with probability of receive TACE compared to potentially curative treatments (LT, LR and ABL) and palliative therapies (SOR and BSC) in BCLC B patients.

Variables		Curative treatment (LT, LR and ABL)		Palliative treatment (SOR and BSC)		Curative treatment (LT, LR and ABL)		Palliative treatment (SOR and BSC)	
		First treatment				Main treatment			
		aOR (95% CI)	p	aOR (95% CI)	p	aOR (95% CI)	p	aOR (95% CI)	p
Sex	Females	Ref	-	Ref	-	Ref	-	Ref	-
	Males	1.17 (0.69-1.99)	0.56	0.87 (0.44-1.74)	0.69	1.07 (0.64-1.80)	0.80	0.84 (0.42-1.70)	0.63
Age (per 10-year increase)		0.78 (0.65-0.93)	0.005	1.05 (0.81-1.35)	0.73	0.73 (0.61-0.88)	0.001	1.03 (0.79-1.34)	0.83
Period of diagnosis	P1	Ref	-	Ref	-	Ref	-	Ref	-
	P2	0.28 (0.06-1.35)	0.11	1.06 (0.23-4.77)	0.94	0.26 (0.05-1.26)	0.09	1.03 (0.23-4.64)	0.97
	P3	3.16 (0.98-10.18)	0.05	3.57 (0.89-14.31)	0.07	3.83 (1.19-12.30)	0.02	3.89 (0.97-15.67)	0.06
	P4	2.37 (0.77-7.29)	0.13	1.02 (0.25-4.23)	0.97	3.48 (1.14-10.67)	0.03	1.21 (0.29-5.01)	0.80
	P5	1.98 (0.66-5.91)	0.22	1.26 (0.33-4.82)	0.73	3.06 (1.03-9.11)	0.04	1.42 (0.37-5.44)	0.61
	P6	1.82 (0.60-5.53)	0.29	0.76 (0.19-3.04)	0.70	3.11 (1.03-9.36)	0.04	0.92 (0.23-3.70)	0.91
Surveillance	No	Ref	-	Ref	-	Ref	-	Ref	-
	Yes	1.39 (0.95-2.03)	0.09	0.72 (0.43-1.20)	0.21	1.14 (0.78-1.65)	0.50	0.65 (0.38-1.10)	0.11
CSPH	No	Ref	-	Ref	-	Ref	-	Ref	-
	Yes	0.44 (0.30-0.66)	<0.0001	0.89 (0.51-1.56)	0.68	0.57 (0.39-0.84)	0.004	0.92 (0.52-1.63)	0.77
MELD		0.94 (0.88-1.02)	0.12	1.10 (1.01-1.20)	0.04	0.91 (0.85-0.98)	0.01	1.08 (0.98-1.18)	0.11
Number		0.87 (0.76-0.99)	0.04	1.09 (0.99-1.19)	0.07	0.88 (0.78-0.99)	0.04	1.08 (0.99-1.18)	0.09
Diameter (cm)		0.95 (0.86-1.05)	0.29	1.13 (1.03-1.23)	0.01	0.91 (0.83-0.99)	0.04	1.10 (1.01-1.21)	0.03
AFP (ng/mL)	≤20	Ref	-	Ref	-	Ref	-	Ref	-
	20-200	1.01 (0.63-1.62)	0.96	0.73 (0.36-1.48)	0.38	0.89 (0.56-1.42)	0.63	0.72 (0.35-1.48)	0.37
	>200	0.82 (0.53-1.26)	0.36	1.19 (0.67-2.10)	0.56	0.81 (0.53-1.23)	0.32	1.22 (0.68-2.18)	0.51

TACE treatment is the reference category of the multinomial logistic regression. OR<1 indicates that the variable is associated with higher probability of being treated with TACE rather than the comparison category (curative treatments or palliative treatments). OR>1 indicates that the variable is associated with higher probability to be treated with potentially curative treatments (or palliative treatments) rather than TACE.

Abbreviations: LT, liver transplantation; LR, liver resection; ABL, ablation; SOR, systemic therapy; BSC, best supportive care; aOR, adjusted odds ratio; CI, confidence interval; CSPH, clinically significant portal hypertension; MELD, Model for End-Stage Liver Disease; MVI, macrovascular invasion; EHS, extrahepatic spread; AFP, alpha-fetoprotein.

Supplementary Table 2. Survival analysis according to the period of diagnosis in BCLC B patients.

Period of diagnosis	Median OS (months)	5-year survival (%)	aHR (95% CI) [a]	p
All patients				
P1	16.0 (12.2-19.8)	15.4	Ref	-
P2	25.0 (20.3-29.7)	13.6	0.75 (0.53-1.07)	0.12
P3	30.0 (24.0-36.0)	23.7	0.75 (0.55-1.03)	0.07
P4	35.0 (29.1-40.9)	26.5	0.60 (0.44-0.81)	0.001
P5	35.0 (30.0-40.0)	33.8	0.54 (0.40-0.74)	0.0001
P6	NE (NE-NE)	51.5	0.40 (0.28-0.57)	<0.0001
Patients treated with TACE as first therapy				
P1	16.0 (11.2-20.8)	6.6	Ref	-
P2	25.0 (21.8-28.2)	8.2	0.85 (0.56-1.30)	0.46
P3	34.0 (25.8-42.2)	23.7	0.60 (0.39-0.90)	0.02
P4	34.0 (27.6-40.4)	25.9	0.53 (0.36-0.79)	0.002
P5	36.0 (30.7-41.3)	33.5	0.52 (0.35-0.77)	0.001
P6	NE (NE-NE)	50.2	0.38 (0.24-0.61)	<0.0001
Patients treated with TACE as main therapy				
P1	15.0 (10.9-19.1)	9.3	Ref	-
P2	25.0 (21.9-28.1)	6.2	0.84 (0.55-1.29)	0.44
P3	30.0 (18.6-41.4)	17.5	0.66 (0.44-1.01)	0.06
P4	29.0 (23.8-34.2)	13.4	0.60 (0.40-0.91)	0.02
P5	31.0 (22.7-39.3)	23.9	0.65 (0.43-0.98)	0.04
P6	47.4 (NE-NE)	53.8	0.39 (0.24-0.65)	0.0003

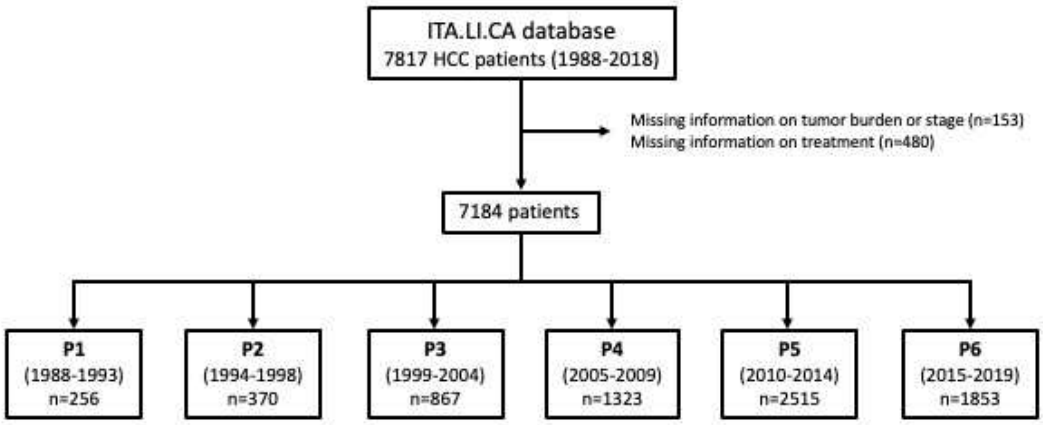
a) Adjusted for: age, etiology, surveillance, CSPH, MELD, AFP level and main treatment (this latter only in the group including all patients).
Abbreviations: OS, overall survival; aHR, adjusted hazard ratio; NE, not estimable; TACE, trans-arterial chemoembolization.

Supplementary Table 3. Univariate and multivariate Cox regression analysis in BCLC B patients.

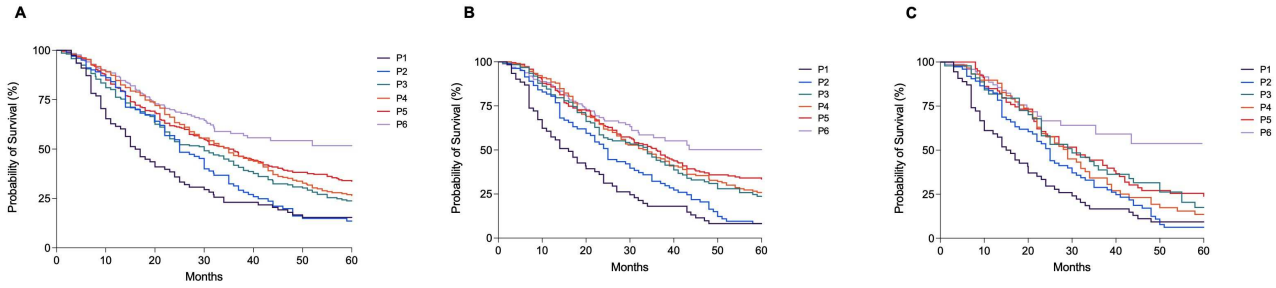
Variables	Univariate		Multivariate	
	HR (95% CI)	p	aHR (95% CI)	p
Period				
P1	Ref	-	Ref	-
P2	0.86 (0.63-1.17)	0.34	0.75 (0.53-1.07)	0.12
P3	0.76 (0.58-1.00)	0.05	0.75 (0.55-1.03)	0.07
P4	0.60 (0.46-0.79)	0.0003	0.60 (0.44-0.81)	0.001
P5	0.52 (0.40-0.68)	<0.0001	0.54 (0.40-0.74)	0.0001
P6	0.41 (0.30-0.56)	<0.0001	0.40 (0.28-0.57)	<0.0001
Sex				
Female	Ref	-	-	-
Male	0.87 (0.73-1.05)	0.14		
Age – 10 years increase	1.06 (1.00-1.13)	0.05	1.03 (0.95-1.12)	0.48
Surveillance				
No	Ref	-	Ref	-
Yes	0.81 (0.71-0.94)	0.005	0.83 (0.71-0.98)	0.03
Etiology				
Viral	Ref	-	Ref	-
Not viral	0.83 (0.71-0.97)	0.02	0.95 (0.78-1.15)	0.59
Viral + other	1.12 (0.91-1.38)	0.30	1.15 (0.90-1.48)	0.25
CSPH				
No	Ref	-	Ref	-
Yes	1.42 (1.23-1.65)	<0.0001	1.21 (1.02-1.45)	0.03
Number	1.02 (1.00-1.04)	0.09	-[a]	-[a]
Diameter (cm)	1.02 (1.00-1.04)	0.09	-[a]	-[a]
Child-Pugh				
A	Ref	-	-[b]	-[b]
B7	1.56 (1.29-1.90)	<0.0001		
B8-9	2.24 (1.83-2.73)	<0.0001		
MELD	1.06 (1.04-1.08)	<0.0001	1.04 (1.01-1.07)	0.006

AFP (ng/mL)				
≤20	Ref	-	Ref	-
20-200	1.03 (0.87-1.23)	0.72	1.00 (0.81-1.23)	0.99
>200	1.27 (1.08-1.49)	0.003	1.35 (1.12-1.63)	0.002
Main treatment				
BSC	Ref	-	Ref	-
LT	0.07 (0.04-0.12)	<0.0001	0.06 (0.03-0.11)	<0.0001
LR	0.18 (0.12-0.26)	<0.0001	0.17 (0.10-0.26)	<0.0001
ABL	0.23 (0.16-0.33)	<0.0001	0.23 (0.15-0.34)	<0.0001
TACE	0.35 (0.25-0.50)	<0.0001	0.31 (0.22-0.46)	<0.0001
SOR	0.46 (0.30-0.70)	0.0003	0.52 (0.32-0.86)	0.01
Other	0.57 (0.38-0.87)	0.009	0.46 (0.28-0.75)	0.002

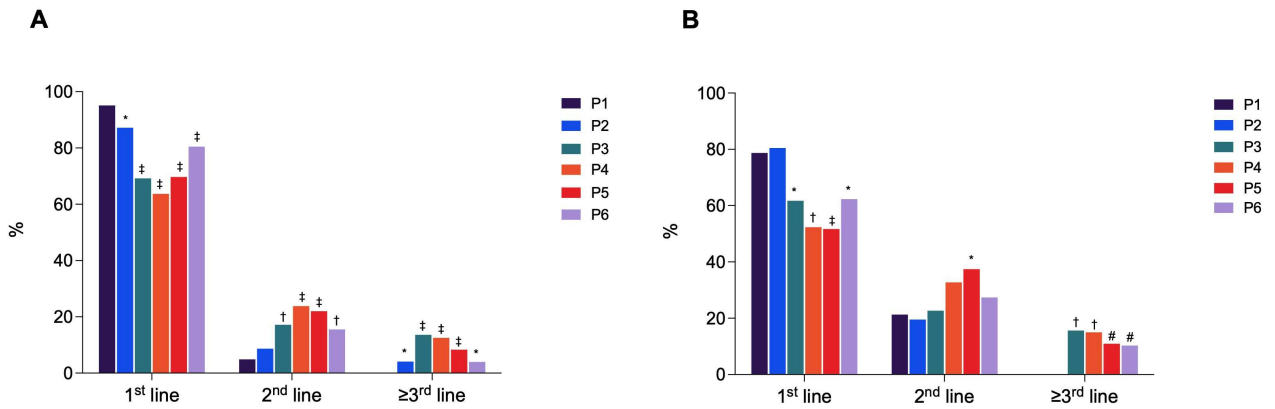
a) Not included in multivariate analysis to avoid collinearity with stage.
b) Not included in multivariate analysis to avoid collinearity with MELD
Abbreviations: HR. hazard ratio; CI, confidence interval; aHR, adjusted hazard ratio; CSPH, clinically significant portal hypertension; MELD, Model for End-stage Liver Disease; AFP, alpha-fetoprotein; BSC, best supportive care; LT, liver transplantation; LR, liver resection; ABL, ablation; TACE, transarterial chemoembolization; SOR, systemic therapies.



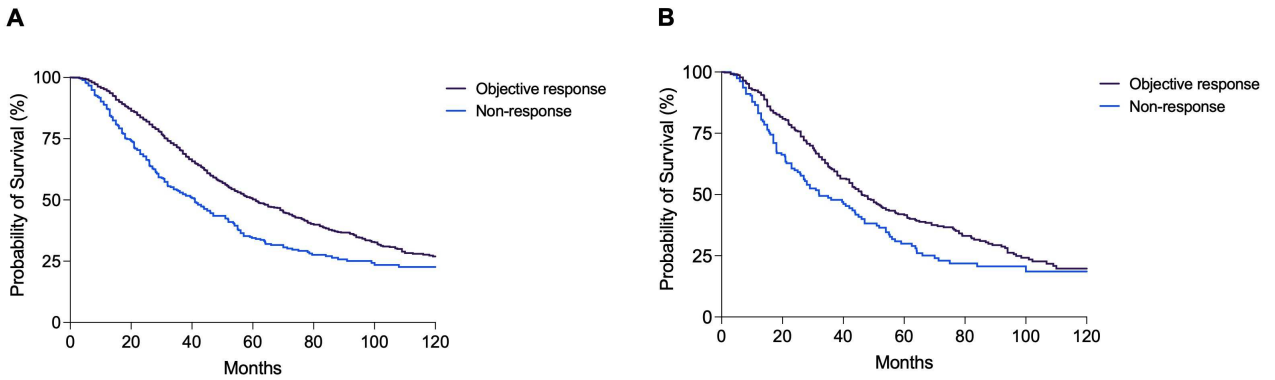
Supplementary Figure 1. Flow chart of patient selection.



Supplementary Figure 2. Kaplan-Meier curves showing overall survival according to the period of diagnosis in BCLC B patients (**A**), in BCLC B patients treated with TACE as first treatment (**B**) and in those treated with TACE as main treatment (**C**) (all p<0.0001).



Supplementary Figure 3. Proportion of patients treated with TACE overall (**A**) and in BCLC B stage (**B**) in 1st, 2nd and ≥3rd line divided according to the period of diagnosis (* p<0.05 and ≥0.01; # p<0.01 and ≥0.001; † p<0.001 and ≥0.0001; ‡ p<0.0001).



Supplementary Figure 4. Kaplan-Meier curves showing overall survival according to the response to the first TACE in the whole patient population (A) and in BCLC B patients (B). Patients with objective response demonstrated a statistically significant longer survival compared to non-responders (p<0.0001 in the whole population and p=0.004 in BCLC B).

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA. Cancer J. Clin. 2021;71:209–249.
2. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology. 2004;127:S35-50.
3. Ioannou GN, Splan MF, Weiss NS, McDonald GB, Beretta L, Lee SP. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. 2007;5:938–45, 945.e1–4.
4. Mittal S, El-Serag HB. Epidemiology of hepatocellular carcinoma: consider the population. J. Clin. Gastroenterol. 2013;47 Suppl:S2–S6.
5. Italian Association of Cancer Registries (AIRTUM) - available at https://www.registri-tumori.it/cms/pubblicazioni/i-numeri-del-cancro-italia-2020.
6. Trevisani F, De Notariis S, Rapaccini G, Farinati F, Benvegnù L, Zoli M, et al. Semiannual and annual surveillance of cirrhotic patients for hepatocellular carcinoma: Effects on cancer stage and patient survival (Italian experience). Am. J. Gastroenterol. 2002;97:734–744.
7. Pelizzaro F, Vitale A, Sartori A, Vieno A, Penzo B, Russo FP, et al. Surveillance as determinant of long-term survival in non-transplanted hepatocellular carcinoma patients. Cancers (Basel). 2021;13:1–16.
8. Pelizzaro F, Peserico G, D'Elia M, Cazzagon N, Russo FP, Vitale A, et al. Surveillance for hepatocellular carcinoma with a 3-months interval in "extremely high-risk" patients does not further improve survival. Dig. liver Dis. Off. J. Ital. Soc. Gastroenterol. Ital. Assoc. Study Liver. 2021;
9. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.
10. Park JW, Chen M, Colombo M, Roberts LR, Schwartz M, Chen PJ, et al. Global patterns of hepatocellular carcinoma management from diagnosis to death: The BRIDGE Study. Liver Int. 2015;35:2155–2166.
11. Bargellini I, Florio F, Golfieri R, Grosso M, Lauretti DL, Cioni R. Trends in utilization of transarterial treatments for hepatocellular carcinoma: results of a survey by the Italian Society of Interventional Radiology. Cardiovasc. Intervent. Radiol. 2014;37:438–444.
12. Lo C-M, Ngan H, Tso W-K, Liu C-L, Lam C-M, Poon RT-P, et al. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. Hepatology. 2002;35:1164–1171.
13. Lammer J, Malagari K, Vogl T, Pilleul F, Denys A, Watkinson A, et al. Prospective randomized study of doxorubicin-eluting-bead embolization in the treatment of hepatocellular carcinoma: results of the PRECISION V study. Cardiovasc. Intervent. Radiol. 2010;33:41–52.
14. Golfieri R, Giampalma E, Renzulli M, Cioni R, Bargellini I, Bartolozzi C, et al. Randomised controlled trial of doxorubicin-eluting beads vs conventional chemoembolisation for hepatocellular carcinoma. Br. J. Cancer. 2014;111:255–264.
15. A comparison of lipiodol chemoembolization and conservative treatment for unresectable hepatocellular carcinoma. N. Engl. J. Med. 1995;332:1256–1261.
16. Cucchetti A, Giannini EG, Mosconi C, Plaz Torres MC, Pieri G, Farinati F, et al. Recalibrating survival prediction among patients receiving trans-arterial chemoembolization for hepatocellular carcinoma. Liver Cancer Int. 2021;2:45–53.
17. Campani C, Vitale A, Dragoni G, Arena U, Laffi G, Cillo U, et al. Time-Varying mHAP-III Is the Most Accurate Predictor of Survival in Patients with Hepatocellular Carcinoma Undergoing Transarterial Chemoembolization. Liver cancer. 2021;10:126–136.
18. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J-F, et al. Sorafenib in Advanced Hepatocellular Carcinoma. N. Engl. J. Med. 2008;359:378–390.
19. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet.

2018;391:1163–1173.

20. Bruix J, Qin S, Merle P, Granito A, Huang YH, Bodoky G, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017;389:56–66.

21. Abou-Alfa GK, Meyer T, Cheng A-L, El-Khoueiry AB, Rimassa L, Ryoo B-Y, et al. Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. N. Engl. J. Med. 2018;379:54–63.

22. Zhu AX, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, et al. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2019;20:282–296.

23. Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim T-Y, et al. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. N. Engl. J. Med. 2020;382:1894–1905.

24. Sieghart W, Hucke F, Pinter M, Graziadei I, Vogel W, Müller C, et al. The ART of decision making: retreatment with transarterial chemoembolization in patients with hepatocellular carcinoma. Hepatology. 2013;57:2261–2273.

25. Hucke F, Sieghart W, Pinter M, Graziadei I, Vogel W, Müller C, et al. The ART-strategy: sequential assessment of the ART score predicts outcome of patients with hepatocellular carcinoma re-treated with TACE. J. Hepatol. 2014;60:118–126.

26. Adhoute X, Penaranda G, Naude S, Raoul JL, Perrier H, Bayle O, et al. Retreatment with TACE: the ABCR SCORE, an aid to the decision-making process. J. Hepatol. 2015;62:855–862.

27. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68:723–750.

28. Roayaie S, Jibara G, Tabrizian P, Park J-W, Yang J, Yan L, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology. 2015;62:440–451.

29. Vitale A, Farinati F, Pawlik TM, Frigo AC, Giannini EG, Napoli L, et al. The concept of therapeutic hierarchy for patients with hepatocellular carcinoma: A multicenter cohort study. Liver Int. 2019;39:1478–1489.

30. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. Semin. Liver Dis. 2010;30:52–60.

31. Cabibbo G, Petta S, Barbàra M, Missale G, Virdone R, Caturelli E, et al. A meta-analysis of single HCV-untreated arm of studies evaluating outcomes after curative treatments of HCV-related hepatocellular carcinoma. Liver Int. Off. J. Int. Assoc. Study Liver. 2017;37:1157–1166.

32. Faber W, Seehofer D, Neuhaus P, Stockmann M, Denecke T, Kalmuk S, et al. Repeated liver resection for recurrent hepatocellular carcinoma. J. Gastroenterol. Hepatol. 2011;26:1189–1194.

33. Chan ACY, Chan SC, Chok KSH, Cheung TT, Chiu DW, Poon RTP, et al. Treatment strategy for recurrent hepatocellular carcinoma: salvage transplantation, repeated resection, or radiofrequency ablation? Liver Transplant. Off. Publ. Am. Assoc. Study Liver Dis. Int. Liver Transplant. Soc. 2013;19:411–419.

34. Sun W-C, Chen I-S, Liang H-L, Tsai C-C, Chen Y-C, Wang B-W, et al. Comparison of repeated surgical resection and radiofrequency ablation for small recurrent hepatocellular carcinoma after primary resection. Oncotarget. 2017;8:104571–104581.

35. Song KD, Lim HK, Rhim H, Lee MW, Kim Y-S, Lee WJ, et al. Repeated Hepatic Resection versus Radiofrequency Ablation for Recurrent Hepatocellular Carcinoma after Hepatic Resection: A Propensity Score Matching Study. Radiology. 2015;275:599–608.

36. Lee S, Jeong WK, Rhim H. Repeated percutaneous radiofrequency ablation for hepatocellular carcinoma in patients with cirrhosis: assessment of safety based on liver function and portal hypertension parameters. J. Vasc. Interv. Radiol. 2014;25:1573–1579.

37. Rossi S, Ravetta V, Rosa L, Ghittoni G, Viera FT, Garbagnati F, et al. Repeated radiofrequency ablation for management of patients with cirrhosis with small hepatocellular carcinomas: a long-term cohort study. Hepatology. 2011;53:136–147.

38. Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, et al. Arterial embolisation or chemoembolisation

versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. Lancet (London, England). 2002;359:1734–1739.

39. Guarino M, Tortora R, de Stefano G, Coppola C, Morisco F, Salomone Megna A, et al. Adherence to Barcelona Clinic Liver Cancer guidelines in field practice: Results of Progetto Epatocarcinoma Campania. J. Gastroenterol. Hepatol. 2018;33:1123–1130.

40. Giannini EG, Bucci L, Garuti F, Brunacci M, Lenzi B, Valente M, et al. Patients with advanced hepatocellular carcinoma need a personalized management: A lesson from clinical practice. Hepatology. 2018;67:1784–1796.

41. Tabrizian P, Jibara G, Shrager B, Schwartz M, Roayaie S. Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis. Ann. Surg. 2015;261:947–955.

42. Sangiovanni A, Triolo M, Iavarone M, Forzenigo L V., Nicolini A, Rossi G, et al. Multimodality treatment of hepatocellular carcinoma: How field practice complies with international recommendations. Liver Int. 2018;38:1624–1634.

43. Kokudo T, Hasegawa K, Matsuyama Y, Takayama T, Izumi N, Kadoya M, et al. Survival benefit of liver resection for hepatocellular carcinoma associated with portal vein invasion. J. Hepatol. 2016;65:938–943.

44. Kokudo T, Hasegawa K, Matsuyama Y, Takayama T, Izumi N, Kadoya M, et al. Liver resection for hepatocellular carcinoma associated with hepatic vein invasion: A Japanese nationwide survey. Hepatology. 2017;66:510–517.

45. Yin L, Li H, Li AJ, Lau WY, Pan ZY, Lai ECH, et al. Partial hepatectomy vs. transcatheter arterial chemoembolization for resectable multiple hepatocellular carcinoma beyond Milan criteria: A RCT. J. Hepatol. 2014;61:82–88.

46. Zhang XP, Gao YZ, Chen ZH, Chen MS, Li LQ, Wen TF, et al. An Eastern Hepatobiliary Surgery Hospital/Portal Vein Tumor Thrombus Scoring System as an Aid to Decision Making on Hepatectomy for Hepatocellular Carcinoma Patients With Portal Vein Tumor Thrombus: A Multicenter Study. Hepatology. 2019;69:2076–2090.

47. Kim KM, Sinn DH, Jung SH, Gwak GY, Paik YH, Choi MS, et al. The recommended treatment algorithms of the BCLC and HKLC staging systems: does following these always improve survival rates for HCC patients? Liver Int. 2016;36:1490–1497.

48. Pecorelli A, Lenzi B, Gramenzi A, Garuti F, Farinati F, Giannini EG, et al. Curative therapies are superior to standard of care (transarterial chemoembolization) for intermediate stage hepatocellular carcinoma. Liver Int. 2017;37:423–433.

49. Vitale A, Trevisani F, Farinati F, Cillo U. Treatment of hepatocellular carcinoma in the Precision Medicine era: from treatment stage migration to therapeutic hierarchy. Hepatology. 2020;72:2206–2218.

50. Vitale A, Farinati F, Noaro G, Burra P, Pawlik TM, Bucci L, et al. Restaging Patients With Hepatocellular Carcinoma Before Additional Treatment Decisions: A Multicenter Cohort Study. Hepatology. 2018;68:1232–1244.

51. Erridge S, Pucher PH, Markar SR, Malietzis G, Athanasiou T, Darzi A, et al. Meta-analysis of determinants of survival following treatment of recurrent hepatocellular carcinoma. Br. J. Surg. 2017;104:1433–1442.

52. Cabibbo G, Celsa C, Calvaruso V, Petta S, Cacciola I, Cannavò MR, et al. Direct-acting antivirals after successful treatment of early hepatocellular carcinoma improve survival in HCV-cirrhotic patients. J. Hepatol. 2019;71:265–273.

53. Kudo M, Han G, Finn RS, Poon RTP, Blanc J-F, Yan L, et al. Brivanib as adjuvant therapy to transarterial chemoembolization in patients with hepatocellular carcinoma: A randomized phase III trial. Hepatology. 2014;60:1697–1707.

54. Giannini EG, Bodini G, Corbo M, Savarino V, Risso D, Di Nolfo MA, et al. Impact of evidence-based medicine on the treatment of patients with unresectable hepatocellular carcinoma. Aliment. Pharmacol. Ther. 2010;31:493–501.

CHAPTER 15

Capecitabine in advanced hepatocellular carcinoma: a multicenter experience

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ABSTRACT

Background: Recent data suggest a potential activity and a good tolerability of capecitabine in advanced hepatocellular carcinoma (HCC).

Aims: To evaluate capecitabine activity and safety in a wide cohort of advanced HCC patients.

Methods: Retrospective analysis of 143 capecitabine-treated patients (January 2010 to December 2017) in three centers of the Veneto Oncology Network.

Results: Capecitabine was administered in second and third line, but also in first line instead of sorafenib in case of Child-Pugh B (70%), compromised clinical conditions (14%) or contraindications to anti-angiogenetics (16%). Median overall survival (OS) and time to progression (TTP) were 6.9 and 2.8 months, respectively. There were no differences in OS and TTP between the 32 patients treated with non- metronomic scheme (2000 mg/day for 14 days) and the 111 patients treated with metronomic scheme (1000mg/day) after correction for prognostic factors at baseline with a propensity score analysis. Capecitabine was more active in patients intolerant to sorafenib than in those progressing during treatment ($p = 0.024$). At least one adverse event (mainly hematological) was experienced by 73% of patients but discontinuation was necessary only in 11 (8%).

Conclusions: Capecitabine can be considered an active and safe option in advanced HCC, especially for patients unfit for other treatments.

INTRODUCTION

Hepatocellular carcinoma (HCC) is globally the sixth most common cancer and the fourth most frequent cause of cancer-related death, with 854.000 incident cases and 810.000 deaths per year (1). The incidence of HCC is progressively increasing, especially in western countries (2). Despite the surveillance in cirrhotics, in many patients (approximately 30%) the diagnosis is achieved when curative treatments are no longer feasible (3–5).

Sorafenib, an oral multi-tyrosine kinase inhibitor, currently represents the standard first-line systemic therapy for advanced HCC (BCLC-C) or tumors progressing after loco-regional therapies, with well-preserved liver function (Child-Pugh A) (6). However, 80–90% of treated patients experience at least one drug-related adverse event (AE), leading to dose reduction in about half cases and to permanent discontinuation in 30–40% of them (7–9). Moreover, sorafenib fails to control cancer progression in about 30–40% of patients (7,10). Many drugs have been tested in first line versus sorafenib (11–13) and in second line after sorafenib failure (14–17). Ten years passed before other drugs proved to be effective for advanced HCC: lenvatinib (18) demonstrated its non-inferiority compared to sorafenib in first line, while regorafenib (19) and cabozantinib (20) were able to determine a survival improvement against placebo in second line after sorafenib. The scenario of systemic therapies in advanced HCC is rapidly evolving, and soon also nivolumab (21), ramucirumab (22) and pembrolizumab (23) could be approved in Europe.

Standard chemotherapy has never been proved effective in the treatment of HCC (24), either because of the refractoriness of the tumor or due to the coexistence of cirrhosis, which impairs both drug metabolism and reduces tolerability.

Capecitabine is an orally administered 5-Fluorouracil (5-FU) prodrug absorbed as an intact molecule via the gastrointestinal tract (25–27). Thymidine phosphorylase, that is more expressed in tumor than in normal surrounding cells, promotes an enzymatic reaction generating 5-FU selectively in

tumor tissue (25,27). Capecitabine has been investigated in the treatment of advanced HCC using either a conventional (28–32) or a metronomic approach (33–38), with no definitive results in term of survival benefit. All the studies however proved the favorable tolerability profile of capecitabine, especially when metronomically administered. The concept of metronomic chemotherapy (39), defined as the chronic administration of chemotherapeutic agents at relatively low, minimally toxic doses, without drug free breaks, has been recently introduced in oncology. The main advantage is toxicity reduction, but metronomic administration seems also to improve antitumoral effect (39–45).

Metronomic chemotherapy is particularly appealing in patients with HCC, who are in most instances fragile and present low tolerability to chemotherapeutics. After the first pioneering work of Farrag et al. (33) on metronomic capecitabine in advanced HCC, a series of studies evaluated that treatment in both naïve and sorafenib-treated patients (34,35,37,38), confirming its safety and suggesting its efficacy in providing a survival benefit.

This multicenter study aims to retrospectively evaluate the activity and the safety of capecitabine in a large group of patients with advanced HCC treated with different therapeutic schemes.

MATERIALS AND METHODS

Study population

We retrospectively analyzed the records of 168 patients with HCC treated with capecitabine between January 1st 2010 and December 31st 2017 in three centers part of Veneto Oncology Network: Padova University Hospital (Gastroenterology Unit), Istituto Oncologico Veneto IRCCS (Unit of Medical Oncology I, Padova) and Feltre Hospital (Medical Oncology Unit). One hundred forty-three patients (85.1%) were included in the study, while 25 (14.9%) were excluded for lack of

follow-up data or for being treated with combination therapy (capecitabine + sorafenib or gemcitabine).

The baseline recorded characteristics of patients treated with capecitabine were: Eastern Cooperative Oncology Group performance status (ECOG-PS) ≤2, Child-Pugh score A or B, total serum bilirubin ≤3 mg/dL, platelets count ≥50 × 109 /L, hemoglobin level ≥9 g/dL, white blood cell count ≥1.5 × 10^9 /L, transaminases ×5 the upper normal level, creatinine ≤1.5 mg/dL, no ascites or ascites controlled by diuretics, encephalopathy grade ≤1 and no history of coronary disease or hearth failure.

Capecitabine was administered orally with two schemes: metronomic scheme (MS, capecitabine at the dosage of 1000 mg/day, continuously without drug-free breaks) and non-metronomic scheme (NMS, capecitabine at the dosage of 2000mg/day administered for 14 days followed by 7 days of interval). NMS was used in oncologic setting, where medical oncologists applied conventional capecitabine scheme in patients with HCC who were more likely to tolerate it.

Patients stopped treatment either because of radiological and/or symptomatic progression of HCC (ECOG-PS ≥3, ≥2 unit increase of Child-Pugh score), or because of the occurrence of unacceptable toxicity. Several patients continued capecitabine despite proved radiological tumor progression because they were still receiving clinical benefit from the treatment. Drug-related adverse events (classified according to the National Cancer Institute Common Terminology Criteria for Adverse events, NCI-CTCAE version 4.03) were properly managed with supportive therapy, dose reduction or drug interruption.

Diagnosis of HCC was obtained according to European guidelines available at the time of diagnosis (46,47).

Before starting capecitabine treatment, HCC was staged by multiphase chest and abdomen compute tomography (CT) scan or magnetic resonance (MRI) according to the BCLC staging system (48). Additional investigations were performed, when clinically indicated.

Patients underwent clinical follow-up every 2 months; for the first 2 months of treatment, patients were monitored with laboratory tests (blood count) every 15 days to assess the onset of early hematological adverse events (AEs). Imaging exams (abdomen ultrasound, CT or MRI) were repeated every 2–3 months or more frequently when clinically indicated.

Tumor response was evaluated according to modified Response Evaluation Criteria in Solid Tumor (mRECIST) (49). In patients who had no imaging after capecitabine treatment (25 patients, 18%), the progression was defined according to biochemical (alpha-fetoprotein [AFP]) or clinical parameters.

All patients, at the beginning of treatment, signed a center specific informed consent agreeing to receive treatment and to the anonymized collection of their clinical data. The study was conducted in accordance to the ethical guidelines of the 1975 declaration of Helsinki.

Statistical analysis

Continuous data were expressed as mean ± standard deviation (SD) or median and intervals, while discrete variables as absolute value and relative frequency. Student's t test was used to compare continuous data, χ^2 Pearson's test and Fisher's test to compare discrete data.

Overall survival (OS) was calculated from the date of the beginning of capecitabine treatment to death, with values censored at 31st December 2017 (end of the study) or at the last evaluation. Time to progression (TTP) was calculated from the date of the beginning of capecitabine treatment to the first evidence of cancer progression. OS and TTP were expressed in months as median values with 95% confidence interval (CI). Survival curves were estimated using Kaplan–Meier method and were compared with the Log-rank test.

To compare MS and NMS groups minimizing the confounding effects of the different distribution of baseline characteristics, a propensity score matching with the method of inverse probability weighting (IPW) was performed. MS and NMS patients were matched for: presence of cirrhosis, etiology of liver disease, Child- Pugh score, ECOG-performance status, number of liver lesions, diameter of the largest liver lesion, presence of macrovascular invasion (MVI), presence of extrahepatic spread (EHS), BCLC stage and line of capecitabine therapy. For variable balance assessment the standardized difference between the two groups and the variance ratio were used. For a proper balance in the variables, an absolute standardized difference ≤0.25 and a variance ratio between 0.5 and 2 were considered (50). Kaplan–Meier method and log-rank tests were used to estimate and compare the curves using the weights obtained with the IPW method.

Variables associated with survival at the univariate analysis were included in the Cox multivariate regression model to establish the independent prognostic predictors.

Statistical significance was met with a 2-tailed p value < 0.05. All statistical analysis were performed using StatsDirect ver. 3.1.14- 2017 (StastDirect Ltd, Cheshire, UK) and SAS/STAT® ver.14.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

The 143 patients were treated with capecitabine in first (30%), second (52%) and third line (18%). Capecitabine was administered instead of sorafenib in first line because of Child-Pugh B (30 pts, 70%), compromised clinical conditions (6 pts, 14%) and other contraindications to anti-angiogenetics (7pts, 16%). Among patients treated with capecitabine in second and third line, 21 (28%) and 3 (12%) were Child-Pugh B, respectively (Table 1).

Table 1. Systemic therapy and line of treatment with capecitabine. In the table are also shown Child-Pugh class and ECOG-PS according to the line of capecitabine treatment.

Systemic Therapy				
	All patients	**First-line**	**Second-line**	**Third-line**
Sorafenib	90	87	3	0
Chemotherapy + RCTs/experimental therapies	31	13	22[a]	0
Capecitabine	143	43	75	25
Child-Pugh class and ECOG-PS according to line of capecitabine treatment				
		First-line (n=43)	**Second-line (n=75)**	**Third-line (n=25)**
Child-Pugh	A B	13 (30) 30 (70)	54 (72) 21 (28)	22 (88) 3 (12)
ECOG-PS	0 1 2	14 (33) 23 (53) 6 (14)	32 (43) 33 (44) 10 (13)	16 (64) 9 (36) -

[a] 4 patients underwent chemotherapy in first and second line
Abbreviations: RCTs, randomized controlled trials; ECOG-PS, Eastern Cooperative Oncology Group performance status.

Biological, clinical and tumor characteristics of included patients are reported in Table 2. Of our cohort, 111 patients (78%) were treated with MS and 32 (22%) with NMS. Male sex accounted for approximately 80% in both groups while MS patients were slightly older than NMS. MS patients had more frequently HCC on a cirrhotic liver (p=0.0017) and had more often a viral etiology of the underling liver disease (p=0.0026). NMS patients had a better Child-Pugh score (p=0.02), better ECOG-PS (p<0.0001) and less advanced tumors, with smaller lesions (p=0.0002) and less frequent MVI (p<0.0001). Lastly, NMS-treated patients had a better cancer stage according to the BCLC stage (p=0.0009).

Table 2. Patients' clinical, biological and tumoral characteristics, also according to their treatment schedule.

		All patients (n=143)	**MS (n=111)**	**NMS (n=32)**	**p**
Sex	Males Females	113 (79) 30 (21)	87 (78) 24 (22)	26 (81) 6 (19)	0.60
Age (mean ± SD)		65.8 ± 10.9	66.7 ± 10.0	62.6 ± 13.0	0.05
Cirrhosis	Yes No HCC post-LT	112 (78) 13 (9) 18 (13)	92 (83) 9 (8) 10 (9)	20 (61) 4 (13) 8 (26)	0.002
Etiology	Alcohol HBV HCV Other causes Multiple causes	36 (25) 12 (8) 57 (40) 14 (10) 24 (17)	23 (21) 8 (7) 48 (43) 13 (12) 19 (17)	13 (41) 4 (12) 9 (28) 1 (3) 5 (16)	0.003

Child-Pugh class	A B	89 (62) 54 (38)	65 (59) 46 (41)	24 (75) 8 (25)	0.02
ECOG-PS	0 1 2	65 (46) 62 (43) 16 (11)	37 (33) 59 (53) 15 (14)	28 (86) 3 (10) 1 (4)	< 0.0001
Number	< 3 3-5 > 5	46 (32) 15 (10) 82 (58)	33 (30) 11 (10) 67 (60)	13 (40) 4 (13) 15 (47)	0.18
Diameter (cm)	< 3 3-5 > 5	44 (31) 35 (24) 64 (45)	28 (25) 27 (24) 56 (51)	16 (50) 8 (25) 8 (25)	0.0002
MVI	Yes No	56 (39) 87 (61)	50 (45) 61 (55)	6 (18) 26 (82)	< 0.0001
EHS	Yes No	74 (52) 69 (48)	54 (49) 57 (51)	20 (63) 12 (37)	0.046
BCLC stage	B C D	12 (8.5) 130 (91) 1 (0.5)	5 (4.5) 105 (94.5) 1 (1)	7 (22) 25 (78) 0 (0)	0.0009

Abbreviations: MS: Metronomic Scheme; NMS: Non-Metronomic Scheme; SD, standard deviation; LT, liver transplantation; HBV, hepatitis B virus; HCV, hepatitis C virus; ECOG-PS: Eastern Oncology Cooperative Group performance status; MVI, macrovascular invasion; EHS, extrahepatic spread; BCLC: Barcelona Clinic Liver Cancer

Survival analysis and response to treatment

Mean length of capecitabine treatment was 5.7 months (95% CI 4.1–7.3). Patients treated with NMS received treatment for a significantly longer period of time (10.2 [95% CI 3.9–16.5] vs. 4.3 months [95% CI 3.4–5.2]; p=0.002).

Considering the entire cohort, the median overall survival (OS) was 6.9 months (95% CI 5.7–8.1) and the median time to progression (TTP) was 2.8 months (95% CI 2.0–3.6). The OS of patients treated in third line was 10.5 months (95% CI 5.3–15.6) vs. 5.4 (95% CI 3.0–7.8) and 6.8 months (95% CI 5.1–8.4) of those treated in first and second line, respectively (p=0.01).

NMS-treated patients achieved a better OS, with a 10.5 (95% CI 7.2–13.7) vs. 5.7 months (95% CI 4.0–7.3) survival (p=0.0005). Patients subsequently treated with additional chemotherapy (i.e., gemcitabine or oxaliplatin) after capecitabine discontinuation had a median OS of 13.8 (95% CI 7.0–20.7) vs. 4.7 months (95% CI 3.6–5.7) of patients undergoing BSC (p<0.0001). Censoring the OS of these patients at the time of the new treatment, the survivals achieved for the NMS and MS treated patients were 6.0 months (95% CI 1.3–10.6) and 4.9 months (95% CI 3.8–5.9), respectively (p=0.03).

Time to progression (TTP) also was significantly longer in NMS than in MS patients: 3.9 months (95% CI 2.2–5.5) vs. 2.5 months (95% CI 1.9–3.1) (p=0.03).

In order to correctly compare the efficacy of MS and NMS, a propensity score analysis with the IPW method was used to minimize the important differences between the two groups at baseline. After balancing for the different prognostic characteristics of patients, the OS of MS and NMS patients was comparable (7.3 months [95% CI 4.6–10.1] vs. 9.6 months [95% CI 5.0–16.8], p=0.9). Also the TTP was not significantly different between the two groups: 4.7 (95% CI 2.6–5.8) for MS and 3.8 months (95% CI 2.2–4.7) for NMS (p=0.9) (Figure 1).

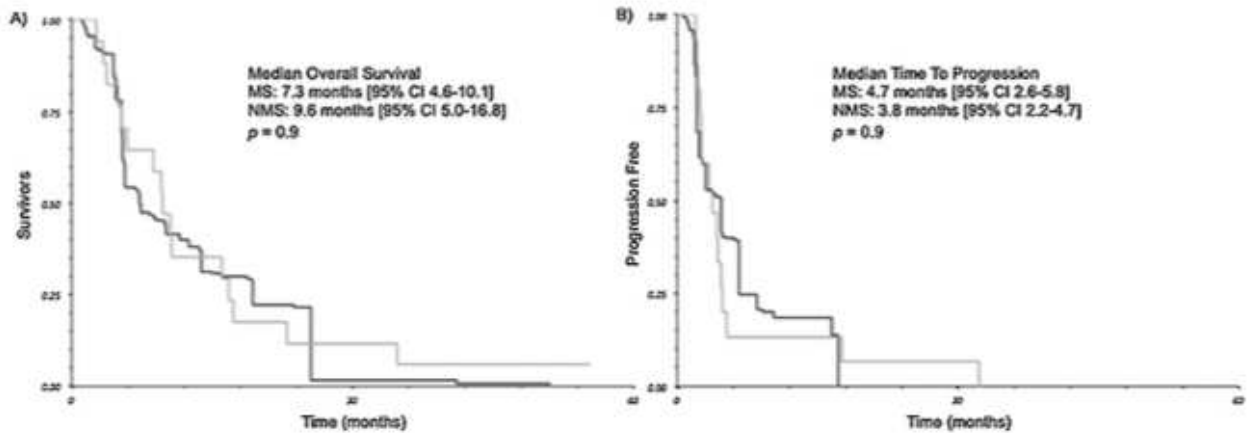

Figure 1. (**A**) Overall survival of patients treated with metronomic scheme (black line) and non-metronomic scheme (grey line) after propensity score matching. (**B**) Time to Progression of patients treated with metronomic scheme (black line) and non-metronomic scheme (grey line) after propensity score matching (MS = metronomic scheme; NMS = non-metronomic scheme).

The response to treatment was assessable in 138 patients (96.5%), because 5 (3.5%) had an early discontinuation due to AEs. The best response to treatment was: complete response (CR) in 2 patients (1.5%), partial response (PR) in 8 patients (5.8%) and stable disease (SD) in 30 patients (21.7%); the other 98 patients (71.0%) progressed during capecitabine treatment. The disease control rate (CR + PR + SD) was 29.0%. NMS guaranteed a better disease control rate than MS (38.8% vs. 26.2%; p=0.04). The response to capecitabine, regardless of the scheme of treatment, correlated

with survival: the OS was 5.4 months in case of progressive disease (PD) (95% CI 4.0–6.8), 9.2 months in SD (95% CI 3.9–14.5), 18.7 months in PR + CR (95% CI 1.9–39.4) (p=0.0002).

Of the 90 patients previously treated with sorafenib, 57 (63%) discontinued this treatment for PD and 33 (37%) for intolerance. A larger survival benefit was demonstrated in those interrupting sorafenib due to intolerance compared to those with cancer progression (8.4 months [95% CI 6.0–10.8] vs. 5.0 months [95% CI 3.6–6.3], p=0.02) (Figure 2).

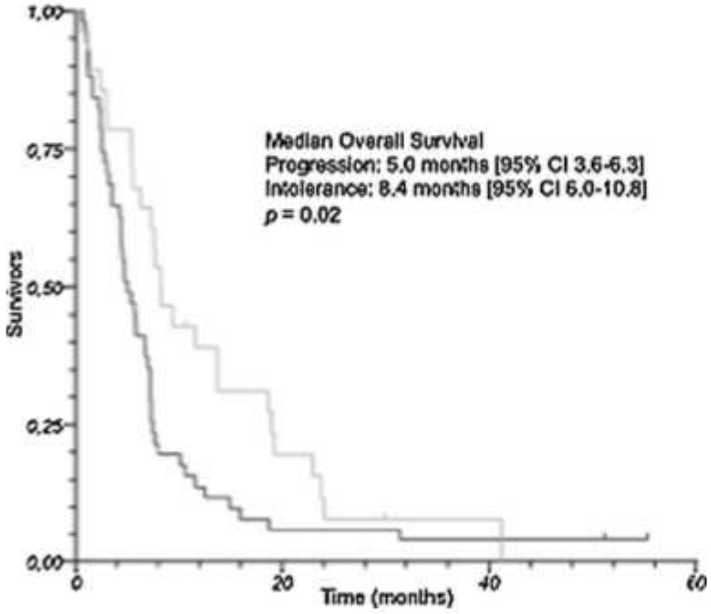

Figure 2. Overall survival of capecitabine treated patients according to causes of sorafenib discontinuation, disease progression (black line) or intolerance (grey line).

At univariate analysis variables associated with OS were Child-Pugh class, ECOG-PS, number of hepatic lesions and diameter of the largest one, presence of MVI, BCLC stage, scheme of capecitabine treatment, response to treatment and additional therapy after capecitabine discontinuation. A multivariate Cox analysis identified only number of hepatic lesions (p=0.002) and therapy after capecitabine (p=0.03) as independent prognostic factors.

A recent study by Giannini et al. (51) describes the “natural history” of patients with advanced hepatocellular carcinoma (BCLC C) undergoing BSC included in the ITA.LI.CA. database. The patients were subgrouped according to the clinical features determining their allocation (ECOG-PS, MVI,

EHS). In our study, due to the lack a control group of BSC patients, we compared the survival of our patients with those reported in the paper by Giannini et al. and depicted in Table 3. Albeit obviously lacking a statistical comparison, the table shows slightly higher survival for capecitabine treated patients. The comparison of the PS1 and PS2 patients is not provided given the limited number of patients belonging to these subgroups.

Table 3. Comparison between BCLC-C patients treated with best supportive care (*Giannini et al.* (51)) and our group of BCLC C patients treated with capecitabine.

BCLC C patients		**Survival (months) in BCLC C patients of *Giannini et al.***	**Survival (months) in our BCLC C group of Capecitabine treated patients (n=130)**
	PS 1	13.2	/
	PS 2	11.2	/
	MVI	4.0	7.0
	EHS	5.2	7.2
	MVI + EHS	2.0	3.5

Abbreviations: BCLC, Barcelona Clinic Liver Cancer; PS, Performance status; MV, Macrovascular invasion; EHS, Extrahepatic spread

Safety

Table 4 reports the adverse events emerged during capecitabine treatment, categorized according to the NCI-CTCAE v 4.03. Overall, 105 patients (73%) experienced at least one AEs of any grade during treatment: 57% had a grade 1–2 AEs while 16% a grade 3–4 AEs. Main drug-related AEs were thrombocytopenia (38%), anemia (34%), fatigue (22%), leucopenia (18%). Abdominal pain, dyspnea, hand-foot skin reaction, nausea/vomiting, diarrhea and mucositis were reported. Drug-related AEs were in most cases mild and were properly managed. In 11 patients (8%), an AE led to treatment discontinuation. In particular, 3 patients interrupted the treatment for anemia, 3 for intolerable fatigue, 2 for thrombocytopenia and 1 patient each for acute kidney injury, cholangitis and pulmonary embolism. No treatment-related deaths were recorded.

AEs were slightly, but not significantly, more frequent in NMS group: 26 NMS patients (81%) developed AEs vs. 80 MS patients (72%) (p=0.133). Grade 3–4 AEs more often occurred in MS patients (14% vs. 6%; p=0.059).

Table 4. Adverse events of capecitabine treatment categorized according to the National Cancer Institute, Common Terminology Criteria for Adverse Events classification.

Adverse events	Any grade	Grade 1-2	Grade 3-4
Overall	105 (73)	82 (57)	23 (16)
Leucopenia	26 (18)	25 (16.3)	1 (0.7)
Thrombocytopenia	54 (38)	50 (35)	4 (3)
Anemia	48 (34)	40 (28)	8 (6)
Fatigue	32 (22)	26 (18)	6 (4)
HFS	5 (3)	5 (3)	/
Nausea/vomiting	5 (3)	5 (3)	/
Abdominal pain	9 (6)	9 (6)	/
Dyspnea	7 (5)	7 (5)	/
Diarrhea	3 (2)	3 (2)	/
Mucositis	3 (2)	3 (2)	/
Pulmonary embolism	1 (0.7)	/	1 (0.7)
Acute kidney injury	1 (0.7)	/	1 (0.7)
Cholangitis	1 (0.7)	/	1 (0.7)
Hypoglycemia	1 (0.7)	/	1 (0.7)

Data are presented as absolute and relative frequency (percentage).

DISCUSSION

Treatment of advanced HCC still represents a difficult issue, mainly due to lack of therapeutic alternatives, given that, at least in Europe, we have only few approved systemic drugs: sorafenib in first line, regorafenib and cabozantinib in second line (10,19,20). Regorafenib has been approved very recently for patients with tumor progression but tolerant to sorafenib. However, a relatively large share of patients (30–40%) are intolerant to sorafenib (7–9) and in these patients only cabozantinib could be an option. Beyond these drugs, other therapeutic alternatives are approaching the market (lenvatinib (18), nivolumab (21), ramucirumab (22), pembrolizumab (23)), but they have still to be approved.

A large number of studies evaluated capecitabine treatment in advanced HCC (28,29,38,30–37). All these studies demonstrated the tolerability of capecitabine, even in patients with slightly impaired hepatic function and the potential activity of the drug in prolonging HCC patient survival.

In our study, we retrospectively analyzed a large series of patients treated with capecitabine: the median OS was 6.9 months and the median TTP was 2.8 months. These results are in line with those previously reported by our group in a smaller series (36), with a shorter survival compared to the literature (34,35,37,38). The explanation for the shorter median survival relies in the more advanced liver disease of our group of patients (38% of patients had a Child-Pugh score B, 54% had an ECOG-PS 1–2, 68% had ≥3 hepatic lesions, 39% had MVI and 52% EHS).

To the best of our knowledge, the conventional and the metronomic scheme have never been compared, even retrospectively. NMS and MS patients in our cohort not only differed markedly in the sample size, but also in their baseline characteristics: patients treated with the NMS had better liver functional reserve, better clinical conditions and less advanced tumors. Without correction, NMS achieved better results in terms of survival, but after balancing the two groups for the different prognostic characteristics with a IPW propensity score analysis, no differences in terms of efficacy were found between the two groups.

Post-capecitabine treatments could influence overall survival. Patients treated with additional systemic chemotherapy after capecitabine discontinuation had a better survival than BSC patients (13.8 vs. 4.7 months; $p<0.0001$). These patients, mostly belonging to the NMS subgroup (53% versus 15% for MS), were apparently fit enough to be treated with additional therapies that prolonged their survival. The impact of the additional therapies was evaluated censoring the OS of patients treated after capecitabine at the time of the new treatment, demonstrating a slightly advantage in terms of OS for NMS.

Response to treatment was associated with survival: patients with complete or partial response had a better survival than patients with stable or progressive disease (18.7 months for PR + CR vs. 9.2 months for SD vs. 5.4 months for PD; p=0.0002).

In our study, we confirmed the findings of the paper by Trevisani et al. (38): in patients previously treated with sorafenib, the greater survival benefit was observed in intolerant patients compared to those with tumor progression (8.4 months vs. 5.0 months; p=0.024). This finding is more relevant if we consider that the only option for patients intolerant to sorafenib is cabozantinib.

Our population of patients has been treated with capecitabine in second-line after sorafenib, in third-line after sorafenib and other chemotherapeutic or experimental drugs and even in first- line for patients that could not be alternatively treated. The longer survival of patients treated in third-line could be again potentially explained by the fact that patients previously treated with sorafenib and included in RCTs, had probably better liver function or slow tumor progression. The higher survival of these patients could be due to the intrinsic characteristics of liver tumor and of cirrhosis, rather than the activity of capecitabine itself.

Capecitabine proved to be a safe option in every study investigating its role in advanced HCC (28,34,35,37,38), even in patients with moderately impaired liver function. Having a therapeutic option for these patients is extremely important because no systemic treatment is currently or will be soon available for advanced HCC patients with impaired liver function (Child-Pugh B). Recently, a paper by De Lorenzo et al. (52) evaluated activity and safety of metronomic capecitabine in a cohort of Child-Pugh B patients and, similarly to what we reported in this study, concluded in favor of the safety and efficacy of the drug.

Despite a higher rate of adverse events compared to other studies (35,37) (73% of patients experienced at least one AEs), these were mainly mild (grade 1–2 in 56% and grade 3–4 in only 16%) and easily manageable. No treatment-related death was observed and only in 11 patients (8%) the

treatment was discontinued for AEs. The higher rate of grade 3–4 adverse events in MS group (MS 14% vs. NMS 6%) can be due to the worse liver function and/or clinical condition of these patients. Nature of adverse events recorded in our study was comparable to that reported in literature (35,37,38), even though with a higher rate of hematological problems. The mean length of treatment in our cohort (5.7 months) confirms once more the tolerability of capecitabine.

Lastly, it should be emphasized that capecitabine is an inexpensive treatment and represent an alternative for advanced HCC easily sustainable by most National Health Systems.

Our study has several limitations, the first of which is its retrospective nature that may have introduced unintentional biases in the analysis. The study population, although the largest in literature at present, is very heterogeneous and this obviously may affect the results interpretation. Nevertheless, our population offers a “real-world” image of the management of patients with advanced HCC, covering most of the clinical situations we are facing in everyday clinical practice.

Another limit of our study is the lack of a control group of patients treated with BSC. The indirect comparison of survivals in our series with that of patients with advanced HCC undergoing BSC belonging to the ITA.LI.CA database (51), seems to suggest that capecitabine could potentially represent an active treatment.

In conclusion, the results obtained in our large retrospective study are in line with what previously reported in literature regarding efficacy and safety of capecitabine in advanced HCC. Metronomic and non-metronomic schemes seem to be equally effective, and the indirect comparison with the BSC-managed ITA.LI.CA patients confirms that capecitabine could be a reasonable option in patients who are not candidate for other treatments. Patients discontinuing sorafenib for intolerance is the group in whom capecitabine is more active. Capecitabine confirmed to be a well-tolerated and a safe treatment in patients with advanced HCC, also in those with impaired liver function. These results need to be confirmed through adequately planned trials, in order to establish the precise

role of capecitabine in the therapeutic management of patients with advanced HCC in relation to sorafenib and other emerging systemic targeted therapies.

REFERENCES

1. Akinyemiju T, Abera S, Ahmed M, Alam N, Alemayohu MA, Allen C, et al. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015. JAMA Oncol. 2017;3:1683–1691.

2. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology. 2012;142:1264-1273.e1.

3. Singal AG, El-Serag HB. Hepatocellular Carcinoma From Epidemiology to Prevention: Translating Knowledge into Practice. Clin. Gastroenterol. Hepatol. 2015;13:2140–2151.

4. Santi V, Trevisani F, Gramenzi A, Grignaschi A, Mirici-Cappa F, Del Poggio P, et al. Semiannual surveillance is superior to annual surveillance for the detection of early hepatocellular carcinoma and patient survival. J. Hepatol. 2010;53:291–297.

5. Trevisani F, Santi V, Gramenzi A, Di Nolfo MA, Del Poggio P, Benvegnu L, et al. Surveillance for early diagnosis of hepatocellular carcinoma: is it effective in intermediate/advanced cirrhosis? Am. J. Gastroenterol. 2007;102:2448–57.

6. Galle PR, Forner A, Llovet JM, Mazzaferro V, Piscaglia F, Raoul JL, et al. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2018;69:182–236.

7. Iavarone M, Cabibbo G, Piscaglia F, Zavaglia C, Grieco A, Villa E, et al. Field-practice study of sorafenib therapy for hepatocellular carcinoma: A prospective multicenter study in Italy. Hepatology. 2011;54:2055–2063.

8. Marrero JA, Kudo M, Venook AP, Ye SL, Bronowicki JP, Chen XP, et al. Observational registry of sorafenib use in clinical practice across Child-Pugh subgroups: The GIDEON study. J. Hepatol. 2016;65:1140–1147.

9. Lencioni R, Kudo M, Ye SL, Bronowicki JP, Chen XP, Dagher L, et al. GIDEON (Global Investigation of therapeutic DEcisions in hepatocellular carcinoma and of its treatment with sorafeNib): Second interim analysis. Int. J. Clin. Pract. 2014;68:609–617.

10. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J-F, et al. Sorafenib in advanced hepatocellular carcinoma. N. Engl. J. Med. 2008;359:378–390.

11. Cheng A-L, Kang Y-K, Lin D-Y, Park J-W, Kudo M, Qin S, et al. Sunitinib Versus Sorafenib in Advanced Hepatocellular Cancer: Results of a Randomized Phase III Trial. J. Clin. Oncol. 2013;31:4067–4075.

12. Johnson PJ, Qin S, Park J-W, Poon RTP, Raoul J-L, Philip PA, et al. Brivanib Versus Sorafenib As First-Line Therapy in Patients With Unresectable, Advanced Hepatocellular Carcinoma: Results From the Randomized Phase III BRISK-FL Study. J. Clin. Oncol. 2013;31:3517–3524.

13. Cainap C, Qin S, Huang W-T, Chung IJ, Pan H, Cheng Y, et al. Linifanib Versus Sorafenib in Patients With Advanced Hepatocellular Carcinoma: Results of a Randomized Phase III Trial. J. Clin. Oncol. 2015;33:172–179.

14. Llovet JM, Decaens T, Raoul JL, Boucher E, Kudo M, Chang C, et al. Brivanib in patients with advanced hepatocellular carcinoma who were intolerant to sorafenib or for whom sorafenib failed: Results from the randomized phase III BRISK-PS study. J. Clin. Oncol. 2013;31:3509–3516.

15. Zhu AX, Kudo M, Assenat E, Cattan S, Kang Y-K, Lim HY, et al. Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: the EVOLVE-1 randomized clinical trial. JAMA. 2014;312:57–67.

16. Zhu AX, Park JO, Ryoo B-Y, Yen C-J, Poon R, Pastorelli D, et al. Ramucirumab versus placebo as second-line treatment in patients with advanced hepatocellular carcinoma following first-line therapy with sorafenib (REACH): a randomised, double-blind, multicentre, phase 3 trial. Lancet Oncol. 2015;16:859–870.

17. Rimassa L, Assenat E, Peck-Radosavljevic M, Pracht M, Zagonel V, Mathurin P, et al. Tivantinib for second-line treatment of MET-high, advanced hepatocellular carcinoma (METIV-HCC): a final analysis of a phase 3, randomised, placebo-controlled study. Lancet Oncol. 2018;19:682–693.

18. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018;391:1163–1173.

19. Bruix J, Qin S, Merle P, Granito A, Huang YH, Bodoky G, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase

3 trial. Lancet. 2017;389:56–66.

20. Abou-Alfa GK, Meyer T, Cheng A-L, El-Khoueiry AB, Rimassa L, Ryoo B-Y, et al. Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. N. Engl. J. Med. 2018;379:54–63.

21. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. Lancet. 2017;389:2492–2502.

22. Zhu AX, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, et al. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2019;20:282–296.

23. Zhu AX, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, et al. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. Lancet Oncol. 2018;19:940–952.

24. Ge S, Huang D. Systemic therapies for hepatocellular carcinoma. Drug Discov. Ther. 2015;9:352–362.

25. Ishikawa T, Utoh M, Sawada N, Nishida M, Fukase Y, Sekiguchi F, et al. Tumor selective delivery of 5-fluorouracil by capecitabine, a new oral fluoropyrimidine carbamate, in human cancer xenografts. Biochem. Pharmacol. 1998;55:1091–1097.

26. Miwa M, Ura M, Nishida M, Sawada N, Ishikawa T, Mori K, et al. Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. Eur. J. Cancer. 1998;34:1274–1281.

27. Schuller J, Cassidy J, Dumont E, Roos B, Durston S, Banken L, et al. Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients. Cancer Chemother. Pharmacol. 2000;45:291–297.

28. Patt YZ, Hassan MM, Aguayo A, Nooka AK, Lozano RD, Curley SA, et al. Oral capecitabine for the treatment of hepatocellular carcinoma, cholangiocarcinoma, and gallbladder carcinoma. Cancer. 2004;101:578–586.

29. Lee JO, Lee KW, Oh DY, Kim JH, Im SA, Kim TY, et al. Combination chemotherapy with capecitabine and cisplatin for patients with metastatic hepatocellular carcinoma. Ann. Oncol. 2009;20:1402–1407.

30. Xia Y, Qiu Y, Li J, Shi L, Wang K, Xi T, et al. Adjuvant therapy with capecitabine postpones recurrence of hepatocellular carcinoma after curative resection: A randomized controlled trial. Ann. Surg. Oncol. 2010;17:3137–3144.

31. He SL, Shen J, Sun XJ, Zhu XJ, Liu LM, Dong JC. Efficacy of capecitabine and oxaliplatin regimen for extrahepatic metastasis of hepatocellular carcinoma following local treatments. World J. Gastroenterol. 2013;19:4552–4558.

32. Abdel-Rahman O, Abdel-Wahab M, Shaker M, Abdel-Wahab S, Elbassiony M, Ellithy M. Sorafenib versus capecitabine in the management of advanced hepatocellular carcinoma. Med. Oncol. 2013;30.

33. Farrag A. Efficacy and Toxicity of Metronomic Capecitabine in Advanced Hepatocellular Carcinoma *. J. Cancer Ther. 2012;2012:71–77.

34. Brandi G, de Rosa F, Agostini V, di Girolamo S, Andreone P, Bolondi L, et al. Metronomic capecitabine in advanced hepatocellular carcinoma patients: a phase II study. Oncologist. 2013;18:1256–1257.

35. Granito A, Marinelli S, Terzi E, Piscaglia F, Renzulli M, Venerandi L, et al. Metronomic capecitabine as second-line treatment in hepatocellular carcinoma after sorafenib failure. Dig. Liver Dis. 2015;47:518–522.

36. Murer F, Pozzan C, Peserico G, Farinati F. Capecitabine in advanced hepatocellular carcinoma. Dig. Liver Dis. 2016;48:1260–1261.

37. Casadei Gardini A, Foca F, Scartozzi M, Silvestris N, Tamburini E, Faloppi L, et al. Metronomic capecitabine versus best supportive care as second-line treatment in hepatocellular carcinoma: a retrospective study. Sci. Rep. 2017;7:42499.

38. Trevisani F, Brandi G, Garuti F, Barbera MA, Tortora R, Casadei Gardini A, et al. Metronomic capecitabine as second-line treatment for hepatocellular carcinoma after sorafenib discontinuation. J. Cancer Res. Clin. Oncol. 2018;144:403–414.

39. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J. Clin. Invest. 2000;105:1045–1047.

40. Kamen BA, Rubin E, Aisner J, Glatstein E. High-Time Chemotherapy or High Time for Low Dose. J. Clin. Oncol.

2000;18:2935–2937.

41. Gasparini G. Metronomic scheduling: the future of chemotherapy? Lancet Oncol. 2001;2:733–740.

42. Cramarossa G, Lee EK, Sivanathan L, Georgsdottir S, Lien K, Santos KD, et al. A systematic literature analysis of correlative studies in low-dose metronomic chemotherapy trials. Biomarkers Med. 2014;8:893–911.

43. Browder T, Butterfield CE, Kräling BM, Shi B, Marshall B, O'Reilly MS, et al. Antiangiogenic Scheduling of Chemotherapy Improves Efficacy against Experimental Drug-resistant Cancer. Cancer Res. 2000;60:1878 LP – 1886.

44. Pasquier E, Kavallaris M, André N. Metronomic chemotherapy: New rationale for new directions. Nat. Rev. Clin. Oncol. 2010;7:455–465.

45. Kareva I, Waxman DJ, Klement GL. Metronomic chemotherapy: An attractive alternative to maximum tolerated dose therapy that can activate anti-tumor immunity and minimize therapeutic resistance. Cancer Lett. 2015;358:100–106.

46. Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J. Hepatol. 2001;35:421–430.

47. Llovet JM, Ducreux M, Lencioni R, Di Bisceglie AM, Galle PR, Dufour JF, et al. EASL-EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. J. Hepatol. 2012;56:908–943.

48. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005;42:1208–1236.

49. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. Semin. Liver Dis. 2010;30:52–60.

50. Stuart EA. Matching methods for causal inference: A review and a look forward. Stat. Sci. 2010;25:1–21.

51. Giannini EG, Bucci L, Garuti F, Brunacci M, Lenzi B, Valente M, et al. Patients with advanced hepatocellular carcinoma need a personalized management: A lesson from clinical practice. Hepatology. 2018;67:1784–1796.

52. De Lorenzo S, Tovoli F, Barbera MA, Garuti F, Palloni A, Frega G, et al. Metronomic capecitabine vs . best supportive care in Child-Pugh B hepatocellular carcinoma : a proof of concept. Sci. Rep. 2018;8:4–10.

CHAPTER 16

Conclusions

Filippo Pelizzaro

CONCLUSIONS

In this thesis, I discussed previous data regarding the current knowledge on circulating biomarkers in HCC patients and I reported the results on several potential circulating molecules that can be useful in stratification of patient prognosis, focusing in particular on patients treated with transarterial chemoembolization (TACE). Indeed, I assessed the prognostic value of SCCA-IgM, a marker that has already demonstrated to be potentially useful in survival prediction, demonstrating that its interpretation should consider also the gender of patients. Subsequently, I demonstrated the utility of VEGF and HIF-1α, two of the most important molecules involved in neoangiogenesis triggered by TACE, in the prediction of response to treatment and survival. Then, miR-21 and miR-122 were investigated as circulating prognostic biomarkers in TACE-treated patients, confirming their potential role as survival predictors. Stimulated by these results, and considering also the inconsistent evidence present in the literature, I also evaluated the changes of circulating miR-21 in patients with chronic liver diseases and HCC. The results obtained demonstrated that this biomarker probably is not ideal for HCC diagnosis, but it is correlated with the severity of liver fibrosis, necroinflammatory activity and residual liver function, potentially being a useful molecule for having some insights on different aspects of chronic liver diseases. Lastly, I moved to evaluate inflammatory biomarkers, demonstrating that prostaglandin E_2 has a potential role in predicting the survival in patients treated with TACE and that inflammatory-based scores (PLR and NLR) accurately reflect the prognosis of patients regardless of treatment received and in some therapy subgroups.

Taken together, these studies provide a solid background to further investigate these molecules as prognostic biomarkers in patients with HCC. Nevertheless, prognostic stratification of HCC patients is a very complicated issue. Therefore, more efforts and prospective validation are needed to identify markers accurate enough for introduction into clinical practice.

In the second part of this thesis, I moved to evaluate some clinical factors that can influence the prognosis of HCC patients. Surveillance in patients at risk of liver cancer is worldwide recommended in order to reduce cancer-related mortality, through early diagnosis and application of potentially curative treatments. In this thesis, I demonstrated that regular surveillance is fundamental for achieving long-term survival of HCC patients. However, I proved also that the tightening of surveillance interval to 3-months in patients considered at higher risk did not reveal to be associated with better prognosis compared to the standard schedule (6 months).

In patients with cancer, staging systems are fundamental to provide an accurate prognostic prediction. Here, I demonstrated that a size threshold should be introduced for monofocal tumors in the BCLC staging system, the most commonly used in HCC. Indeed, despite being classified in early stage, large monofocal tumors have a prognosis more similar to intermediate stage cancers. Nevertheless, according to the principle of "therapeutic hierarchy", liver resection in associated with longer survival compared to other treatments also in these patients and should be offered whenever possible.

Lastly, I presented some data regarding treatment of HCC patients, which is the most important determinant of prognosis. TACE has still an important role in the management of liver cancer patients, even though its application declined compared to the past and, at least in expert centers, deviated from guidelines recommendations in a large proportion of patients. Even though survival of TACE-treated patients improved over time, suggesting a better patient selection as well as technical advancements, potentially curative therapies provide a greater survival benefit and should be preferred whenever feasible. Then, I reported data on capecitabine treatment in advanced HCC, showing that it is safe and effective. Despite the remarkable expansion of systemic therapy possibilities, capecitabine could be considered an option for patients who cannot be otherwise treated.

In conclusion, in this thesis I provided some evidence in favor of several prognostic biomarkers and investigated some aspects of surveillance, staging and treatment, all well-known prognostic factors. Despite HCC is still burdened by a poor prognosis in the great majority of cases, research efforts to identify biomarkers useful in stratifying patients' survival and possibly in guiding the management, define of the optimal surveillance strategy, refine staging and develop appropriate treatments are essential to improve the prognosis of these patients.

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Chapter 3

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Chapter 5

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Chapter 7

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Chapter 9

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Chapter 11

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Chapter 12

This chapter is in press in Digestive and Liver Disease, Filippo Pelizzaro, Giulia Peserico, Marco D'Elia, Nora Cazzagon, Francesco Paolo Russo, Alessandro Vitale, Edoardo G. Giannini, Manuela Piccinnu, Gian Ludovico Rapaccini, Maria Di Marco, Eugenio Caturelli, Marco Zoli, Rodolfo Sacco, Giuseppe

Cabibbo, Fabio Marra, Andrea Mega, Filomena Morisco, Antonio Gasbarrini, Gianluca Svegliati-Baroni, Francesco Giuseppe Foschi, Andrea Olivani, Alberto Masotto, Gerardo Nardone, Giovanni Raimondo, Francesco Azzaroli, Gianpaolo Vidili, Filippo Oliveri, Franco Trevisani, Fabio Farinati for the Italian Liver Cancer (ITA.LI.CA) Study Group. Surveillance for hepatocellular carcinoma with a 3-months interval in "extremely high-risk" patients does not further improve survival. doi: 10.1016/j.dld.2021.08.025. Copyright Elsevier Ltd (2021)

Chapter 13

This chapter was published in Liver International, Vol. 41, Filippo Pelizzaro, Barbara Penzo, Giulia Peserico, Angela Imondi, Anna Sartori, Alessandro Vitale, Umberto Cillo, Edoardo G. Giannini, Antonella Forgione, Gian Ludovico Rapaccini, Maria Di Marco, Eugenio Caturelli, Marco Zoli, Rodolfo Sacco, Giuseppe Cabibbo, Fabio Marra, Andrea Mega, Filomena Morisco, Antonio Gasbarrini, Gianluca Svegliati-Baroni, Francesco Giuseppe Foschi, Andrea Olivani, Alberto Masotto, Gerardo Nardone, Giovanni Raimondo, Francesco Azzaroli, Gianpaolo Vidili, Filippo Oliveri, Franco Trevisani, Fabio Farinati on behalf of Italian Liver Cancer (ITA.LI.CA) group. Monofocal hepatocellular carcinoma: how much does size matter? Pages 396-407, doi: 10.1111/liv.14718. Published by John Wiley & Sons Ltd (2021)

Chapter 14

Material from: Filippo Pelizzaro, Selion Haxhi, Barbara Penzo, Alessandro Vitale, Edoardo G. Giannini, Vito Sansone, Gian Ludovico Rapaccini, Maria Di Marco, Eugenio Caturelli, Donatella Magalotti, Rodolfo Sacco, Ciro Celsa, Claudia Campani, Andrea Mega, Maria Guarino, Antonio Gasbarrini, Gianluca Svegliati-Baroni, Francesco Giuseppe Foschi, Andrea Olivani, Alberto Masotto, Gerardo Nardone, Giovanni Raimondo, Francesco Azzaroli, Gianpaolo Vidili, Maurizia Rossana

Brunetto, Franco Trevisani, Fabio Farinati for the Italian Liver Cancer (ITA.LI.CA) group. Transarterial chemoembolization for hepatocellular carcinoma in clinical practice: temporal trends and survival outcomes of an iterative treatment. Frontiers in Oncology, accepted manuscript.

Chapter 15

This chapter was published in Digestive and Liver Disease, Vol. 51, Filippo Pelizzaro, Ambra Sammarco, Vincenzo Dadduzio, Davide Pastorelli, Petros Giovanis, Caterina Soldà, Mario Domenico Rizzato, Giuseppe Lombardi, Sara Lonardi, Giulia Peserico, Angela Imondi, Anna Sartori, Gemma Maddalo, Fabio Farinati. Capecitabine in advanced hepatocellular carcinoma: a multicenter experience. Pages 1713-1719, doi: 10.1016/j.dld.2019.06.015. Copyright Elsevier Ltd (2019)