

## 2016 Hepatocellular Carcinoma: Global view

# Prediction of hepatocellular carcinoma biological behavior in patient selection for liver transplantation

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## Abstract

Morphological criteria have always been considered the benchmark for selecting hepatocellular carcinoma (HCC) patients for liver transplantation (LT). These criteria, which are often inappropriate to express the tumor's biological behavior and aggressiveness, offer only a static view of the disease burden and are frequently unable to correctly stratify the tumor recurrence risk after LT. Alpha-fetoprotein (AFP) and its progression as well as AFP-mRNA, AFP-L3%, des-γ-carboxyprothrombin, inflammatory markers and other serological tests appear to be correlated with post-transplant outcomes. Several other markers for patient selection including functional imaging studies such as <sup>18</sup>F-FDG-PET imaging, histological evaluation of tumor grade, tissue-specific biomarkers, and molecular signatures have been outlined in the literature. HCC growth rate and response to pre-transplant therapies can further contribute to the transplant evaluation process of HCC patients. While AFP, its progression, and HCC response to pre-transplant therapy have already been used as a part of an integrated prognostic model for selecting patients, the utility of other markers in the transplant setting is still under investigation. This article intends to review the data in the literature concerning predictors that could be included in an integrated LT selection model and to evaluate the importance of biological aggressiveness in the evaluation process of these patients.

**Key words:** Hepatocellular carcinoma; Liver Alpha-fetoprotein; Transplantation; Biomarkers; Histopathology; Recurrence; Integrated prognostic tool

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**Core tip:** An integrated model predicting post-transplant survival of hepatocellular carcinoma patients after

liver transplantation has not yet been defined. Current selection criteria for liver transplantation that do not consider its biological aggressiveness are mainly based on morphological tumor markers that offer only a static view of the tumor. Many biomarkers predicting post-transplant outcome and stratifying those patients who are candidates for liver transplantation are under evaluation. An integrated prognostic model will make it possible to quantify the tumor burden *via* functional imaging modalities as well as biological markers.

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## INTRODUCTION

Liver transplantation is the gold standard treatment for selected patients with hepatocellular carcinoma (HCC) as it cures both the tumor and the underlying liver cirrhosis. Since widespread use of liver transplantation (LT) is still limited due to organ shortage<sup>[1]</sup>, reliable patient selection criteria are critical to maximize LT survival benefit. The equipoise between the impact that decision will have on the patients remaining on the waiting list and on the recipient him/herself must be based on reliable predictors of post-transplant outcome.

Explant pathology features constitute a direct expression of the tumor's biological aggressiveness and, in particular, of micromacrovascular invasion and dedifferentiated grading. The former, in particular, represents the most important marker of HCC aggressiveness<sup>[2-6]</sup>, Iwatsuki *et al.*<sup>[7]</sup> demonstrated a more than 4-fold increased risk of recurrence following transplant when microvascular invasion is detected. Although associated with an excellent ability to predict post-transplant HCC recurrence, microvascular invasion is difficult to detect at the pre-operative biopsy. Crucial information needed to predict the outcome and to guide the decision-making process about listings becomes available, therefore, only after the explant specimen has undergone pathologic examination. Although research has been attempting to identify new makers, gross morphology has until now been considered the primary prognostic parameter. Strict adherence to macromorphological criteria (*i.e.*, the Milan criteria) has been considered the best selection criteria for HCC patients in the transplant setting since the sizes and the number of the nodules are considered the best surrogates for microvascular invasion. Indeed, nodule size and number are the worldwide standard for patient selection in most centers. The Milan, the University of California, San

Francisco (UCSF), the total tumor volume (TTV) and the Up-to-7, which<sup>[8,9]</sup> are associated with good overall middle to long term disease-free survival (DFS) rates after transplantation<sup>[10,11]</sup>, are the most frequently used and scientifically validated morphology-based criteria.

It is widely recognized, nevertheless, that use of pre-transplant macromorphological criteria in the selection process of LT candidates poses a number of relevant drawbacks. It has likewise been shown that in a large proportion of patients (up to 15%-25%)<sup>[12]</sup>. There is a significant discrepancy between pre-transplant radiologic staging and explant pathology. These findings are partially attributable to the time-lapse between the last radiologic evaluation and the transplant itself during which the tumor and the staging may have progressed. The discrepancy may also be explained by inaccurate radiological data leading to an underestimation of nodule number/size. There could also be an incomplete overlapping of macromorphological traits and absence of microvascular invasion. In fact, although infrequent, small paucinodular HCC may present biologically aggressive features that would seem to predict an unfavorable post-transplant outcome. Finally, and more importantly, the strict adoption of macromorphological criteria could lead to the exclusion of a relevant number of patients who could, instead, benefit from transplant<sup>[13-17]</sup>. Despite being outside current transplant criteria, some multinodular HCCs and also, to a lesser extent, relatively large tumors, have been shown to possess a favorable biological behavior and an acceptable long term post-transplant DFS. In view of these findings, several clinical trials have focused on expanding the Milan criteria<sup>[9,18]</sup>. According to "the metroticket concept"<sup>[8]</sup>, nevertheless, the further HCC staging criteria is expanded for LT, the greater the cost will be in terms of higher recurrence. Conversely, when morphological selection protocols such as the Milan one include other recurrence predictors, their performance in predicting tumor recurrence seems to be improved<sup>[19,20]</sup>, thus suggesting that other prognostic factors do exist and may be useful in improving prognostic accuracy. These considerations underline how a better understanding of the proliferative activity of HCC tumors can help to improve the selection criteria for LT and optimize resource allocation.

Although a definitive characterization is at yet unattainable, much light has recently been shed on the assessment of HCC tumor aggressiveness; these data and our own findings are the object of the present article, which has been divided into five sections: (1) biohumoral markers; (2) radiologic features; (3) histology; (4) response to therapy; and (5) tumor doubling times.

## BIOHUMORAL MARKERS

### AFP

First described by Abelev *et al.*<sup>[21]</sup> in 1963, alpha-fetoprotein (AFP) is a usually fetal-specific glycoprotein

**Table 1** Hepatocellular carcinoma patients' pre-transplant  $\alpha$ -fetoprotein level, outcome, and biological features according to some recent studies

Ref.	No. of patients	Static AFP cut-off (mg/L)	Dynamic AFP	Outcome for increasing AFP ranges	P value	Other biological features
Berry <i>et al</i> <sup>[31]</sup> (2013)	8659	$\leq 15$ , 16-66, 66-320, > 320	-	6 yr OS: from 70% to 60%, 57%, 51%	-	-
Toso <i>et al</i> <sup>[26]</sup> (2011)	6478	$\leq 100$ , 100-500, > 500	-	3 yr OS: from 71% to 60%, 51%	< 0.001	-
Mailey <i>et al</i> <sup>[32]</sup> (2011)	2253	$\leq 20$ , > 400	-	4 yr OS: from 76% to 54%	< 0.001	-
Duvoux <i>et al</i> <sup>[20]</sup> (2012)	1033	$\leq 100$ , 100-1000, > 1000	-	5 yr OS: from 68% to 51%, 39%	< 0.001	VI, poor differentiation
Todo <i>et al</i> <sup>[30]</sup> (2007)	653	$\leq 200$ , > 1000	-	5 yr OS: from 73% to 34%	< 0.001	-
Fujiki <i>et al</i> <sup>[41]</sup> (2009)	144	$\leq 200$ , > 800	-	5 yr RFS: from 90% to 40%	0.003	VI, poor differentiation
Sotiropoulos <i>et al</i> <sup>[12]</sup>	100	$\leq 20$ , 20-200, 200-1000, > 1000	-	5 yr RFS: from 97% to 60%, 57%, 51%	0.0003	-
Hameed <i>et al</i> <sup>[45]</sup>	211	$\leq 1000$ , > 1000	-	5-yr RFS: from 80.3% to 52.7%	0.025	VI
Kondili <i>et al</i> <sup>[46]</sup>	32	-	grate increasing, low increasing	In 5 Patients with recurrence AFP increased at a greater magnitude than in 27 without recurrence	-	-
Han <i>et al</i> <sup>[28]</sup>	48	-	$\leq 50$ mg/L per month, > 50 mg/L per month	1 yr RFS: from 90% to 40%	< 0.001	VI
Vibert <i>et al</i> <sup>[29]</sup>	153	-	$\leq 15$ mg/L per month, > 15 mg/L per month	5 yr RFS: from 76% to 54%	0.01	VI
Merani <i>et al</i> <sup>[47]</sup>	6817	-	Stable, $\geq 400$ , downstaged to < 400	ITT survival: from 81% to 48%	< 0.001	-

OS: Overall survival rate; RFS: Recurrence-free survival rate; ITT: Intention-to-treat; VI: Vascular invasion.

whose importance in the diagnosis of HCC is well established<sup>[22-25]</sup>. Increasing evidence<sup>[8,11,26-29]</sup> suggests, moreover, that it also has a role in predicting outcome after LT. Since many studies<sup>[30-33]</sup> have reported a correlation between pre-LT serum AFP and post-LT overall survival in patients with HCC, AFP is considered an independent predictor of post-transplant survival (Table 1). Todo *et al*<sup>[30]</sup> reported 1, 3, and 5-year survival rates, respectively, of 84%, 77%, 72% for AFP < 200  $\mu$ g/L vs 65%, 42%, 34% for AFP > 1000  $\mu$ g/L. Mailey *et al*<sup>[32]</sup> likewise showed that the 1, 3, and 5-year absolute survival rates of 92%, 82%, 74%, respectively, in patients whose AFP level was lower than 20  $\mu$ g/L decreased to 82%, 63%, 52% among those with an AFP > 400  $\mu$ g/L.

Berry *et al*<sup>[31]</sup> reported that transplant recipients with HCC and serum AFP levels  $\leq 15$  ng/mL at transplant did not have a higher post-transplant mortality (AHR = 1.03) with respect to those without HCC. Patients with 16 to 65 ng/mL (AHR = 1.38), 66-320 ng/mL (AHR = 1.65), and > 320 ng/mL (AHR = 2.37) serum AFP levels had progressively worse post-transplant mortality rates in comparison with recipients without HCC. Those investigators also reported that patients outside the Milan criteria had excellent outcomes if their AFP levels were < 15 ng/mL, while those who fulfilled the Milan criteria but had high AFP serum levels had poor survival rates.

Other investigators have reported that HCC recurrence after LT was correlated to pre-transplant

AFP levels<sup>[30,34-41]</sup>. Fujiki *et al*<sup>[41]</sup> demonstrated that the 1, 3, and 5-year recurrence-free survival (RFS) rates in 144 patients was, respectively, 97%, 91%, 90% when AFP was 200  $\mu$ g/L. However when the AFP was higher than 800  $\mu$ g/L the RFS were 65%, 40%, 40%. Evaluating 100 HCC transplant patients, Sotiropoulos *et al*<sup>[37]</sup> found 1, 3, and 5-year RFS rates were 100%, 97% and 97% for AFP < 20  $\mu$ g/L vs 68%, 23% and 23%, respectively, for AFP values higher than 100  $\mu$ g/L.

Biological behavior features such as vascular invasion and tumor grade<sup>[2-7,42]</sup> have also been shown to be correlated with AFP levels. Fujiki *et al*<sup>[41]</sup> demonstrated that AFP > 800  $\mu$ g/L was associated with an increased risk of microvascular invasion and poor differentiation of HCC with respect to AFP < 200  $\mu$ g/L. In addition, vascular invasion and tumor differentiation had the highest odds ratios (OS) with AFP levels in a multivariate analysis by Duvoux *et al*<sup>[20]</sup>.

In view of this evidence, new transplant selection criteria that include AFP have been investigated. Carrying out a study on a population of 6478 patients, Toso *et al*<sup>[26]</sup> reported that both total TTV and AFP levels were significant predictors of survival. A combined patient selection score based on TTV and AFP was thus developed. Compared to all the other criteria systems tested, that score was found to be the best predictor of outcome (Table 2). Duvoux *et al*<sup>[20]</sup> subsequently studied a 2 cohort population (with training and validation groups) of patients who had undergone LT for HCC within the context of a multicentric retro-

**Table 2** Integrated selection criteria schemes for liver transplantation in hepatocellular carcinoma patients

Ref.	Model	No. of Patients	Parameters	Cut-off (points)	Criteria	Endpoint	Criteria-in outcome	Criteria-out outcome	Validation
Toso <i>et al</i> <sup>[26]</sup>	TTV/AFP	6478	TTV	$\leq 115 \text{ cm}^3$ , $> 115 \text{ cm}^3$	$\text{TTV} \leq 115 \text{ cm}^3$ AND $\text{AFP} \leq 400 \text{ ng/mL}$	Corrected posttransplant 3-yr OS	$> 65\%$	$< 50\%$	Grat <i>et al</i> : 104 patients with similar results
Duvoux <i>et al</i> <sup>[20]</sup>	The AFP Model	Training cohort: 597 Validation cohort: 435	Longest Diameter No. of nodules AFP	$< 3 \text{ cm}$ (0), $3\text{--}6 \text{ cm}$ (1), $> 6 \text{ cm}$ (4) $1\text{--}3$ (0), $\geq 4$ (2) $< 100 \text{ ng/mL}$ (0), $100\text{--}1000$ (2), $> 1000$ (3)	Sum of individual points $\leq 2$	posttransplant 5-yr RFS	7.7 % (Milan-in) 14.4 % (Milan-out)	53.3 % (Milan-in) 47.6 % (Milan-out)	Notarpaolo <i>et al</i> <sup>[44]</sup> : 560 patients with similar results
Lai <i>et al</i> <sup>[211]</sup>	-	422	mRECIST AFP slope	Progression <i>vs</i> No progression $\leq 15 \text{ ng/mL}$ per mo, $> 15 \text{ ng/mL}$ per mo	No progression AND AFP slope $\leq 15$	5-yr RFS 5-yr OS	RFS: 90% (Milan-in), 87% (Milan-out) OS: 88% (Milan-in), 83.5% (Milan-out)	RFS: 67.7% (Milan-in) 47% (Milan-out) OS 67.3% (Milan-in) 55.4% (Milan-out)	Not yet validated

AFP: Alpha-fetoprotein.

spective study and identified 3 independent pre-LT predictors of recurrence: the number of tumors, the tumor size, and the AFP level. These parameters were incorporated to develop a model stratifying low and high risk of recurrence. A 3-tier AFP level score was included in the model and cut-offs of 100  $\mu\text{g/L}$  and 1000  $\mu\text{g/L}$  were adopted to identify the 3 AFP groups. The model proved to have an impact on recurrence and on survival, and net reclassification improvement showed that its predictability was superior to the Milan criteria<sup>[20]</sup> (Table 2). Duvoux's AFP model is, in fact, currently used in France ([www.agence-biomedecine.fr](http://www.agence-biomedecine.fr)) and United Kingdom ([www.odt.nhs.uk/pdf/advisory\\_group\\_papers/LAG/HCC\\_recommendations\\_IR\\_TS\\_b\\_NAS\\_Work\\_in\\_Progress.pdf](http://www.odt.nhs.uk/pdf/advisory_group_papers/LAG/HCC_recommendations_IR_TS_b_NAS_Work_in_Progress.pdf) 19); a value  $\leq 2$  is used as inclusion criteria for LT in HCC patients. The model has recently received an external validation in Spain<sup>[43]</sup> as well as in Italy<sup>[44]</sup>, and a recent United States study confirmed the strong prognostic power of an  $\text{AFP} > 1000 \text{ ng/mL}$  threshold in HCC patients undergoing LT meeting the Milan Criteria<sup>[45]</sup>.

Although static, and despite the fact that a clear, unanimous cut-off level has yet to be defined, it has been seen AFP does indeed predict outcomes of HCC patients undergoing LT. It is important to remember, however, that a single assessment of serum AFP levels are unable express dynamic changes in the tumor's biological behavior. Since tumor aggressiveness shows a tendency to progress, at least two time-spaced evaluations are needed to determine if the biological course is stationary or progressing. A study by Kondili *et al*<sup>[46]</sup> published in 2007 showed that a rapid increase in AFP levels before LT represents a risk factor for tumor recurrence. Another study by Han *et al*<sup>[28]</sup> demonstrated that AFP progression exceeding 50  $\mu\text{g/L}$  per month was significantly correlated to

both vascular invasion and poorly differentiated tumor grade. Vibert *et al*<sup>[29]</sup> who measured AFP levels once a month in 153 patients on waiting lists for LT with the intent of demonstrating the relevance of dynamic AFP variations, found that its progression was more predictive of tumor recurrence and poor survival after LT than any static value. A slope  $> 15 \mu\text{g/L}$  per month was identified as the cut-off value. As suggested by Merani *et al*<sup>[47]</sup> decreasing AFP values also seem to have a clinical significance. Studying 6817 HCC cases, they reported that patients successfully downstaged from  $\text{AFP} > 400 \mu\text{g/L}$  to  $\text{AFP} \leq 400 \mu\text{g/L}$  had better post-transplant outcomes than patients whose AFP remained  $> 400 \mu\text{g/L}$  after downstaging. In addition, both increasing and decreasing AFP levels were found to be relevant to the evaluation of the oncological behavior of HCC, identifying tumors tending toward either a positive or a negative evolution. In view of the biomarker's potential relevance, the investigators concluded that further studies are warranted to standardize the cut-off values and assessment time points.

Despite abundant data on AFP found in the literature, any conclusions for the time being can only be tentative in view of many unsolved issues. Firstly, since AFP is a biomarker, biases linked to different laboratory methods and processing techniques are unavoidable and comparisons of results from multiple laboratories/studies are uncertain. Secondly, it is probable that the frequent exclusion of LT patients who die within 30 post-operative days has restricted data regarding the most aggressive tumors. According to Hakeem *et al*<sup>[48]</sup>, moreover, patients included in AFP studies are highly heterogeneous. Finally, prognostic evaluations based on AFP levels are made only with regard to patients whose serum levels are higher



than normal ( $> 20 \mu\text{g/L}$ )<sup>[49]</sup> despite the fact that a considerable percentage of HCC patients are AFP-negative. In a study by Yang *et al.*<sup>[50]</sup> focusing on novel prognostic biomarkers for HCC, 48.3% of the 305 patients studied had AFP  $< 20 \mu\text{g/L}$ . Zhang *et al.*<sup>[51]</sup> likewise reported that 30%-40% of HCC patients studied were AFP-negative. Although a dynamic evaluation of the biomarker (AFP slope) could partially obviate this problem, further studies specifically addressing HCC patients with in-range AFP values are warranted.

### Des- $\gamma$ -carboxyprothrombin

Des- $\gamma$  carboxyprothrombin (DCP), also known as protein induced by vitamin K absence or antagonist II (PIVKA-II), was described more than twenty years after the first description of AFP<sup>[52]</sup>. The role of DCP as a biomarker for the diagnosis of HCC has been confirmed over the years<sup>[53-56]</sup>, just as has been its potential to detect HCC early, given the highly sensitive immune assay that has been developed<sup>[57-59]</sup>. DCP's sensitivity and specificity in diagnosing HCC appear to be better than those of AFP<sup>[60-62]</sup>, and simultaneous testing of both markers has been proposed for tumor detection<sup>[63]</sup>.

Interestingly, DCP has also been shown to be predictive of outcomes regardless of treatment<sup>[62,64-67]</sup>. Encouraging predictive values were first reported after ablative therapies for HCC<sup>[64,65,68]</sup>. According to Imamura *et al.*<sup>[66]</sup> DCP was able to predict recurrences after resection for small HCCs and, similarly, Sakaguchi *et al.*<sup>[67]</sup> demonstrated that DCP  $> 100 \text{ mAU/mL}$  was associated to a negative prognosis in HCC patients within the Milan criteria undergoing resection. Some studies have shown that DCP has a predictive significance also with regard to LT outcome and that it is a powerful predictive serum marker. Basing their data on a cohort of 124 patients undergoing living donor liver transplantation (LDLT), Shindoh *et al.*<sup>[69]</sup> found that the prognosis of these patients strongly depended on maximum pre-LT AFP or DCP values. Multivariate analysis performed on 144 HCC patients who underwent LDLT at the Kyoto University showed that DCP  $> 400 \text{ mAU/mL}$  was an independent risk factor for tumor recurrence after transplant.

Fujiki *et al.*<sup>[41]</sup> subsequently published the Kyoto expanded criteria for LDLT which included preoperative DCP levels  $< 400 \text{ mAU/mL}$ , tumor size, and number<sup>[41]</sup>. A similar proposal was made by Taketomi *et al.*<sup>[70]</sup> who suggested a different cut-off value for DCP (DCP  $< 300 \text{ mAU/mL}$ ). The role of DCP was recently confirmed in a United States population. A serum DCP  $\geq 7.5 \text{ mAU/mL}$  in 127 HCC patients undergoing LT significantly correlated with tumor recurrence (HR = 3.5; 1.9-6.7). The HR increased when DCP was combined with AFP and the Milan criteria<sup>[71]</sup>. In addition, finding DCP expression in the liver of HCC patients, especially in the peritumoral tissue, both Inagaki *et al.*<sup>[72]</sup> and Tang

*et al.*<sup>[73,74]</sup> suggested that a combination of serum and tissue DCP expression be utilized.

DCP's prognostic role seems to be linked to its association with elevated cellular proliferation and tumor growth rates<sup>[64,75]</sup> as well as high infiltrative growth and vascular invasion values<sup>[62,72,73,76]</sup>. Recently, Poté *et al.*<sup>[62]</sup> reported that a serum level of DCP  $> 90 \text{ mAU/mL}$  was an independent predictor of vascular invasion, while high DCP tissue expression was associated with poor tumor differentiation. *In vitro* studies have proven that PIVKA-II is able to promote cellular proliferation and migration<sup>[77,78]</sup> just as it induces expression of angiogenic factors such as endothelial growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF)<sup>[79,80]</sup>.

While both clinical and biological reports and *in vitro* studies support the view that DCP is an index of HCC aggressiveness, its relative clinical relevance is still under debate.

### AFP mRNA

Post-transplant tumor recurrences are almost certainly due to residual cancer cells from the removed tumor, and detection of HCC cells in the peripheral blood seems to be a direct and accurate method to predict tumor recurrence<sup>[81]</sup>. At the same time, AFP mRNA expression in the peripheral blood is a reliable marker of circulating cancer cells<sup>[82]</sup>.

In 2005 our research group reported that the pre-operative AFP mRNA level is a significant predictor of survival after radical therapy for HCC<sup>[83]</sup>. Marubashi *et al.*<sup>[84]</sup> likewise reported that a positive pre-operative test for peripheral blood AFP mRNA was found to be an independent risk factor for the recurrence of HCC after LDLT.

Using a nested-polymerase chain reaction (PCR) technique, Ijichi *et al.*<sup>[85]</sup> reported, instead, that the pre-operative presence of AFP mRNA-expressing cells in the peripheral blood was not associated with after resection HCC recurrence.

Data supporting AFP mRNA's role as a predictor of HCC recurrence are as yet inconclusive. Toso *et al.*<sup>[86]</sup> hypothesized that strategies to decrease the engraftment of circulating tumor cells could lower the risk of recurrence. Some of the strategies they proposed were selecting recipients with low baseline circulating HCC cells by adding biological markers to the accepted combination of morphological criteria and decreasing the perioperative release of HCC cells *via* careful perioperative handling of the tumor.

### AFP-L3%

Given its high specificity and sensitivity in detecting tumors, in the early 90s some investigators began to consider lens culinaris agglutinin-reactive fraction of  $\alpha$ -fetoprotein (AFP-L3%), the percentage of a fucosylated form of AFP over the total AFP level, an adjunctive marker for HCC diagnosis of<sup>[87-90]</sup>.

The biomarker also seems to be able to predict prognosis. High AFP-L3% levels have, in fact, been reported to be correlated with poor outcome after transcatheter arterial chemoembolization (TACE)<sup>[91]</sup> and with a higher risk of recurrence after local ablation<sup>[92,93]</sup> and hepatectomy<sup>[92,94]</sup>. According to Kobayashi *et al.*<sup>[95]</sup> AFP-L3% values are useful in predicting the outcome of patients with normal serum AFP levels<sup>[96]</sup>. Considering it a potential new generation tumor marker, Kusaba demonstrated that liver cancer cells expressing AFP-L3% showed a tendency towards early vascular invasion and intra-hepatic metastasis, staining more positive with Ki67 and less with alpha-catenin<sup>[97]</sup>. Chaiteerakij *et al.*<sup>[71]</sup> found that AFP-L3% was significantly associated with tumor recurrence in a population of 127 HCC patients undergoing transplantation. Interestingly, the HR increased from 2.6 (1.2-6.6) to 4.5 (1.9-10.6) when that parameter was added to the Milan criteria. A prognostic value was also attributed by that same study to the absolute AFP-L3% value.

There is still little evidence, nevertheless, to support the prognostic relevance of AFP-L3% in the LT context.

**Other biomarkers:** Many other HCC biomarkers after liver resection, loco-regional treatments or LT have been cited in medical literature but their potential prognostic role in the transplant population has yet to be well defined.

Furthermore, the systematic citation of each individual marker is beyond the scope of this review, which focuses on the most accessible and reproducible markers used in daily clinical practice. Nevertheless a brief mention is made for potential subsequent studies into their prognostic value in patients with HCC undergoing LT.

However, one particular biomarker worthy of mention is glypican-3 (GPC3). This is a membrane glycoprotein which is involved in cell cycle regulation and which is detected in HCC patients. Although there is as yet no unanimous agreement on this, high levels of GPC3 in HCC tissue after curative resection and LT seem to lead to poor prognosis in terms of both disease free and overall survival<sup>[98,99]</sup>. In addition, a link has been found between high GPC3 expression and high tumor grade (moderate and poor differentiation), late TNM stage (III, IV), vascular invasion, tumor multifocality and metastasis in patients with HCC. Research has also shown high GPC3 expression to be associated with the presence of large tumors (5 cm or more)<sup>[100]</sup>. The importance of glypican 3 in patients with HCC undergoing liver transplantation has already been proved to be useful in prognosis stratification and several authors propose a cut-off value of  $3.5 \times 10^{-2}$ <sup>[101,102]</sup>.

Another important biomarker to mention is human telomerase reverse transcriptase mRNA (h-TERT mRNA). Several studies have demonstrated that high

h-TERT mRNA expression is a prognostic indicator of poor outcome in HCC patients. The prognostic power of h-TERT mRNA has been evaluated also in a liver transplant setting: HCC patients with an elevation of human telomerase reverse transcriptase mRNA (preoperatively in the blood or after neoadjuvant immunochemotherapy) suffered of higher tumor recurrence and lower survival rates than those without h-TERT mRNA in the blood<sup>[103,104]</sup>. More studies are required to validate the prognostic power of h-TERT mRNA due to an absence of an unanimous consensus<sup>[105,106]</sup>.

High levels of alpha 1 fucosidase (AFU) and transforming growth factor beta-1 (TGF-B1) seem to be associated with poor prognosis in patients with HCC. Although more studies are required to evaluate the importance of said biomarkers after LT<sup>[107-113]</sup>.

Furthermore, biomarkers such as human cervical cancer oncogene (HCCR), tumor specific growth factor (TSGF) and gamma-glutamyl transferase II (GGT II) have been proved to have an important role in diagnosis, but more studies are required to clarify its prognostic role<sup>[56,114-120]</sup>.

### Systemic inflammatory markers

Systemic host inflammation is another factor that has been evaluated as a parameter to assess tumor aggressiveness<sup>[121,122]</sup>. Depending on the tumor micro-environment, pro-inflammatory macrophages, cytokines and chemokines seem to be factors responsible for tumor progression given their ability to induce metastatization and to inhibit apoptosis, thus facilitating angiogenesis and DNA damage<sup>[123]</sup>.

Neutrophil-to-lymphocyte ratio (NLR) is a serum inflammatory marker that has been attracting increasing interest since it has been found to be a predictor of recurrence and poor prognosis in patients with colorectal-liver metastasis<sup>[123-125]</sup>. High neutrophil levels are able to enhance the propensity for vascular invasion and metastatization by increasing the production of VEGF<sup>[126,127]</sup>. Conversely, low lymphocyte numbers seem to be responsible for impaired immunosurveillance against disease development and progression<sup>[128]</sup>.

In 2009, Halazun *et al.*<sup>[123]</sup> demonstrated that NLR predicts outcome in HCC patients after LT. They reported that patients meeting the Milan criteria with a  $NLR \geq 5$  had significantly worse recurrence-free survival (RFS) and lower survival rates than patients with a  $NLR < 5$ . Similar effects on tumor recurrence and survival were later reported by other groups both for cadaveric and for living donor liver transplantation (LDLT)<sup>[129-132]</sup>. A recent meta-analysis evaluating the prognostic significance of NLR in HCC patients confirmed, moreover, that high NLR was associated with poor OS and DFS of liver transplanted patients with HRs of 3.42 (2.41-4.85) and 5.90 (3.99-8.70), respectively. Notably, conventional prognostic indexes such as vascular invasion, multiple tumors, and AFP

$\geq 400$  ng/mL were also reported to be correlated with NLR<sup>[133]</sup>. Despite discordant findings<sup>[69,134]</sup>, the data uncovered until now suggest that NLR can contribute to the LT selection process of HCC patients. Further data are needed to confirm the marker's effectiveness.

Other inflammatory-related prognostic markers are under evaluation. In a retrospective intention-to-treat analysis on 181 HCC patients listed for LT, Lai *et al.*<sup>[135]</sup> demonstrated that the platelet-to-lymphocytes ratio (PLR)  $> 150$  was more efficacious than NLR in predicting the risk of HCC recurrence after LT and that it can be used to stratify patients for tumor-free-survival (91.6% vs 80.7%,  $P = 0.02$ ). The usefulness of PLR was recently confirmed by a Chinese study focusing on a cohort of 343 HCC in whom a PLR = 125 was found to be the most appropriate cut-off to predict tumor-free survival after LT (sensitivity 61.6%, specificity 62.7%)<sup>[136]</sup>. Unanimous agreement has, however, yet to be reached<sup>[134]</sup>.

Some have hypothesized that an inflammatory response is implicated in the pathogenesis of cancer-related malnutrition<sup>[137]</sup>. The prognostic nutritional index (PNI) has been proposed as a further marker of inflammation and HCC-related prognosis. Chan *et al.*<sup>[138]</sup> demonstrated that PNI is an independent prognostic index of OS and DFS after surgical resection of Barcelona Clinic Liver Cancer (BCLC) Stage 0/A, but its potential role in the liver transplant setting is as yet unestablished.

Due to a clear lack of single, self-sufficient prognostic biomarkers, some attempts have been made to assess prognosis using an integrated combination of more than one of these. Toyoda *et al.*<sup>[139]</sup> for example, used an Asian population to develop the so-called BALAD staging score which is based on 5 serum markers including AFP, AFP-L3% and DCP; the scoring system's predictive power was found to be similar to that of the BCLC staging system. Although the model was recently validated on a British population<sup>[140]</sup>, it has yet to be validated in a transplant setting.

Retrospectively studying 185 patients who underwent hepatectomy for HCC, Kiriya concluded that triple positive tumor markers for HCC (AFP, AFP-L3% and DCP) correlated with the poorest prognosis and the most invasive characteristics in pathological findings<sup>[141]</sup>.

As a final consideration, research on miRNA plasma expression is arousing interest in view of the potential role of miRNA signature profiling in HCC prognosis stratification<sup>[142]</sup>.

## RADIOLOGICAL FEATURES: THE ROLE OF <sup>18</sup>F-FDG-PET IMAGING

Morphological imaging studies have proven to be effective in predicting outcome after orthotopic liver transplantation (OLT). While both the Milan and the UCFS criteria are based on the size and number of

radiologically detected tumors, functional imaging studies appear to be able to provide even further information about the tumor. F-18 fluoro-2-deoxy-d-glucose positron emission topography (<sup>18</sup>F-FDG PET) estimates the tumor growth and metabolism based on calculated tumor volumes and maximum standardized uptake values. The different ratio of glucose-6-phosphatase and hexokinase in the normal liver and tumor cells results in an increased accumulation of <sup>18</sup>F-FDG in primary HCC lesions<sup>[143]</sup>.

While <sup>18</sup>F-FDG-PET has demonstrated suboptimal sensitivity in detecting new HCC ( $< 50\%$ )<sup>[144]</sup>, there are reports that it is useful in uncovering the presence of extra-hepatic metastasis<sup>[145]</sup>, in providing information about HCC prognosis, and in predicting tumor recurrence after LT<sup>[146-149]</sup>. In a retrospective analysis, Yang *et al.*<sup>[146]</sup> demonstrated that <sup>18</sup>F-FDG-PET positive patients (PET +) showed an overall greater risk of tumor recurrence with respect to PET negative (PET-) patients (OR = 7.6). Its ability to predict prognosis and tumor recurrence was confirmed in 2009 by Kornberg *et al.*<sup>[147]</sup> who carried out a retrospective analysis on 42 liver transplanted patients. Their results demonstrated that PET+ patients had a significantly worse 3-year DFS (35%) and a higher recurrence rate (RR 50%) than PET- patients (DFS = 93%, RR = 3.8%). The same research group recently demonstrated that HCC patients meeting the Milan criteria with a non-avid <sup>18</sup>F-FDG PET achieved an excellent DFS after LT ( $> 80\%$  at 5-year follow-up). An avid uptake of <sup>18</sup>F-FDG was found to be an independent predictor of tumor related drop-out from waiting lists. This finding confirms the potential advantages of this technique in the LT setting<sup>[148]</sup>.

As far as biological findings are concerned, Yang *et al.*<sup>[146]</sup> demonstrated that PET+ (greater PET lesion uptake) HCCs were significantly associated with some poor prognostic factors such as AFP  $> 200$  ng/mL ( $P < 0.001$ ) and vascular invasion ( $P = 0.003$ )<sup>[146]</sup>. Kornberg likewise demonstrated that PET+ status was an independent predictor of microvascular invasion. The uptake of <sup>18</sup>F-FDG in HCC patients is reported, moreover, to vary according to the degree of tumor differentiation<sup>[146]</sup>. Well- and well-to-moderately differentiated HCCs, in particular, exhibit an <sup>18</sup>F-FDG metabolism that is similar to normal liver tissue while moderate-to-poorly and poorly differentiated HCCs demonstrate an enhanced one<sup>[146,150,151]</sup>.

New tracers (e.g., <sup>18</sup>F-fluoropropoxy-L-tryptophan and L-methyl-<sup>3</sup>H-methionine, <sup>11</sup>C-metomidate, <sup>11</sup>C-acetate, <sup>18</sup>F-fluorocholine and <sup>11</sup>C-choline) aiming to improve PET's specificity and sensitivity in detecting HCC and its metastases are presently under investigation<sup>[152-154]</sup>. Dual Tracer PET-CT imaging is also under examination: <sup>11</sup>C-acetate and <sup>18</sup>F-FDG have already demonstrated high sensitivity and specificity in detecting HCC (about 95% and 100% respectively) in candidates for LT or liver resection<sup>[155,156]</sup>; the potential role of these new tracers and the recently introduced oncologic PET-MRI<sup>[157]</sup> in the

transplant scenario and their ability to detect biological aggressiveness remain to be established.

## HISTOLOGY: THE PRE-TRANSPLANT LIVER BIOPSY

As mentioned above, microvascular invasion is an established independent prognostic factor for HCC recurrence after LT<sup>[2-7]</sup>. A strong correlation between microvascular invasion and the histologic tumor grade of HCC has, in fact, been reported by many authors<sup>[2,14,19,158,159]</sup>, and high tumor grade has been found to be an independent predictor of vascular invasion<sup>[160]</sup>. In addition, Tamura *et al.*<sup>[42]</sup> reported that histologic differentiation itself represents an independent predictor of survival following transplantation since a low tumor grade increases the 3-year survival rate both in patients with small ( $\leq 5$  cm) and large ( $> 5$  cm) tumors.

A very low post-transplant tumor recurrence rate was found by our group when a pre-transplant biopsy grade-based selection protocol that did not include the patient's Milan criteria standing was utilized in 145 HCC patients. When the G3 HCCs were excluded from LT, the 5 year survival free recurrence was 92% and none of the patients with tumors  $> 5$  cm had recurrence<sup>[19]</sup>. When poorly differentiated HCCs were excluded from our patient database, only 12% of the tumors were  $> 5$  cm which, of course, confirms the relevance of a tumor grading selection system. DuBay *et al.*<sup>[161]</sup> who subsequently validated this approach, studied 294 HCC patients for 8 years during which time they gradually tested and developed a transplant selection protocol. Their findings showed results gradually shifting away from the Milan criteria and towards a biopsy-based system (the extended Toronto criteria). A comparison between the two periods of the study confirmed that the exclusion of poorly differentiated tumors irrespective of macromorphological features (Milan criteria) achieved excellent survival rates.

Findings from Pawlik *et al.*<sup>[162]</sup> single-center study did not, however, confirm the independent prognostic power of a pre-operative biopsy for outcome after LT. While the tumor grade associated with vascular invasion or disease-specific death was not significant, the tumor grade on the final pathologic examination was found to be a reliable predictor of vascular invasion and outcome. The disparity between the preoperative histology and the final surgical specimen analysis may be explained by the fact that well or moderately-differentiated tumor areas can coexist adjacent to poorly differentiated ones. Sampling bias should, therefore, always be taken into consideration. The median tumor size in Pawlik *et al.*<sup>[162]</sup> study was 7.0 cm, and half of the patients had very large tumors with a markedly increased risk of heterogeneity and dedifferentiation<sup>[163,164]</sup>. More generally, while the biopsy reading is strictly related to

the quality of the tissue sample, the histologic grade is subject to inter-observer variability. Notwithstanding the existence of the Edmondson and Steiner grading system<sup>[165]</sup>, and although automated grading systems have been proposed<sup>[166]</sup>, HCC biopsy imaging grading is still visual, qualitative, and subjective. Indeed, the inter-observer variability has been shown to be relevant, with a K statistic from 0.32 to 0.66, respectively, for moderately and well differentiated HCC<sup>[167]</sup>. Lack of concordance clearly limits the widespread use of a grade-based selection protocol of HCC patients for LT.

On the other hand, if one goes beyond the possibility of "false negative" results, virtually all G3 biopsies in HCC patients refer to true poorly differentiated tumors, and all high grade tumors prove to be strictly correlated with poor outcome after LT. Excluding these patients from LT listing may contribute to reducing the prevalence of patients with aggressive HCC and this, of course, will lead to beneficial results in terms of overall post-transplant outcome, as has been reported by one of our studies as well as by a Canadian study.

Multiple fine needle aspiration biopsies (FNABs) are not, however, recommended given the complication rates that vary from 0.75% to 13.6%<sup>[168]</sup>. Cases of bleeding have also been described in 1/500 biopsies and those requiring urgent hospitalization and blood transfusion range from 1 /2500 to up to 1/10000 biopsies. There is, in addition, a 0 to 3% risk of needle tract seeding in most studies although it did not seem to influence the oncological outcome<sup>[169-171]</sup>.

A visual liver and biopsy site assessment could overcome these sampling issues. Core needle biopsy (CNB) under laparoscopic ultrasonography (LUS) guidance makes it possible to directly examine the area to be sampled<sup>[172,173]</sup>. Helmreich-Becker *et al.*<sup>[174]</sup> described LUS guided CNB as a safe and feasible procedure. LUS appears to be extremely promising tool in the transplant setting; biopsy specimens and high diagnostic accuracy can be obtained, and HCC can be treated (downstaging - bridging) all at the same time and potentially repeatedly. Further studies are needed to establish its role in the pre-transplant assessment of HCC patients.

While current guidelines do not include liver biopsy to diagnose radiologically typical<sup>[175]</sup> HCC, given the tendency to characterize tumors focusing on their molecular features, biopsies will presumably play a key role in the near future. Large steps forward have, in fact, been made in the molecular signatures field thanks to the development of microarray technologies which permit the tumor expression of several molecular markers associated with deregulation of genes or pathways to be tested at the same time by processing a tumor tissue sample<sup>[176,177]</sup>. Deregulation of these genes and pathways has, in turn, been proven to affect some of the tumor's biological features such as vascular invasion<sup>[178,179]</sup> and growth rate<sup>[180,181]</sup>,



and several studies have shown that it plays a role in worsening prognosis<sup>[181-184]</sup>. In a recent paper, Villa *et al.*<sup>[181]</sup> demonstrated that a five-gene transcriptomic hepatic signature including angiopoietin-2, NETO2, DLL4 ESM1 and NR4A1 was able to rapidly identify growing HCCs and was independently associated with an increase in mortality. Several signatures, including the miRNA expression pattern<sup>[185]</sup> have been proposed and studied until now, but agreement on the best predictive molecular signature pattern has yet to be reached.

It is worth mentioning that epigenetic features have also been investigated as prognostic biomarkers. In fact, as with other cancers, HCC has demonstrated a distinct methylation profile. The hypermethylated form of p16 (CDKN2A), for example, a tumor-suppressor gene involved in cell cycle regulation, has already been associated with advanced stages of HCC, vascular invasion, poor tumor differentiation and, finally, with worse prognosis<sup>[186]</sup>.

In view of its relatively recent appearance, the molecular signature still lacks clinical relevance. Evidence gathered until now suggests that it can be potentially used in the LT evaluation workup. Even more importantly, in the same way that a great deal of information can be gained from a biopsy specimen, the same can be said for the tumor's histological features. In addition, recent technologies have made it possible to obtain genomic profiling on formalin fixed, paraffin embedded samples making the molecular signature a feasible, reproducible tool for tumor evaluation in the near future<sup>[184]</sup>.

## RESPONSE TO THERAPY

Loco-regional therapy response in LT candidates with HCC has been extensively studied over the last 10 to 15 years, but an in-depth analysis of the topic does not fall within the aims of the current work<sup>[15,29,175,187-200]</sup>. Despite the fact that there is a paucity of randomized clinical trials regarding downstaging or bridging therapies, most centers throughout the world adopt loco-regional therapies before or after placing a patient on a waiting list<sup>[188,196,201-203]</sup>.

Resection, ablation (either percutaneous or laparoscopic), or TACE alone or together with other therapies, which are the most widely used therapeutic strategies, aim to reduce the dropout rate while patients are on waiting lists and/or tumor recurrence after LT<sup>[204-206]</sup>. Although downstaging strategies focus on reducing the tumor burden until the patient meets transplantation criteria (*i.e.*, Milan), it is widely accepted that a prolonged response to downstaging therapies can itself be considered a selection criteria that mirrors the biologic behavior of the tumor and predicts a relatively low risk of recurrence after LT. Similarly, as reported by a number of studies, a good response to bridging therapies can serve as a surrogate marker of a favorable tumor biology (Table 3).

In an intention-to-treat analysis, Millonig *et al.*<sup>[187]</sup> demonstrated that patients with a complete response to TACE had 1-, 2-, and 5-year survival rates of 89.1%, 85.1%, and 85.1%, respectively, compared with 68.6%, 51.4%, and 51.4% in non-responders ( $P = 0.02$ ). Similar results were also found by our group while studying recurrence after LT in patients who were stratified into responder and non-responders to pre-transplant HCC therapy. The probability of post-transplant HCC recurrence was shown to be higher in the non-responder ( $P = 0.04$ ) group<sup>[15]</sup>. In a seminal study by Otto *et al.*<sup>[207]</sup> 136 HCC patients who underwent TACE were assessed during the waiting period for LT in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The authors reported a 22% 5-year freedom from recurrence rate in the TACE-with-progression group and a 92% rate in the TACE-without-progression one ( $P < 0.0001$ ). Response to therapy was also more predictive of outcome than was the Milan criteria ( $P = 0.0001$ ).

Different 5-year recurrence rates (19.4% in patients with partial or no response to bridging therapy vs 5.5% in responders) were also detected by Cucchetti *et al.*<sup>[199]</sup> who studied the data of 315 LT candidates. A strict correlation between tumor responsiveness and outcome was also found when progression or no-progression was found at the pathological examination<sup>[208,209]</sup>. Ho *et al.*<sup>[210]</sup> assessed loco-regional treatment (both as downstaging or bridging strategies) before LT in 86 HCC patients by dividing the population into 3 subgroups depending on the degree of tumor necrosis at the pathological examination (group I : 10%-50%; group II : 50%-90%; and group III : > 90). The patients with a higher necrosis rate after therapy (group III) were found to survive longer ( $P = 0.003$ ) and had significantly lower recurrence rates than the patients in the other two groups ( $P = 0.001$ ). Finally, when response to therapy was integrated with higher AFP levels, its power to predict prognosis in terms of RFS and OS was found to be enhanced. Absence of progression as far as tumor burden and the AFP (slope  $\leq 15$ ) were concerned identified a subgroup of patients with excellent prognosis, irrespective of conventional criteria<sup>[211]</sup> (Table 2).

With regard to downstaging, some studies have shown that the outcome of downstaged HCCs patients was similar to that of patients meeting the Milan criteria. Ravaioli *et al.*<sup>[17]</sup> reported a 3-year RFS in patients fulfilling the Milan criteria and in downstaged patients of 83% and 75%, respectively ( $P = \text{NS}$ ). As outlined in Table 3, similar results were found by Chapman *et al.*<sup>[212]</sup> and De Luna *et al.*<sup>[213]</sup>. Yao *et al.*<sup>[214]</sup> recently reported long term results after LT in HCC patients downstaged to T2 and compared their data to those of patients meeting the Milan criteria: the 5-year post-transplant and recurrence-free survival were 77.8% and 90.8%, respectively, in the downstaged group vs 81% and 88% in the T2 group ( $P = \text{NS}$ ). Since the

**Table 3** Response to therapy: comparison of outcomes following different pre-transplant strategies

Ref.	Treatment	Response assesemnt	Transplant criteria	No. of patients		Outcome		Comparison between responders and non responders	Comparison between downstaged patient vs conventional criteria
Millonig <i>et al</i> <sup>[187]</sup>	TACE	RECIST	UCSF	Total	116	Total	-	0.02	NS <sup>1</sup>
				Downstaging (DS)	NA	DS responders	5-yr OS = 25%		
				Bridging (B)	NA	B responders	5-yr OS = 85.7%		
						B non responders	5-yr OS = 51.4%		
Chapman <i>et al</i> <sup>[212]</sup>	Resection, ablation, TACE	RECIST	Milan	Total	136	Total	-	NA	NS
				DS	76	DS responders	5-yr RFS = 50%		
				B	60	B responders	5-y RFS = 62.6%		
						B non responders			
Vitale <i>et al</i> <sup>[15]</sup>	Resection, ablation, TACE	RECIST	Milan	Total	147		5-yr ITT survival = 74%	< 0.01	NA
				DS	NA	DS responders	-		
				B	NA	B responders	5-yr ITT survival = 83%		
						B non responders	5-yr ITT survival = 63%		
Cucchetti <i>et al</i> <sup>[199]</sup>	Resection, ablation, TACE	mRECIST	Milan	Total	315	Total	-	0.017	NS <sup>1</sup>
				DS	53	DS responders	5-yr RR = 19.2%		
				B	240	B responders	5-yr RR = 5.5%		
						B non responders	5-yr RR = 19.4%		
Ravaioli <i>et al</i> <sup>[17]</sup>	Resection, ablation, TACE	RECIST	Milan	Total	177	Total	3-yr RFS = 82%	NA	NS
				DS	48	DS responders	3-yr RFS = 75%		
				B	NA	B responders	3-yr RFS = 83%		
						B non responders			
Yao <i>et al</i> <sup>[214]</sup>	Resection, ablation, TACE	mRECIST	Milan	Total	606	Total	-	NA	NS
				DS	118	DS responders	5-yr RFS = 90.8%		
				B	NA	B responders	5-yr RFS = 88%		
						B non responders			
De Luna <i>et al</i> <sup>[213]</sup>	TACE	NA	Milan	Total	122	Total	3-yr OS = 82.7%	NA	NS
				DS	27	DS responders	3-yr OS = 84.1%		
				B	NA	B responders	3-yr OS = 84.7%		
						B non responders			
Graziadei <i>et al</i> <sup>[190]</sup>	TACE	NA	Milan	Total	63	Total	NA	NA	NA
				DS	15	DS responders	4-yr OS = 41%		
				B	48	B responders	5-yr OS = 94%		
						B non responders	na		
Otto <i>et al</i> <sup>[195]</sup>	TACE	mRECIST	Milan	Total	136	Total	-	< 0.0001	NA
				DS	49	DS responders	5-yr RFS = 92%		
				B	87	B responders	5-yr RFS = 22%		
						B non responders			

De Giorgio <i>et al</i> <sup>[198]</sup>	Resection, ablation, TACE	NA	Milan	Total DS B	206 NA 83	Total DS responders B responders B non responders	NA	NA
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<sup>1</sup>Computed not stastically significant. NA: Not available; NS: Not statistically significant; OS: Overall Survival; RFS: Recurrence free survival or freedom from recurrence; RR: Recurrence rate; RECIST: Response Evaluation criteria in solid tumors; mRECIST: Modified response evaluation criteria in solid tumors; TACE: Trans arterial chemo-embolization; TACI: Trans Arterial chemo-infusion; UCSF: University of California San Francisco.

2-year cumulative probability for dropout was 34.2% in the downstaged group vs 25.6% in the T2 LT recipient group, this suggests that downstaging has an important impact on tumor biology. Interestingly, the prevalence of microvascular invasion and of poorly differentiated grade was found to be similar in the two groups when the final pathological features were compared.

These findings provide further evidence that non-responsive HCCs could conceal an aggressive biology while responsive ones could mirror a milder behavior. At the same time, they emphasize the relevance of response to therapy as an index of tumor biologic aggressiveness and the need to develop standardized guidelines to evaluate it. Several studies suggest using the modified version of Response Evaluation Criteria In Solid Tumours (mRECIST)<sup>[215-218]</sup> which constitute the modified version of the RECIST criteria for the assessment of response to therapy in solid tumors<sup>[219]</sup>. With respect to conventional criteria, modified ones include an oncological assessment of tumor viability and focus on reducing the viable tumor volume, defined by enhanced areas on imaging. Standardized criteria could help to improve homogeneity in response to therapy assessments.

There are, nevertheless, still some limitations with regard to the reliability and reproducibility of mRECIST. Since tumor assessment is subjective, its accuracy depends both on the technician's ability as well as on the quality of imaging<sup>[220]</sup>. Furthermore, its criteria are only applicable to HCC with typical features because assessment of response in atypical HCC remains obscure<sup>[220]</sup>. Finally, vascular shunt and alterations (especially for infiltrative tumors) can alter enhancement and thus lead to interpretation errors. In view of these and other considerations, some authors suggest evaluating AFP variations together with radiological features for an objective assessment of response to therapy<sup>[47,211]</sup>.

A potential drawback of bridging and downstaging therapies is, however, tumor dedifferentiation. Kojiro *et al*<sup>[221]</sup> reported that tumors with sarcomatoid changes were more frequent in patients who were treated with TACE before transplant than those who were not. Sarcomatoid modification has also been described in some case reports after radiofrequency ablation<sup>[222,223]</sup>. Zen *et al*<sup>[224]</sup> reported that only pretreated HCCs showed dedifferentiation towards a biliary phenotype when they analyzed explanted specimens. Yamamoto *et al*<sup>[225]</sup>

reported a high recurrence rate after radiofrequency ablation (RFA) in tumors that had aggressive biological features.

Selection pressure on resistant cells, phenotypic adaptative changes, and the protein expression normally triggered by hypoxia may explain therapy-induced histopathological changes<sup>[224,226]</sup>. Regardless of etiology, caution should be used when applying pre-LT therapies as investigation tools given the biological switch phenomenon.

### Test of time

A complete or partial response to therapy does not guarantee that it will be stable over time, and a rapid recurrence after response could uncover an aggressive tumor biology which would contraindicate the transplant. Not only the assessment of the response to therapy but also the course following that response (or more generally the overall tumor growth rate) are important considerations when HCC patients are being evaluated for LT as far as the so-called "test of time" is concerned. Reporting good outcomes when tumors were closely evaluated for 8 mo from the time of ablation to the date of enlisting a patient for transplant<sup>[210]</sup>, Roberts *et al*<sup>[210]</sup> proposed scheduling a waiting period following downstaging procedures to assess tumor behavior. Toso *et al*<sup>[227]</sup> also stressed the importance of an observation period between entering a downstaging program and being placed on a transplantation waiting list and suggested utilizing at least a 6-mo minimum test of time. Cescon *et al*<sup>[122]</sup> instead, proposed using a 3-mo waiting period with re-staging at the end to verify the new status. Despite the drawback of having to wait and the intrinsic increase in drop-out risk, the test of time appears to be an efficacious surrogate marker of tumor aggressiveness that could be integrated with other "static" prognostic tools (histology, response to therapy, morphologic studies). Further studies using standardized response assessments and homogeneous periods are needed to evaluate the parameter's true potential.

### VOLUME DOUBLING TIME

In 1961 Mordecai Schwartz proposed a biomathematical approach to clinical tumor growth and the formula he outlined to calculate the doubling time (DT) was:

$$DT = t \ln 2 / (\ln V_2 - \ln V_1)$$

Where  $t$  is the time interval between measurements and  $V_2$  and  $V_1$  are the tumor volumes detected at imaging, respectively, at the end and at beginning of the time interval<sup>[228]</sup>.

Subsequent studies described a wide variability in DTs with values ranging from < 30 d up to 600 d<sup>[229-234]</sup>. The assumption that various growth velocities reflect different tumor behaviors led researchers to search for correlations between HCC DT and other tumor or patient characteristics. The close relationship between DTs and prognosis has recently been investigated by Villa *et al.*<sup>[181]</sup> in a prospective study on 78 patients with newly diagnosed HCC. Study data confirmed that tumor doubling time ranged from 30 to 621 d. When the study population was divided into quartiles according to HCC growth rate, different survival profiles depending on the speed of tumor DT were found: 25% of the patients demonstrated DTs less than or equal to 53 d and had a significantly worse prognosis than the patients with DTs in other quartiles, regardless of the treatment prescribed<sup>[181]</sup>.

Static macromorphological parameters such as initial tumor diameter and ultrasound features have been demonstrated to be correlated with DT<sup>[231,234]</sup>. More interestingly, however, DT seems to be correlated with tumor differentiation<sup>[229,231,232]</sup>, mitotic activity<sup>[229,230]</sup>, vascular invasion and direct indexes of biological aggressiveness. Nakajima *et al.*<sup>[229]</sup> studied 34 patients with small HCCs using some markers of cell division such as Ki-67, Apo-I and their histologic grade to classify the tumors as slowly, moderately, or rapidly growing. They concluded that the more rapid the tumor growth, the higher the cell production and the less differentiated the tumor<sup>[229]</sup>. Moreover, as reported by several studies<sup>[232,234-236]</sup>, AFP levels were found to be correlated with tumor growth velocity, and this finding has confirmed the indirect link between DT and biological aggressiveness.

In addition, the direct influence of DT on outcome after surgery has also been reported. Okazaki *et al.*<sup>[230]</sup> outlined poor outcomes after hepatectomy in those patients whose DT was short. Similarly, Cucchetti *et al.*<sup>[232]</sup> calculated that the 3-year recurrence rate after liver resection was significantly higher in patients with DT < 100 d than in those with DT > 100 d ( $P = 0.008$ ). Even if there are no reports on the effect of DT on the post-transplant outcome, the studies mentioned above clearly demonstrate that a tumor's growth velocity is a faithful mirror of its intrinsic aggressiveness. It seems reasonable then, although there are no studies to prove it, that rapidly growing tumors have poor outcomes after LT.

While the strict correlation between AFP levels and tumor growth velocity has been repeatedly demonstrated<sup>[232,234-236]</sup>, the lack of agreement about this link<sup>[237]</sup> and the ease of obtaining the DT parameter point the way to further research on the role of DT in the evaluation of HCC aggressiveness in LT candidates.

## CONCLUSION

Predicting post-transplant HCC recurrence on the basis of the tumor size and the number of nodules can only seem simplistic and imprecise in the light of the disease's complexity. A number of recent studies have confirmed the predictive accuracy of other parameters used to assess the biological behavior of HCC and in particular with reference to tumor progression and response to therapy. Prospective randomized studies designed to validate the prognostic role of each of these parameters present relevant feasibility issues. Repeatable, multiparametric, integrated models developed on the basis of large multicentric prognostic studies are no doubt the best strategy to improve our ability to select the most appropriate HCC patients for liver transplant.

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