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ORIGINAL ARTICLE

# Morphophenotypic Classification of Hepatocellular Carcinoma: the Biliary/Stem Cell Subgroup and Worst Outcome—Implications on Patient Selection

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## Abstract

**Background** Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the third cause of cancer-related death. Current clinical/pathological criteria contribute to risk stratification, but are far from the desired on individualized medicine. Recently, HCC classifications have been published based on immunohistochemical and morphological features.

**Methods** A retrospective review of patients submitted to surgical treatment—partial hepatectomy (PH) or liver transplantation (LT), with pathological diagnosis of HCC, in a 9-year period (2007–2015) was performed.

**Results** Applying the classification of Srivastava et al. (#1), based on the expression of CD31, p53, AFP and CD44, tumour size and presence of vascular invasion, HCC were categorized as low- and high-risk HCC. With the classification of Tsujikawa et al. (#2), HCC were classified into biliary/stem cell marker positive, Wnt signalling positive and the "all negative" HCC, according to the expression of CK19, SALL4, β-catenin glutamine synthetase, EpCAM and p53. There were sixty-six patients (53 males; 13 females), with median age of 64.5 ± 9.46 years (range 38–86), with solitary HCC, comprehending 37 PH (56.1%) and 29 LT (43.9%). The mean overall survival (OS) was 75.4 ± 6.9 months. Biliary/stem cell type of HCC was a predictive factor of worse OS on the overall population (24.4 versus 78.3 months, $p = 0.032$) and in PH cohort (11.5 versus 64.01 months, $p = 0.016$), on uni- and multivariate analyses.

**Conclusion** These results support the relevance of a risk stratification classification of HCC. Classification #2 seems adequate to our reality demonstrating OS impact, allowing its application in future biopsies, prompting individualized medicine.

**Keywords** Hepatocellular carcinoma · Individualized medicine · Morphophenotypic classification · Stem cells

**MeSH Terms** Carcinoma · Hepatocellular · Liver neoplasms · Hepatectomy · Liver transplantation · Precision medicine · Biopsy · Stem cells

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## Introduction

Hepatocellular carcinoma (HCC) corresponds to 90% of all hepatic primary tumours, being one of the most common cancers in the world and the 4th cause of cancer-related death.[1,2] The majority of HCC cases are associated with a recognized risk factor, namely chronic viral infection (hepatitis B, hepatitis C), alcohol consumption and exposure to aflatoxin; in the last years, there has been an increasing role of the non-alcoholic fatty liver disease, obesity and diabetes, with consequent development of HCC.[3]

HCC treatment with curative intent usually requires surgical intervention—hepatectomy or liver transplantation, commonly with good results.[4] However, these options have some limitations, specifically concerning organ availability, number of nodules and also the hepatic function and future liver remnant of the patient.[5]

Nevertheless, even in selected patients with single and/or small lesions, there are tumour relapses and tumour-related deaths, pointing out the distinct biological behaviour with different tumoural aggressivenesses.[6] In the last years, several markers have been proposed to identify more aggressive tumours: serum parameters like alpha fetoprotein (AFP), *vascular endothelial growth factor* (VEGF) and glypican 3;[4] clinical parameters, including portal vein thrombosis, model for end-stage liver disease (MELD) score, Child-Pugh classification and uncompensated cirrhosis;[7,8] and pathological criteria such as HCC gross classification,[9] microvascular invasion and tumoural differentiation.[6] Nonetheless, the prognostic value and the ability to stratify patients have not been the satisfactory and do not fulfil the need to clinically manage HCC patients, moreover in a pre-operative setting.

In order to overcome this limitation, several molecular classifications have emerged, with different clinical and therapeutic interventions,[10–13] which require fresh or frozen tumoural tissue with ensuing molecular classification not available in the majority of the institutions.

Recent studies have tried to establish morphological and immunohistochemical patterns, feasible in the mainstream of pathology departments, using routine-based antibody techniques, with simple and reproducible classification.[6,14] The application of this prognostic and stratification scores, allowing a morphophenotypic approach to the molecular classification, would be of extreme importance to an individualized medicine with proper surgical procedure selection and appropriate follow-up. The most recent scores based on formalin-fixed paraffin-embedded tissue were the ones developed by Srivastava et al.[14] and Tsujikawa et al.[6] They apply widely available antibodies that reflect aggressive tumour characteristics such as vascularization, P53 overexpression and biliary/stem cell markers.

The objectives of this work are to perform a clinical and pathological analysis of HCC and apply two distinct morphophenotypic classification scores, with overall survival correlation in order to assess the one that better suits our reality, allowing a posterior application in patient stratification in routine practice.

## Material and Methods

Clinical and pathological analyses of patients submitted to partial hepatectomy (PH) or liver transplantation (LT), with pathological examination revealing a single HCC, in a 9-year period (2007–2015) at the Serviço de Cirurgia A and Unidade de Transplantes Hepáticos Pediátricos e de Adulto, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal, were performed. The study was approved by the institutional ethics committee.

Clinical data were collected from patients' medical records—age, gender, tumour location, tumour recurrence, date of death and AFP serum levels.

The histological characteristics of HCC were determined in a retrospective manner, in representative tissue sections of each patient available in archive, by two experienced hepatopancreatobiliary pathologists (RCO and MAC) in haematoxylin and eosin (H&E)–stained slides, blinded to clinical and prognostic data.

The pathologic review took into consideration the tumour size (cm), surgical margin, gross subtype, microvascular invasion and capsule.

Gross subtype was considered as nodular, diffuse, multiple and satellite, as defined by the World Health Organization (WHO)[15]; microvascular invasion and capsule are defined as present or absent; tumour differentiation was defined according to morphological characteristics, stated by WHO.[15]

**Morphophenotypic Classification of HCC** For this purpose, immunohistochemical (IHC) study was performed with a paraffin-embedded tissue cut into 4-μm sections adherent on Superfrost Plus Slides (Thermo Fisher Scientific®Plus, Braunschweig, Germany). All glass slides with tissue sections were preheated at 60 °C in an oven prior to IHC staining for 40 min and staining was carried out on Ventana Benchmark Ultra equipment (Ventana Medical System, Tucson, USA).

For classification #1, the expression of CD31 (JC70, Ventana Medical System, Tucson, USA), p53 (DO-7, Ventana Medical System, Tucson, USA), AFP (anti-alpha-fetoprotein, Ventana Medical System, Tucson, USA) and CD44 (SP37, Ventana Medical System, Tucson, USA) were evaluated. The value of each one of the proteins represents their expression (negative = 0; positive = 1) and the histological characteristics classified as absent = 0 and present = 1; tumour size was quantified in centimetres. With these results, the score was calculated using the following formula: (0.800 ×

CD31) + (0.597 × p53) + (0.662 × AFP) + (0.485 × CD44) + (0.583 × tumour size) + (1.001 × vascular invasion).

Depending on the result, HCC is classified as $HCC^{high\text{-}risk}$ if the value is equal or superior to 3.240 and $HCC^{low\text{-}risk}$ if the value is inferior to 3.240, according to the description by Srivastava et al.[14]

For classification #2, the evaluation included the expression of CK19 (A53-B/A2.26, Ventana Medical System, Tucson, USA)—membrane and/or cytoplasmic in more than 5% of tumour cells; SALL4 (6E3, Ventana Medical System, Tucson, USA)—nuclear staining in more than 5% of tumour cells; β-catenin (anti-beta-catenin 14, Ventana Medical System, Tucson, USA)—nuclear staining in more than 5% of tumour cells; glutamine synthetase (GS-6, Ventana Medical System, Tucson, USA)—strong and diffuse cytoplasmic expression in tumour cells; EpCAM (Ber-EP4, Ventana Medical System, Tucson, USA)—membrane and diffuse staining in more than 5% of tumour cells; and p53 (DO-7, Ventana Medical System, Tucson, USA)—nuclear staining in more than 5% of tumour cells.

Accordingly, HCC was classified into three subgroups: one with positivity for at least one of the following markers—SALL4, CK19 and EpCAM, so-called B/S group (*biliary/stem cells*); other with staining for β-catenin and/or glutamine synthetase and also P53 staining, denominated as W/S group (*Wnt signalling*); and the last group the "all negative", with no staining for any of the markers, as stated by Tsujikawa et al.[6]

The interaction with the immune system was also studied, with immunohistochemistry staining for programmed death 1 (PD-1, SP142, Ventana Medical System, Tucson, USA) and programmed death ligand 1 (PD-L1, 22C3, Dako, Hamburg, Germany).

### Statistical Study

The statistical study was performed resorting to the Statistical Package for the Social Sciences, version 21.0. Non-parametric studies, such as $\chi^2$/Fisher's exact test, were used for independent measures. Survival studies were made with Kaplan-Meier, log rank and Cox regression. Statistical significance was considered for $p < 0.05$.

## Results

### Patient Samples

We have a total of 66 patients, 53 males and 13 females, with single HCC, encompassing 37 PH (56.1%) and 29 LT (43.9%). The median of age was of 64.5 ± 9.46 years old (range 38–86) and overall actuarial survival (OS) was 75.36 ± 6.9 months.

Of the 41 (61.1%) patients with AFP serum determination, only three patients were over 200 μg/ml. On gross examination, the majority of HCC was of nodular type ($N = 63$, 91.5%), with one of diffuse type (1.5%) and one of satellite type (1.5%). Average size was 3.5 ± 4.4 cm (1–22 cm), with size equal or superior to 5 cm in 36.4%. In 20 cases (30.3%), HCC had a capsule. Microvascular invasion was present in 24 cases (36.4%).

Using classification #1, HCC were classified as $HCC^{high\text{-}risk}$ and $HCC^{low\text{-}risk}$ with 33 (50%) for each group; an example of the parameters used is shown in Fig. 1.

Applying classification #2, 5 HCC (7.6%) were classified in the B/S group, 26 (39.4%) in the W/S group and 35 (53%) in the "all negative" group; examples of the immunostaining are exhibited in Fig. 2.

Regarding evaluation of PD-1/PD-L1 axis, the immunohistochemical study for PD-1 and PD-L1 was negative in all HCC.

The clinical and pathological data are shown in Table 1.

### LT Group

This group compreends 29 patients that undergone LT (23M:6F), with median of 61 ± 6.18 years, with only one patient over 70 years, six patients (20.7%) had HCC equal or larger than 5 cm, seven (24.1%) had microvascular invasion and five (17.2%) had a capsule. Using classification #1, 9 (31%) were $HCC^{high\text{-}risk}$ and 20 (69%) were $HCC^{low\text{-}risk}$. Applying classification #2, 3 HCC (10.3%) were classified as B/S group, 11 HCC (37.9%) as W/S group and 15 HCC (51.7%) in the "all negative" group.

### PH Group

Thirty-seven patients were submitted to PH (30M:7F) with median age of 70 ± 10.16 years; 21 patients (56.8%) with 70 or more years. HCC was equal or larger than 5 cm in 18 patients (48.6%), vascular invasion was present in 17 (45.9%) and capsule in 15 (40.5%).

Resorting to classification #1, 24 (64.9%) were $HCC^{high\text{-}risk}$ and 13 (35.1%) were $HCC^{low\text{-}risk}$. When classification #2 was applied, 2 HCC (5.4%) were classified as B/S group, 16 HCC (43.2%) as W/S group and 19 HCC (51.4%) in the "all negative" group.

The clinical and pathological data are shown in Table 2.

### Factors with Impact in Overall Survival

There was a positive correlation between absence of vascular invasion and better OS in the global population of the study ($p = 0.02$), and in the PH cohort ($p = 0.01$)—Fig. 3. Multivariate analysis confirms these findings with favourable OS in the absence of vascular invasion on the global

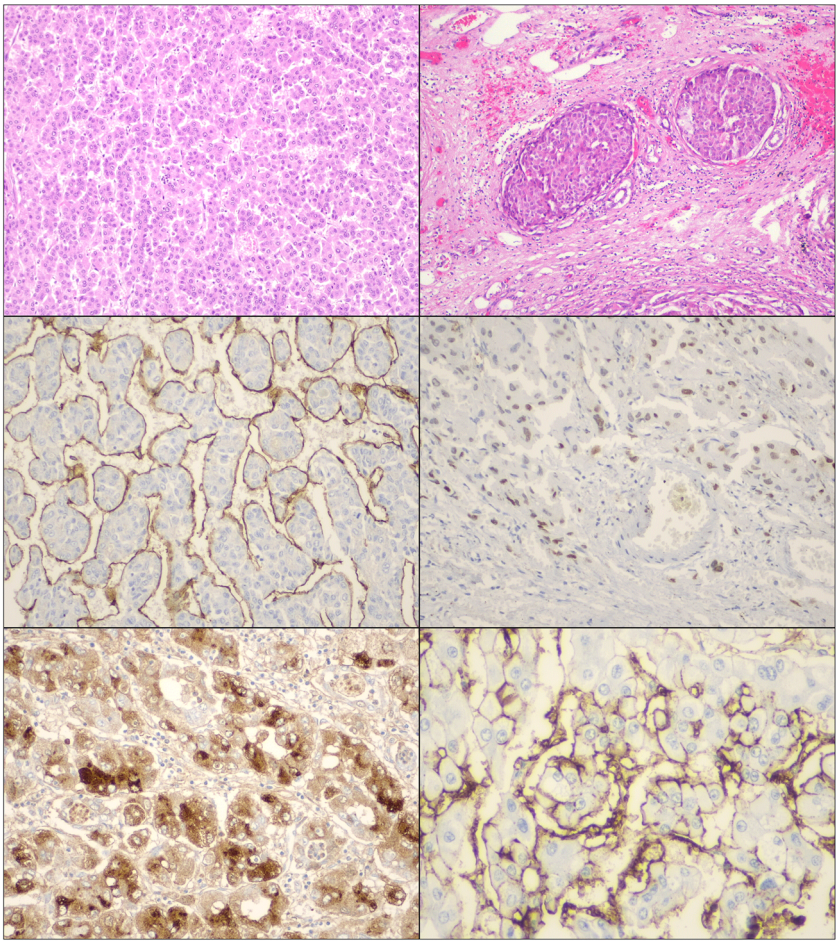

**Fig. 1** Hepatocellular carcinoma with a trabecular pattern, H&E × 100 (**a**); microvascular invasion, H&E × 100 (**b**); immunostaining for CD31 (**c**), P53 (**d**), alpha-fetoprotein (**e**) and CD44 (**f**)—pictures of the ancillary studies on a × 200 magnification. H&E, haematoxylin and eosin

population: $p = 0.04$, hazard ratio (HR) 0.358 and 95% confidence interval (CI) 0.179–0.714; and in the hepatectomy cohort: $p = 0.03$, HR 0.269, 95% CI 0.112–0.641.

The presence/absence of capsule and the size did not show influence on OS; age of patient was also not related to OS ($p > 0.05$).

When analysing the influence of classification #1 on OS, patients with $HCC^{high\text{-}risk}$ had a lower OS (71 months) when compared with $HCC^{low\text{-}risk}$ (77 months) in overall population and in the PH cohort (58 versus 67 months)—Fig. 4, however without statistical significance ($p > 0.05$).

In the evaluation of classification #2, the B/S group had a worse OS in the overall population (24.4 versus 83.75 and 71.47 months, in the W/S and all negative group respectively), in the LT cohort (33 versus 101.73 and 67.68 months, in the W/S and all negative group respectively) and in the PH cohort (11.5 versus 58.18 and 66.14 months, in the W/S and all negative group respectively), but there is no statistical significance ($p > 0.05$).

Nevertheless, this situation acquires statistical impact when we compare the effect on OS of the B/S group versus others on the overall population (24.4 versus 78.8 months, $p = 0.032$) and on the PH cohort (11.5 versus 64.01 months, $p = 0.016$) on univariate analysis—Fig. 5. This effect was confirmed by multivariate analysis with $p = 0.023$, HR 3.154 and 95% CI 1.190–10.379 for overall population and $p = 0.035$ for PH cohort—HR 5.369 and 95% CI 1.129–25.525.

There was no association between the B/S group and vascular invasion, with HCC size equal or superior to 5 cm, existence of chronic liver disease and respective aetiology—$\chi^2$ test, $p > 0.05$.

Tumoural relapse was observed in 12 patients (18.2%), only in the PH cohort, without association with vascular invasion, capsule, $HCC^{high\text{-}risk}$ and B/S group.

## Discussion and Conclusion

HCC is a malignant neoplasm with a high incidence and mortality, despite the development of adequate therapeutics and optimal surgical approaches.

It is intimately related to chronic liver diseases—mainly with viral and alcohol aetiologies, and in the last years, there is a strong association with non-alcoholic fatty liver disease, which is becoming increasingly prevalent in our population,

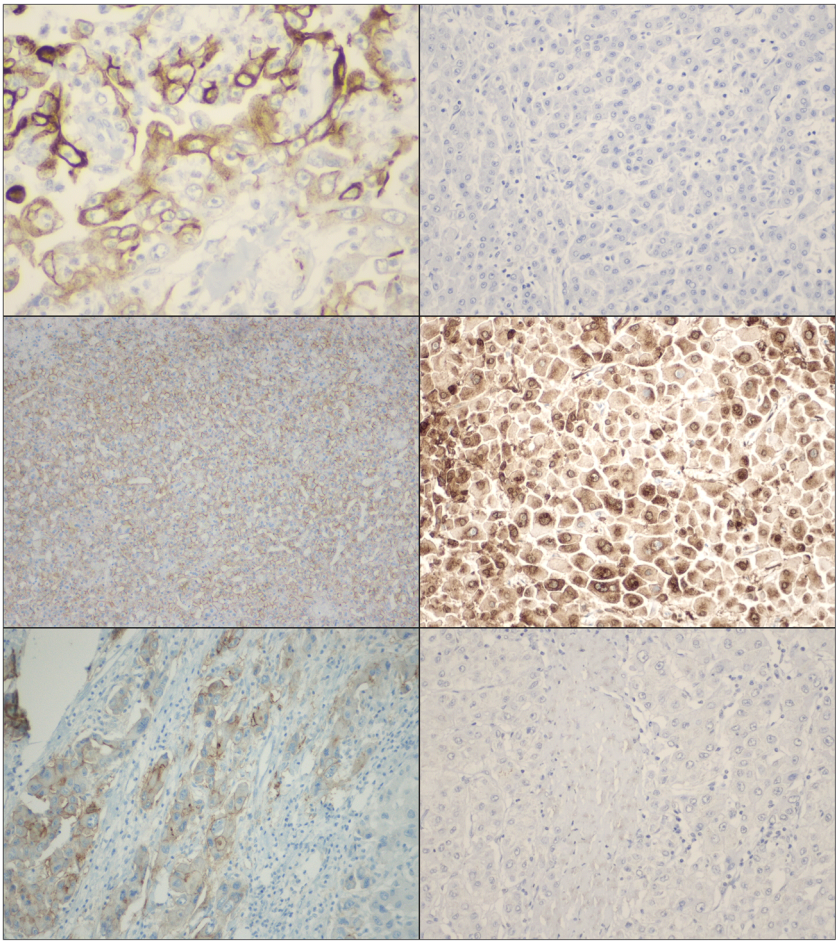

**Fig. 2** Ancillary studies for the classification described by Tsujikawa et al: positive staining for CK19, × 400 (**a**), absence of staining for SALL4, × 200 (**b**), membrane staining for β-catenin, × 100 (**c**), diffuse staining for glutamine synthetase, × 200 (**d**), positive staining for EpCAM, × 200 (**e**), and negative staining for P53, × 200 (**f**)

and is considered nowadays a well-established factor for HCC development.[16]

There have been several proposed clinical and pathological characteristics to predict the biological course of disease, but without the desired strength—except for immunostaining for CK19 and the identification of microvascular invasion. Furthermore, the small number of pre-operative biopsies does not allow a correct stratification and an individualized approach of patients before surgical procedure. Most of the pathological studies are performed on surgical specimens, way out of time for the pre-operative decision.[1,15,16]

Nowadays, the current clinical and pathological criteria allow some evaluation and risk stratification of patients, but far away, from what is intended to be an individualized medicine, especially in the pre-operative decision.

Development in oncobiology has led to molecular classifications of HCC: some are based on the concept of metabolic zonation, with more aggressive perivenular HCC and a less aggressive periportal HCC, the former associated with less differentiated HCC and with upregulation of genes associated with cellular proliferation.[12,13] Other classifications are more complex and involve multiple integrated molecular analysis with gene clustering.[11,17] The integrated morphological and molecular classifications are robust and validated in controlled cohorts, with strong clinical and pathological correlation and precise therapeutic implications;[13] however, they demand complex and specific studies, as well as fresh/frozen tumoural tissue, which is not available in the majority of institutions.

In the last years, some HCC classifications have been published that resort on immunohistochemical staining and morphological characteristics that can be potentially performed on any pathology department, namely the classification proposed by Srivastava et al.[14] (#1) and Tsujikawa et al.[6] (#2), both able to predict HCC biological behaviour. These two classifications are recent, based on formalin-fixed paraffin-embedded tissue, and use widely available antibodies.

When applied to our study population, both had influence regarding OS, with worse outcome in the aggressive subtypes—$HCC^{high\text{-}risk}$ and in the B/S group; however, the $HCC^{high\text{-}risk}$ classification did not obtain statistical significance.

In our population, classification #2 with the identification of the B/S group and poorer OS had statistical power, in uni- and multivariate analyses, in agreement with the findings reported by Tsujikawa et al.[6] More interestingly, it remained

**Table 1** Clinical and pathological characteristics of the study population. *Classification #1* classification by Srivastava et al.; *Classification #2* classification by Tsujikawa et al.; *HCC* hepatocellular carcinoma; *B/S* biliary/stem cells; *W/S* Wnt signalling; *N/A* non-applicable

| | Number of patients | Percentage (%) |
|---|---|---|
| Type of surgery | | |
| Partial hepatectomy | $N = 37$ | 56.1 |
| Liver transplant | $N = 29$ | 19.7 |
| Gender | | |
| Male | $N = 53$ | 80.3 |
| Female | $N = 13$ | 19.7 |
| Age | | |
| < 70 years | $N = 44$ | 66.7 |
| ≥ 70 years | $N = 22$ | 33.3 |
| Median age ± standard deviation | 64.5 ± 9.46 | N/A |
| Chronic liver disease | | |
| No | $N = 11$ | 16.7 |
| Yes | $N = 55$ | 83.3 |
| Chronic liver disease aetiology | | |
| Alcohol | $N = 39$ | 70.9 |
| HCV | $N = 9$ | 16.4 |
| NASH | $N = 4$ | 7.3 |
| HBV | $N = 2$ | 3.6 |
| Other | $N = 1$ | 1.8 |
| Size | | |
| < 5 cm | $N = 42$ | 63.6 |
| ≥ 5 cm | $N = 24$ | 36.4 |
| Capsule | | |
| Absent | $N = 46$ | 69.7 |
| Present | $N = 20$ | 30.3 |
| Vascular invasion | | |
| Absent | $N = 42$ | 63.6 |
| Present | $N = 24$ | 36.4 |
| Classification #1 | | |
| $HCC^{low\text{-}risk}$ | $N = 33$ | 50 |
| $HCC^{high\text{-}risk}$ | $N = 33$ | 50 |
| Classification #2 | | |
| B/S group | $N = 5$ | 7.6 |
| W/S group | $N = 27$ | 40.9 |
| "All negative" group | $N = 34$ | 51.5 |

significant in the partial hepatectomy cohort, reinforcing the role of liver transplantation as major treatment of HCC. Liver transplantation works in a dual manner, treating both HCC and a non-healthy liver, fertile ground for the development of new neoplasias, which does not occur in the partial hepatectomy. Classification #2 did not show association with existence of chronic liver disease nor the aetiology of HCC, which reinforces the role of biopsy for tumoural tissue sampling and posterior characterization.

The fundament of classification #1, from Srivastava et al., seems to be related to the angiogenic properties of the HCC, a characteristic established as a biologic risk factor, since it integrates in its formula microvascular invasion—a powerful risk factor for worse OS,[18] and also the increase in tumour vascularization in the form of higher CD31 expression, translating a sinusoidal capillarization with loss of fenestrae, and increased HCC aggressiveness.[19]

The major drawback of this classification is the fact that is necessary to evaluate microvascular invasion for the outcome of the formula. Microvascular invasion is not always present in the liver biopsy but is rather easy to evaluate in the surgical specimen. It could be possible to adapt this feature and integrate in algorithms with radiology assumption of microvascular invasion;[20–22] however, they are still far from the desirable concordance.

Classification #2, proposed by Tsujikawa et al., presents a good reproducibility and resorts to antibodies widely used in pathology laboratories, easy to use and to evaluate, and possible to determine in pre-operative biopsy. It is based on the identification of cells with more aggressive phenotype, with worse OS,[23–26] allowing the definition of more aggressive strategies and design individualized therapeutics.[27–29]

The use of morpho- and phenotypic classifications may represent tailored approaches with a correct identification of patients with more aggressive tumours, leading to better therapeutic options. Classification #2, by Tsujikawa et al., has the potential to be applied in liver biopsy tissue, with a tremendous role in the pre-operative scenario. This may also imply changes in the diagnostic and staging routines. The number of hepatic nodule biopsies has been decreasing, due to radiology improvements and to the complications of the procedure; yet the biopsy represents a unique opportunity for obtaining biological material, allowing a detailed classification of the nodule, not possible by non-invasive methods.[30] Besides the classification into more aggressive groups, the biological material also represents a potential for identification of biomarkers with posterior application for targeted therapy.[31–35] In the last years, some specific treatment options have been reported specifically for liver cancer stem cells, namely RNA regulation, interruption of signalling pathways, blocking autophagy and inactivation of drug resistance genes present in cancer stem cells, among others.[36,37] The correct identification of the biliary/stem cell HCC subgroup would allow better understanding of tumoural biology and personalized medicine, eventually with reference to clinical trials.

In the age of immunotherapy, the liver biopsy may also provide an opportunity for identification of tumours that may respond to anti PD-1/PD-L1 agents.

The anticancer immune activity, mediated by T cytotoxic lymphocytes, plays an important role, reason why the inhibition or negative regulation of the immune cycle is fundamental for tumour development. The production of PD-L1 has an

**Table 2** Clinical and pathological characteristics of the population submitted to liver transplantation and partial hepatectomy. *HCV* hepatitis C virus; *NASH* non-alcoholic steatohepatitis; *HBV* hepatitis B virus; *Classification #1* classification by Srivastava et al.; *Classification #2* classification by Tsujikawa et al.; *HCC* hepatocellular carcinoma; *B/S* biliary/stem cells; *W/S* Wnt signalling

| | Liver transplantation cohort | Partial hepatectomy cohort | |
|---|---|---|---|
| Gender | | | |
| Male | $N = 23$ (79.3%) | $N = 30$ (81.1%) | $p > 0.05$ |
| Female | $N = 6$ (20.7%) | $N = 7$ (18.9%) | |
| Age | | | |
| < 70 years | $N = 28$ (96.6%) | $N = 16$ (43.2%) | $p < 0.001$ |
| ≥ 70 years | $N = 1$ (3.4%) | $N = 21$ (56.8%) | |
| Median age ± standard deviation | 67.76 ± 10.16 | 59.79 ± 6.18 | $p < 0.001$ |
| Chronic liver disease | | | |
| No | $N = 0$ (0%) | $N = 11$ (40.7%) | $p = 0.001$ |
| Yes | $N = 29$ (100%) | $N = 26$ (70.3%) | |
| Chronic liver disease aetiology | | | |
| Alcohol | $N = 22$ (75.9%) | $N = 17$ (65.4%) | $p > 0.05$ |
| HCV | $N = 5$ (17.3%) | $N = 4$ (15.4%) | |
| NASH | $N = 0$ (0%) | $N = 4$ (15.4%) | |
| HBV | $N = 1$ (3.4%) | $N = 1$ (3.8%) | |
| Other | $N = 1$ (3.4%) | $N = 0$ (0%) | |
| Size | | | |
| < 5 cm | $N = 23$ (79.3%) | $N = 19$ (51.4%) | $p = 0.017$ |
| ≥ 5 cm | $N = 6$ (20.7%) | $N = 18$ (48.6%) | |
| Capsule | | | |
| Absent | $N = 24$ (82.8%) | $N = 22$ (59.5%) | $p = 0.036$ |
| Present | $N = 5$ (17.2%) | $N = 15$ (40.5%) | |
| Vascular invasion | | | |
| Absent | $N = 22$ (75.9%) | $N = 20$ (54.1%) | $p = 0.057$ |
| Present | $N = 7$ (24.1%) | $N = 17$ (45.9%) | |
| Classification #1 | | | |
| $HCC^{low\text{-}risk}$ | $N = 20$ (69%) | $N = 13$ (35.1%) | $p = 0.006$ |
| $HCC^{high\text{-}risk}$ | $N = 9$ (31%) | $N = 24$ (64.9%) | |
| Classification #2 | | | |
| B/S group | $N = 3$ (10.3%) | $N = 2$ (5.4%) | $p > 0.05$ |
| W/S group | $N = 11$ (37.9%) | $N = 16$ (43.2%) | |
| "All negative" group | $N = 15$ (51.7%) | $N = 19$ (51.4%) | |

immune suppressive activity by connecting with the PD-1 in T cytotoxic lymphocytes and in B7.1 in antigen-presenting cells. An immunostaining for PD-L1/PD-1 would allow to select patients candidates to PD-L1/PD-1 blockade,[38] which may reveal good responses and increase in OS;[39,40] however, in our study population, we did not had expression of PD-1 and PD-L1. The role of PD-L1 is still controversial in HCC management: the expression of PD-L1 in tumour tissue has

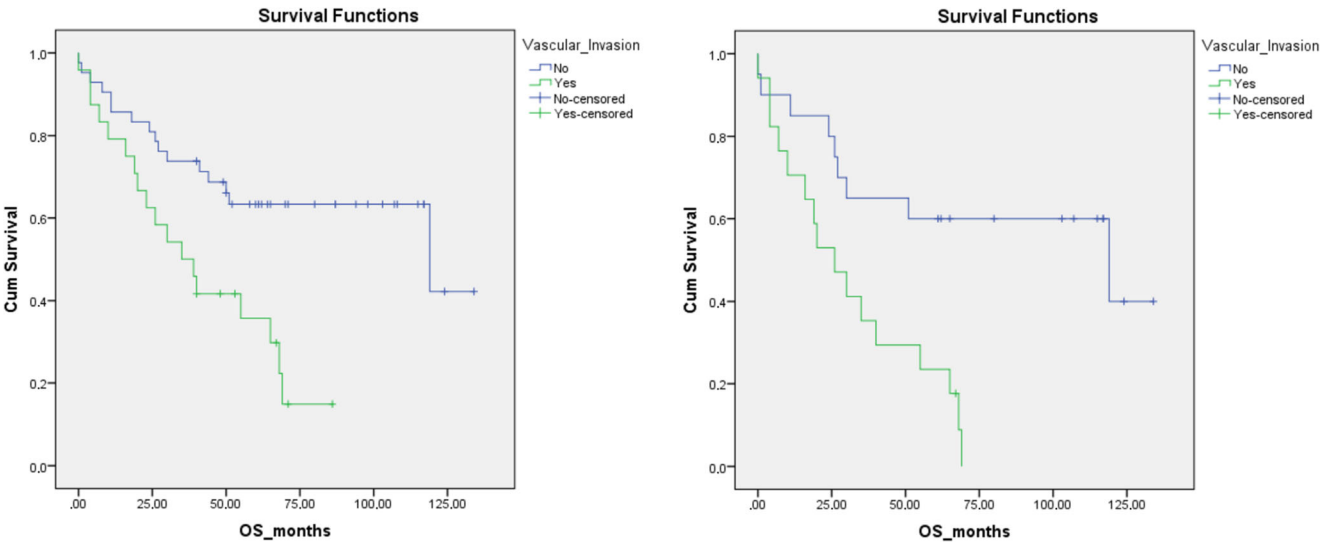


**Fig. 3** Overall survival curves regarding vascular invasion, global cohort (left side, $p = 0.02$) and partial hepatectomy cohort (right side, $p = 0.01$)

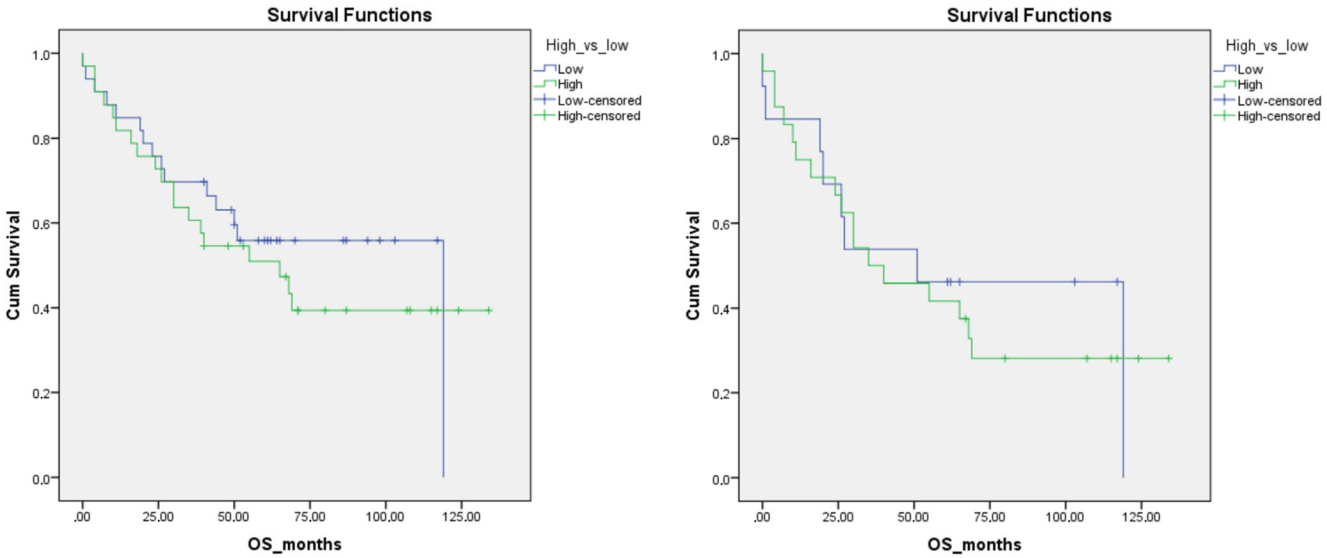


**Fig. 4** Overall survival curves regarding $HCC^{low\text{-}risk}$ and $HCC^{high\text{-}risk}$ in the overall population (left side) and partial hepatectomy (right side). Patients with $HCC^{high\text{-}risk}$ had worse survival, however without statistical significance ($p > 0.05$)

been linked to higher efficiency of immune checkpoint inhibitors, and currently, several clinical trials are in course with nivolumab, pembrolizumab and durvalumab, among others, in monotherapy regimen or in combination between them or with other molecular targeted agents;[41,42] other studies have found no difference between expression of PD-L1 and prognosis in patients submitted to surgery.[43] The expression of PD-L1 is widely variable—between 1 and 30% of HCC register PD-L1 expression[44] and this may be due to interobserver variability and the use of different PD-L1 clones;[45] PD-L1 expression is also modulated by macrophage M1[46] and this may provide different results in PD-L1 expression.

That does not necessary mean that immunotherapy does not have a role in HCC, and the recent knowledge that the evaluation of tumour mutation burden (TMB) is more effective in identifying patients more responsive to checkpoint inhibitors,[47] reinforcing the role of the biopsy as a tissue obtaining method. TMB is linked to prognosis in patients with HCC and high TMB has been reported as more reliable in predicting response to checkpoint inhibitors than PD-l1 expression;[48,49] however, the low number of patients with TMB determination and checkpoint inhibitor therapy does not allow to take solid conclusions.[47,50]

The risks of biopsy—haemorrhage and seeding—have been reported as infrequent and easy to handle, without considerable impact in OS.[16] The risk of seeding is estimated in 2.7% and may be even inferior in reference centres, according to the description by Silva et al.,[51] and concerning

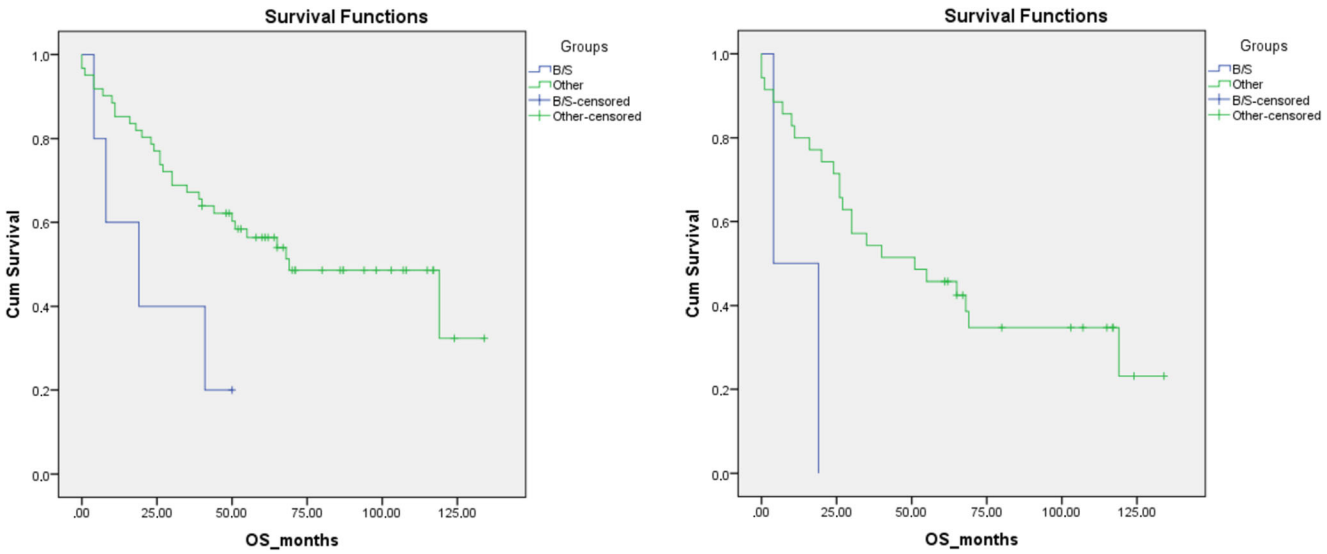


**Fig. 5** Survival curves regarding the worst survival of the B/S group versus other groups on the overall population (left side, $p = 0.032$) and partial hepatectomy population ($p = 0.016$)

haemorrhage, Rockey et al. refer a low risk—3 to 4%, the majority with mild bleeding, with only need for transfusion in 0.5%.[52] Therefore, the risks concerning biopsy are low, easy to cope with and should not be dissuasive, and the biopsy should not be reserved only for problematic imaging cases, but also used for prognostic implications. The use of local therapy and its impairment in performing the ancillary techniques was not an issue since they were performed on viable tumour areas.

This study has some limitations, namely its retrospective design, limiting the amount of information obtained—only 41 patients (62.1%) had AFP serum level determined, and the selection criteria with only patients with one HCC may had induced bias in the selection of the patients.

The results support the necessity of a morphophenotypic classification of patients with HCC, especially in the preoperative context; it may help to better select patients with well-compensated chronic liver disease and without portal hypertension to undergo liver transplant or partial hepatectomy according to stratification risk. Patients with aggressive HCC may benefit from hepatic transplantation, if they remain inside the transplant criteria after a waiting period, while in patients with more indolent HCC and compensated liver disease (low grade of fibrosis), partial hepatectomy may be enough.

The classification by Tsujikawa et al. seems adequate to our reality with effects in OS, feasible in biopsies, with routine antibodies, easy to interpret, allowing an individualized medicine, that in a situation with no expression of PD-1 and PD-L1 may identify patients that would benefit from aggressive treatments, especially the ones within the B/S group.[27,29,53]

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**Authors' Contribution** RCO and RM designed the study, collected clinical data and wrote the manuscript. AMA performed statistical analysis. AJ and PT performed ancillary tests. CC, PG, BC and MRS collected clinical and pathological data. JGT and MAC were senior supervisors and revised the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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## Compliance with Ethical Standards

The study was approved by the institutional ethics committee.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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