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Clinical and Pathological Analysis of Patients Undergoing Liver Transplantation for Hepatocellular Carcinoma

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Abstract

Introduction**:** Hepatocellular carcinoma (HCC) is an aggressive tumor with raising incidence worldwide and represents a major global health problem. Liver transplant (LT) is an optimal therapeutical option with potential to cure HCC and subjacent liver disease. However, HCC recurrence is a major problem and hepatocarcinogenesis and tumor biological behavior is still poorly understood. The aim of this study was to identify clinical, analytical and histological prognostic factors with impact in post-LT overall survival (OS) and disease free survival (DFS) and to determine post-LT prognostic and tumor recurrence value of immunohistochemical markers such as cytokeratin 19 (CK19), glypican-3 (GPC3) and podoplanin (D2-40), on patients that underwent LT for HCC.

Patients and Methods**:** Retrospective study of clinical and outcome data of 80 patients who underwent LT for HCC in our center from 1st January of 2010 to 31st December of 2015.

Results**:** Follow-up was at least 1 year and the mean period was 33.6±21.9 (0-84) months. One and 5-year OS was 88.6% and 85.2%, respectively and DFS was 87.4% and 83.6%, respectively. Ninety-day post-LT morbidity was 63.7% and mortality 5%. Recurrence of HCC was observed in 10%. In univariate analysis ($p<0.05$) presence of 4-5 nodules ($p=0.017$), alpha-fetoprotein (AFP) serum levels ≥ 200ng/ml ($p=0.027$), microvascular invasion (MVI) ($p=0.018$) and HCC recurrence ($p<0.001$) were predictors of worse OS. Presence of 4-5 nodules ($p=0.022$) and MVI ($p=0.006$) were predictors of worse DFS ($p<0.05$). There was no statistical difference on OS ($p=0.717$) and DFS ($p=0.794$) of patients with BCLC (Barcelona Clinic Liver Cancer) 0-C stages when compared to stage D. In patients beyond Milan Criteria (MC), 3-year OS when MVI was observed was 50%, whereas without MVI was 93.8% ($p=0.026$). On multivariate analysis, we found that AFP serum levels ≥200ng/ml ($p=0.035$) and MVI ($p=0.021$) were independent predictor factors of worse OS and MVI was also a predictor of worse DFS ($p=0.027$).

Conclusion**:** LT is an optimal therapeutical option for patients with severe liver disease complicated with HCC, even in those with end-stage liver disease. Further work must be done to assess and identify predictive factors that clarify biological tumor behavior of HCC, that can be applied in a better selection of patients for LT.

Keywords: hepatocellular carcinoma, liver transplant, prognostic factors, histopathology, tumor recurrence.

Abbreviations:

ACE	arterial chemoembolization
AFB1	aflatoxin B1
AFP	alpha-fetoprotein
AJCC	American Joint Committee on Cancer
BCLC	Barcelona Clinic Liver Cancer
BMI	body mass index
CK19	cytokeratin 19
CLD	chronic liver disease
D2-40	podoplanin
DCP	des-gamma-carboxyprothrombin
DFS	disease free survival
DMII	diabetes mellitus type II
GPC3	glypican 3
HBV	hepatitis B virus
HCV	hepatitis C virus
HCC	hepatocellular carcinoma
HGDN	high-grade dysplastic nodules
IHC	immunohistochemical
INR	international normalized ratio

LGDN	low-grade dysplastic nodules
LT	liver transplant
MC	Milan criteria
MELD	Model for End-stage Liver Disease
MS	metabolic syndrome
MVI	microvascular invasion
NAFLD	non-alcoholic fatty liver disease
NAT	neo-adjuvant treatments
OS	overall survival
PBC	primary biliary cirrhosis
PF	prognostic factor
PSC	primary sclerosing cholangitis
RF	risk factors
RFA	radiofrequency ablation
SH-HCC	steatohepatitic hepatocellular carcinoma
TIPS	Transjugular Intrahepatic Portosystemic Shunt
UCSF	University of California, San Francisco

I. Introduction

Hepatocellular carcinoma (HCC) is an aggressive tumor with raising incidence worldwide and represents more than 90% of primary hepatic malignancies (1, 2); is the second leading cause of death by cancer worldwide (more than 700.000 deaths per year) and the fifth most common malignancy.(3, 4) HCC is more common in men with a peak of incidence in the seventh decade, however, it can occur earlier in Chinese and African populations.(1, 4-6)

The etiology of HCC is multifactorial; the major environmental risk factors (RF) for HCC are infection by hepatitis B virus (HBV) and C (HCV), alcohol, aflatoxin B1(AFB1), metabolic syndrome (MS) and diabetes *mellitus* type II (DMII). Hereditary Hemochromatosis, Alpha-1 antitrypsin deficiency and Wilson's disease are inherited metabolic RF.(1, 5, 7) Geographic distribution of HCC is largely determined by RF prevalence and about 85% of the cases emerge in developing countries. Life style modifications associated with MS and alcohol abuse are responsible for recent HCC increasing prevalence, mainly in developed countries.(1, 5, 8)

GLOBOCAN 2012 data for Portugal showed about 1000 new cases of HCC each year, overall age-adjusted incidence of 5 cases/100.000 and a mortality above 900 cases each year.(4)

HCC normally grows on a background of chronic liver inflammation and cirrhosis; the cumulative risk of developing HCC in a cirrhotic liver is 3-5% per year (one-third over lifetime).(7, 9) High grade dysplastic nodules (HGDN) are pre-neoplastic lesions and its malignization occurs in 30% over a period of 1-5 years.(9, 10) Tumor extension, liver function and performance status are the most important prognostic factors (PF).(1)

Hepatocarcinogenesis is a complex process that stills poorly understood but genetic heterogeneity suggests involvement of multiple intracellular pathways.(2) More frequent mutations are related with activation of CTNNB1 (20-40%) and AKT/mTOR or inactivation of TP53 and RB1.(11, 12) Other proteins involved in hepatocarcinogenesis are IL-6, APC, PTEN,

BRCA2, SMAD2 and -4, c-Myc, cyclin-D1; VEGF overexpression was also associated with angiogenesis and tumor proliferation.(2, 13) Some series described microRNA's as future potential biological markers/ molecular therapeutic targets considering their role as oncogenes and/or tumor suppressor agents in hepatocarcinogenesis.(10, 12, 13)

Liver transplant (LT) is the only therapeutic option with potential to simultaneously cure HCC and subjacent chronic liver disease (CLD) and if Milan criteria (MC) are met, 5-year overall survival (OS) above 70% and tumor recurrence below 15% is typically described.(7, 14-16) Scarcity of organs and drop-out in waiting list due to tumor progression are the major drawbacks of LT.(17) Neo-adjuvant treatments (NAT) are recommended to prevent tumor progression when expected waiting time is longer than 6 months in waiting list.(3, 8, 16)

In fact, gross morphology criteria seem to be inappropriate to evaluate tumor biological behavior and factors associated with poor survival and pathological evaluation are rarely evaluated preoperatively. Selection criteria for LT do not include histopathological and molecular PF for tumor aggressiveness, such as microvascular invasion (MVI), which is important to predict recurrence and survival and it is directly associated with histologic differentiation, number and size of nodules, but cannot be evaluated on pre-LT stage, by imaging studies or liver biopsy.(1, 5, 8, 18, 19) Some series suggested the "test of time" for MELD (Model for End-stage Liver Disease) prioritized HCC patients in waiting list for LT, because a longer time of observation allowed tumor biology to manifest and was associated with decreased post-LT recurrence.(20, 21)

The primary objective of this study was to determine PF with impact in post-LT OS and disease-free survival (DFS) in patients with HCC. The secondary objective was to determine the post-LT prognostic and tumor recurrence value of immunohistochemical (IHC) markers such as cytokeratin 19 (CK19), glypican-3 (GPC3) and podoplanin (D2-40).

II. Patients and methods

1. Study design

This retrospective study included clinical, pathological and IHC analysis of 80 patients who underwent LT for HCC at Unidade de Transplantação Hepática Pediátrica e de Adultos, Centro Hospitalar e Universitário de Coimbra (UTHPA, CHUC), from 1st January 2010 to 31st December 2015. These patients were diagnosed with HCC based on preoperative imaging studies and/or diagnostic confirmation by pathologic examination of total hepatectomy specimens. Demographic, clinical and pathological data were collected from patient's records. Exclusion criteria were other LT indications, insufficient clinical information, poor histological material and incidental pathological confirmation of cholangiocarcinoma diagnosis (Figure 1). A formal ethics committee approval was not required considering the retrospective nature of this study.

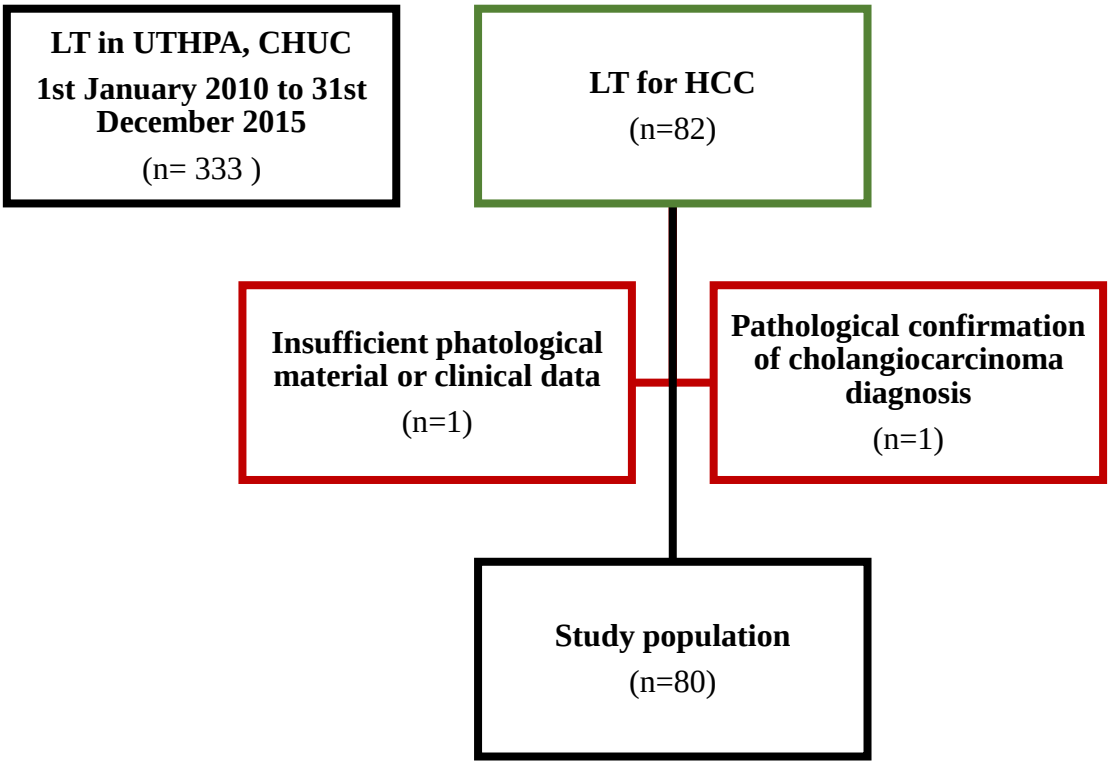

Figure 1. Exclusion criteria and study population.

2. Patients and tumor characteristics

The study included 80 patients, 69 men and 11 women. All cases had CLD and 65% with associated symptoms. Mean age was 58.8 ±6.8 (35-72) years and 14 had above 65 years-old.

Alcohol abuse was the most common etiology of underlying CLD, accounting for 60% of all cases when isolated (68.8% when associated with HBV or HCV infections). Other etiologies for CLD were HCV (13.8%) and HBV (11.3%) infections, non-alcoholic fatty liver disease (NAFLD) (2.5%), primary biliary cirrhosis (PBC) (2.5%) and primary sclerosing cholangitis (PSC) (1.3%).

Comorbidities more frequently described were DMII (59.5%), arterial hypertension (81%), dyslipidemia (46.3%) and overweight. Applying body mass index (BMI), 45% were overweight, and 26.3% obese. Clinical characteristics are described with more detail in Table 1.

Pre-LT serum levels of alpha-fetoprotein (AFP), total bilirubin and alkaline phosphatase, platelets counting, INR (international normalized ratio), prothrombinemia and albuminemia are presented in Table 2.

MELD score was calculated based on pre-LT serum levels of bilirubin, creatinine and INR (22, 23), with a median value of 13, mean of 13.9±5.5 (range 6-35), MELD ≥10 in 77.5% and ≥15 in 42.5% of patients (Table 3). Child-Pugh classification was calculated based on pre-LT serum levels of bilirubin, albumin, INR and existence of encephalopathy or ascites (24), and 49.4% were classified into class A, 35.4% were class B and 15.2% were class C (Table 3).

Characteristic	No. of patients (%)
Gender	
Male	69 (86.3%)
Female	11 (13.7%)
Age	
Mean ± SD (range) years	58.8 ±6.8 (35-72)
> 65 years	14 (17.5%)
CLD etiology	
Alcohol	48 (60%)
HCV	11 (13.8%)
HBV	9 (11.3%)
HCV + Alcohol	6 (7.5%)
HBV + Alcohol	1 (1.3%)
NAFLD	2 (2.5%)
PBC	2 (2.5%)
PSC	1 (1.3%)
Comorbidities	
DMII	47 (59.5%)
Arterial hypertension	64 (81%)
Dyslipidemia	37 (46.3%)
BMI ≥25 Kg/m2	57 (71.3%)

Table 1. Clinical characteristics of study population.

Characteristic	No. of patients (%)
Alpha-fetoprotein	
>100ng/mL	6 (7.9%)
>200ng/mL	5 (6.6%)
Total bilirubin	
≥3 mg/dl	15 (18.8%)
Serum albumin	
<3.5 g/dl	42 (52.5%)
Alkaline phosphatase	
≥120U/L	44 (55%)
Platelets	
$<120x10^9$/L	56 (70%)
INR	
≥1.20	62 (77.5%)
Prothrombinemia	
<75%	63 (78.8%)

Table 2. Pre-LT laboratorial assessment.

Classification	Score
MELD score	
median	13
mean ± SD (range)	13.9±5.5 (6-35)
≥10	62 (77.5%)
≥15	34 (42.5%)
Child-Pugh class	
A	39 (49.4%)
B	28 (35.4%)
C	12 (15.2%)

Table 3. Liver disease assessment.

MC defined as a solitary lesion ≤ 5cm or 2 to 3 nodules ≤ 3cm in the absence of macrovascular invasion on imaging staging (14, 25), were met in 57.5% of cases (Table 4). Up-to-seven criteria, an expansion of MC, defined as the sum of the size of the largest lesion in centimeters with the number of nodules (15, 26), were met in 72.5% of cases (Table 4). University of California, San Francisco (UCSF) criteria defined as 1 lesion ≤ 6.5cm or 2 to 3 lesions ≤ 4.5cm with a total diameter ≤ 8cm (15, 27), were met in 70% of cases (Table 4). In 21 (26.3%) patients, none of CM, Up to Seven or UCSF criteria were met. Barcelona Clinic Liver Cancer (BCLC) classification has 5 stages (0, A, B, C, D) related to tumor stage, liver function, performance status and symptoms related with the malignant disease and links each stage to a treatment algorithm.(1, 9) BCLC classification in our study population was: 8.8% very early stage (class 0), 45% early stage (class A), 31.2% intermediate stage (class B), 0% advanced stage (class C) and 15% terminal/end stage (class D) (Table 4). All morphological criteria were applied according to the total hepatectomy specimen pathological analysis data to a more precise, uniform and correct interpretation of the results.

Criteria	**No. of patients (%)**
Milan criteria	
In	46 (57.5%)
Up to Seven	
In	58 (72.5%)
UCSF	
In	56 (70%)
BCLC	
0 (very early stage)	7 (8.8%)
A (early stage)	36 (45%)
B (intermediate stage)	25 (31.2%)
C (advanced stage)	0 (0%)
D (terminal stage)	12 (15%)

Table 4. Selection criteria for liver transplantation.

Pathological analysis of total hepatectomy specimens allowed diagnostic validation, staging and histological characterization of tumor nodules, and was performed without awareness of clinical data, treatment details or patient outcome (Table 5). In this study, small HCC was defined as a single lesion < 3cm in diameter. Dysplastic nodules were identified in 27 (33,8%) patients; 15 patients with low-grade dysplastic nodules (LGDN), 6 patients with high-grade dysplastic nodules (HGDN) and 6 patients with LGDN and HGDN. Tumor differentiation of the larger nodule was defined in accordance with Edmonson-Steiner grading system: G1- well differentiated, G2 – moderately differentiated, G3 – poorly differentiated. (11). MVI was present in 36.8% of cases (venous in 95.8% and mixed- venous and lymphatic – in 4.2%). IHC staining showed that CK19 was negative in 93.3%, positive in 4.4% and positive/negative in 2.3%; GPC3 was negative in 66.7% and positive in 33.3%. The most common histological pattern of larger nodule was trabecular (37.5% macrotrabecular and 32.5% microtrabecular). (11, 28) Other histological patterns identified were acinar or pseudoglandular (13.8%), steatohepatitic (10%) and clear cell variant (1.3%). Two variants of HCC were identified – classic (87.5%) and steatohepatitic (SH-HCC) (12.5%).(28) Steatosis was present in 62.5%, steatohepatitis in 10%, cirrhosis in 97.5%. According to American Joint Committee on Cancer (AJCC) classification (9), 27.5% were stage I, 55.1% were stage II, 14.4% were stage III and 2.9% were stage IV.

Characteristic	No. of patients (%)
Number of nodules	
Single	37 (46.3%)
2-3	26 (32.5%)
4-5	11 (13.8%)
6-7	6 (7.5%)
≥ 3	28 (35%)
Small HCC	21(26.3%)
Larger tumor size±SD, cm	3.7±2.4 (1-8)
Tumor differentiation	
G1 - Well	4 (5%)
G2 - Moderately	63 (78.8%)
G3 – Poor	9 (11.3%)
Encapsulation	
Larger tumor	43 (57.3%)
In any nodule	46 (59.7%)
MVI	
Larger tumor	24 (32.9%)
In any nodule	28 (36.8%)
Histological pattern	
Macrotrabecular	30 (37.5%)
Microtrabecular	26 (32.5%)
Pseudoglandular	11 (13.8%)
Steatohepatitic	8 (10%)
Clear cell variant	1 (1.3%)
HCC classification	
Classic	70 (87.5%)
SH-HCC	10 (12.5%)

Table 5. Tumor characteristics.

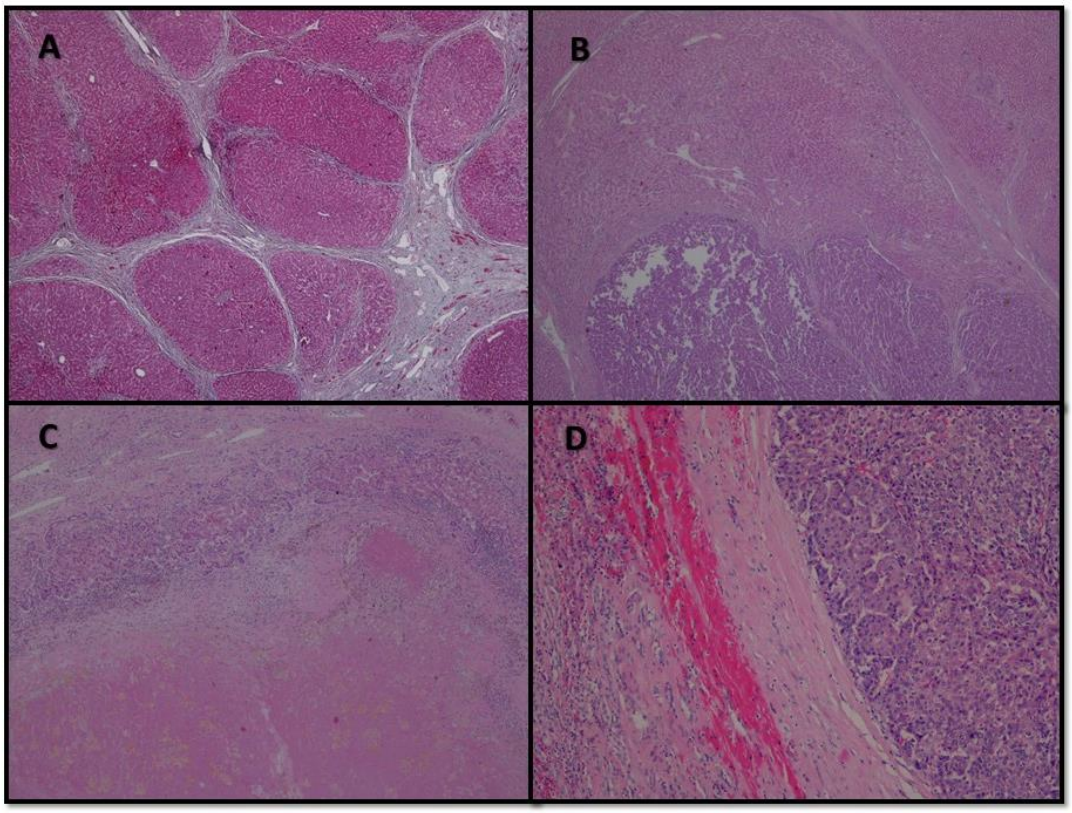

Figure 2. Pathological analysis on optic microscopy: A – Masson's trichrome staining (20x) of cirrhotic liver with regenerative nodules; B- Hematoxylin and Eosin (H&E) staining (20x) of HCC showing nodule-in-nodule growth; C- H&E staining (40x) of post-therapeutical necrosis of HCC; D- H&E staining (100x) of nodule encapsulation.

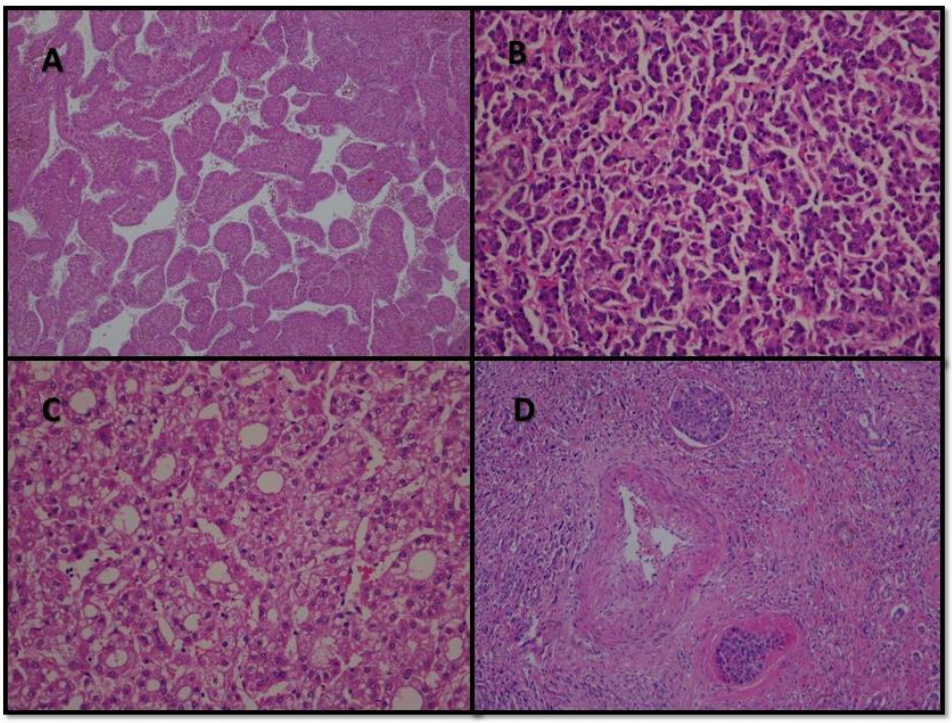

Figure 3. Pathological analysis on optic microscopy: A- H&E staining (100x) of macrotrabecular pattern; B- H&E staining (200x) of microtrabecular pattern; C- H&E staining (200x) of pseudoglandular pattern; D- H&E staining (100x) of microvascular invasion.

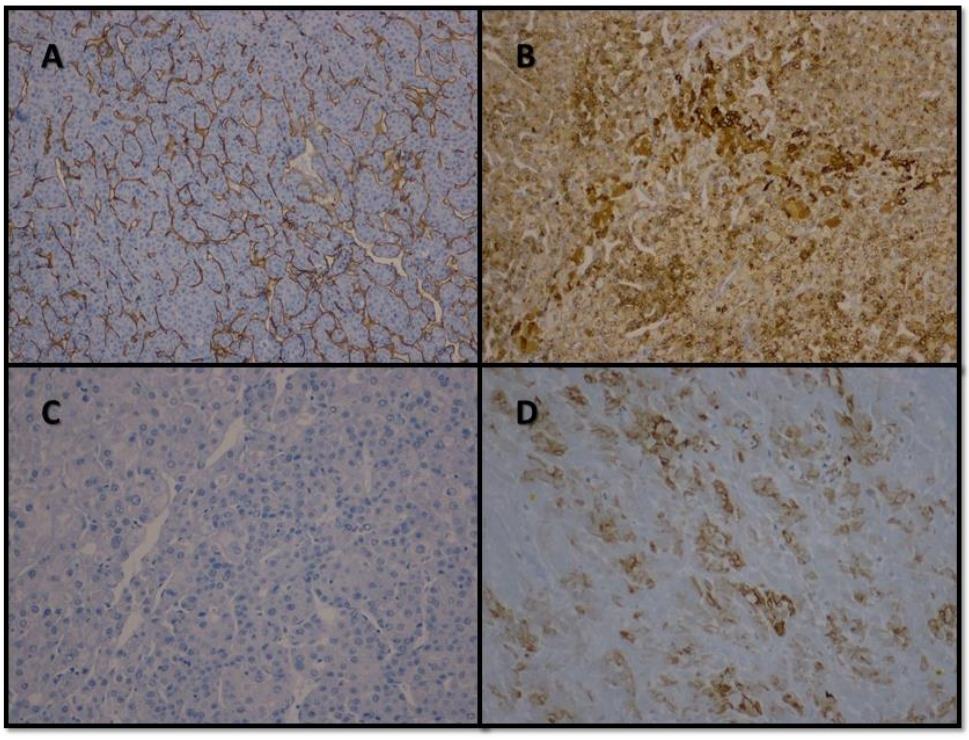

Figure 4. Immunohistochemical analysis: A- CD34 positive staining (100x); B- GPC3 positive staining (100x); C- negative CK19 staining (200x); D- positive CK19 staining (200x).

3. Treatment

NAT by arterial chemoembolization (ACE) or radiofrequency ablation (RFA) was performed in 46.3% and necrosis achieved in the largest nodule is described in Table 6. Other treatments used before LT were: sorafenib in 2 patients and TIPS (Transjugular Intrahepatic Portosystemic Shunt) in 4 patients. LT was performed with Piggyback technique in 96.3% and with classic in the remaining cases. All patients received deceased-donor organs. The median of total ischemic time was 474.5 minutes, mean of 483,5±120 (264-873) minutes, more than 7 hours (420 minutes) in 65%. The median of portal vein ischemic time was 418.5 minutes, mean of 431.8±115.6 (227-800) minutes; median of warm ischemia time was 49 minutes, mean of 51.7±16.4 (24-105) minutes. Hemoderivative transfusions in perioperative period was seen in 68.8% of the patients.

Characteristic	**No. of patients (%)**
NAT	37 (46.3%)
ACE	24 (64.9%)
RFA	13 (35.1%)
Large nodule necrosis	
0-25%	3 (8.6%)
26-50%	4 (11.4%)
51-75%	5 (14.3%)
76-100%	23 (65.7%)
>50%	28 (80%)
100%	5 (14.3%)

Table 6. Neo-adjuvant treatment.

4. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS™) software (version 22.0 for Windows; SPSS Inc., Chicago, IL, USA). We made a descriptive

analysis of metric variables and quantitative data were expressed as numbers ± standard deviation (SD) and range.

Survival was evaluated with Kaplan-Meier method and differences between groups with log-rank test. Multivariate analysis of factors associated with survival were evaluated with Cox regression model. The differences were considered as statistically significant when $p<0.05$.

III. Results

1. Post-transplant mortality and morbidity

Post-LT hospitalization mean time was 13±12.8 (6-94) days. In the first 90 days after LT observed mortality was 5%, due to cardiogenic shock and infectious complications (Table 7). Ninety-day morbidity was 63.7%; 37.5% major morbidity, classes IIIa, IIIb, IV and V of Clavien-Dindo classification (Fig.5 and Table 7). (29, 30) Recurrence of HCC was confirmed in 10% (5% extra-hepatic – pulmonary and bone -, 2.5% in hepatic graft and 2.5% with hepatic and extra-hepatic recurrence) and in those, MVI was present in 85.7%.

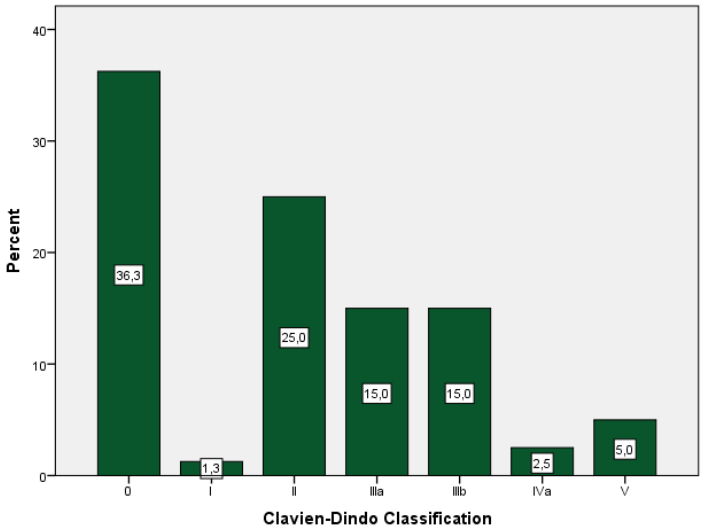

Figure 5. Surgical complications up to the 90th post-LT day according to Clavien-Dindo classification.

Surgical Complications	No. of patients (%)
Biliary	
Leak	5 (6.25%)
Stricture	11 (13.75%)
Vascular	
Hepatic artery stenosis	4 (5%)
Splenic artery steal syndrome	7 (8.75%)
Renal	
Acute renal failure	5 (6.25%)
Urinary tract infection	3 (3.75%)
Pulmonary	
Effusion	23 (28.75%)
Pneumonia	4 (5%)
Edema	1 (1.25%)
Pneumothorax	1 (1.25%)
Infectious	
Intra-abdominal abscess	6 (7.5%)
Surgical site infection	3 (3.75%)
Bacteremia	3 (3.75%)
Peritonitis	4 (5%)
Graft reinfection (HCV)	3 (3.75%)
Cytomegalovirus infection	4 (5%)
Gastrointestinal	
Incisional hernia / dehiscence	9 (11.25%)
Cardiac	
Atrial fibrillation	3 (3.75%)
Post-LT Mortality	4 (5%)
Infectious complication	2 (2.5%)
Cardiogenic shock	1 (1.25%)
Peri-operatory death	1 (1.25%)

Table 7. Post-LT surgical complications.

2. Overall survival and disease free survival

After discharge, patients were followed up at the outpatient clinic. The length of follow-up was at least 1 year and the mean period was 33.6±21.9 (range 0-84) months.

OS defined as the time between LT and the date of tumor-related death or end of follow-up period of the study if patients were alive, and mean was 73.0±3.1 months (Fig. 6). DFS defined as the period between LT and tumor recurrence and mean was 71.7±3.3 months (Fig. 7).

In patients with HCC recurrence, the mean OS was 15.3±3.3 (8.8-21.7) months, 1- and 3-year OS were 37.5% and 12.5%, respectively; compared to mean OS of 79.4±2.2 (75.0-83.8) months and 1- and 3-year OS of 94.4% for those without HCC recurrence (p<0.001) (Fig. 8).

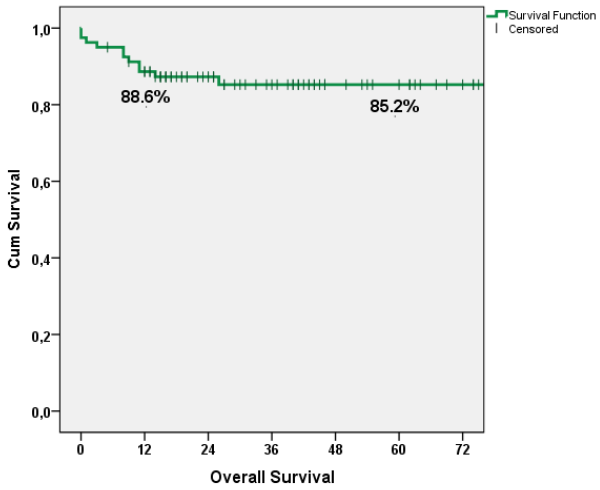

Figure 6. Overall Survival (OS) of the entire cohort: 1- and 5-year were 88.6% and 85.2%, respectively.

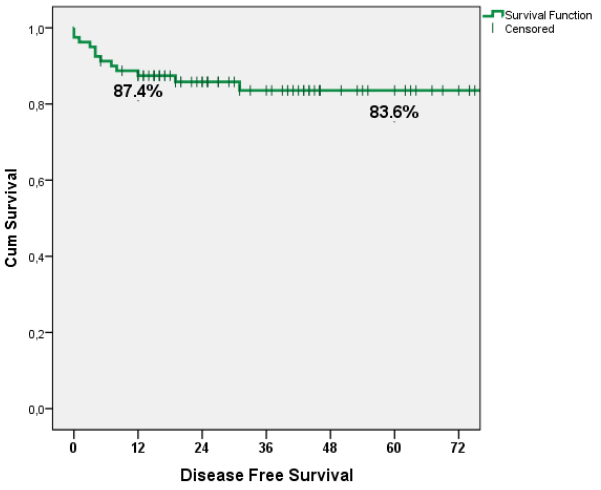

Figure 7. Disease Free Survival (DFS) of the entire cohort: 1- and 5-year were 87.4% and 83.6%, respectively.

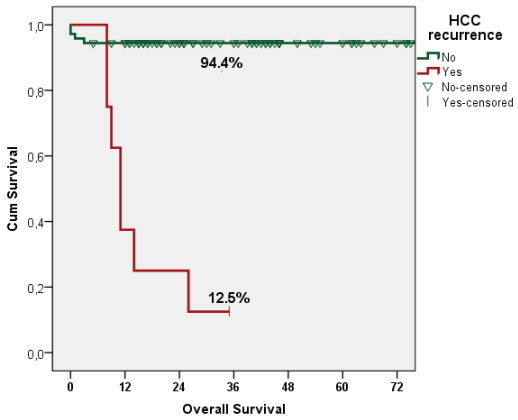

Figure 8. 3-year OS was 12.5% for patients with HCC recurrence, compared to 94.4% for those without recurrence (p<0.001).

3. Prognostic factors with impact on OS and DFS – univariate analysis

The presence of 4 or 5 nodules was a worse prognostic factor for OS (p=0.017), with mean OS of 45.8±10.3 (27.7-65.9) months compared to 76.0±2.9 (70.3-81.6) months for patients without 4-5 nodules (Fig. 9). This parameter was also associated with worse DFS (p=0.022), with mean DFS of 46.7±10.0 (27.0-66.3) months compared to 74.6±3.1 (68.4-80.7) months (Fig.10).

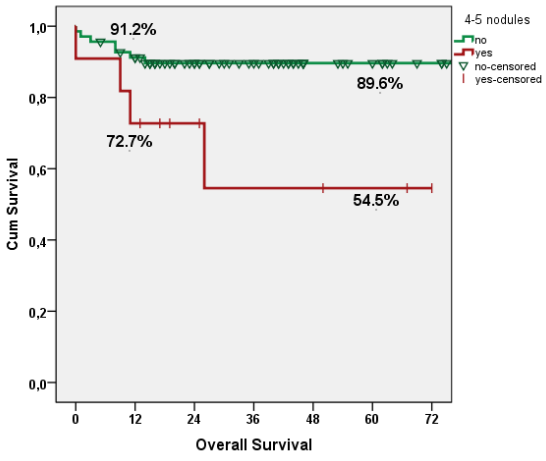

Figure 9. 5-year OS was 54.5% for patients with 4-5 nodules and 89.6% for those without 4-5 nodules (p=0.017)

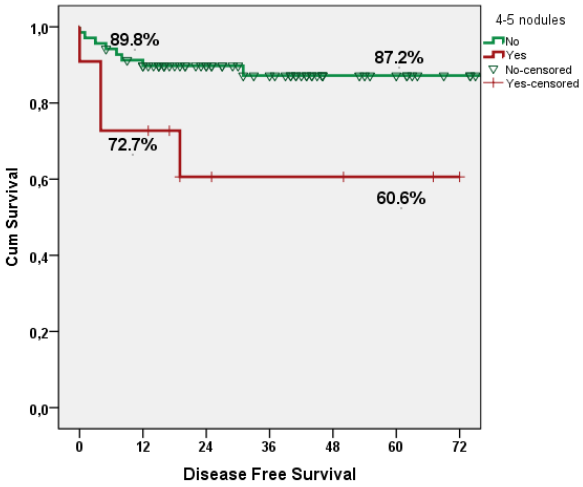

Figure 10. 5-year DFS was 60.6% for patients with 4-5 nodules and 87.2% for those without 4-5 nodules (p=0.022).

AFP serum levels ≥ 200ng/ml showed significant impact on OS (p=0.027) - mean OS of 39.4±13.0 (14.0-64.8) months compared to 74.0±3.1 (67.9-80.1) for those with AFP < 200ng/ml (Fig. 11). AFP serum levels ≥200ng/ml had tendency for worse DFS but with no statistical significance (p=0.057) (Fig. 12).

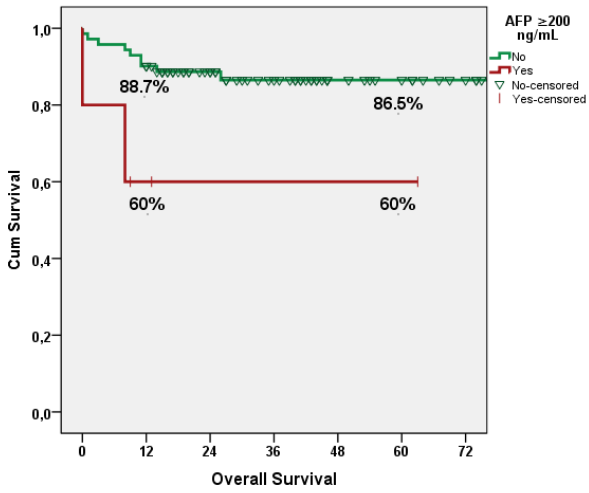

Figure 11. 5-year OS for patients with AFP serum levels ≥ 200ng/ml was 60% and was 86.5% for those with AFP serum levels < 200ng/ml (p=0.027).

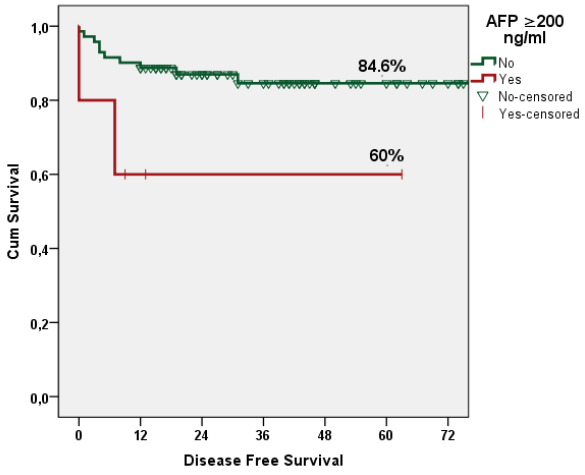

Figure 12. 5-year DFS for patients with AFP serum levels ≥ 200ng/ml was 60% and was 84.6% for those with AFP serum levels < 200ng/ml (p=0.057).

MVI in any nodule represented a PF on OS (p=0.018), mean OS of 47.1±4.9 (37.5-56.7) months compared to 78.9±2.9 (73.3-84.5) months for those without MVI (Fig.13). MVI also represented a PF on DFS (p=0.006), mean DFS of 44.1±5.3 (33.7-54.5) months compared to 78.9±2.9 (73.3-84.5) months for those without MVI (Fig.14). MVI was present in 2 patients (10%) with small HCC, in 9 (25.7%) patients with single lesion, in 12 (27.3%) patients meeting MC, in 16 (28.6%) patients meeting Up-to-seven criteria and in 16 (29.6%) patients meeting UCSF criteria.

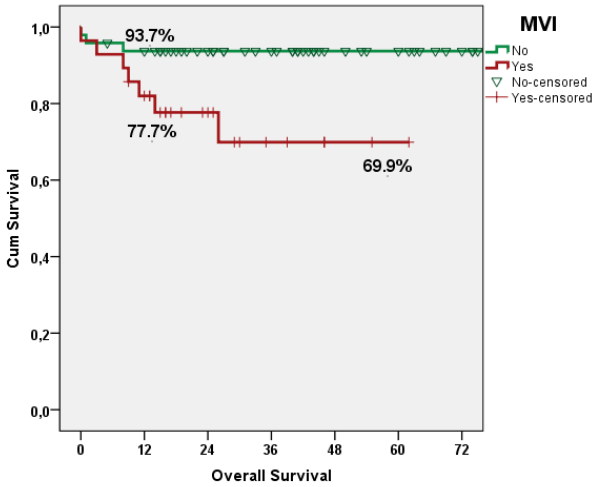

Figure 13. 5-year OS was 69.9% for patients with MVI and 93.7% for those without MVI (p=0.018).

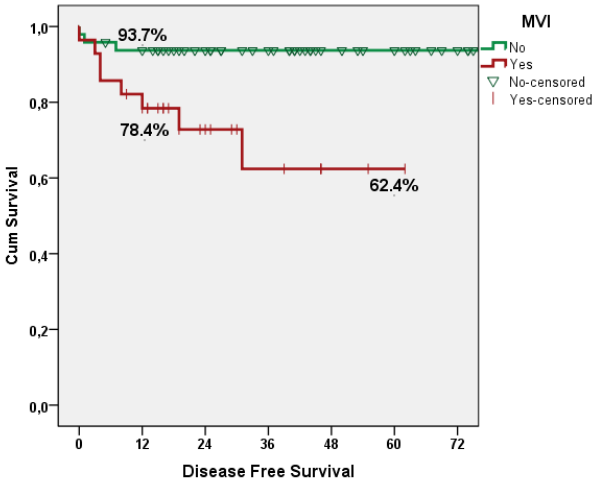

Figure 14. 5-year DFS was 62.4% for patients with MVI and 93.7% for those without MVI (p=0.006).

There was no statistical difference in OS and DFS between BCLC classification stages (0-D). When comparing stage D with others (0-C), there were no differences in OS (p=0.717) and DFS (p=0.794). Mean OS for stage D was 64.9±7.8 (49.6-80. 2) months compared to 73.4±3.3 (66.9-79.8) months for those in BCLC 0, A, B or C stages (Fig.15). Mean DFS was 64.5±8.1 (48.7-80.3) months for stage D compared to 72.0±3.5 (65.2-78.9) months for other stages (Fig.16).

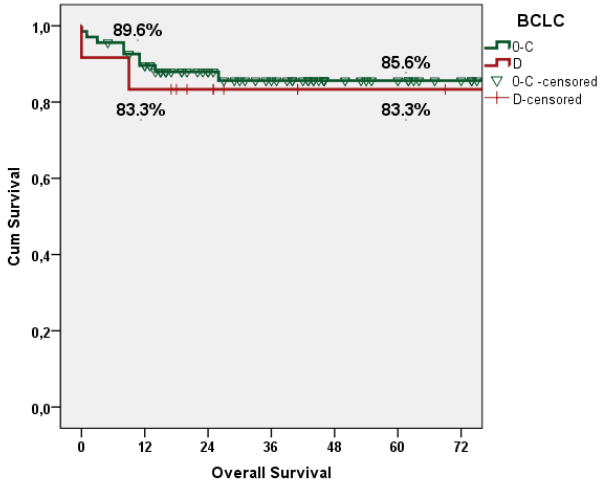

Figure 15. 5-year OS of 83.3% for patients with BCLC stage D and 85.6% for other stages (p=0.717).

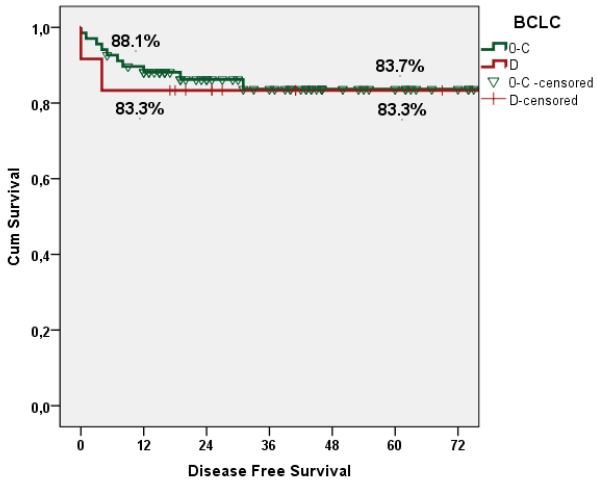

Figure 16. 5-year DFS of 83.3% for patients with BCLC stage D and 83.7% for other stages (p=0.794).

There was no significant impact in OS or DFS by different CLD etiologies, including between alcoholic, the most frequent in our study population, and other etiologies. Other factors that had no impact on OS and DFS are: small HCC, different Child-Pugh classes, NAT and dominant histological patterns.

The presence of a solitary nodular lesion showed tendency to a better OS and DFS but, when its diameter is larger than 5cm, it was associated with worse outcomes (OS and DFS). Dysplastic nodules, HGDN but not LGDN, showed tendency for worse outcomes (OS and DFS) but without statistical significance. Tumor differentiation had impact on outcomes, better for more differentiated lesions (G1) and worse for poor differentiated (G3), but also without statistical significance. Other factors that showed tendency for better outcomes but without statistical significance were: patients who met MC, Up-to-seven and UCSF criteria, MELD score ≥10, complete necrosis of the larger nodule accomplished by NAT, nodule encapsulation (in any nodule or in the larger nodule) and SH-HCC variant of HCC.

Patients meeting MC showed 5-year OS of 91.1% compared to 76.4% for those exceeding the criteria (p=0.131) (Fig.17), and 5-year DFS of 91.3% compared to 71.5% of those exceeding the criteria (p=0.064) (Fig.18). The patients beyond MC with MVI had a mean OS of 30.3±4.9 (20.6-39.9) months compared to 72.3±4.6 (63.2-81.3) for those without MVI (p=0.026) (Fig.19). Similar results were observed for DFS (p=0.009): patients with MVI had a mean DFS of 26.0±5.1 (16.1-35.9) months compared to those without MVI with 72.3±4.6 (63.2-81.3) months (Fig.20). For patients who met MC, the presence or absence of MVI did not seem to affect OS (p=0.816) and DFS (p=0.816). (Fig. 21 and 22).

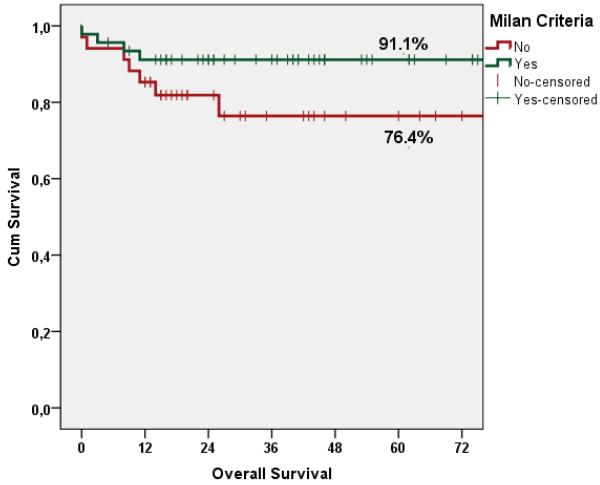

Figure 17. 5-year OS of 91.1% in patients meeting MC compared to 76.4% for those exceeding these criteria (p=0.131).

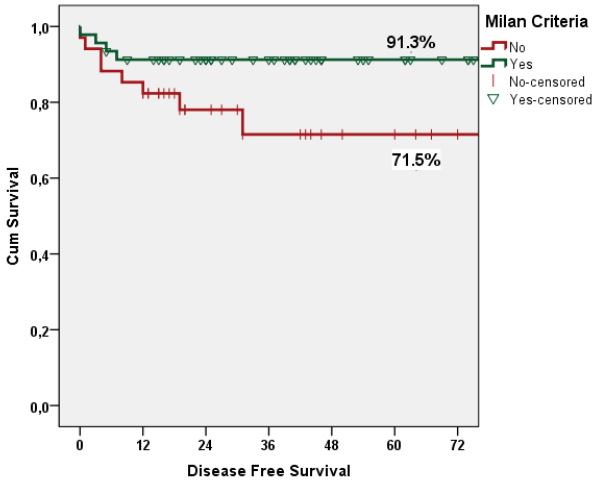

Figure 18. 5-year DFS of 91.3% in patients meeting MC compared to 71.5% for those exceeding these criteria (p=0.064).

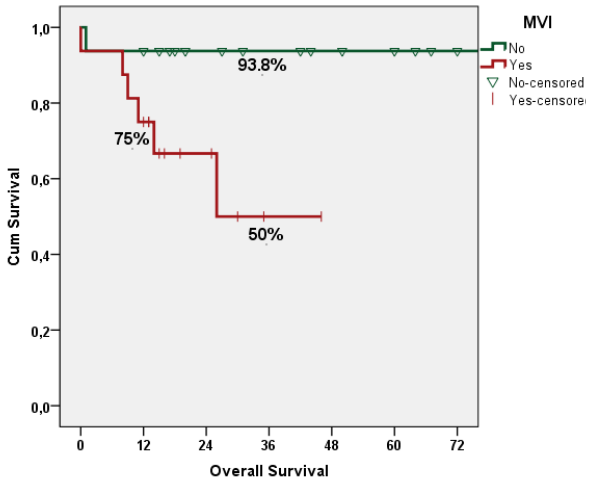

Figure 19. Patients beyond MC: 3-year OS of 50% in patients with MVI compared to 93.8% for those without MVI (p=0.026).

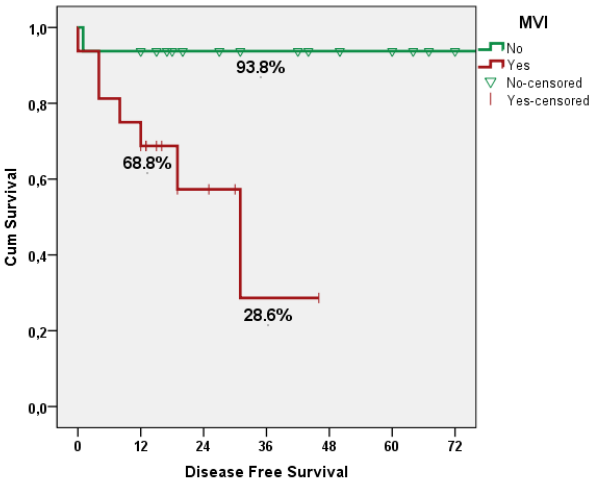

Figure 20. Patients beyond MC: 3-year DFS of 28.8% in patients with MVI compared to 93.8% for those without MVI (p=0.009).

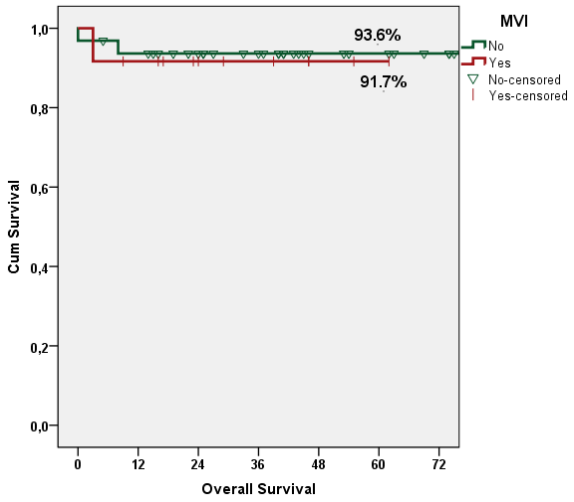

Figure 21. Patients meeting MC: 5-year OS of 91.7% in patients with MVI compared to 93.6% in patients without MVI (p=0.816).

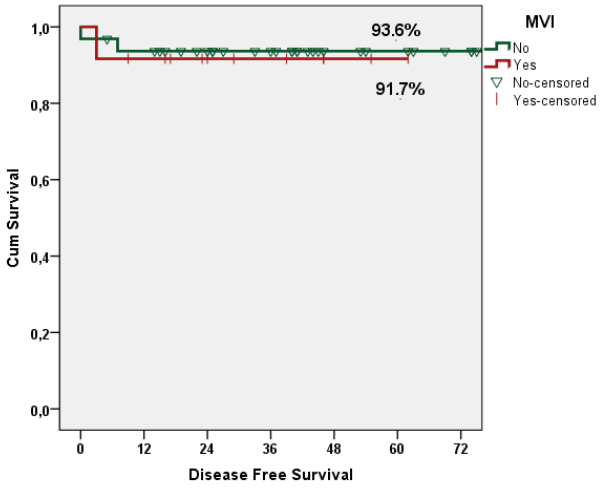

Figure 22. Patients meeting MC: 5-year OS of 91.7% in patients with MVI compared to 93.6% in patients without MVI (p=0.816).

		Overall Survival		Disease Free Survival	
	No. of patients (%)	**5-year OS**	**P**	**5-year DFS**	**P**
MELD score					
≥ 10	62 (77.5%)	93.9%	0.265	94.1%	0.273
< 10	18 (22.5%)	83.3%		83.3%	
Milan Criteria					
In	46 (57.5%)	91.1%	0.131	91.3%	0.064
Out	34 (42.5%)	76.4%		71.5%	
Up-to-seven criteria					
In	58 (72.5%)	89.5%	0.163	86.7%	0.213
Out	22 (27.5%)	72.0%		75.0%	
UCSF criteria					
In	56 (70%)	91.0%	0.061	88.0%	0.096
Out	24 (30%)	70.6%		73.1%	
MC, Up-to-seven and UCSF exclusion					
Yes	21 (26.3%)	71.4%	0.135	74.2%	0.176
No	59 (73.7%)	89.7%		86.9%	
BCLC classification					
0 – C stages	68 (85%)	85.6%	0.717	83.7%	0.794
D (terminal stage)	12 (15%)	83.3%		83.3%	
Larger nodule necrosis (NAT)					
Complete	5 (13.5%)	100%	0.279	100%	0.254
Incomplete	32 (86.5%)	78.9%		72.4%	

Table 8. Clinical and histopathological parameters and its impact on OS and DFS – univariate analysis.

		Overall Survival		Disease Free Survival	
	No. of patients (%)	**3-year OS**	**P**	**5-year DFS**	**P**
Milan criteria out					
MVI present	**16 (50%)**	**50%**	**0.026**	**28.6%**	**0.009**
MVI absent	16 (50%)	93.8%		93.8%	
Milan criteria in					
MVI present	12 (27.3%)	91.7%	0.816	91.7%	0.816
MVI absent	32 (72.7%)	93.6%		93.6%	
HCC recurrence					
Present		**12.5%**	**<0.001**	**-**	**-**
Absent		94.4%			

Table 9. Clinical and histopathological parameters and its impact on OS and DFS – univariate analysis.

		Overall Survival		Disease Free Survival	
	No. of patients (%)	**5-year OS**	**P**	**5-year DFS**	**P**
Number of nodules					
1	37 (46.3%)	91.7%	0.175	87.6%	0.305
>1	43 (53.7%)	79%		80.6%	
Single lesion					
> 5cm	20 (54%)	79.3%	0.352	70%	0.141
< 5cm	17 (46%)	87.3%		87.9%	
4-5 nodules					
Yes	**11 (13.8%)**	**54.6%**	**0.017**	**60.6%**	**0.022**
No	69 (86.2%)	89.6%		87.2%	
Dysplastic nodules					
HGDN	12 (44.4%)	66.7%	0.237	71.4%	0.311
HGDN absent	15 (55.6%)	88%		85.6%	
AFP					
≥200ng/ml	**5 (6.6%)**	**60%**	**0.027**	60%	0.057
<200ng/ml	75 (93.4%)	86.5%		84.6%	
Larger nodule differentiation					
G1	4 (5%)	100%		100%	
G2	63 (78.8%)	86.2%	0.227	83.9%	0.242
G3	9 (11.3%)	66.7%		66.7%	
MVI					
Yes	**28 (36.8%)**	**69.9%**	**0.018**	**62.4%**	**0.006**
No	48 (63.2%)	93.7%		93.7%	
Larger nodule encapsulation					
Yes	43 (57.3%)	89.3%	0.228	90.1%	0.128
No	32 (42.7%)	80.2%		75.6%	
HCC classification					
Classic	70 (87.5%)	83.3%	0.207	81.4%	0.185
SH-HCC	10 (12.5%)	100%		100%	

Table 10. Clinical and histopathological parameters and its impact on OS and DFS – univariate analysis

4. Independent predictors of OS and DFS

On multivariate analysis MVI was an independent predictor of OS (p=0.021) and DFS (p=0.027). AFP serum levels ≥ 200ng/m were independently associated with decreased OS (p=0.035). The presence of 4 or 5 nodules had no significant impact on OS and DFS on multivariate analysis.

	Overall Survival			**Disease Free Survival**		
	HR	**95% CI**	**P**	**HR**	**95% CI**	**P**
Microvascular invasion	**0.296**	**0.105-0.834**	**0.021**	**0.214**	**0.055-0.839**	**0.027**
AFP ≥200ng/ml	**0.244**	**0.066-0,907**	**0.035**	-	-	-
4-5 nodules	0.737	0.226-2.405	0.613	0.340	0.096-1.201	0.094

Table 11. Independent predictors of overall and disease-free survival (multivariate analysis).

IV. Discussion

The presence of MVI, poor tumor differentiation, number of nodules > 3, tumor size and AFP levels were independent PF for HCC recurrence after LT in several studies.(31-33) In our study, the presence of 4-5 nodules was a PF for OS (p=0.017) and DFS (p=0.022) (Fig. 9 and 10; Table 10). Although tumor differentiation was previously related with HCC recurrence and MVI, in our series, poor histological differentiation (G3) was associated with worse outcomes but without statistical significance (Table 10).

HCC might have local invasive behavior at a relatively early stage and MVI was identified in 20% of lesions up to 2cm in diameter, in 30-60% of lesions of 2-5 cm and up to 60-90% when diameter is >5cm. (1, 19) MVI is a critical hallmark in HCC progression and worldwide accepted as the strongest prognostic predictor for OS, DFS and tumor recurrence after LT for

HCC.(3, 18) In fact, conventional imaging techniques and liver biopsy have been so far unsuccessful for preoperative detection of MVI but some serum markers such as des-gamma-carboxyprothrombin (DCP) – protein induced by vitamin K absence or antagonist II (PIVKA-II) -, had been suggested as predictors of MVI and HCC recurrence after LT. (19, 32-35) In our study, patients with MVI compared to those without MVI had worse 5-year OS (69.9% vs 93.7%, p=0.018) and 5-year DFS (62.4% vs 93.7%, p=0.006) (Fig.13 and 14; Table 10). MVI was also associated with worse OS and DFS when MC were not met (Fig.19 and 20; Table 9) and it was present in the majority (85.7%) of patients that had HCC recurrence. However, when MC were met, the presence or absence of MVI did not seem to affect outcomes (Fig. 21 and 22; Table 9). Thus, it seems that MVI does not represent a contraindication for LT if patients are within MC but it is associated with undesirable survival rates when tumors exceed MC, which can be a contraindication for LT.

AFP was the most widely used serum marker for HCC screening but was partially abandoned due to its suboptimal sensitivity; its serum values remain normal in up to 30% of advanced HCC, only 10-20% of early HCC presenting with abnormal high AFP serum levels. (1, 3, 36) AFP has a sensitivity rate of 60-80% in HCC and false positive results during pregnancy, any active liver disease, embryonic or other gastrointestinal tumors. (35) In fact, the increase rate of AFP level was described to be a preoperative predictor of tumor biological behavior and aggressiveness and as an independent PF for tumor recurrence and post-LT outcomes.(19, 32, 33) In our study, AFP serum levels ≥ 200ng/mL was a PF for 5-year OS (60% vs 86.5%, p=0.027), and had tendency for worse DFS (p=0,057) (Fig. 11 and 12; Table 10).

NAT are used to reduce drop-out rate from waiting list but was also associated with lower risks of tumor recurrence after LT and increased long term survival; some series described a 5-year OS of 87% in patients with complete response to NAT compared to 62% in patients non-treated. (3, 37) In fact, tumor response to locoregional bridging therapy could also predict favorable

HCC biological behavior and could be helpful in patient's selection for LT. (3, 32, 33) In our study, complete tumor necrosis achieved by NAT was associated with better outcomes, but without statistical significance (Table 8).

End-stage HCC patients (BCLC stage D) presents with poor performance status and/or Child-Pugh C with an estimated OS less than 3 months and no curative treatment is recommended (best supportive care).(1, 9) A recent meta-analysis suggested that intra-arterial chemotherapy or LT may be superior to other treatments in patients BCLC stage D.(38) In our study, there was no statistical difference on outcomes between BCLC stages. Similarly, there was no difference on 5-year OS (83.3% vs 85.6%, p=0,717) and 5-year DFS (83.3% vs 83.7%, p=0,794) between BCLC stage D and others (0, A, B, C) (Fig. 15 and 16; Table 8). A good selection of patients for LT, that initially had indication for symptomatic treatment, showed similar outcomes to other patients.

Tumor recurrence rates remains up to 10-20% and once it is established, therapeutic options available are scarce, without or with little impact on prognosis.(31) In our study, recurrence occurred in 10% of patients and about 75% with extrahepatic disease, which is possibly related to immunosuppressed status after LT. Additionally, HCC recurrence was a strong predictor of worse OS ($p<0.001$), with 3-year OS of 12.5% compared to 94.4% for those without recurrence. (Fig.8; Table 9)

V. Conclusion

Despite all the efforts, HCC still have a poor prognosis, mainly depending in tumor's stage at presentation, patient's poor clinical status and vascular invasion.

LT is an optimal therapeutic option for patients with liver disease and HCC, and a careful selection of patients for LT is one of the most effective strategies for treatment and prevention of HCC recurrence. Due to organ scarcity, receptors selection must be careful and with caution

when applying expanded criteria, in the presence of MVI and AFP ≥ 200ng/ml. Given that MVI cannot be determined preoperatively, the achievement of an imaging, biologic and molecular signature of tumors with MVI is of extreme importance, since it has been identified as one of the most important PF and predictors of outcomes for HCC after LT.

Morphological parameters showed to be insufficient to predict tumor recurrence and biological behavior may be variable between tumors with similar dimensions. So, strict adoption of morphological selection criteria can lead to exclusion of patients that could potentially benefit from LT. In fact, LT could be performed in advanced HCC if biological parameters and slow progression of the disease suggest favorable tumor behavior.

In our series, even in patients with some poor PF (MVI, BCLC stage D, patients exceeding selection criteria), when compared with other therapeutical options, LT was associated with better OS and DFS. Thus, we suggest the use of marginal organs as well as use of perfusion mechanisms as source of organs for LT in these patients.

The collection/analysis of data retrospectively and the small sample size are some of the limitations of the study and could account for the lack of statistical significance in some of the analyzed correlations. Further investigations are required to achieve reliable understanding on tumor biological behavior and to realize how this knowledge can lead to an optimization of patient's selection for LT. IHC analysis results were not available at the time of this writing but is ongoing and will be added later.

VI. Acknowledgments

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