FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

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CLINICAL AND PATHOLOGIC FACTORS WITH SURVIVAL IMPACT AFTER HEPATECTOMY FOR HEPATOCELLULAR CARCINOMA

ARTIGO CIENTÍFICO

ÁREA CIENTÍFICA DE CIRURGIA GERAL

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MARÇO/2017

Resumo

Introdução: A hepatectomia (HP) é, juntamente com o transplante hepático, o único tratamento potencialmente curativo para o Carcinoma Hepatocelular (CHC). Dada a prevalência do Síndrome Metabólico (SM), o espectro do CHC pode estar a mudar. A clampagem do pedículo hepático (CPH), técnica utilizada para reduzir a hemorragia intra-operatória, pode associar-se a maior recidiva. A citoqueratina 19 (CK19) e o glipicano-3 (GLP-3) podem ser marcadores de pior prognóstico no CHC.

Material de Métodos**:** Revisão clínica e patológica de 59 doentes submetidos a HP por CHC (2005-2013). Hepatopatia crónica em 53 doentes (89,8%), com cirrose em 54,2%; etiologias mais frequentes: etilismo (47,5%), HCV (25,4%) e HBV (11,9%). SM em 36% dos doentes. 95% dos doentes Child-Pugh classe A e 5% classe B; MELD mediano de 8 (6-18). Nódulo único em 46 doentes (78%); tamanho médio de 5,4 cm. Invasão vascular microscópica (MiVI) em 49% e macroscópica (MaVI) em 17%. CPH em 43 doentes (74,1%). Análise estatística: SPSS 21.0 (Kaplan-Meier, log rank e regressão de Cox). Significado estatístico: $p<0.05$.

Resultados**:** Morbilidade major em 22% dos doentes. Mortalidade em 5,1%. Sobrevida global (SG) mediana de 71 meses e sobrevida livre de doença (SLD) mediana de 37. Análise multivariada: MaVI ($p=0.001$), MiVI ($p=0.005$) e Infecção HCV ($p=0.002$) associados a pior SG; SM associado a melhor SG ($p=0.001$); MaVI ($p=0.000$), MiVI ($p=0.035$) e CPH ($p=0.012$) associados a pior SLD. Doentes CK19+/GLP-3- ($p=0.007$) e CK19-/GLP-3+ ($p=0.029$) associados a pior SLD e doentes CK19-/GLP-3- ($p=0.031$) a melhor SLD.

Discussão/Conclusão: A realização de CPH foi um factor independente de pior SLD. A lesão de isquémia-reperfusão que produz poderá desencadear mecanismos de angiogénese e angioinvasão das células tumorais, resultando em maior recidiva. A etiologia HCV associou-se a pior SG. O SM

associou-se a melhor SG, reforçando o papel da hepatectomia nestes casos. A detecção combinada de CK19 e GLP-3 foi um factor de prognóstico independente em doentes com CHC, permitindo a identificação de tumores mais agressivos.

Palavras-chave: carcinoma hepatocelular, hepatectomia, factores de prognostico, clampagem do pedículo hepático, manobra de Pringle, histopatologia.

Abstract

Introduction: Hepatectomy (HP) is, along with liver transplantation, the only potentially curative treatment for Hepatocellular Carcinoma (HCC). The high prevalence of Metabolic Syndrome (MS) may be causing a shift in the CHC spectrum. Hepatic Pedicle Clamping (HPC), used to reduce perioperative bleeding during HP, has been theorized to increase risk of recurrence. Citokeratin 19 (CK19) and glypican-3 (GLP-3) have been identified as markers of worse prognosis in HCC.

Material and Methods**:** Clinical and pathological review of 59 patients undergoing HP for HCC between 2005 and 2013. Chronic liver disease in 53 patients (89,8%), with cirrhosis in 54,2% [most frequent etiologies: ethylism (47,5%), HCV (25,4%) and HBV (11,9%)]. MS in 36% of patients. 95% of patients Child-Pugh class A and 5% class B; Median MELD of 8 (6-18). A single nodule in 46 patients (78%); average size of 5,4 cm. Microscopic vascular invasion (MiVI) in 49% of patients and macroscopic (MaVI) in 17. HPC in 43 patients (74.1%). Statistical analysis with SPSS ™ 21.0. Survival tests (Kaplan-Meier, log rank and Cox regression). Statistical significance with $p<0.05$.

Results**:** Major morbidity in 22% of patients. Mortality in 5,1%. Median overall survival (OS) of 71 months and median disease-free survival (DFS) of 37. In multivariate analysis: MaVI ($p=0.001$), MiVI ($p=0.005$) and HCV infection ($p=0.002$) were associated with worse OS; MS was associated

with better OS (p=0.001); MaVI (p=0.000), MiVI (p=0.035) and HPC (p=0.012) were associated with worse DFS. CK19+/GLP-3- (p=0.007) and CK19-/GLP-3+ (p=0.029) patients were associated with worse DFS and CK19-/GLP-3- (p=0.031) with better DFS.

Discussion/Conclusions: HPC was an independent factor of worse DFS. The ischemia-reperfusion injury (IRI) produced by HPC could promote a more angiogenic and angioinvasive phenotype of tumor cells, resulting in higher recurrence. HCV etiology was associated with worse OS. MS was associated with better OS, highlighting the importance of hepatectomy in these cases. The combined detection of CK19 and GLP-3 was an independent prognostic factor in HCC patients allowing the identification of more aggressive tumours.

Keywords: hepatocellular carcinoma, hepatectomy, prognostic factors, hepatic pedicle clamping, Pringle manoeuvre, histopathology.

I. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer, the third leading cause of cancer-related death worldwide and one of the leading causes of death in patients with cirrhosis (1). Hepatitis B virus (HBV) infection is the most common risk factor for HCC worldwide (2), however, in western countries, HCC incidence has been increasing because of hepatitis C virus (HCV) advanced infection and the growing incidence of non-alcoholic steatohepatitis (1-3).

Numerous basic science and clinical studies have documented a strong association between HCC and metabolic syndrome (MS). These studies have documented that, in most patients, NASH disease is the hepatic manifestation of the MS, which may progress to HCC through the cirrhotic

process, nevertheless, a minority of patients with non-alcoholic fatty liver disease may progress to HCC without cirrhosis (4).

Liver transplantation and hepatectomy are the two curative surgical treatments available for HCC. During hepatectomy, intraoperative bleeding is a major risk. To prevent major bleeding during the surgery, intermittent hepatic pedicle clamping (HPC) - clamping of the hepatic artery and portal vein (Pringle manoeuvre) is used (5). However, the ischemia-reperfusion injury (IRI) caused by HPC results in complex immunological and microvascular changes, through activation of complex cell signaling pathways and can result in a poorer prognosis (6).

Various histopathological factors are well studied and known to have prognostic implications: macroscopic and microscopic vascular invasion, tumor-free resection margin, underlying liver cirrhosis, tumor necrosis, tumor size and absence of tumoral capsule (7).

Along with the growing incidence of MS, a new histological subtype – steatohepatitic HCC (SH-HCC) has been recently identified, and it has been appointed a correlation between these two. This subtype has histological features of steatohepatitis, namely hepatocellular ballooning, Mallory-Denk hyaline bodies, inflammation and fibrosis (8). It is still controversial whether this subtype has impact on the patients' survival.

Hepatic progenitor cells can differentiate in hepatocytes and cholangiocyte cells. Cytokeratin 19 (CK19) is a molecular marker of cholangiocyte cells and currently well-accepted as a biomarker for HCC with hepatic progenitor cells origin, besides the cholangiocyte carcinoma (9). CK19 + HCC was reported to show features related to aggressive behavior, such as lesser tumor cell differentiation and a higher proliferative index (10).

Glypican-3 (GLP-3) is a member of the glypican family of glycosil-phosphatidylinositol-anchored cell surface heparan sulfate proteoglycans and is currently used as a diagnostic molecular marker of HCC and immature hepatocytes. GLP-3 is never expressed in cholangiocyte, cholangiocarcinoma, and is low expressed in well-differentiated HCC (11). It is known that GLP-3 plays different rolls in different cancers, but in HCC it acts as an oncogene and can impact the patients' outcome (12).

Our goal in this study is to investigate the clinical and pathological factors that influences HCC patients' prognosis after hepatectomy.

II. Material and Methods

1. Study Design

This study is a clinical and pathological review of 65 patients undergoing hepatectomy for hepatocellular carcinoma between January 2005 and December 2013 at Serviço de Cirurgia A from Centro Hospitalar de Coimbra (Head of Department: Prof. Doutor Franscisco Castro e Sousa, Coimbra, Portugal).

Four patients were excluded because of insufficient histological material for evaluation and two for undergoing rehepatectomies (Figure 1). The study was approved by the institution's ethical committee and complied with the principles of the Declaration of Helsinki.

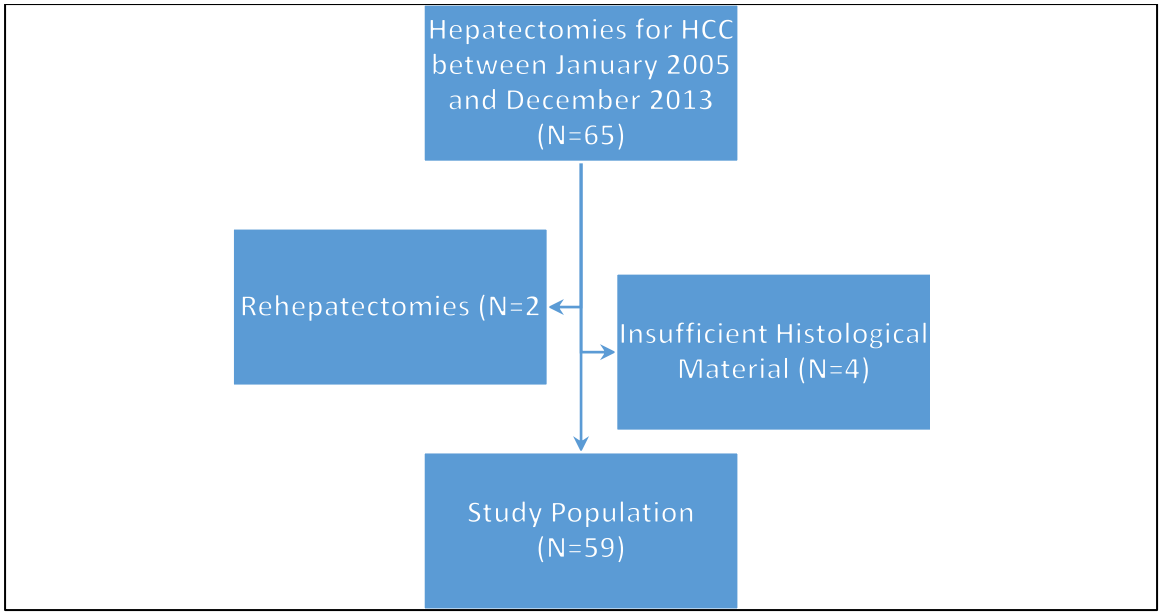

Figure 1 - Final study population

2. Study Population

Of the 59 patients included in this study, 49 were male and 10 female, with a mean age of 71 ± 9 years, with a range from 38 to 86 years.

53 patients had chronic liver disease (89,8%), with established cirrhosis in 54,2%, with the following etiologies: HCV infection – 25,4%, HBV infection – 11,9%, concomitant HCV and HBV infection - 1%, ethylism – 47,5%, NASH – 8,5% and concomitant human immunodeficiency virus (HIV+) and HBV infection - 1,7%

Patients' most prevalent comorbidities were: diabetes mellitus (DM) – 52,5%, systemic arterial hypertension (SAH) – 67,8%, dyslipidaemia – 35,6%, abdominal obesity – 28,8%, smoking habits – 10,2% and MS – 35,6%.

The liver function reserve was assessed by calculation of the Child-Pugh and MELD Scores. 56 patients (94,9%) were classified as Child-Pugh A and 3 (5,1 %) as Child-Pugh B. The median MELD was 8 (range 6 – 18).

A single nodule was present in 78% (46) of the patients, with a mean size of 5,44 ± 4,31cm (range 0,80 - 20 cm), and in 22 patients (37,3%) the largest nodule was greater than or equal to 5 cm in diameter. Regarding location, 38 (64,4%) of the nodules were located in the right lobe, 17 (28,8%) in the left lobe and 4 (6,8%) were billobar. 34 patients (57,5%) accomplished the Milan criteria.

Nine patients (15,3%) underwent neoadjuvant chemoembolization.

3. Operative Details

A right subcostal incision was performed to expose the abdominal cavity, but in 4 cases, resection was performed by laparoscopy (6,8%).

The anatomical transection of the liver parenchyma was done using the Cavitron Utrasonic Surgical Aspirator (CUSA®). Intraoperative ultrasonography was routinely performed. Whenever possible, an anatomical resection consisting of at least one Couinaud segment was performed.

Surgical procedures are summarized in Table 1. Major hepatectomy, defined by the resection of ≥ 3 liver segments, was performed in 20 patients (33,9%), and 16 patients (27,6%) needed blood transfusion during the procedure.

Intermittent HPC or Pringle Manoeuvre was used 29 patients (74,1%), with a mean time of 20,3 ± 18,4 minutes (range 0 – 79).

Three patients (5,1%) underwent pre-operative portal vein embolization.

Table 1 –List of hepatectomies performed (per type of surgery) in 59 patients for hepatocellular carcinoma

Hepatectomies	
Left Hepatectomy	7 (11,9%)
Right hepatectomy	8 (13,6%)
Segments	
One segmentectomy	34 (57,6%)
Bisegmentectomy	5 (8,5%)
Major hepatectomy (≥ 3 segments)	20 (33,9%)

4. Morbidity and Mortality

During the first 90 postoperative days, surgical complications were assessed by the Dindo-Clavien classification (13), and patients were divided in three groups: minor or no morbidity (no morbidity, grade I or II), major morbidity (grade IIIa to IVb) and mortality (grade V).

The definitions and grading systems by the International Study Group of Liver Surgery (ISGLS) (14-16) were used to evaluate the presence of post-operative liver-specific complications: biloma, ascites, abscess, vascular complications, haemorrhage and liver failure.

5. Post-operative follow-up

The clinical follow-up of the patients enrolled was achieved from the medical records or by telephone call interviews.

Overall survival (OS) was defined as the time between hepatectomy and the date of tumoral death or the most recent follow-up registration if the patient was alive.

Disease-Free Survival (DFS) was defined as the time between hepatectomy and the first new tumoral lesion detected by imaging studies, proven to be a liver or distant recurrence.

6. Histopathological Analysis

Two experienced hepatobiliary pathologists reviewed archival histologic material from each patient and evaluated tissue samples from tumor nodule and tumor-free liver parenchyma, without knowledge of clinical data, neoadjuvant treatment or patient outcome.

The Haematoxylin and Eosin (H&E) slides were observed in light microscope - Nikon Eclipse 50i, and images obtained using a Nikon-Digital Sight DS-Fi1 camera.

6.1 Tumoral parenchyma

The tumoral parenchyma was analysed according to the World Health Organization (WHO) (17) and included the following aspects:

Resection margins were measured and classified as R0 (margin ≥ 10 mm), R1 (margin inferior to 10 mm) and R2 (macroscopic tumor in the resection margin).

Macroscopic type of tumor was defined as: nodular, diffuse, satellite and massive.

Tumor size was assessed in centimetres and classified as < 5 cm or ≥ 5 cm. Capsule (Figure 3), macroscopic necrosis, macroscopic (MaVI) and microscopic (MiVI) vascular invasion were also examined and determined as present or absent.

Tumor grading was performed by applying the Edmonson grading system (18) – G1 to G4

The mitotic index was measured by counting the number of mitotic cells (Figure 2) in 10 high-power fields (< 5 mitotic cells – low mitotic index and ≥ 5 mitotic cell – high mitotic index) as described by Ha et al (19).

The histological subtype of tumor was classified as defined by Shibahara et al (20), in conventional-HCC (C-HCC) and steatohepatitic-HCC. The diagnosis of SH-HCC (Figure 4) was made if the tumour fulfilled four of the following five criteria in at least 50% of the tumor main nodule: steatosis (>5% tumour cells), ballooning or Mallory–Denk body formation, interstitial fibrosis and inflammatory infiltrates, no minimal was required for each of the criteria above. (20)

The histological analysis of the tumoral parenchyma are summarized in Table 2.

Table 2- Histological analysis of the tumoral parenchyma of 59 patients undergoing hepatectomy for hepatocellular carcinoma.

Resection margins	
R0	42 (71,2%)
R1	14 (23,7%)
R2	3 (5,1%)
Mascrocopic type of tumor	
Nodullar	54 (91,5%)
Diffuse	2 (3,4%)
Satellite	3 (5,1%)
Massive	0

Tumor size	
< 5cm	37 (62,7%)
≥ 5cm	22 (37,3%)
Capsule	
Yes	29 (49,2%)
No	30 (50,8%)
Macroscropic necrosis	
Yes	26 (44,1%)
No	33 (55,9%)
Macroscopic vascular invasion (MaVI)	
Yes	10 (16,9%)
No	49 (83,1%)
Microscopic vascular invasion (MiVI)	
Yes	29 (49,2%)
No	30 (50,8%)
Edmonson's grading	
G1	8 (13,6%)
G2	41 (69,5%)
G3	9 (15,3%)

G4	1 (1,7%)
Mitotic index	
< 5 mitotic cells – low mitotic index	50 (84,7%)
≥ 5 mitotic cell – high mitotic index	9 (15,3%)
Histological subtype of tumor	
C-HCC	44 (74,6%)
SH-HCC	15 (25,4%)

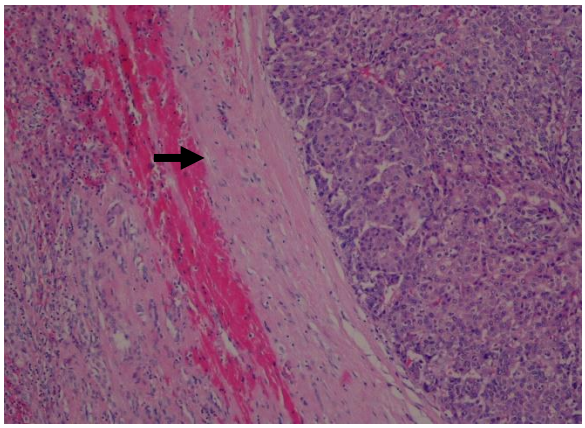

Figure 3 - Presence of fibrous tumoral capsule (arrow) H&E 100x.

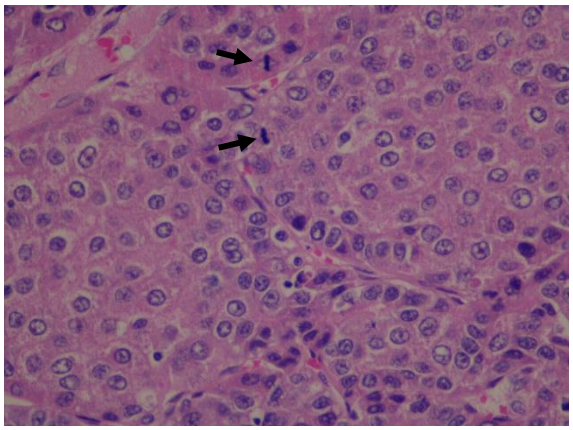

Figure 2 – Mitotic activity (arrow) H&E 400x

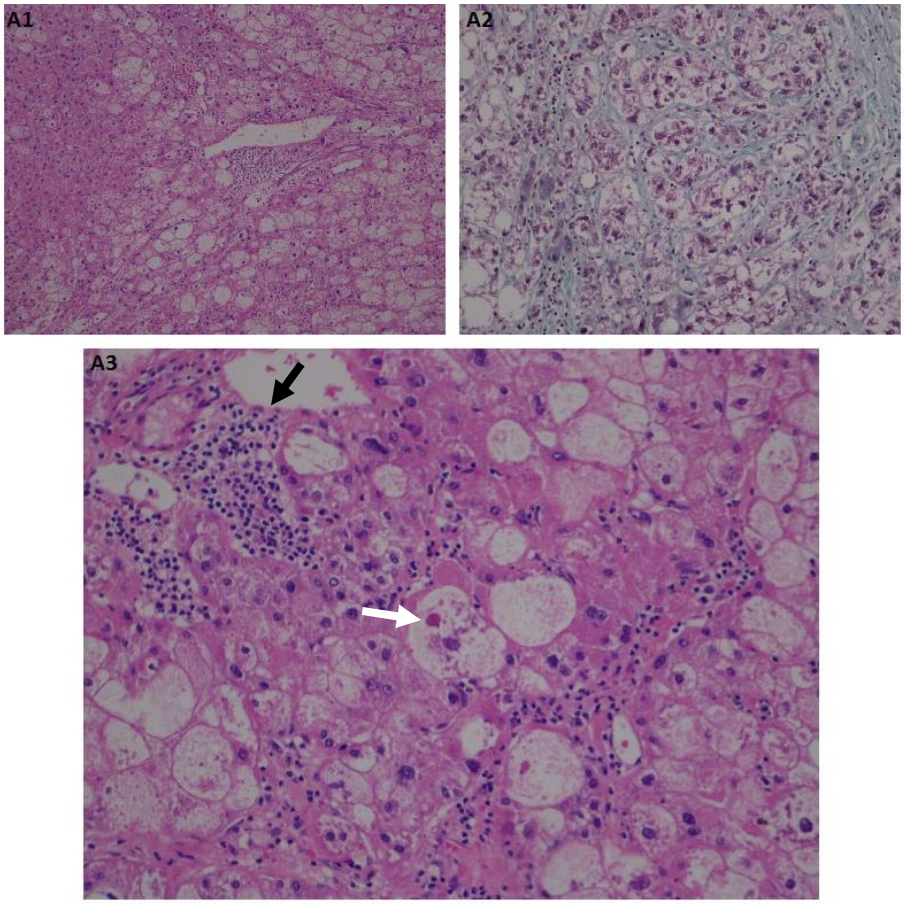

Figure 4 – Steatohepatitic histological subtype of HCC (A1: Diffuse hepatocellular balloning H&E 200x; A2: pericellular fibrosis MT 200x; A3: inflammatory infiltrate (black arrow), hepatocellular ballooning and Mallory-Denk hyaline body (white arrow) H&E 400x).

6.2 Non-tumoral parenchyma

The non-tumoral parenchyma analysis was performed by examination of the slides on Masson's trichrome to assess fibrosis. Metavir (21) and Ishak (22) grading systems were applied to quantify the fibrosis and to evaluate the presence of cirrhosis, defined as M4 (Metavir) and 5-6 (Ishak) (Figure 5).

A histological scoring system for NASH was also applied (Figure 6), as proposed by Kleiner et al (23), by examination of steatosis, lobular inflammation, hepatocellular ballooning and fibrosis.

The histological analysis of the non-tumoral parenchyma is summarized in Table 3.

Table 3 - Histological analysis of non-tumoral parenchyma of 59 patients undergoing hepatectomy for hepatocellular carcinoma.

Metavir	
0	16 (27,1%)
M1	4 (6,8%)
M2	5 (8,5%)
M3	9 (15,3%)
M4	25 (42,4%)
Ishak	
0	16 (27,1%)
F1	1 (1,7%)
F2	4 (6,8%)
F3	4 (6,8%)
F4	5 (8,5%)
F5	4 (6,8%)
F6	25 (42,4%)

NASH	
Yes	5 (8,5%)
No	28 (47,5%)
Non-applicable	26 (44,1%)

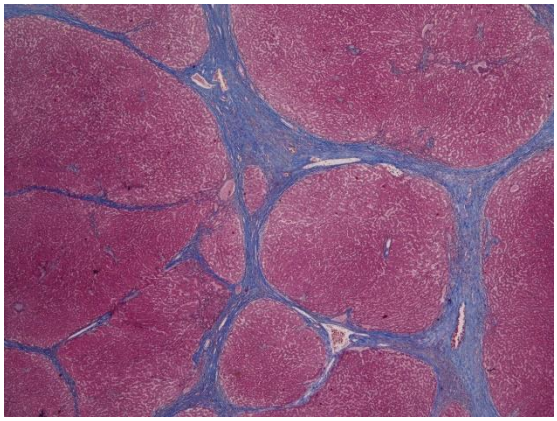

Figure 5- Cirrhosis – thick and complete fibrotic septea around nodules of hepatocytes MT 20x.

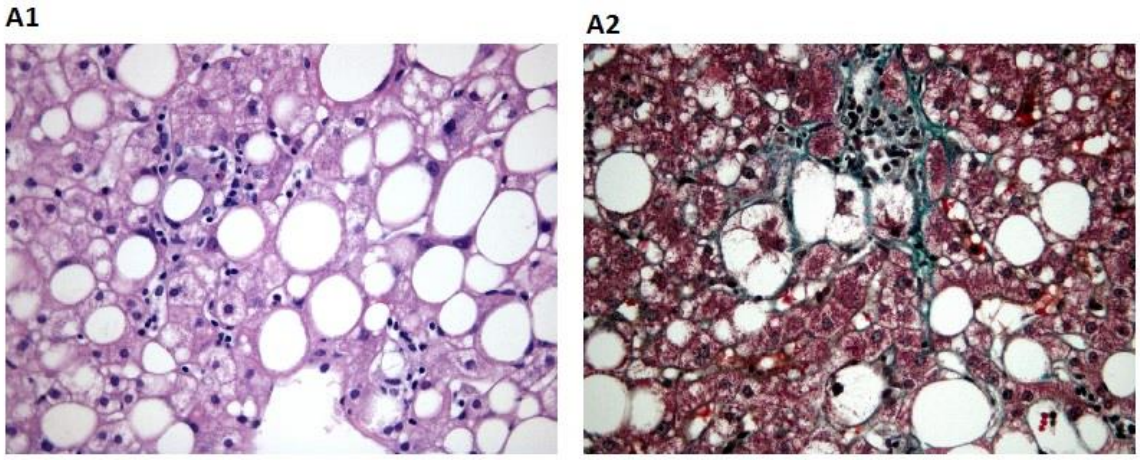

Figure 6 – Non-alcoholic steatohepatitis (A1: macrovacuolar steatosis and mild inflammatoy infiltrate H&E 400x; A2: pericellular fibrosis MT 400x).

6.3 Immunohistochemical staining

Immunohistochemical studies were performed on a representative block of the lesion, resorting to avidin-biotin-peroxidase complex detection system and performed on Ventana Marker Platform Bench Mark ULTRA IHC/ISH using the following antibodies: Cytokeratin 19 (CK19) (A53-B/A2.26, Ventana, AZ-USA) and Glypican-3 (GLP-3) (GC33, ventana, AZ-USA).

CK19 staining (Figure 7) in the tumoral parenchyma was evaluated and semi-quantified. The immunostaining was classified as positive if ≥ 5% immunoreactive cells and as negative if < 5% immunoreactive cells.

GLP-3 staining (Figure 8) in the tumoral parenchyma was evaluated and semi-quantified. The immunostaining was classified as follows: positive - ≥ 10% immunoreactive cells; negative - < 10% immunoreactive cells.

The Immunohistochemical analysis is summarized in Table 4.

Table 4- Immunohistochemical analysis of 59 patients undergoing hepatectomy for hepatocellular carcinoma.

CK19	
Positive (≥ 5%)	6 (10,2%)
Negative (< 5%)	51 (86,4%
Absence	2 (3,4%)
GLP-3	
Positive (≥ 10%)	38 (64,4%)

Negative (< 10%)	18 (30,5%)
Absence	3 (5,1%)

After the immunohistochemical staining, the enrolled cases were divided into 4 groups, as follows:

Group 1 (CK19+/ GLP-3+): cases where tumor cells coexpress CK19 and GLP-3;

Group 2 (CK19+/ GLP-3−): cases where tumor cells express CK19 singly;

Group 3(CK19−/ GLP-3+): cases where tumor cells express GLP-3 singly; and

Group 4 (CK19−/ GLP-3−): cases with negative expression of both CK19 and GLP-3

Table 5 summarizes the groups´ formation in immunohistochemical analysis.

Table 5 - Immunohistochemical groups´ formation of 59 patients undergoing hepatectomy for hepatocellular carcinoma.

Group 1 (CK19+/ GLP-3+)	4 (6,8%)
Group 2 (CK19+/ GLP-3−)	2 (3,4%)
Group 3(CK19−/ GLP-3+)	34 (57,6%)
Group 4 (CK19−/ GLP-3−)	16 (27,1%)
Absence	3 (5,1%)

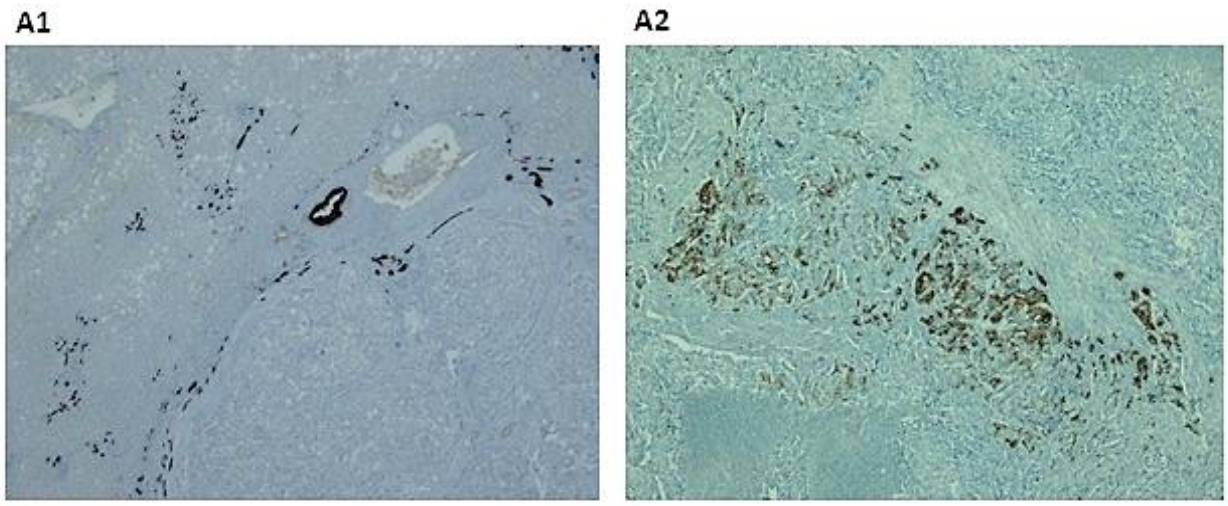

Figure 7- CK19 immunohistochemical staining (A1: negative staining 40x; A2: positive and diffuse staining 40x).

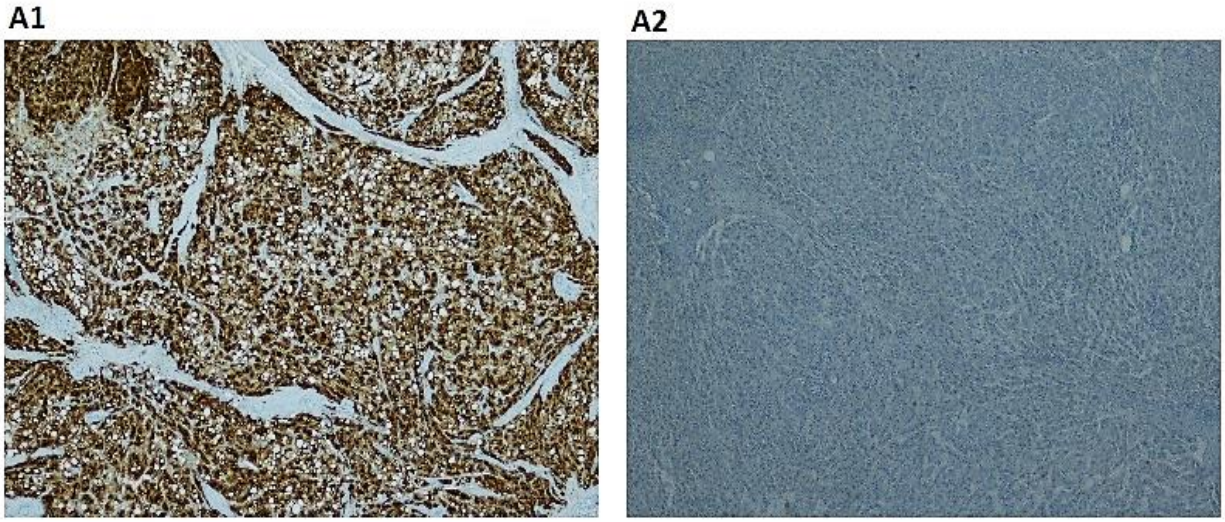

Figure 8 - GLP-3 immunohistochemical staining (A1: positive and diffuse staining 40x; A2: negative staining 40x).

7. Statistical Analysis

Statistical analysis with Statistical Package for the Social Sciences (SPSS ™) 21.0 for Windows.

The quantitative data were expressed as mean ± standard deviation and range.

The survival probabilities were calculated using the Kaplan-Meier method and log-rank test. Factors associated with survival were evaluated with Cox regression. Statistical significance was considered with $p < 0.05$.

III. Results

1. Morbidity and Mortality

Major morbidity in 13 patients (22%). Mortality in 3 patients (5,1%), caused by liver failure (1 patient) and haemorrhage (2 patients).

Major morbidity consisted of Clavien IIIa in 7 patients (11,9%), IIIb in 5 patients and IVb in one patient (1,7%). The clinical conditions of each grade are summarized in Table 6.

Table 6 - Morbidity and mortality in 59 patients undergoing hepatectomy for Hepatocellular Carcinoma

Grade	**n**	**Clinical Conditions**	
No complication	28 (47,5%)	-	
Clavien I	0 (0%)	-	
Clavien II	15 (25,4%)	-	
Clavien IIIa	7 (11,9%)	Ascites [1]	3 (5,1%)
		Abscess [2]	2 (3,4%)

		Biloma [2]	1 (1,7%)
		Pleural effusion [3]	1 (1,7%)
Clavien IIIb	5 (8,5%)	Abscess [4]	4 (6,8%)
		Surgical incision infection and evisceration	1 (1,7%)
Clavien IVa	0 (0%)	-	
Clavien IVb	1 (1,7%)	Haemorrhage and hemodynamic instability [5]	1 (1.7%)
Clavien V	3 (5,1%)	Liver failure	1 (1,7%)
		Haemorrhage and hemodynamic instability	2 (3,4%)

[1] required paracentesis

[2] required percutaneous drainage

[3] required pleural drainage

[4] required surgical drainage

[5] required intensive care unit management

2. Overall and disease-free survival

With a median follow-up of 68 months, the median overall survival (OS) after hepatectomy was 71 ± 20 months (range 0 – 136 months), with 3 and 5-year overall survival of 57,5% and 49,6% respectively.

In the follow-up period, 28 patients (47,5%) experienced recurrence while the other 31 (52,5%) were recurrence-free. Of the 28 patients that developed recurrent disease, 22 patients (78,5%) had only hepatic recurrence, 3 patients (10,7%) developed extra-hepatic recurrence, and 3 patients (10,7%) patients developed hepatic and extra-hepatic concomitant recurrence.

The distant recurrence was registered in stomach (2 patients), bones (2 patient), lungs (1 patient) and pancreas (1 patient).

The median disease-free survival (DFS) after hepatectomy was 37 ± 14 months (range 0 - 103 months), and the 3 and 5-year disease-free survival was 49,2% and 35,7% respectively.

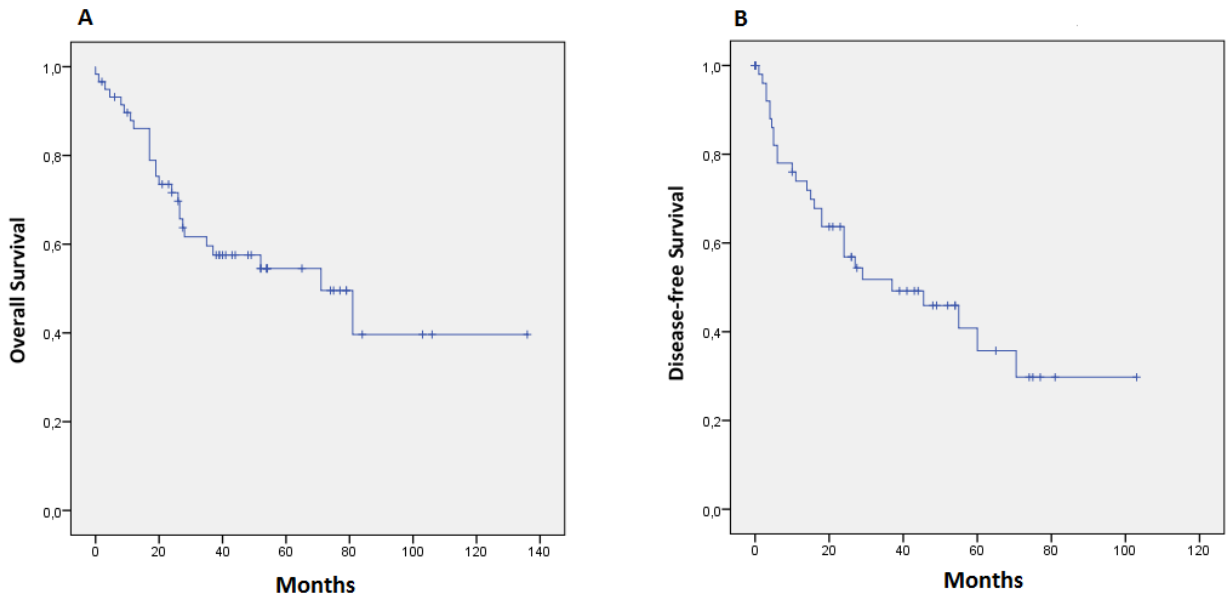

Figure 9- Kaplan-Meier curves of median overall survival (A) and median disease-free survival (B) of 59 patients after hepatectomy for hepatocellular carcinoma.

3. Clinical and operative factors with impact on survival

Two clinical parameters were associated with worse OS: HCV (p = 0.02; HCV+ median OS: 35 ± 7 months vs. HCV- median OS: 132 ± 7 months) and extra-hepatic recurrence (EHR) (p = 0.01; EHR+ median OS: 17 ± 3,5 months vs. EHR- median OS: 81 ± 20 months). Metabolic syndrome was associated with better OS (p = 0.02; MS+ median OS: 132 ± 12 months vs. MS- median OS: 34 ± 12 months) (Figure 10).

No other preoperative clinical feature had statistically significant impact on disease-free survival in Kaplan-Meier test.

Intermittent HPC was associated with worse DFS (p = 0.008; HPC+ median DFS: 24 ± 6 months vs. HPC- median DFS: 96 ± 11 months) (Figure 11).

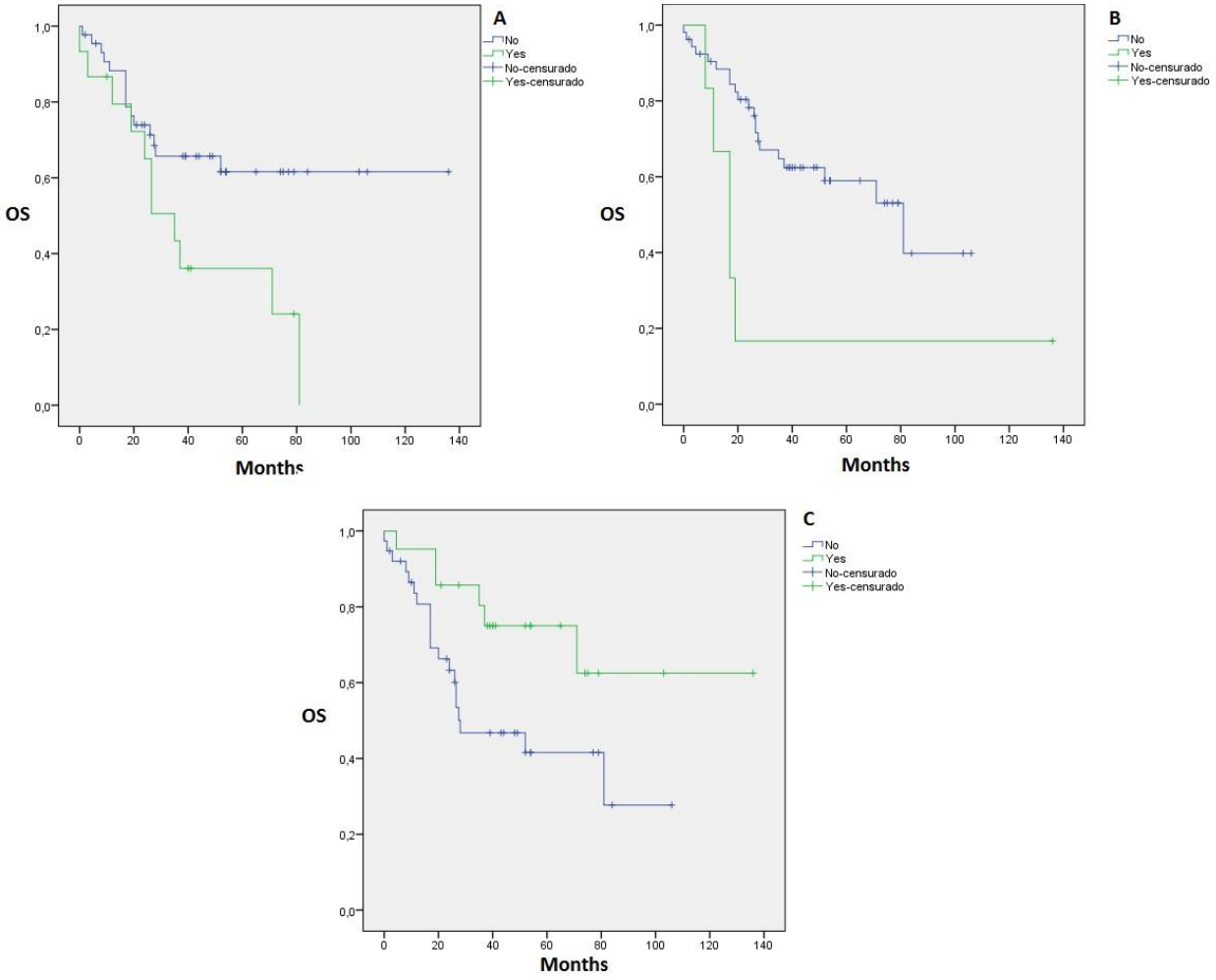

Figure 10 –Survival curves of clinical factors that impact overall survival of 59 patients after hepatectomy for hepatocellular carcinoma (A: Hepatitis C virus infection as a factor of worse overall survival, p = 0.02, HCV+ 5-year OS: 23% vs. HCV- 5-year OS: 61%; B: Extrahepatic recurrence as a factor of worse overall survival, p = 0.01, EHR+ 5-year OS: 17% vs. EHR- 5-year OS: 53%; C: Metabolic Syndrome as a factor of better overall survival, p = 0.02, MS+ 5-year OS: 63% vs. MS- 5-year OS: 42%).

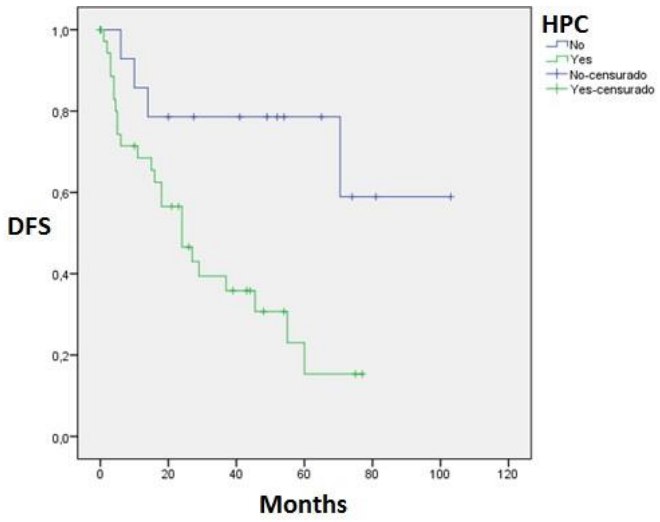

Figure 11 - Survival curve of operative factor that impact disease-free survival of 59 patients after hepatectomy for hepatocellular carcinoma: intermittent hepatic pedicle clamping (HPC) as a factor of worse disease-free survival (p = 0.008, HPC+ 5-year DFS: 17% vs. HPC- 5-year DFS: 61%).

4. Pathologic factors with impact on survival

4.1. Resection margins

OS was not affected by tumor-free resection margin.

Patients with R2 had worse DFS than R1 patients and R0, respectively (p = 0.001; R0 median DFS: 60 ± 17 months vs. R1 median DFS: 11 ± 9 months vs. R2 median DSF: 5 ± 2,5 months) (Figure 12).

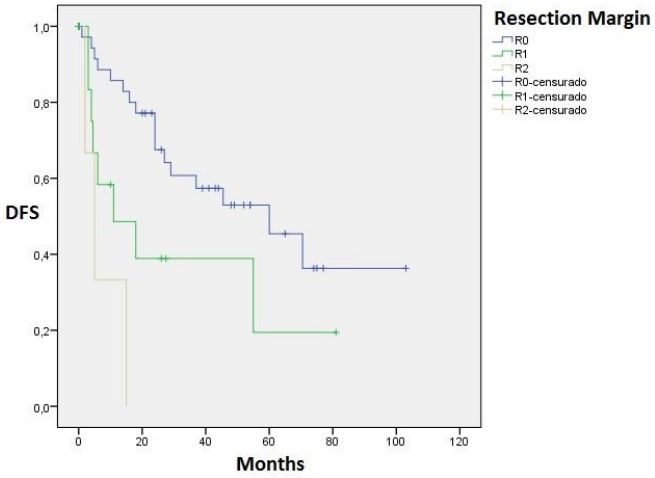

Figure 12 - Survival curve of tumor-free resection margin as a factor that impact disease-free survival of 59 patients after hepatectomy for hepatocellular carcinoma: R2 margin as a factor of worse disease-free survival (p = 0.001, R0 5-year DFS: 45,4% vs. R1 5-year DFS: 19,4% vs. R2 5-year DFS: 0%).

4.2. Non-tumoral parenchyma

None of the parameters studied were associated with worse OS or worse DFS.

4.3. Tumoral parenchyma

Macroscopic (MaVI) and microscopic (MiVI) vascular invasion were associated with either worse OS ($p < 0.001$ and $p = 0.01$, respectively) (Figure 13) and worse DFS ($p < 0.001$ for both) (Figure 14). Table 7 summarizes the statistical analysis of MaVI and MiVI impact in the patients' OS and DFS.

Table 7 - Macroscopic and microscopic vascular invasion as factors of worse overall survival and worse disease-free survival of 59 patients after hepatectomy for hepatocellular carcinoma.

Overall survival (OS)

	Median OS	5-year OS	p value
MaVI+	17 ± 4 months	0%	0.000
MaVI-	81 ± 11 months	56%	
MiVI+	28 ± 9 months	36%	0.01
MiVI-	132 ± 12 months	63%	

Disease-free survival (DFS)

	Median DFS	5-year DFS	p value
MaVI+	5 ± 1 months	0%	< 0.001
MaVI-	55 ± 11 months	38%	
MiVI+	15 ± 4 months	25%	< 0.001
MiVI-	71 ± 11 months	44%	

Diffuse tumoral macroscopic type was associated with worse DFS (p = 0.01; Nodular median DFS: 45 ± 7 months vs. Satellite median DFS: 24 ± 18 months vs. Diffuse median DSF: 12 ± 6 months) Figure 15).

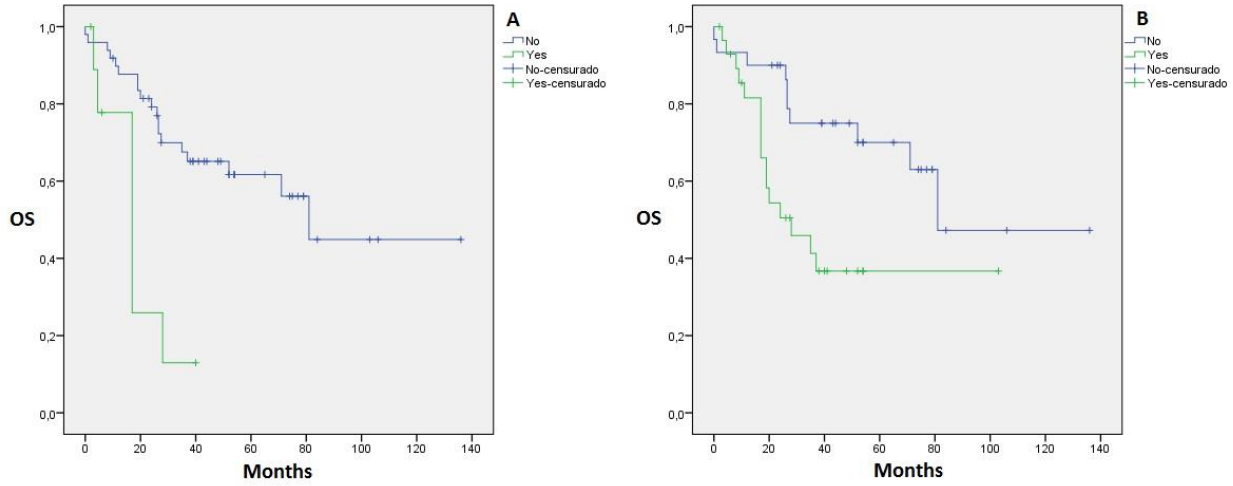

Figure 13- Survival curves of worse overall survival for macroscopic vascular invasion (A) and microscopic vascular invasion (B) of 59 patients after hepatectomy for hepatocellular carcinoma.

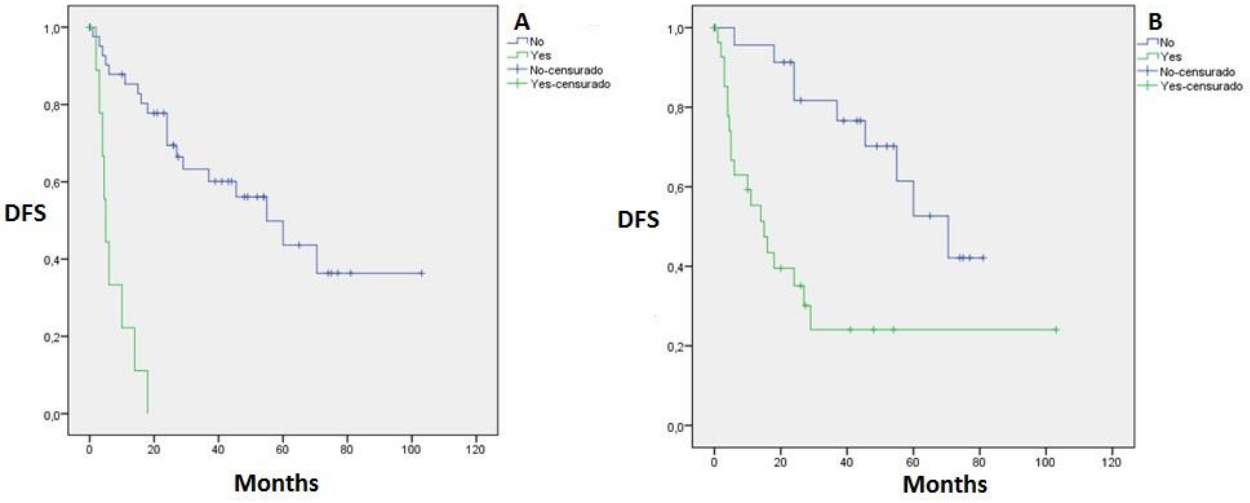

Figure 14 - Survival curves of worse disease-free survival for macroscopic vascular invasion (A) and microscopic vascular invasion (B) of 59 patients after hepatectomy for hepatocellular carcinoma.

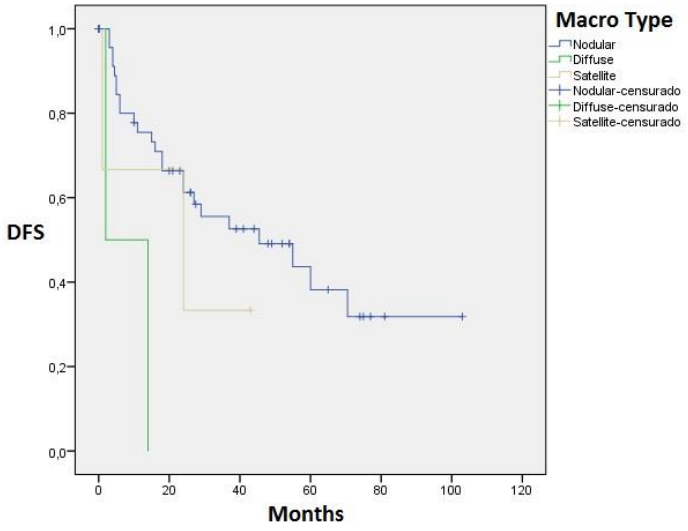

Figure 15- Survival curve for tumoral macroscopic type as a factor that impact disease-free survival of 59 patients after hepatectomy for hepatocellular carcinoma: Diffuse macroscopic type as a factor of worse disease-free survival (p = 0.01, Nodular 5-year DFS: 34% vs. Satellite 5-year DFS: 0% vs. Diffuse 5-year DFS: 0%).

There was no statistical significance that associates the histological subtype with worse OS (p = 0.2) and DFS (p = 0.5), although C-HCC has worse OS than SH-HCC (Figure 16).

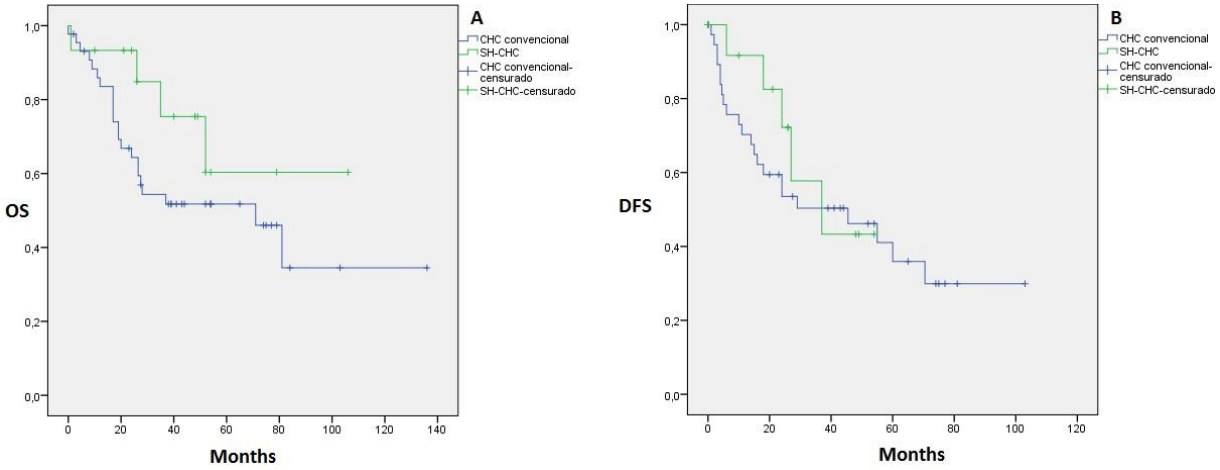

Figure 16 - Survival curves of overall survival (A) and disease-free survival (B) for histological subtype of 59 patients after hepatectomy for hepatocellular carcinoma.

There is no correlation between SH-HCC and HCV (p = 0.5), HBV (p = 0.1), ethylism (p = 0.6), estabilished cirrhosis (p = 0.1), NASH (p = 0.7), MS (p = 0.5) and diabetes (p = 0.2). There is a correlation between SH-HCC and female gender (p = 0.01).

No other parameter studied was associated with worse OS or worse DFS.

4.4. Immunohistochemical staining

There was no association between CK19 or GLP-3 and worse OS or worse DFS when studied singly.

Regarding to DFS, group 2 (CK19+/ GLP-3−) had worse DFS and group 4 (CK19−/ GLP-3−) had better DFS (p = 0.01) (Figure 17).

Table 8 summarizes the statistical analysis of the groups' impact in disease-free survival.

Table 8 - Immunohistochemical groups formation survival analysis: Group 4 as a factor of better disease-free survival and Group 2 as a factor of worse disease-free survival of 59 patients after hepatectomy for hepatocellular carcinoma.

Disease-free survival (DFS)

	Median DFS	**5-year DFS**	**p value**
Group 1 (CK19+/ GLP-3+)	38 ± 14 months	0%	0.01
Group 2 (CK19+/ GLP-3−)	4 ± 3,5 months	0%	

Group 3(CK19−/ GLP-3+)	27 ± 8 months	15%
Group 4 (CK19−/ GLP-3−)	71 ± 11 months	49%

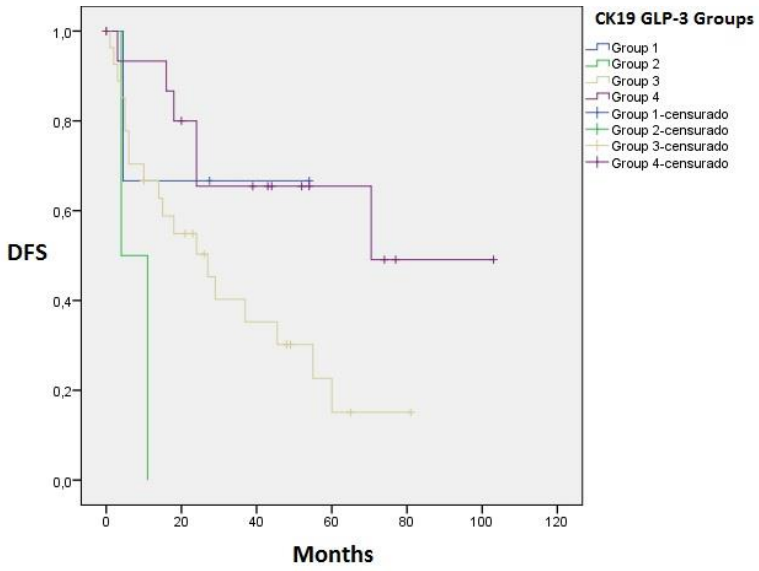

Figure 17 - Survival curves of worse disease-free survival for immunohistochemical staining of CK19 and GLP-3 of 59 patients undergoing hepatectomy for hepatocellular carcinoma: group 4 (CK19-/GLP-3-) with better disease-free survival and group 2 (CK19+/GLP3-3-) with worse disease-free survival.

5. Cox Regression

5.1. Overall survival

In multivariate analysis, HCV was associated with worse OS (HZ = 4,02 p = 0,002). MS was a predictor of better OS (HZ = 0,19 p = 0,001).

Macroscopic (MaVI) and microscopic (MiVI) vascular invasion were also associated with worse OS (HZ = 4,48 p = 0,001 and HZ = 3,91 p = 0,005, respectively).

The cox regression for OS is summarized in Table 9.

Table 9 - Multivariate analysis for overall survival of 59 patients after hepatectomy for hepatocellular carcinoma.

	HZ	**95% CI**	**P VALUE**
HCV	4,02	1,66 – 9,77	0,002
MaVI	4,48	1,79 – 11,20	0,001
MiVI	3,91	1,52 – 10,04	0,005
MS	0,19	0,07 – 0,52	0,001

5.2. Disease-free survival

Intermittent HPC and R2 resection margin were associated with worse DFS in multivariate analysis (HZ = 4,07 p = 0,012 and HZ = 2,60 p = 0,002).

Macroscopic and microscopic vascular invasion were associated with worse DFS (HZ = 6,16 p < 0,001 and HZ = 2,83 p = 0,035, respectively).

Group 2 (CK19+/ GLP-3−) and group 3 (CK19−/ GLP-3+) were identified as independent factors of worse DFS (HZ = 9,99 p = 0,007 and HZ = 2,67 p = 0,029), and group 4 (CK19−/ GLP-3−) as a factor of better DFS (HZ = 0,36 p = 0,031).

The cox regression for DFS is summarized in Table 10.

Table 10- Multivariate analysis for disease-free survival of 59 patients after hepatectomy for hepatocellular carcinoma.

	HZ	95% CI	P VALUE
HPC	4,07	1,37 – 12,12	0,012
R2	2,60	1,44 – 4,71	0,002
MaVI	6,16	2,21 – 17, 14	0,000
MiVI	2,83	1,08 – 7, 42	0,035
Group 2 (CK19+/ GLP-3−)	9,99	1,89 – 52,76	0,007
Group 3 (CK19−/ GLP-3+)	2,67	1,11 – 6,44	0,029
Group 4 (CK19−/ GLP-3−)	0,36	0,14 – 0,91	0,031

IV. Discussion

Hepatocellular carcinoma is a challenging disease. Management is difficult because of the underlying liver disease and due to lack of adequate systemic therapy. Resection, alongside liver transplantation, is still the most important curative option (24). A study recently reported that hepatectomy is an important surgical management procedure in intrahepatic recurrence cases after liver transplantation, and should be considered in selected cases (25). Regarding HCC resection there are many clinical and pathological parameters to be taken into account.

This study was design to identify clinical and pathologic prognostic factors that impact the overall and disease-free survival of HCC patients in hepatectomies.

Many studies (26, 27) and meta-analysis of observational studies (28) have reported worse prognosis in patients with HCV and HBV infection undergoing hepatectomy for HCC when compared with patients with negative serology. In our study, only HCV infection was identified as independent factor of worse OS. It is generally accepted that virus-induced chronic inflammation and hepatocyte necrosis might cause the hepatocytes to undergo proliferation and thus increase the occurrence of genetic aberrations, which may be the main mechanism responsible for late intrahepatic recurrence (28).

It is also generally accepted that HCV-related HCC develops at a more advanced stage of baseline liver disease than does HBV-related HCC (29). This means that even though patients underwent hepatectomy, they will have a worse HCC, since their livers have advanced baseline disease. HCV-related HCC is more likely to be multifocal, whereas HBV leads predominantly to unifocal tumors (29), suggesting that the risk of developing a recurrent and more aggressive lesion after hepatectomy is higher in patients with HCV than in those with HBV.

Metabolic syndrome was identified as an independent factor of better overall survival in this study. Although the association between metabolic syndrome and HCC is not readily identifiable in a significant percentage of HCC cases, it is now well established, that metabolic syndrome is contributing to the development of HCC (4). With the current rising epidemic of obesity and metabolic syndrome in the general population, this is becoming the most common cause of HCC in developed countries (2). In the liver, metabolic syndrome may cause inflammatory and angiogenic changes due to underlying insulin resistance and fatty liver disease (30). It is thought that HCC secondary to metabolic syndrome may have better prognosis than its other counterparts

partly because of early diagnosis with favorable prognostic markers and easier management of their comorbidities (4). Furthermore, resection is probably more easily performed in NASH livers than in other etiologies due to a preserved hepatocellular function and absence of cirrhosis (4).

It is still controversial whether HPC is safe during HP. Tralhão et al (31) reported that HPC or selective hemihepatic continuous portal clamping are safe methods of vascular control during liver resection, with no adverse effects on hepatocellular function. However, in other studies, HPC has been associated with worse prognosis (5, 32-34), and Hamaguchy et al (6) showed in a rat HCC model that longer HPC (15 minutes), during major hepatectomy, followed by reperfusion, induced the secretion on various cytokines (TFN-α, IL-1β, IL-6, VEGF) and accelerated HCC growth through the upregulation of hypoxia inducible factor (HIF)-1α and the activation of the IL-6-JAK-STAT3 signaling pathway. Man et al (35) reported that hepatic ischemia for 60 minutes followed by reperfusion for 60 minutes exacerbated liver tumor growth and metastasis through the activation of cell adhesion, invasion, and angiogenesis pathways. Our current study correlates HPC with worse DFS, in univariate and multivariate analysis, since the ischemia-reperfusion injury (IRI) produced by HPC could promote a more angiogenic and angioinvasive phenotype of tumor cells, and can activate signaling angiogenic pathways resulting in higher recurrence. Strategies aiming at reducing IRI are critically important in liver surgery, and more studies should be carried to access the impact of HPC, such as the one suggested by Xiaobin et al (36), a randomized, prospective and controlled multicenter trial to access whether HPC has a negative effect on the long-term outcome of HCC patients. This trial will also provide prognostic differences, safety, advantages and disadvantages between HPC and non-HPC surgical procedures.

It is known that macroscopic vascular invasion, microscopic vascular invasion and a compromised resection margin result in earlier recurrence of tumor and a worse prognosis, since these factors

compromise the curative intention of the surgical resection (37-39). Our results were in agreement with this, since macroscopic vascular invasion and microscopic vascular invasion were identified as independent factors of worse OS and DFS and R2 resection margin was identified as an independent factor of worse DFS in our study's population.

Recently it was identified a new histological subtype of HCC – steatohepatitic HCC (SH-HCC). The SH-HCC morphology has similar features of steatohepatitis, namely hepatocellular ballooning, Mallory-Denk hyaline bodies, inflammation and fibrosis. Some studies relates an association between SH-HCC subtype and the metabolic condition of the patient (20, 40, 41) and others indicate that HCC can also develop steatohepatitic morphology outside the setting of fatty liver disease or metabolic syndrome (8). In our study there was no correlation between SH-HCC and metabolic syndrome. This may happen because SH-HCC subtype is more likely to result from genetic changes to shared genes or metabolic pathways within the tumor (8).

In our study there was no prognostic significance of SH-HCC when compared with the C-HCC, and this has been reported from Shibahara et al (20), but Chan et al (41) reported that SH-HCC was associated with late tumor recurrence despite having more favorable baseline tumor features. Since this results are divergent in the literature it is necessary to do more research about SH-HCC's survival impact in a larger population sample.

In our study there was a correlation between SH-HCC and female sex. Currently, there is no literature review supporting this association, which suggests a necessity to carry more investigations to further explore and understand this.

CK19 (42-44) and GLP-3 (11, 45, 46) have been identified as markers of worse prognosis in HCC. However, in our study, when they were analyzed as a single marker, there was no correlation with the patients' prognosis. A recent study, carried by Feng et al (9) reported that CK19 and GLP-3

expression, when analyzed together, had impact on patients' prognosis. In our study, patients from group 4 (CK19-/GLP-3-) independently presented better DFS, while patients from group 3 (CK19-/GLP-3+) and group 2 (CK19+/GLP-3-) experienced worse DFS. This may happen because these markers represent a state of low cell differentiation, correlated with higher aggressive potential of the tumor, and can result in a higher risk of intrahepatic metastasis, microvascular invasion, regional lymph node involvement, and distant metastasis (9). In our study, group 1 (CK19+/GLP-3+) patients were not associated with worse prognosis in univariate and multivariate analysis. In theory this group should have the most aggressive behavior. This suggests that a larger sample of patients is needed to investigate these markers, since our population with CK19+ was small.

V. Conclusions

Despite the difficulty in the management of HCC patients, hepatectomy is one of the potential curative treatments available. During surgical resection, hepatic pedicle clamping results in an ischemia-reperfusion injury which could promote a more angiogenic and angioinvasive phenotype of tumor cells, and can activate signaling angiogenic pathways resulting in higher and more aggressive recurrence. In our series HPC was associated with worse DFS. Compromised resection margins during hepatectomy are also a factor that impacts the patients' prognosis.

There are another factors associated with a worse prognosis: HCV etiology, macroscopic and microscopic vascular invasion and diffuse macroscopic type.

Patients with metabolic syndrome can have a better prognosis even though it causes inflammatory and angiogenic changes due to underlying insulin resistance and fatty liver disease, but their comorbidities are easier to manage and they have favorable prognostic markers.

The combined staining of CK19 and GLP-3 could be a valuable asset to identify patients with poorer cell differentiation since it can result in a more aggressive tumoral behavior, affecting the patients' prognosis and even their response to treatment. This staining combined with other immunohistochemical and morphology markers will allow a good correlation with molecular classification and consequently personalized treatment and follow-up.

Agradecimentos

Ao meu orientador, Dr. Henrique Alexandrino, um obrigada muito especial por ter sido um apoio constante, um orientador presente e disponível, que tornou todo este trabalho possível e muito melhor, mas acima de tudo por ser uma pessoa que me inspira a ser um profissional tão bom e competente quanto ele.

Ao meu co-orientador, Dr. Rui, pelo entusiasmo contagiante e por tornar tudo mais alegre, além do apoio e disponibilidade sem fim, por ter me ensinado tanto e me ter feito gostar de anatomia patológica.

Ao Professor Doutor Franscisco Castro e Sousa e a toda a equipa médica do serviço de cirurgia A por terem tornado possível o desenvolvimento deste estudo. Agradeço também à D. Isabel, porque sem ela, sem a sua ajuda e disponibilidade não teria conseguido desenvolver este trabalho.

À Prof. Isabel Carreira, Prof. Marília Dourado e Prof. João Relvas, um agradecimento muito especial, porque o meu caminho na FMUC começou com vocês.

À D. Leonor, por ser a pessoa mais inspiradora e boa que encontrei nesta faculdade, e que me tocou profundamente.

Aos meus amigos: Ju, Sal, Tri, Du, Chris, Pinheiro, Catarina, Marta, Sílvia, João, Raquel e Mafalda, por serem a família que escolhi, todos os dias, incondicionalmente e pacientemente. Vocês tornam a minha vida melhor todos os dias.

À D. Graça, Sr. Donaciano, D. Esperança, Catarina, António, Duarte e André por me acolherem na sua família e por todo o apoio e ajuda contantes.

Aos meus pais, Júlio e Andreia, aos meus irmãos, Larissa, Victor e Rafaela, aos meus avós, Fátima e Levi, aos meus tios, Claúdia, Eduardo, Levi Eduardo e Cíntia, e aos meus primos, Adler e Raquel,

porque mesmo longe, vocês estão comigo aonde estou e fazem parte de mim. Porque me apoiam e amam incondicionalmente e porque me incentivam a ser sempre melhor e, principalmente, a ser feliz e buscar o meu caminho.

Por fim, ao meu namorado, José Pedro, por viver esta aventura comigo todos os dias, por me apoiar, e me ajudar de perto como ninguém, por me incentivar sempre a caminhar mais longe e a lutar pelos meus sonhos, mas sem me deixar esquecer de que o mais importante é ser uma pessoa que acrescenta algo ao mundo.

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