Histology and Histopathology

From Cell Biology to Tissue Engineering

Hepatocellular carcinoma in non-alcoholic fatty liver disease (NAFLD) - pathological evidence for a predominance of steatohepatitic inflammatory non-proliferative subtype

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Summary. Objectives. This study evaluated clinical and pathological aspects of patients with hepatocellular carcinoma (HCC) secondary to non-alcoholic fatty liver disease (NAFLD) and related these factors to immunohistochemical markers representative of the proliferative class.

Methods. We evaluated 35 HCC nodules from 21 patients diagnosed with NAFLD undergoing liver resection (n=12) or liver transplantation (n=8) or both (n=1). Demographic, clinical and biochemical data were compared to histological features and to immunohisto-chemical reactivity for K19 and Ki-67.

Results. Cirrhosis was present in 58% of patients. Ages ranged from 50 to 77 years. Sixteen patients (76%) were male and had type 2 diabetes mellitus, 81% had arterial hypertension, and 90% had BMI above 25 kg/m^2. Alpha-fetoprotein levels were normal in 62% of patients. Twenty-five (70%) nodules were diagnosed as "steatohepatitic HCC". Only 32% of the nodules presented high levels of Ki-67 (>10%) and/or K19 (>5%), although 63% were poorly differentiated (G.3/G.4) according to Edmondson & Steiner grading system. K19 positivity (>5%) was associated with higher degree of intratumoral inflammation (G.2/G.3), and with fibrosis, both at the center of the tumor and at the tumor front, whereas Ki-67 positivity (>10%) was associated with ballooning of neoplastic cells and occurred in more than 70% in non-cirrhotic patients.

Conclusion. NAFLD-related HCC was found in non-cirrhotic patients in 42% of cases, alpha-fetoprotein level was normal in 63% and "steatohepatitic HCC" was the predominant histological type. Immunoexpression of K19 and/or Ki-67 occurred in 32% of the nodules and were associated with intratumoral inflammation and ballooning, suggesting that HCC in MtS may be preferentially "an inflammatory, non-proliferative subtype of HCC".

Abbreviations. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; AP, prothrombin activity; ATP III, National Cholesterol Education Program Adult Treatment Plan III; BCLC, Barcelona Clinic Liver Cancer; BMI, body mass index; T2DM, type 2 diabetes mellitus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HC-FMUSP, University of Sao Paulo School of Medicine Hospital; K19, keratin 19; Ki-67, cell cycle antigen Ki-67; LT, liver transplantation; NAFLD, non-alcoholic liver disease; NASH, non-alcoholic steatohepatitis; MtS, metabolic syndrome; TMA, tissue microarray.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and one of the most prevalent neoplasms worldwide (Forner et al., 2018). The incidence of HCC is increasing still due to advanced stage chronic hepatitis C and especially due to the increase in incidence of obesity, type 2 diabetes (T2DM) and non-alcoholic fatty liver disease (NAFLD) (Bugianesi, 2007; Llovet et al., 2016; Wong et al., 2016).

Few studies have evaluated the pathological and immunohistochemical features of HCC secondary to NAFLD. Salomão et al. first reported "steatohepatitic HCC" as a HCC variant presenting histological pattern of steatohepatitis in the neoplastic hepatocytes, occurring in patients with HCC secondary to hepatitis C virus (HCV) infection, mostly when NAFLD was found as a comorbidity. Histological definition of this variant requires the presence of at least 3 of the following 4 criteria: neoplastic hepatocellular ballooning, Mallory-Denk bodies, inflammation and intratumoral fibrosis in more than 50% of the nodule (Salomao et al., 2010). Shibahara et al. retrospectively evaluated the presence of this variant in the 382 HCC nodules from 293 patients surgically treated at the University Hospital of Tokyo from 2005 to 2010. Steatohepatitic histological type was found in 120 HCC nodules (31.4%) from 106 patients (36.4%) who exhibited a higher frequency of T2DM, arterial hypertension, and dyslipidemia (DLP) as compared to patients presenting conventional histological pattern of HCC (Shibahara et al., 2014).

A retrospective study by Hernandez-Alejandro et al. assessed clinical and pathological features of 64 patients undergoing liver transplantation (LT) for HCC secondary to HCV and 17 patients undergoing transplantation for HCC secondary to non-alcoholic steatohepatitis (NASH). HCV-related HCC presented higher vascular invasion (23.4% vs. 6.4%, p=0.002) and more poorly differentiated tumors (4.7% vs. 0%, p<0.001) compared to the HCC-NASH group (Hernandez-Alejandro et al., 2012).

Genomic assessment of all types of HCC profiling from several groups converged to the identification of 2 major molecular clusters: "proliferative" and "non-proliferative", based on differential enrichment in prognostic signatures, pathway activation and tumor phenotypes (Hoshida et al., 2010; Zucman-Rossi et al., 2015; Llovet et al., 2016; Forner et al., 2018). Multivariate regression approach to simultaneously clustering data from five platforms published from The Cancer Genome Atlas Research Network (TCGA) (Ally et al., 2017) defined 3 major molecular subtypes, one of them closely related to the "proliferative" type suggested by Hoshida et al. and by Zucman-Rossi et al., characterized by younger age, Asian ethnicity and normal body weight, higher tumor grade and presence of macrovascular invasion, as well as a low frequency of CTNNB1 mutation and a low frequency of TERT promoter mutation. This "proliferative" subtype was associated with overexpression of proliferation marker genes such as MYBL2, PLK1 and MKi67 (Ally et al., 2017).

By allowing topographical and cellular localization of selected gene products, immunohistochemistry has been shown as a valuable bridge integrating morphological to molecular concepts. Therefore, the present study is aimed at further histological characterization of HCC secondary to clinical evidence of NAFLD, as related to main clinical features and to the immunohistochemical expression of K19 and Ki-67, well-known surrogate markers for the "proliferative class of HCC".

Materials and methods

Patients and samples

This study is based on a retrospective series of adult patients (≥18 years) with HCC secondary to NAFLD or to cryptogenic cirrhosis in the presence of metabolic syndrome (MtS) components according to the National Cholesterol Education Program Adult Treatment Plan III guidelines (ATP III), who were operated and followed up at the Liver Transplant Division or Liver Surgery and Biliary Tract Division of the University of Sao Paulo School of Medicine Hospital (HC-FMUSP) and Division of Gastroenterology of the University of Porto Alegre School of Medicine Hospital from 2005 to 2015. These patients were classified as having NAFLD when histological or ultra-sonographic features of fatty liver were present and all other known causes of liver disease were excluded. Staging of hepatic architectural changes in NAFLD patients followed the criteria presented by Kleiner et al. (2005). The diagnosis of HCC was performed according to the international guidelines of the American Association for Study of Liver Diseases (AASLD) (Bruix et al., 2016) or European Association for Study of the Liver (EASL) (Llovet et al., 2012) and histologically confirmed on surgical/explant specimens by an experienced liver pathologist (VAFA) according to the criteria defined by the International Consensus Group for Hepatocellular Neoplasia (Kojiro et al., 2009).

From a total of 423 patients who underwent liver resection or LT for HCC, the following cases were excluded: positive serology for hepatitis C (n=264) or B (n=44), positive history of alcohol abuse (n=50), chronic liver disease from other well-defined causes (n=21), cryptogenic liver disease without risk factors for MtS (n=16), and cases with insufficient data to participate in

the study (n=7). Thus, twenty-one patients were eligible for the present analysis, encompassing 35 HCC nodules, which were individually assessed for the histopathological and immunohistochemical variables (Fig. 1).

All slides from the 35 HCC nodules from the 21 patients retrieved from the files of the Division of Anatomical Pathology (DAP-HC-FMUSP) were jointly reviewed by two observers (VAFA and PBC). As presented at Table 1, seven histological variables were assessed in non-tumoral liver and 8 variables were assessed in each HCC nodule. Tumor fibrosis and inflammation was assessed independently in the center of the tumor ("intratumor") and at the "tumor front".

Tissue microarray (TMA) construction

Aiming at a generous representation of each area of interest, TMAs were produced via the extraction of tissue 1 mm-diameter cores from original paraffin-donor blocks from hepatocellular carcinoma samples. Three representative areas from the central region and three from the tumoral border of each nodule were assessed in up to 4 nodules per patient.

Table 1. Semiquantitative assessment of histopathological variables.

Variable	Low grade	High grade
Non-Tumoral Liver		
Liver Architecture Changes	Mild (0+1+2)	Advanced (3+4)
Portal infiltrate	Absent or low (0+1+2)	High (3+4)
Periportal/interface activity	Absent or low (0+1)	High (2+3)
Parenchymal activity	Absent or low (0+1)	High (2+3)
Ballooning	Absent or low (0+1)	High (2)
Steatosis	Absent or low (0+1)	Moderate/ severe (2+3)
Mallory-Denk bodies	Absent or few (0+1)	Numerous (2)
Intratumoral		
Architectural grade	Lower (GO/G1/G2)	Higher (G3/G4)
Nuclear grade	Lower (G1/G2)	Higher (G3/G4)
Edmondson-Steiner grading	Well-differentiated (G1/G2)	Poorly differentiated (G3/G4)
Intra tumoral steatosis	Absent or low (0+1)	Moderate/severe (2+3)
Intra tumoral ballooning	Absent or low (0+1)	High (2)
Intra tumoral inflammation	Absent or low (0+1)	High (2+3)
Intra tumoral fibrosis	Absent or low (0+1+2)	High (3+4)
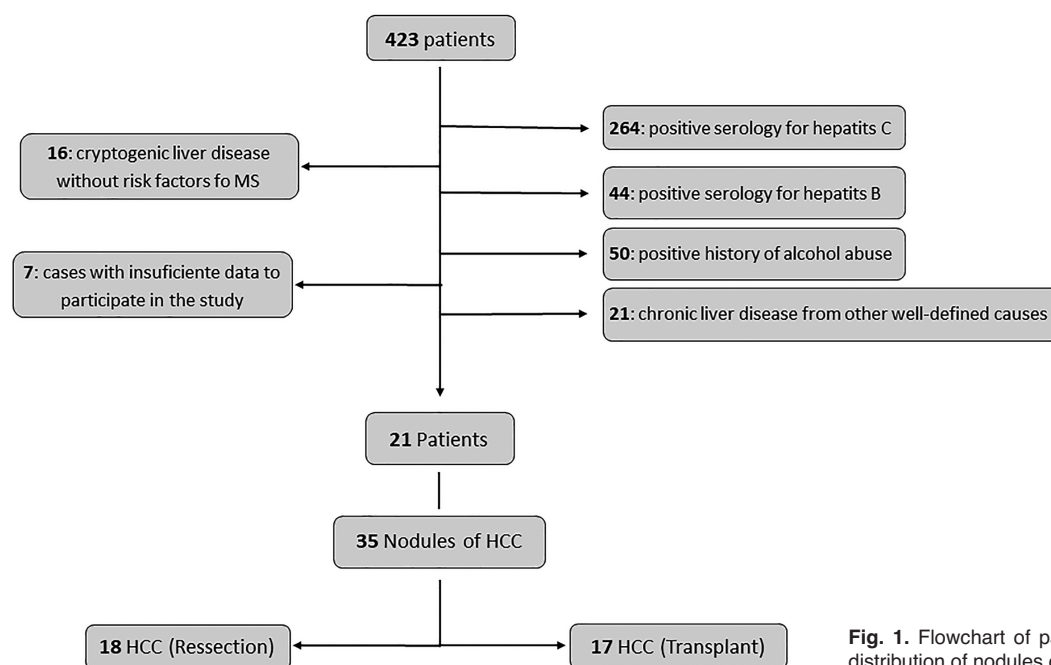
Fig. 1. Flowchart of patients included in the study and distribution of nodules of HCC.

Immunohistochemistry reactions

Primary monoclonal antibodies against K19 and Ki-67 were standardized in our laboratory at USP (K19: clone B-170-1: 300; Novocastra, United Kingdom; Ki-67: clone MIB-1, 1: 400; Dako, Denmark). Briefly, slide-mounted sections of the paraffin-embedded tissues were deparaffinized, rehydrated and immersed in 10-mM citrate buffer (pH 6.0) and steam heated for 40 min for antigen retrieval. Slides were washed with distilled water, and endogenous peroxidase was blocked via incubation in a 6% hydrogen peroxide solution in methanol at room temperature 3 x 10 min. Protein blocking was performed via incubation in CASBlock™ solution (Invitrogen, United States) at 37°C for 10 min. Primary antibodies were incubated at 37°C for 30 min followed by incubation at 4°C for 18 h. The detection of K19 and Ki-67 was amplified using the Novolink™ polymer system (Novocastra, United Kingdom) at 37°C

for 30 min. All immunoreactive signals were visualized with chromogen 3-3-diaminobenzidine (60 mg/dL in a phosphate buffer, pH 7.4) at 37°C for 5 min, followed by washing with distilled water, and counterstaining with Harris' hematoxylin at room temperature for 1 min. Slides were dehydrated in a progressive alcohol series, cleared in xylene, and mounted with Entellan™ (Merck, United States). The biliary ducts in a normal liver section and lymphoid follicles in tonsils served as positive controls for K19 and for Ki-67, respectively. Substitution of primary antibody by non-relevant antibodies served as negative controls.

Immunohistochemical evaluation

The assessment of immunohistochemical reactivity above the cut-offs of K19 and Ki-67, selected as surrogate markers for the "proliferative class" was possible in available blocks from 31 nodules from 19 patients, since samples from 2 patients did not provide sufficient material to construct the TMA. K19 and Ki-67 expression was evaluated at the periphery and center of the tumor, and the highest value ("hot spot") was chosen to represent the nodule.

The number of Ki-67 antigen expressing nuclei was counted in each visualized representative field from TMA samples by one experimented observer (ALF). As many neoplastic cells as possible, up to 1000 nuclei were counted in each area, and the results were presented as a percentage of labeled cells. Nodules with Ki-67>10% were considered "highly proliferative".

Samples were semiquantitated for K19 using the following expression scores: 0: absent; 1, estimate of up to 5%, and 2, over 5%. Scores 0 and 1 were considered "negative", whereas "positive" reactions were those with more than 5% immunostained for K19. The spot which yielded the highest score was considered the "hot spot" to represent the nodule.

Statistical analysis

Data were stored and analyzed using the IBM SPSS version 20.0 for statistical analysis. Frequency and percentage variables were described and compared using Fisher's Exact test. The threshold for significant P values was established as $P<0.05$.

Results

Clinical and laboratorial findings

Major clinical and laboratorial findings are depicted in Table 2. Twelve of the 21 patients were cirrhotic (58%). The 9 non-cirrhotic patients (42%) presented NAFLD stages: F2: 6 patients, F3=3 patients. Thirty-five HCC nodules were studied: 18 nodules (51%) from liver resection and 17 nodules (49%) from explants. Patient age ranged from 50 to 77 years, and 76% were male. A total of 76% patients had T2DM, 81% were

Table 2. Demographic, clinical and laboratory features of patients with hepatocellular carcinoma (HCC).

Feature	n=21(%)
Age (years)	
Mean (Min-Max)	64.9(50-77)
Gender male	16(76%)
Gender female	5(24%)
BMI> 25	19(90%)
Diabetes	16(76%)
Dyslipidemia	8(38%)
Hypertension	17(81%)
AST*	47(39-55)
ALT*	46(33-73)
FA*	119(92-158)
GGT*	163(73-262)
Bilirubin*	0.79(0,62-1,5)
Albumin*	4.1(3.6-4.3)
Platelets*	160(95-222)
AP (%)*	81(67-90)
AFP*	5.7(2.6-19)
Number of nodules	
1	12(57%)
2	6(28%)
3	1(5%)
4 or more	2(10%)
Cirrhosis	
Yes	12(58%)
No	9(42%)
Child Pugh	
A	8(38%)
B	4(20%)
C	0
NA	9(42%)
Ecog	
0	19(90%)
1	2(10%)
2	0

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FA, alkaline phosphatase; GGT, gamma glutamyl transferase; AFP, alpha-fetoprotein; TP, prothrombin activity in %; ECOG, Eastern Cooperative Oncology Group *Median with interquartile.
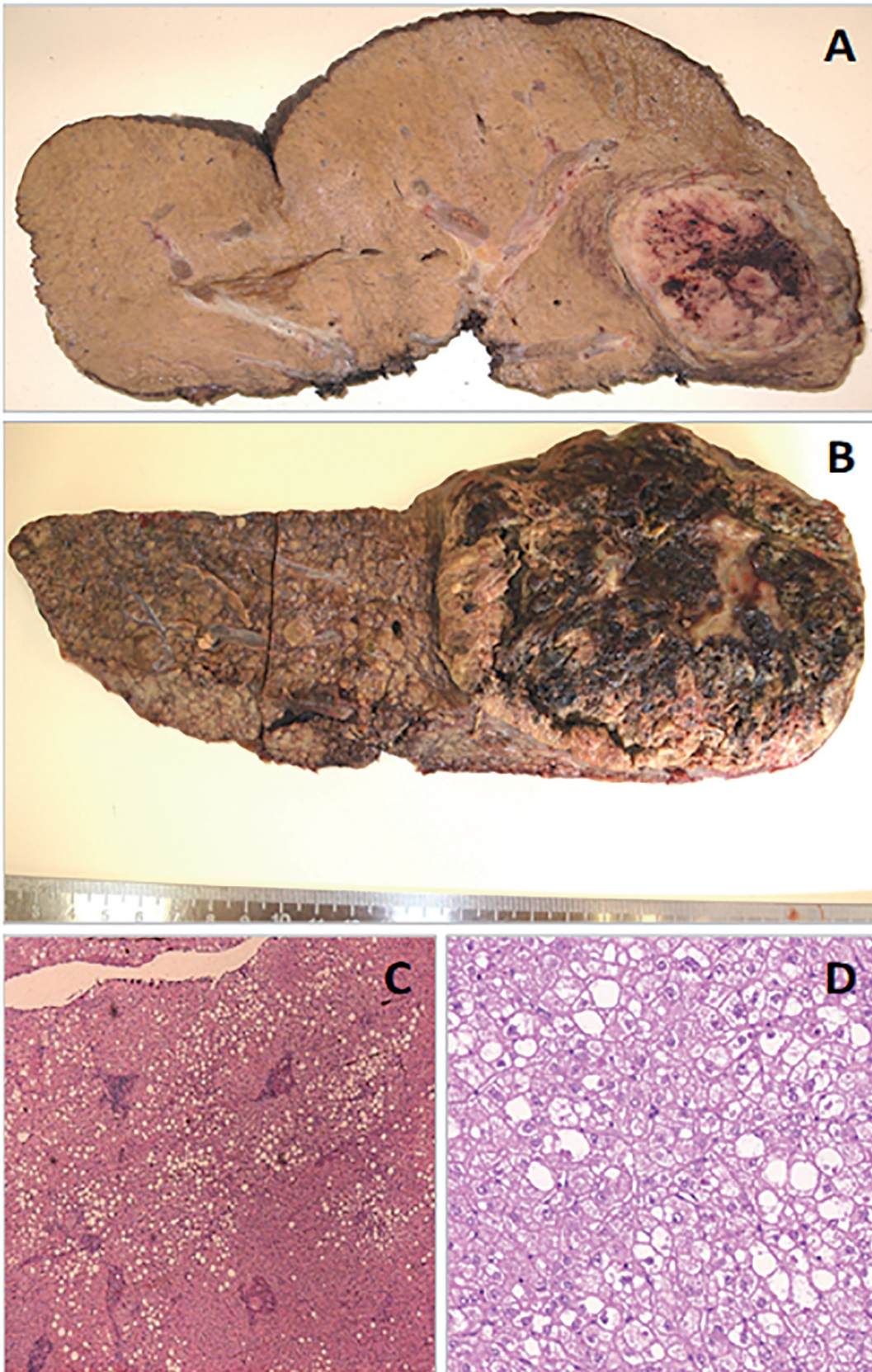
Fig. 2. Pathological findings, **A.** Explant from a large, heterogeneous HCC occurring in a yellowish enlarged liver with minor fibrosis (NASH, F2). **B.** HCC in a cirrhotic liver. **C.** Non-cirrhotic liver with NASH F2 (same case fig. 2A). **D.** Non-neoplastic liver presenting Steatosis, Ballooning and Mallory-Denk Bodies. HE: C, x 40; D, x 200.

hypertensive, and 38% had dyslipidemia. The BMI ranged from 22 to 35.7 kg/m^2. Most patients (90%) were overweight (BMI above 25 kg/m^2) or obese and only 2 diabetic patients (10%) exhibited BMI below 25 kg/m^2. Nine of the 21 patients (42%) had been under HCC screening with imaging and AFP. The level of alpha-fetoprotein was normal in 13 patients (62%).

Pathological findings

Twelve patients had 1 HCC nodule, 6 patients had 2 nodules, 1 patient had 3 nodules and 2 patients had 4 nodules or more. The nodule size ranged from 4 cm to 15 cm. Histopathological assessment of the 35 HCC nodules revealed steatosis in 26 nodules, moderate/severe in 9 of them. Ballooning of neoplastic hepatocytes was found in 31 nodules, grade 2 in 22 nodules. Moreover, Mallory-Denk Bodies grade 2 was observed in 23 nodules, usually in the most ballooned tumor cells. Thus, 25 nodules (71%) fulfilled histological criteria for "steatohepatitic HCC" according to Edmondson & Steiner grading system, thirteen (37%)
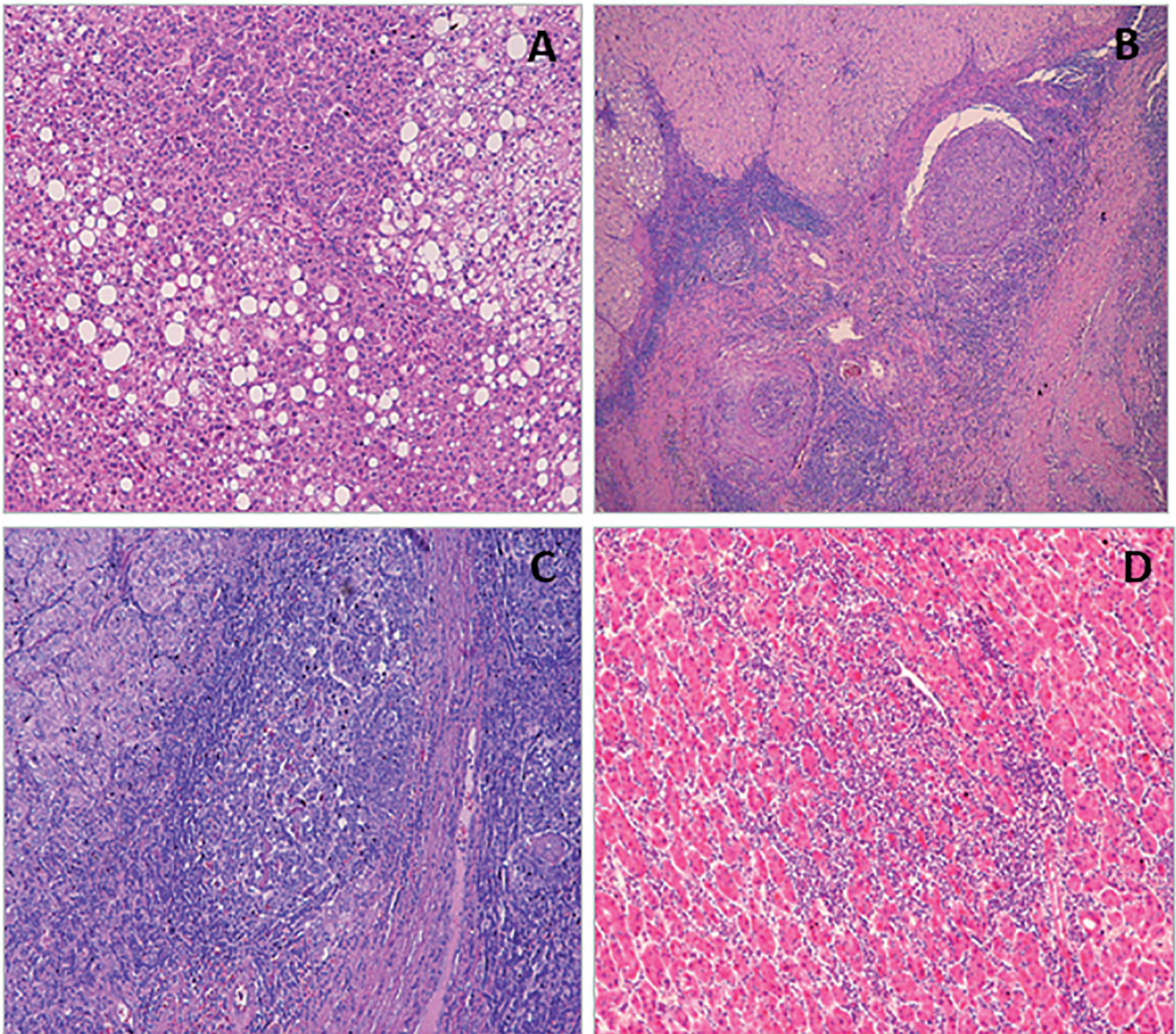
Fig. 3. Histopathological aspects of NASH-related HCC. **A.** Steato-hepatitic variant of HCC depicting macro- and microvesicular steatosis, ballooning, Mallory-Denk bodies and intratumoral lymphocyte-rich inflammation. **B.** Intratumoral and tumor invasion front lymphocyte-rich inflammation and fibrosis in grade 2 vascular invasion. **C.** Hepatocellular carcinoma presenting intratumoral fibrosis grade 3. **D.** Major intratumoral lymphocyte-rich inflammation, grade 3. HE. A, x 200; B, x 40; C, D, x 100.
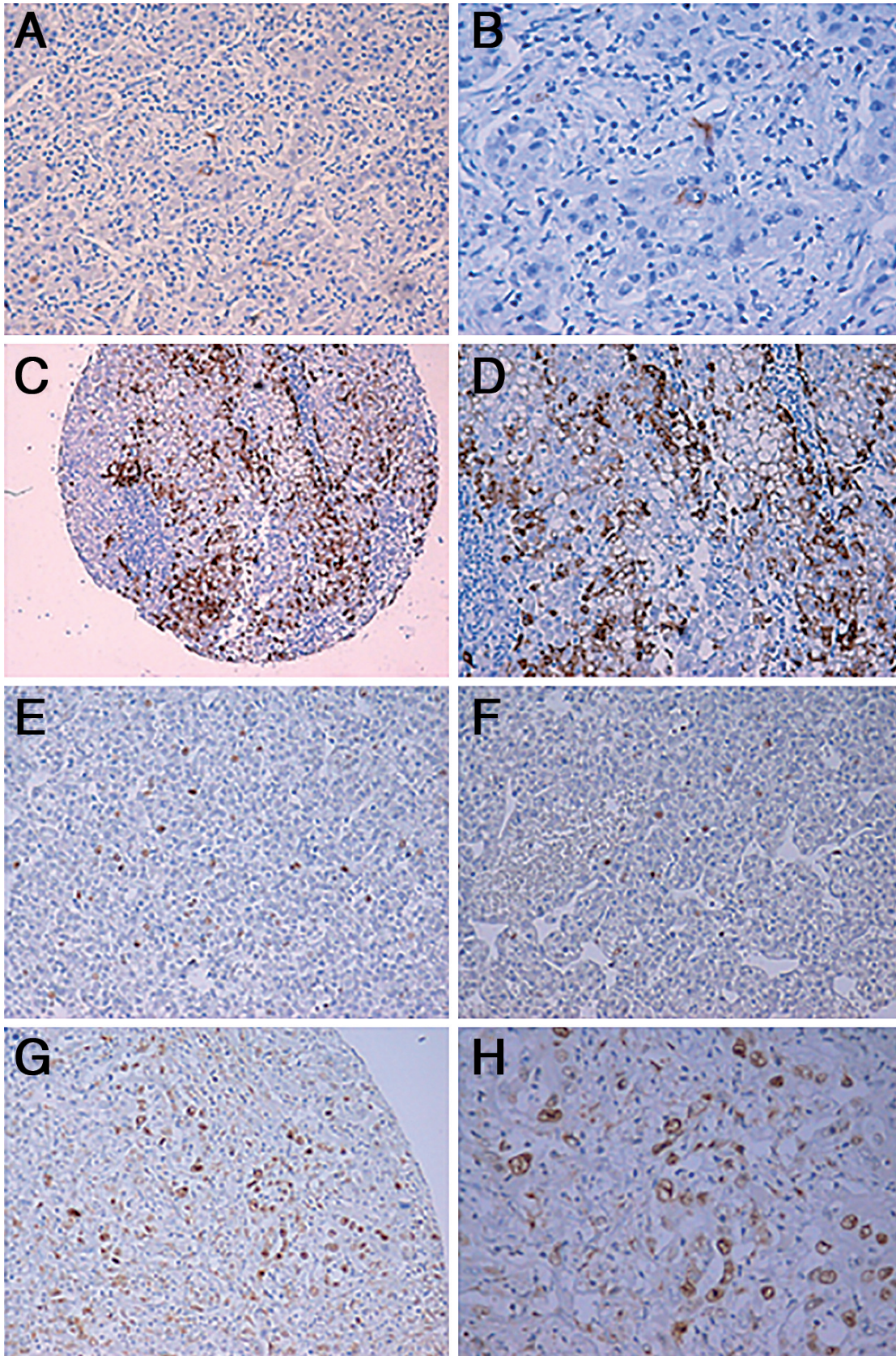
Fig. 4. NASH-related HCC - Representative immunoreactions. **A.** Expression of K19 in less than 1 % of cells. **B.** Expression of K19 in less than 1 % of cells .**C.** Expression of K19 in approximately 50% of cells in an Steatohepatitic HCC. **D.** Expression of K19 in approximately 60% of cells in an Steatohepatitic HCC. **E.** Ki-67 in 3% of neoplastic cells. **F.** Ki-67 in 3% of neoplastic cells. **G.** Ki-67 in 74% of neoplastic cells. **H.** Ki67 in 74% of neoplastic cells. A, D, E, G, x 200; B, F, H, x 400; C, x 100.

nodules were G.1/G.2 HCC, called herein "well-differentiated", whereas 22 (63%) nodules were G.3/G.4, called herein "poorly differentiated" (Edmondson and Steiner, 1954). Figure 2 illustrates the pathological findings. The assessment of specific criteria of the 35 HCC nodules revealed low architectural grade (G.0-G.2)

Table 3. Association of histopathological variables to expression of Keratin 19 (K19).

	K19≤5%	K19>5%	P Value
NON-TUMORAL LIVER			
Liver architecture changes			0.241
0+1+2	4(15.4)	2(40.0)	
3+4	22(84.6)	3(60.0)	
Portal infiltrate			0.301
0+1+2	25(96.2)	4(80.0)	
3+4	1(3.8)	1(20.0)	
Periportal/interface activity			0.625
0+1	16(61.5)	2(40.0)	
2+3	10(38.5)	3(60.0)	
Parenchymal activity			0.999
0+1	18(69.2)	3(60.0)	
2+3	8(30.8)	2(40.0)	
Ballooning			0.241
0+1	22(84.6)	3(60.0)	
2	4(15.4)	2(40.0)	
Steatosis			0.241
0+1	22(84.6)	3(60.0)	
2+3	4(15.4)	2(40.0)	
Mallory-Denk bodies			0.999
0+1	22(84.6)	5(100.0)	
2	4(15.4)	-	
TUMORAL LIVER			
Architectural grade			0.058
0+1+2	13(50.0)	-	
3+4	13(50.0)	5(100.0)	
Nuclear grade			0.128
1+2	12(46.2)	-	
3+4	14(53.8)	5(100.0)	
Edmonson-Steiner grade			0.147
G1+G2	10(38.5)	-	
G3+G4	16(61.5)	5(100.0)	
Intratumoral steatosis			0.147
0+1	16(61.5)	5(100.0)	
2+3	10(28.5)	-	
Intratumoral ballooning			0.999
0+1	10(38.5)	2(40.0)	
2	16(61.5)	3(60.0)	
Intratumoral inflammation			**0.018**
0+1	16(61.5)	-	
2+3	10(38.5)	5(100.0)	
Intratumoral fibrosis			**0.027**
0+1+2	20(76.9)	1(20.0)	
3+4	6(23.1)	4(80.0)	
Tumor invasion front inflammation			0.999
0+1	9(34.6)	1(20.0)	
2+3	17(65.4)	4(80.0)	
Tumor invasion front fibrosis			**0.042**
0+1+2	19(73.1)	1(20.0)	
3+4	7(26.9)	4(80.0)	

Data presented as n (%) and compared using Fisher's exact test.

Table 4. Association of histopathological variables to the expression of Ki-67.

	Ki-67≤10%	Ki-67>10%	P Value
NON-TUMORAL LIVER			
Liver architecture changes			**0.001**
0+1+2	1(4.2)	5(71.4)	
3+4	23(95.8)	2(28.6)	
Portal infiltrate			0.999
0+1+2	22(91.7)	7(100.0)	
3+4	2(8.3)	-	
Periportal/interface activity			0.625
0+1	14(58.3)	4(57.1)	
2+3	10(41.7)	3(42.9)	
Parenchymal activity			0.172
0+1	18(75.0)	3(42.9)	
2+3	6(25.0)	4(57.1)	
Ballooning			0.241
0+1	22(97.1)	3(42.9)	
2	2(8.3)	4(57.1)	
Steatosis			0.11
0+1	21(87.5)	4(57.1)	
2+3	3(12.5)	3(42.9)	
Mallory-Denk bodies			0.212
0+1	22(91.7)	5(71.4)	
2	2(8.3)	2(28.6)	
TUMORAL LIVER			
Architectural grade			0.667
0+1+2	11(45.8)	2(28.6)	
3+4	13(54.2)	5(71.4)	
Nuclear grade			**0.026**
1+2	12(50.0)	-	
3+4	12(50.0)	7(100.0)	
Edmonson-Steiner grade			0.066
G1+G2	10(41.7)	-	
G3+G4	14(58.3)	7(100.0)	
Intratumoral steatosis			0.652
0+1	17(70.8)	4(57.1)	
2+3	7(29.2)	3(42.9)	
Intratumoral ballooning			0.999
0+1	9(37.5)	3(42.9)	
2	15(62.5)	4(57.1)	
Intratumoral inflammation			0.685
0+1	13(54.2)	3(42.9)	
2+3	11(45.8)	4(57.1)	
Intratumoral fibrosis			**0.022**
0+1+2	19(79.2)	2(28.6)	
3+4	5(20.8)	5(71.4)	
Tumor invasion front inflammation			0.999
0+1	8(33.3)	2(28.6)	
2+3	16(66.7)	4(71.4)	
Tumor invasion front fibrosis			0.067
0+1+2	18(75.0)	2(28.6)	
3+4	6(25.0)	4(71.4)	

Data presented as n (%) and compared using Fisher's exact test.

in 17 nodules (48%) and high architectural degree (G.3/G.4) in 18 nodules (52%). Fifteen nodules (43%) had nuclear grade 1 or 2 and 20 nodules (57%) presented nuclear grade 3 or 4. Intratumoral fibrosis G.1/G.2 was found in 24 cases (69%), G.3/G.4 in 11 (31%) (Fig. 3).

Tumor invasion front fibrosis was G.1/G.2 in 24 nodules (69%) and G.3/G.4 in 11 nodules (31%). Tumor invasion front inflammation was G.0/G.1/G.2 in 27 nodules (77%) and G.3/G.4 in 8 (23%) (Figs. 2, 3).

Markers of proliferative class

Twenty-one nodules (68%) were classified as "non-proliferative" HCC, not showing significant expression of either K19 or Ki-67. Ten nodules were classified as the "proliferative class" (10/31=32%), 3 of them presenting only K19 expression in more than 5% of neoplastic cells, another 5 presenting only Ki-67 expression over 10% in neoplastic cells and two nodules positive for both markers. All nodules with K19 expression over 5% exhibited intratumoral inflammation G.2/G.3 (p=0.018); 80% of K19-positive nodules exhibited intratumoral fibrosis G.3/G.4 (p=0.027) and 80% of K19-positive nodules exhibited tumor invasion front fibrosis G.3/G.4 (p=0.042) (Table 3). Expression of K19 was concordant in different regions of 34 HCC nodules, positive in the center and at the periphery (>5%) in 4 nodules and negative in both in 30 nodules. Only one nodule expressed K19 in spots from the center but negative at the periphery of the tumor.

Comparison of Ki-67 immunohistochemistry with the pathological variables of non-tumoral and tumoral livers showed that 71% of the nodules with Ki-67 >10% occurred in non-cirrhotic patients, whereas only 28% of HCC nodules in livers with a more marked degree of fibrosis (G.3/G.4) exhibited Ki-67 >10% (p=0.001). Fifty-seven percent of the nodules with Ki-67 >10% exhibited a marked degree of cell ballooning (G.2) (p=0.014). All nodules with Ki-67 >10 % had high nuclear grade (G.3/G.4) (p=0.026) and 71% had higher intratumoral fibrosis (G.3/G.4) (p=0.022) (Table 4). Regarding Ki-67, 4 nodules presented higher expression of Ki-67 (above 10%) in the tumor front compared to the center, whereas 3 nodules showed a similar pattern of expression of ki-67 in the central region and in tumor front (both above 10%). No nodule had the expression in the center superior to that found at tumor front. Expression of K19 was found >10% in 7 cases, 4 of them higher in the tumor front and 4 of them both at the front and at the tumor center. All other 24 cases had concordant Ki-67 <10% both at the tumor center and at the front (Fig. 4).

Discussion

This study approaches clinical and histopathological features in one of the largest series of HCC secondary to NAFLD, as compared to immunohistochemical evaluation "proliferative markers" as surrogate markers of the molecular subtypes of HCC. The majority of nodules were found related to inflammation/fibrosis and not to the "proliferative subtype of HCC". This finding may serve as further evidence for different carcinogenic mechanisms in the context of NAFLD when compared to HCC cases due to other causes, especially due to HCV. Another important difference is the rather high rate of cases of HCC occurring in non-cirrhotic NAFLD patients (Chagas et al., 2009; Kawada et al., 2009; Paradis et al., 2009; Ertle et al., 2011; Alexander et al., 2013; Cotrim et al., 2016; Kikuchi et al., 2016, Mittal et al., 2016), contrasting to more than 90% of HCC in cirrhosis in HCV patients (Llovet et al., 2016; Forner et al., 2018). Piscaglia et al. performed a prospective, multicenter observational study and compared patients with HCC secondary to NAFLD (n=145) and HCC secondary to HCV (n=611) who were enrolled in Italian secondary care centers between 2010 and the end of 2012. They evaluated the clinical and anatomo-pathological characteristics of the 2 groups. Small HCCs (i.e., single HCC or 2 or 3 nodules up to 3 cm) and BCLC 0 were more frequent in HCC-related HCV. On the other hand, HCC BCLC C and infiltrative HCC was significantly (p<0.0001) more common in patients with NAFLD (infiltrative HCC: 21% vs 4% in NAFLD and HCV, respectively) (Piscaglia et al., 2016). In this series, 75% were BCLC A, and 25% were BCLC B or C. BCLC was not used in patients without cirrhosis, but they exhibited a greater tumor burden. The Italian study demonstrated that patients with HCC-NAFLD exhibited lower survival compared to patients with HCC-HCV, primarily because the previous combination is generally detected at a later stage and with a higher tumor burden and not because HCC-NAFLD is more aggressive. Only 42% of the 21 patients in our series were under follow-up (compared to 48% of the Italian study), and we found that the tumor was more advanced in non-cirrhotic patients, which supports the conclusion that these tumors in NAFLD etiology are diagnosed in later stages, primarily due to the lack of surveillance strategies in patients with NAFLD, MtS or T2DM.

The incidence of liver-related events (cardiovascular events and type 2 diabetes mellitus) in patients with histopathologically confirmed NAFLD remains unclear. Insulin sensitizing drugs (pioglitazone/metformin) and a sodium-glucose cotransporter 2 inhibitor (SGLT2I) seem to have long-term beneficial effects in reducing cardiovascular risk and HCC in NAFLD patients (Carulli et al., 2013; Athyros et al., 2017; Akuta et al., 2018).

Our histopathological review detected 37% of HCC G.1/G.2 according to Edmondson and Steiner. Hernandez-Alejandro et al. also compared HCC-NAFLD (n=17) and HCC-HCV (n=64) patients submitted to liver transplantation and reported that a significantly higher proportion of HCC-HCV had vascular invasion (23.4% vs 6.4%, p=0.002) and poorly differentiated tumors (4.7% vs 0%, p<0.001) compared to HCC-NAFLD

(Hernandez-Alejandro et al., 2012). However, only patients eligible for transplantation were selected for that study. Other studies also demonstrated that HCCs in NAFLD were well or moderately differentiated (Duan et al., 2012). Perhap, the high rate of G.3/G.4 (63%) cases in the present series may be related to a later diagnosis, corroborated by our finding of larger tumors.

Remarkably, 25 (71%) of our 35 nodules fulfilled the histopathological criteria for "Steatohepatitic HCC Subtype" thus reinforcing the association of the presence of metabolic factors (T2DM/ obesity/ hypertension and dyslipidemia) in this subtype (Salomão et al., 2010; Shibahara et al., 2014).

Immunohistochemical detection of K19 >5% of the cells, a marker of progenitor cell component, was found in 16% of the nodules in our series, lower than the rates of 20-50 % in all subtypes of HCCs (Durnez et al., 2006; Feng et al., 2016). Activation of progenitor cells was demonstrated recently in adult and pediatric NAFLD populations, which increases the possibility that these cells contribute to the initiation of HCC in MtS (Guy et al., 2012; Nobili et al., 2012; Lade et al., 2014). In our series, K19 expression was associated with more pronounced degrees of intratumoral inflammation (G.2/G.3) and with more pronounced degrees of intratumoral and tumor invasion front fibrosis (G.3/G.4), coherent with previous studies reporting relating K19 expression in HCC with worse prognosis (Yang et al., 2008; Lee et al., 2012).

Only 22% of the HCC nodules in the present series showed Ki67 expression over 10% of the neoplastic cells, which denotes a low proliferative profile in these nodules. However, notably, Ki67 expression was associated with less severe architecture disturbances of the non-neoplastic liver, i.e., it was more pronounced in HCC than occurred in non-cirrhotic patients. These results reinforce the suspicion that the hepatocarcinogenesis in HCC secondary to NAFLD in the non-cirrhotic patient may be different from patients with cirrhosis (Leung et al., 2015). Ki67 >10% was associated with more intense hepatocellular ballooning (G.2) and to more pronounced nuclear grade and intratumoral fibrosis (G.3/G.4), histological findings also representative of worse prognosis (Bai et al., 2017; Cao et al., 2017). Taken together, lower rates of expression of K19 and Ki-67 suggest that HCC in MtS is preferentially "an inflammatory, non-proliferative subtype of HCC".

In conclusion, the present series of HCC occurring in the clinical context of NAFLD further showed lower rates of cirrhosis. Histological markers of "steatohepatitic HCC" were highly prevalent. Most cases showed high architectural and nuclear degrees, possibly due to lack of surveillance, since many cases were not cirrhotic. Progenitor cell component marked by immunoexpression of K19 and/ or Ki-67 occurred in 32% of the nodules, which suggests that HCC in MtS is preferentially "an inflammatory, non-proliferative subtype of HCC".

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