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HEPATOCELLULAR ADENOMA - MORPHO-PHENOTYPICAL CLASSIFICATION: CHARACTERIZATION OF A POPULATION OF PATIENTS UNDERGOING HEPATECTOMY

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TRABALHO FINAL DO 6º ANO MÉDICO COM VISTA À ATRIBUIÇÃO DO GRAU DE MESTRE NO ÂMBITO DO CICLO DE ESTUDOS DE MESTRADO INTEGRADO EM MEDICINA

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Abstract

Introduction: Hepatocellular adenomas (HCAs) are rare benign liver neoplasms mostly associated with oral contraception and anabolic steroid consumption. The role of metabolic disorders has been increasingly implicated. HCAs are divided in six subgroups (HHCA, IHCA, b^{ex3}HCA, $b^{ex7,8}$HCA, shHCA and UHCA), according to the current molecular classification, based on genetic and histological features. The aim of this study is to make a full clinical and pathological characterization of a Portuguese population of patients who underwent hepatectomy for HCA.

Patients and Methods: Review of clinical, operative and pathological data of 12 patients undergoing hepatic resection for HCA, between 2004 and 2016. Five patients (41.7%) were overweight and four (33.3%) presented grade I obesity. Type 2 diabetes mellitus in 25% of the patients and metabolic syndrome in 41.7%. Of all the nine women included in the study, seven had history of long-term oral contraception. One male was a bodybuilder with previous anabolic steroid consumption. Statistical analysis: SPSS 22.0.

Results: No postoperative mortality but major postoperative morbidity in two (16.7%) patients. The most frequent subtype was inflammatory HCA (IHCA), described in nine (75%) cases, followed by β-catenin activated HCA (βHCA) in three (25%). The presence of a β-catenin mutation in exon 3 was inferred by a diffuse and intense glutamine synthetase staining pattern which was observed in the three βHCA and two IHCA.

Discussion/Conclusion: This is the first full morpho-phenotypical characterization of HCA in a Portuguese surgical cohort. Currently, the management of HCA is based on its size, pattern of progression and gender. The new molecular classification and the recent discoveries concerning the signalling pathways involved in the tumorigenesis have the potential to modify patient-care by identifying the HCA subtypes associated with a higher risk of complications.

Keywords: hepatocellular adenoma, metabolic syndrome, hepatectomy, tumorigenesis.

Resumo

Introdução: Os adenomas hepatocelulares (AHC) são tumores hepáticos benignos associados sobretudo a mulheres sob contraceção oral ou ao uso de esteroides anabolizantes. Distúrbios metabólicos têm sido implicados de forma crescente. A atual classificação molecular divide os AHC em seis subgrupos (HHCA, IHCA, b^{ex3}HCA, $b^{ex7,8}$HCA, shHCA e UHCA), de acordo com características genéticas e patológicas. Este trabalho consiste na caracterização clínica e patológica completa de uma população portuguesa de doentes submetidos a hepatectomia por AHC.

Material e Métodos: Revisão clínica e patológica de 12 doentes submetidos a resseção hepática por AHC (2004-2016). Excesso de peso em cinco doentes (41,7%) e obesidade grau I em quatro (33,3%). Diabetes mellitus tipo 2 em 25% dos doentes e síndrome metabólica em 41,7%. Antecedentes de contraceção oral em sete de um total de nove mulheres. Um caso de uso de esteroides anabolizantes num ex-culturista do sexo masculino. Análise estatística: SPSS 22.0.

Resultados: Morbilidade major em dois casos (16,7%) e ausência de mortalidade pós-operatória. O subtipo histológico mais frequente foi o inflamatório (IHCA), presente em nove casos (75%), seguindo-se o β-catenina mutado (βHCA) em três doentes (25%). Um padrão de expressão de glutamina sintetase difuso e intenso é sugestivo de mutação no exão 3 do gene da β-catenina, tendo sido observado nos três βHCA e em dois IHCA.

Discussão/Conclusão: Trata-se da primeira caracterização morfo-fenotípica de AHC numa série cirúrgica em Portugal. Atualmente, a abordagem dos AHC baseia-se no tamanho, padrão de progressão e género. A nova classificação molecular e as descobertas recentes relativamente às vias de sinalização envolvidas na oncogénese hepatocelular têm o potencial de modificar a terapêutica destes doentes, através da identificação dos subtipos de AHC associados a um maior risco de complicações.

Palavras-chave: adenoma hepatocelular, síndrome metabólica, hepatectomia, oncogénese.

List of Abbreviations

APC – adenoma polyposis coli

b^{ex3}HCA – β-catenin exon 3 mutated hepatocellular adenoma

$b^{ex7,8}$HCA – β-catenin exon 7/8 mutated hepatocellular adenoma

BMI – body mass index

CK7 – cytokeratin 7

COCP – combined oral contraceptive pill

CRP – C-reactive protein

CT – computed tomography

FNH – focal nodular hyperplasia

GS – glutamine synthetase

HCA – hepatocellular adenoma

HCC – hepatocellular carcinoma

HH – hedgehog

HHCA – HNF1A inactivated hepatocellular adenoma

HNF1A – hepatocyte nuclear factor 1 alpha

HSP70 – heat-shock protein 70

IHCA – inflammatory hepatocellular adenoma

LFABP – liver fatty acid binding protein

MRI – magnetic resonance imaging

OC – oral contraception

PVE – portal vein embolization

SAA – serum amyloid A

Shh – sonic hedgehog

shHCA – sonic hedgehog activated hepatocellular adenoma

UHCA – unclassified hepatocellular adenoma

Introduction

Hepatocellular adenomas (HCA) are rare benign liver tumours that affect mainly young women taking oral contraceptives (OC), [1-4] whose risk increases with the type of oral contraceptive and length of contraception. [2] Adenomas, despite being rare in males [1,2] can occur associated with anabolic steroid use. [1-3] The estimated incidence of these neoplasms is 3/100.000 in women who had been exposed to high doses of oestrogen. With the development of third and fourth generation contraceptive pills, containing lower doses of oestrogen, the current incidence is unknown. [3]

Besides hormonal exposure, other risk factors have been implicated, such as obesity, diabetes mellitus, alcohol consumption, glycogenosis, liver vascular disease, and familial liver adenomatosis. [1,3]

Haemorrhage and malignant transformation are two possible complications of liver adenomas [3-6] and are the main indications for surgical resection.

The current molecular classification divides HCAs in six subgroups, according to different genetic and pathological characteristics: hepatocyte nuclear factor 1 alpha (HNF1A) - inactivated HCA (HHCA), inflammatory HCA (IHCA), β-catenin exon 3 mutated HCA (b^{ex3}HCA), β-catenin exon 7/8 mutated HCA ($b^{ex7,8}$HCA), sonic hedgehog activated HCA (shHCA) and unclassified HCA (UHCA). [3,5] (Figure 1)

The subtyping of hepatocellular adenomas is of the utmost importance, especially considering the strong association of the β-catenin activated subtype with the development of hepatocellular carcinoma (HCC). [1-6] Nevertheless, recent studies showed that not all mutations in the β-catenin gene are associated with a higher incidence of HCC, but only those which occur in exon 3. [3,5,7]

Furthermore, it is known that approximately 10% of the patients with IHCA have a mutation in the β-catenin gene, also conferring a higher risk of HCC transformation. [1,2,6]

The objective of this study is to evaluate the clinical and pathological characteristics in a population of patients who underwent hepatic resection for HCA, in the light of the current morphologic and immunohistochemical classification.

To the authors' knowledge, this is the first full morphologic and immunohistochemical characterization of HCA in a surgical Portuguese cohort. The increased understanding of the clinical and pathological features of this disease, in particular in the context of the Portuguese population, will likely enhance our knowledge of this disease.

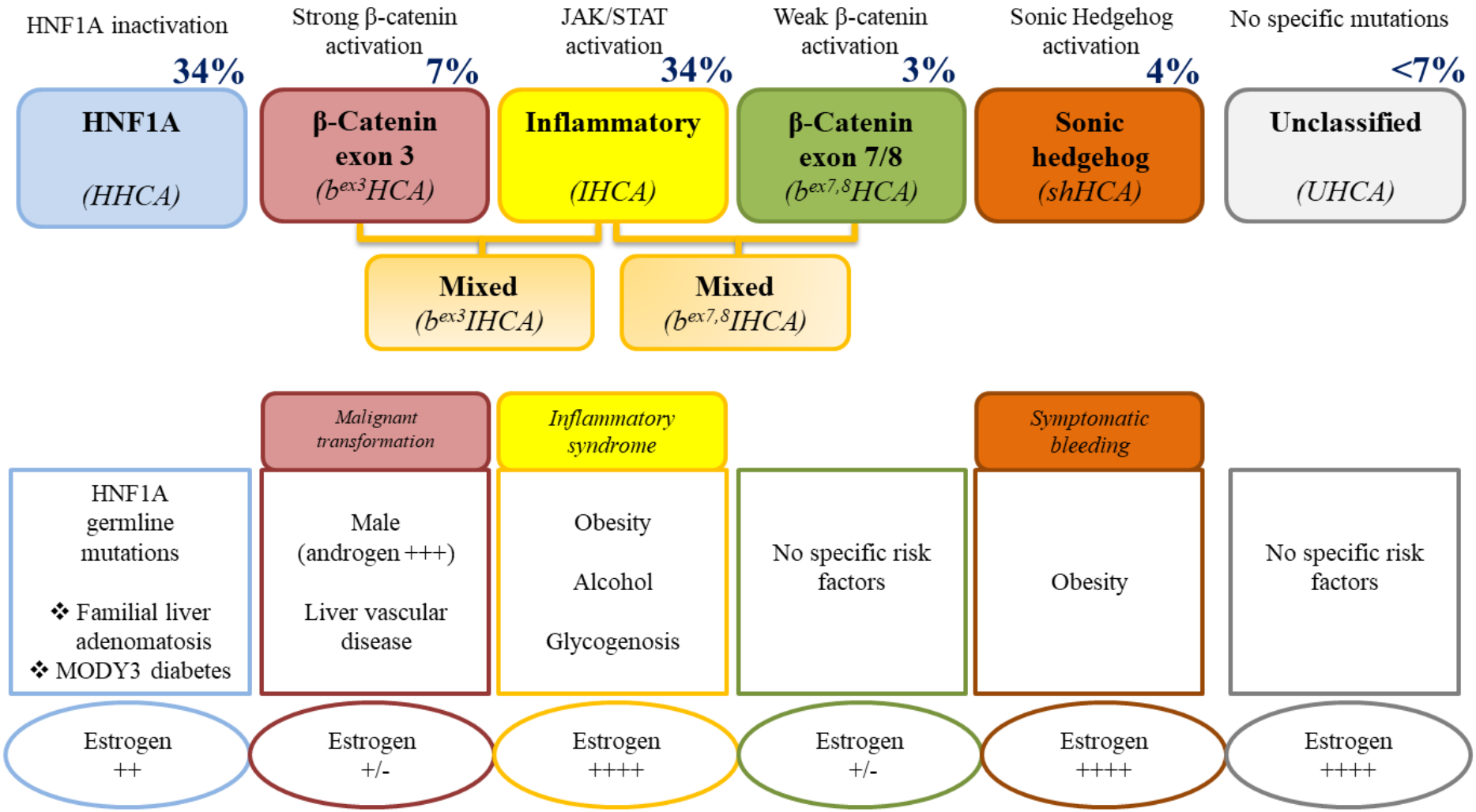

Figure 1 The molecular classification of HCA subtypes associated with risk factors and most frequent complications. (Adapted from Nault et. al [3]).

Patients and Methods

1. Study design

This project consists of a retrospective study of clinical, operative and pathological data of patients undergoing hepatic resection for hepatocellular adenomas at Serviço de Cirurgia A from Centro Hospitalar e Universitário de Coimbra (Head of Department: Prof. Doutor Júlio Soares Leite, Coimbra, Portugal) from January 2004 to May 2016.

The information regarding demographic and clinical features was collected through the patients' medical records and the pathological data was obtained by consulting archived material.

One patient was excluded due to poor histological quality of the surgical specimen and two more were excluded since they underwent HCA resection in other hospitals and, for that reason, the clinical and pathological information was insufficient (Figure 2). A formal approval by the ethics committee was required, despite the retrospective nature of the study.

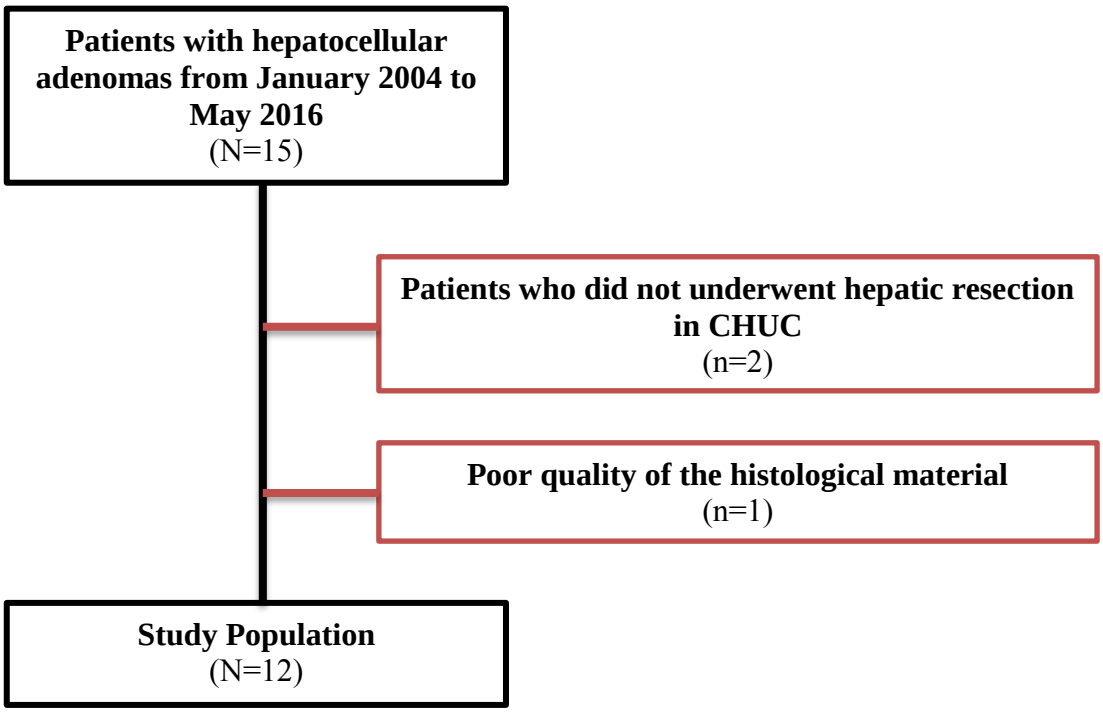

Figure 2 Exclusion criteria and study population. Included for analysis were 12 patients undergoing resection for Hepatocellular Adenoma in our centre.

2. Study population

A total of 12 patients met the inclusion criteria for this study, three of them male and nine female. The mean age was 52.6±12.9 years (range 41-74).

Clinical presentation was abdominal pain in three patients (25%) and haemorrhage in two (16.7%). The other seven patients were asymptomatic (58.3%) and the diagnosis was incidental.

All patients underwent a radiological workup that included abdominal ultrasound, computed tomography (CT) scan and magnetic resonance imaging (MRI). The number of HCAs (or potential HCAs) detected by imaging was recorded and classified as solitary or multiple (≤5 or >5). Nine patients (75%) presented a solitary potential HCA, two HCAs were observed in the radiological exam of two patients (16.7%) and one male presented four nodules compatible with HCA.

Mean body mass index (BMI) was 28.8±4.7 kg/m^2 (range 22.2-37.6), thus, the patients were distributed in four different classes (Figure 3). Five patients (41.7%) were considered overweight, four patients (33.3%) presented grade I obesity and one patient (8.3%) had a BMI value of 37.6 kg/m^2, grade II obesity. However, this patient was a bodybuilder with a recent history of steroid abuse and therefore obesity cannot be assumed.

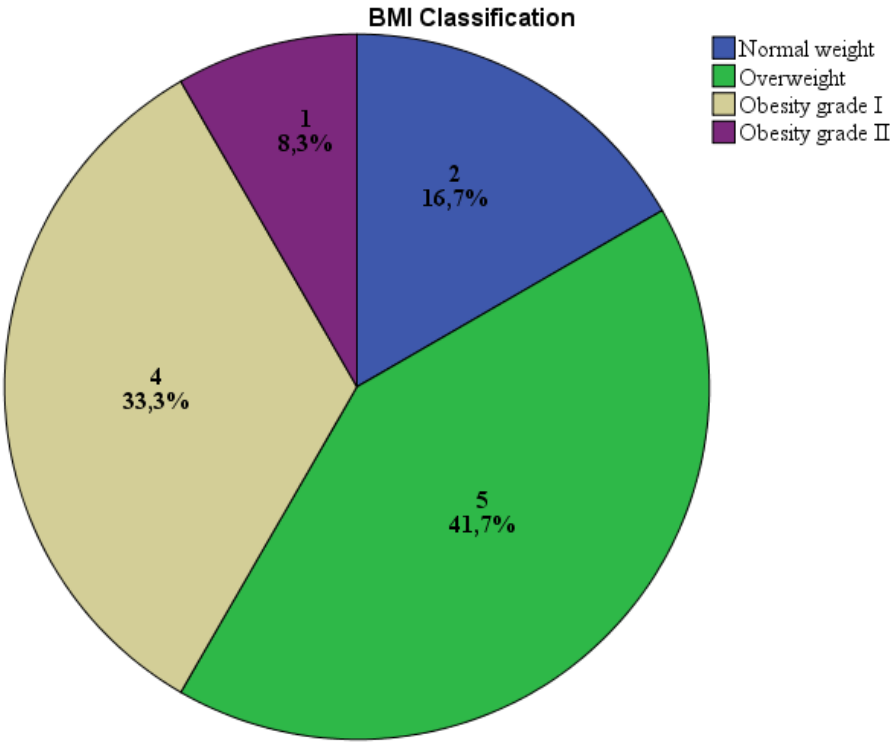

Figure 3 Distribution of patients according to the Body Mass Index (BMI) Classification in the study population of (N=12 patients) undergoing hepatectomy for Hepatocellular Adenoma. Five patients were overweight and four patients presented grade I obesity.

Regarding other relevant comorbidities, three patients (25%) presented type 2 diabetes mellitus, eight patients (66.7%) had arterial hypertension and five of the patients (41.7%) suffered from dyslipidaemia.

Furthermore, five of the patients (41.7%) met the criteria for metabolic syndrome, according to the National Cholesterol Education Program – Adult Treatment Panel III definition. [8]

Of the nine female patients, seven (77.8%) had used the combined oral contraceptive pill (COCP) for a mean of 23 years (range 20-30) and two (22.2%) have never used this contraceptive method. Regarding the patients who had used the COCP, six (85.7%) were taking a third-generation pill and one patient (14.3%) was taking a fourth-generation. All of them had suspended the oral contraceptive at the time of the intervention.

There was also one male patient (8.3%) who had a history of anabolic steroid consumption, suspended four years prior to the diagnosis.

There was no record of relevant alcohol or tobacco consumption in any of the patients and none had allergies. There was no family history of liver cancer, no patient presented any history of chronic liver disease and liver function tests were normal in all patients.

In what concerns past obstetric history in the female population, the median number of pregnancies was 2 (range 0–5) and the median number of deliveries was 2 (range 0-3).

3. Diagnosis

The median number of nodules found was one (range 1-4) with the largest lesion having a mean diameter of 5.2±3.1cm (range 1.8-13). In terms of distribution, five patients (41.7%) presented nodules in the left hemi-liver, four patients (33.3%) had nodules in the right hemi-liver and three patients (25%) had a bilobar distribution.

Radiological diagnosis was inconclusive in eight cases (66.7%), despite suggesting the hypothesis of HCA, other lesions, especially HCC, could not be safely excluded.

Abdominal ultrasound and CT scan suggested the hepatic nodule was a HCA in just one patient, each (8.3%).

MRI supported the diagnosis of HCA in four (33.3%) of all 12 patients. Only one MRI record suggested the subtype of the HCA (HHCA), based on the morphological characteristics, but this was not in agreement with the pathological analysis of the surgical specimen that revealed a βHCA.

Percutaneous liver biopsy was performed in five patients (41.7%) and the results are presented along with the pathological analysis (see Results section).

4. Operative details

The indication for surgical treatment was: suspicion of HCC in six patients (50%), one of them male; size of adenoma >5cm in four patients (33.3%); and spontaneous rupture of the adenoma with hemoperitoneum in two patients (16.7%), one of them male.

One patient (8.3%) had to undergo emergency surgery due to a spontaneous ruptured hepatocellular adenoma. Urgent angioembolization was not necessary in any patient. As preoperative measures, portal vein embolization (PVE) was conducted in one patient (8.3%).

The surgical technique used in our department was previously described by Martins et al. [9]

All of the patients underwent a single minor hepatectomy, defined as resection of up to three Couinaud segments. The surgical procedures are detailed in Table 1 and consisted of anatomical resection in 50% and non-anatomical in the other 50%. A laparoscopic approach was used in three patients (25%).

Two patients (16.7%) required a red blood cells transfusion and three patients (25%) required fresh-frozen plasma. Hepatic pedicle clamping was performed in three patients (25%) for a mean time of 29.3±6.0 minutes (range 23-35).

Table 1 Type of hepatectomy performed in the study population (N=12 patients).

Surgical Procedure	n (%)
Bissegmentectomy	4 (33.3)
Segmentectomy	3 (25)
Atypical resection	5 (41.7)

5. Outcome

Postoperative complications were defined and graded using the Dindo-Clavien score [10]. Patients who presented a score greater than II were considered to have major morbidity. The complications were also divided in hepatic and extrahepatic. The hepatic complications, which included post-hepatectomy haemorrhage, post-hepatectomy liver failure and bile leakage were defined and graded according to the International Study Group of Liver Surgery consensus. [11-13]

6. Pathology

Examination of the surgical specimen was performed in order to determine the final diagnosis and subtype of the adenoma. Formalin-fixed paraffin embedded tissue 4μm sections were stained with haematoxylin-eosin. A gross examination (number, size, colour, limits, texture) and histological analysis were conducted, both in the tumoral and nontumoral liver.

Histological examination evaluated presence of necrosis, haemorrhagic foci (microscopic or macroscopic), congestion, inflammatory infiltrates, peliosis, fibrotic bands, constitutional or repairing fibrosis, scars with abnormal vases, ductular reaction, steatosis (graded according to the scale mentioned below), steatohepatitis, sinusoidal dilatation/telangiectasia (degree and location), pseudo-portal tracts, cytological atypia, bile production and pseudoglandular structures.

The immunohistochemical markers included glutamine synthetase (GS–6, Ventana, AZ-USA), β-catenin (14, Ventana, AZ-USA), liver fatty acid binding protein (LFABP–polyclonal, GeneTex, CA-USA), C-reactive protein (CRP–UG1, Sigma, MO-USA) and serum amyloid A (SAA–monoclonal, Thermo Fisher, MA-USA) for diagnostic purposes and subtyping; anti-cytokeratin 7 (CK7–OV-TC, Cell Marque, CA-USA) antibody and GS staining pattern for differential diagnosis with focal nodular hyperplasia (FNH) and classical

criteria, as well as glypican-3 and heat-shock protein 70 (HSP70), for differential diagnosis with HCC. [14]

In the nontumoral liver, we searched for undetected additional nodules (microadenomas, hemangiomas, FNH and HCC) and the presence of steatosis or other parenchymal abnormalities.

The degree of steatosis was assessed by a morphological semiquantitative technique based on a four-graded scale: grade I (0-5%), grade II (6-33%), grade III (34-66%) and grade IV (≥67%).

Iron accumulation was also assessed (Prussian blue stain–Perls' method) as well as other pigments, such as lipofuscin granules (Sudan Black B stain), melanin (Fontana-Masson stain) and copper (rhodanine stain).

7. Statistical Analysis

Data was analysed using the SPSS™ software version 22.0 for Windows. The quantitative variables were expressed by mean±standard deviation and range. Binary variables were determined by percentages.

Categorical variables were analysed with Chi-squared test. Statistical significance was defined as $p < 0.05$.

Results

1. Postoperative morbidity and outcome

The median length of hospital stay was 6.5 days (range 4-33).

Major postoperative morbidity was observed in two (16.7%) patients, one of them presenting a grade IIIA complication and the other a IVA (Figure 4).

Regarding liver-specific complications, one patient (8.3%) had an infected biloma, another presented grade A post-hepatectomy haemorrhage and a third patient had a grade B bile leakage. There were no cases of post-hepatectomy liver failure.

Additionally, two patients (16.7%) presented respiratory complications: one case of pleural effusion requiring pleural drainage; and one case of acute respiratory failure managed conservatively (Table 2).

We do not report any postoperative mortality.

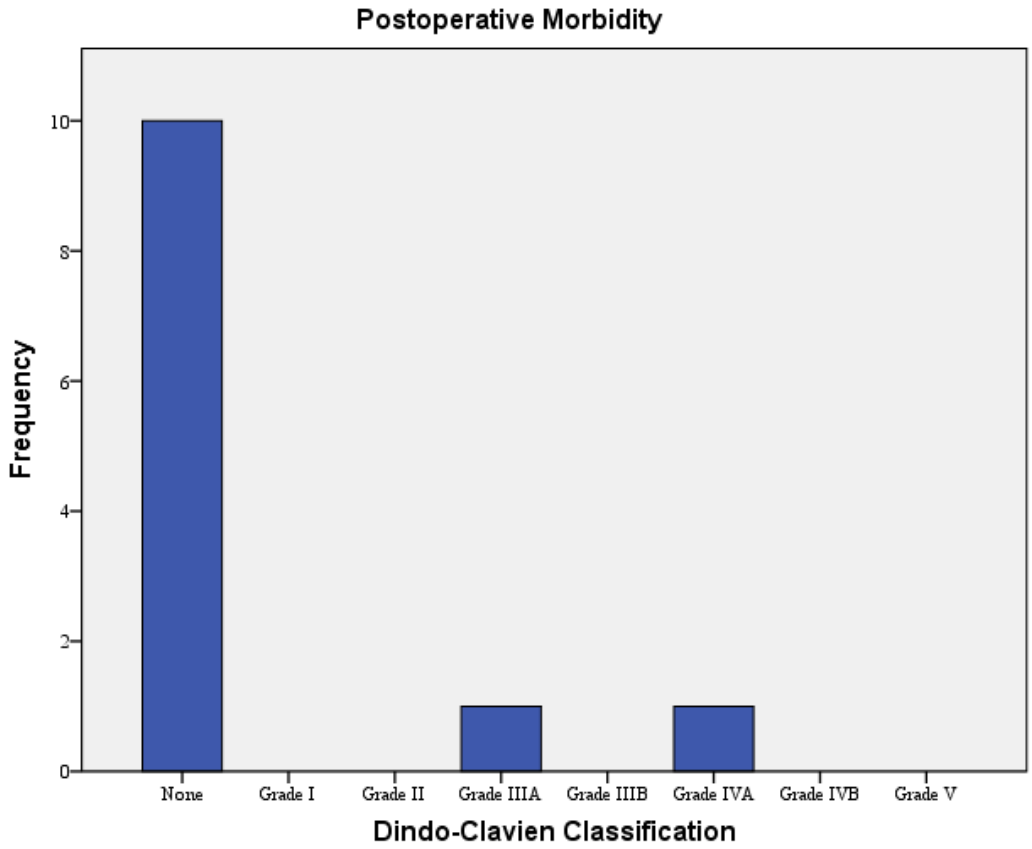

Figure 4 Postoperative morbidity (according to the Dindo-Clavien Classification) in the study population (N=12 patients) submitted to hepatectomy for Hepatocellular Adenoma.

Table 2 Postoperative complications after hepatectomy for HCA in the study population (N=12).

Postoperative complications	**n (%)**
None	10 (83.3)
Hepatic	
Infected biloma	1 (8.3)
Post-hepatectomy haemorrhage – grade A	1 (8.3)
Bile leakage – grade B	1 (8.3)
Extrahepatic	
Pleural effusion	1 (8.3)
Acute respiratory failure	1 (8.3)

2. Pathological findings

The histological examination of the surgical specimen revealed one nodule per patient. In terms of subtyping, nine nodules (75%) were classified as IHCA and three (25%) as βHCA.

2.1 *Previous biopsy*

A percutaneous preoperative liver biopsy was performed in five patients (41.7%). The fragment obtained was not representative of the lesion in two cases.

HCA subtyping according to the analysis of the fragment revealed an inflammatory adenoma (IHCA) in one patient (8.3%) and an unclassified adenoma (UHCA) in another case. In a third patient, the biopsied fragment suggested the nodule had benign characteristics, compatible with HCA, but no subtype was proposed.

2.2 *Global macroscopic features*

The mean macroscopic size of the nodule was 4.9±3.4cm (range 1.5-14). Categorically, the size of the adenoma was lower than 5 centimetres in nine patients (75%), between 5 and 10 centimetres in two (16.7%) and above 10 centimetres in one case (8.3%).

In what concerns the colour, seven HCA (58.3%), including all the βHCA and four IHCA, were mainly red/haemorrhagic, two IHCA (16.7%) were rosy/pale, two (16.7%) were yellow and one (8.3%) was necrotic.

The limits were well defined and the texture was soft in 11 cases (91.7%), which is compatible to the usual characteristics of a HCA. However, the margins of the lesion were ill-defined and the texture was firm in one IHCA, resected from a male patient.

2.3 *Histological analysis according to subtype*

2.3.1 Inflammatory Hepatocellular Adenoma (IHCA)

In the inflammatory subgroup (n=9), eight HCA (88.8%) had haemorrhagic foci, 50% of which were macroscopic and the other 50% microscopic. Necrosis was present in four HCA (44.4%), inflammatory infiltrates were seen in six cases (66.7%), congestion in eight (88.9%) and peliosis in three specimens (33.3%). No fibrotic bands or scars were found but four nodules had constitutional fibrosis and one showed fibrotic remodelling. Pseudo-portal tracts were found in two HCA (22.2%) and other two nodules exhibited ductular reaction, with numerous and thick-walled arteries.

Sinusoidal dilatation was present in seven HCA (77.8%), being mild in four cases (44.4%) and moderate to severe in three (33.3%). Three cases of mild sinusoidal dilatation had a focal distribution and one had a diffuse pattern, which was also present in the cases of moderate to severe dilatation. Regarding the location of these dilated sinusoids, they were zonal in two specimens (22.2%) and non-zonal in five (55.6%).

Curiously, some cytological atypia was observed in two IHCA, one of which also revealed bile production. No pseudoglandular structures were found.

Steatosis was present in four cases (44.4%), one of them classified as grade I, two as grade II and another as grade III.

An illustrative example of this subtype can be seen in figure 5.

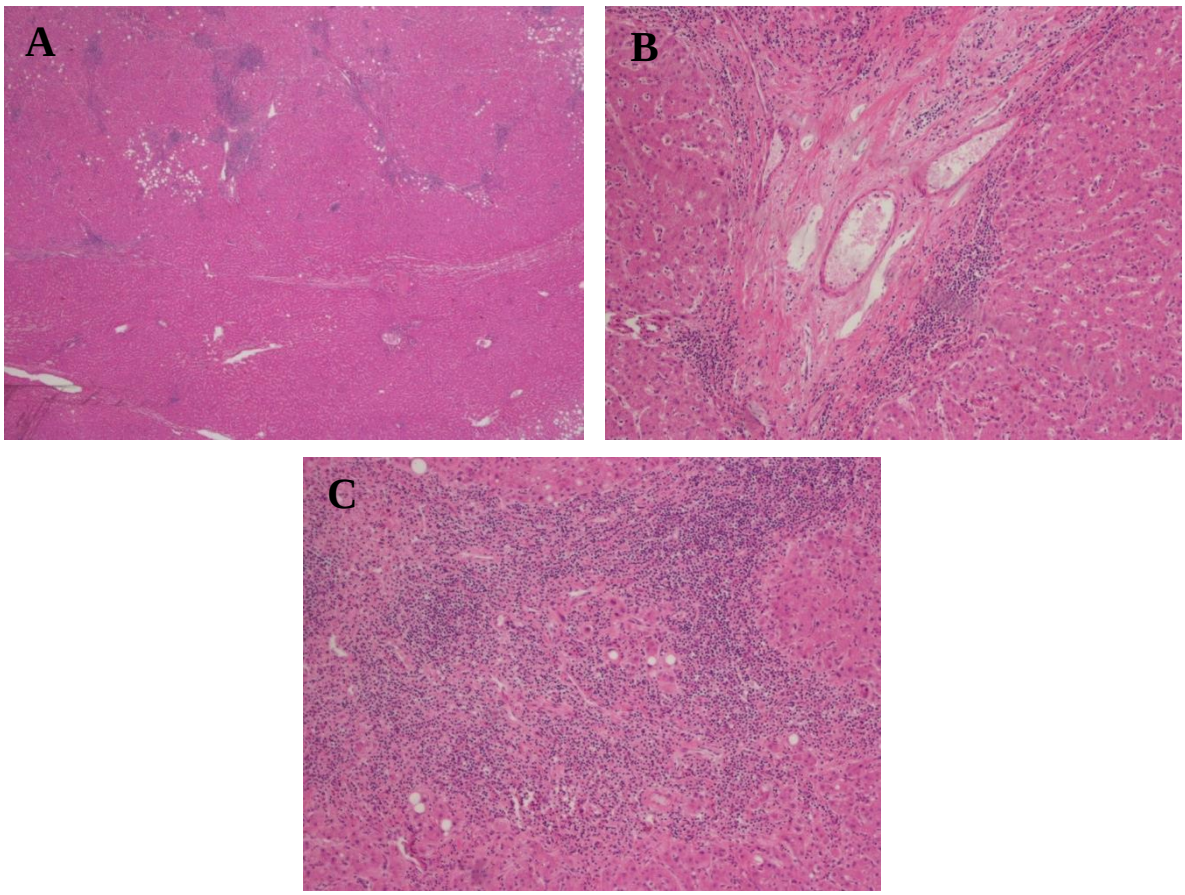

Figure 5 Illustrative example of inflammatory HCA. (A) Well delimited nodule with mild steatosis and inflammation, H&E 20x; (B) Pseudoportal spaces with arteries and mild inflammation, H&E 100x; (C) Pronounced lymphoplasmacytic inflammatory infiltrate, H&E 100x.

2.3.2 β-catenin activated Hepatocellular Adenoma (β-HCA)

Considering the β-catenin activated subtype (n=3), all of these adenomas had haemorrhagic areas, which were microscopic in two cases (66.7%) and macroscopic in one. Consequently, congestion was present in the three cases, as well. Although it is not characteristic, inflammatory infiltrates were found in one nodule (33.3%). One specimen also presented fibrotic bands and two others revealed constitutional fibrosis.

Despite being typically rare in this subtype, [6] steatosis was found in the three specimens, being grade I in one adenoma and grade II in the other two cases.

Sinusoidal dilatation was present in all samples and it was mild, focal and zonal in the three adenomas. Two specimens (66.7%) revealed bile production.

Necrosis, peliosis, ductular reaction, scars with abnormal vases, pseudo-portal tracts, cytological atypia and pseudoglandular structures were absent.

2.4 *Immunohistochemical findings by subtype (Table 3)*

2.4.1 Inflammatory Hepatocellular Adenoma (IHCA)

Liver fatty acid binding protein (LFABP) was positive in all adenomas discarding the HHCA subtype (Figure 6).

Glutamine synthetase (GS) staining was abnormal in three (33.3%) of the nine IHCA. One of them had a heterogeneous and faint GS staining but the GS was strongly and diffusely expressed in the other two cases, suggesting the presence of a β-catenin mutation in exon 3. Moreover, one of these nodules also presented nuclear expression of β-catenin, which supports the existence of the mutation.

CRP staining was positive in all IHCA with a sharp demarcation from the surrounding parenchyma (Figure 7), which is characteristic of this subtype. On the other hand, SAA staining was positive only in two cases (22.2%), but this does not exclude the inflammatory subtype, considering the CRP staining is often stronger. [6]

Prussian blue stain (Perl's method) revealed the presence of iron in three cases (33.3%), rhodamine staining was positive for copper in one adenoma (11.1%) and lipofuscin granules were found in another specimen. Melanin granules were absent.

CK7-positive ductular elements were observed in one nodule (11.1%) which could be a source of difficulty in the differential diagnosis with FNH. However, considering the absence of a map-like distribution in the GS expression, this entity was excluded.

Glypican-3 and HSP70 were negative in all adenomas thus excluding the diagnosis of HCC.

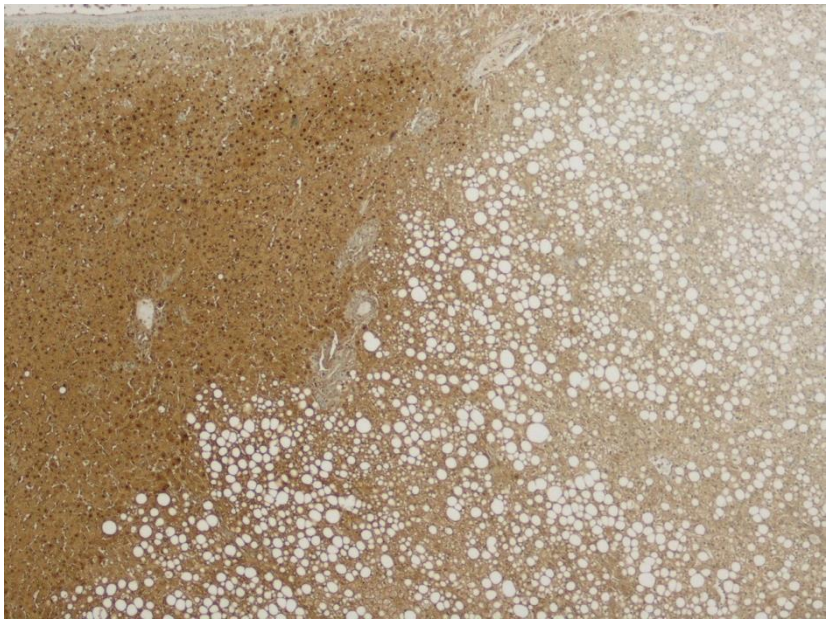

Figure 6 LFABP immunoexpression – despite the severe steatosis on the nodule (right side) there was no loss of LFABP expression both in the tumoral and in the nontumoral tissue, which discarded the diagnosis of a HHCA, 40x.

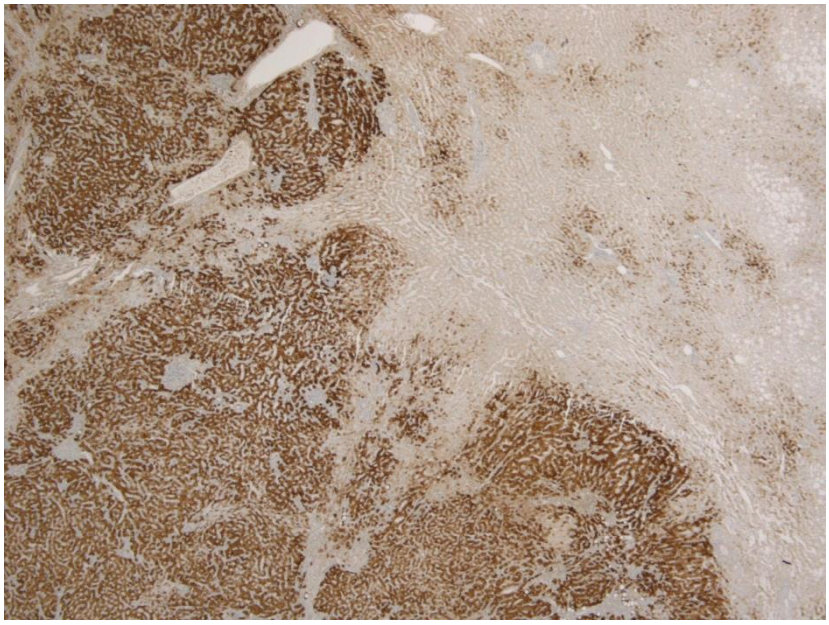

Figure 7 PCR diffuse and strong immunoexpression in the nodule (left side), compared with the nontumoral tissue, providing a diagnosis of IHCA, 20x.

2.4.2 β-catenin activated Hepatocellular Adenoma (βHCA)

GS was strongly expressed in a diffuse pattern (Figure 8) in the three adenomas of this subtype, a fact that allowed us to infer the presence of a mutation in exon 3 of the β-catenin gene, despite a negative nuclear expression of β-catenin.

Again, LFABP was positive in all cases, excluding the HHCA subtype.

Inflammatory markers, like CRP and SAA were negative both in the nodule and in the surrounding parenchyma.

Regarding other pigments, one HCA (33.3%) was positive for iron and two (66.7%) had lipofuscin granules. No copper or melanin pigments were found.

The differential diagnosis with FNH and HCC was not difficult since in the first, GS had no map-like pattern, and in the second, there was no CK7-positive ductular reaction and glypican-3 and HSP70 staining were negative.

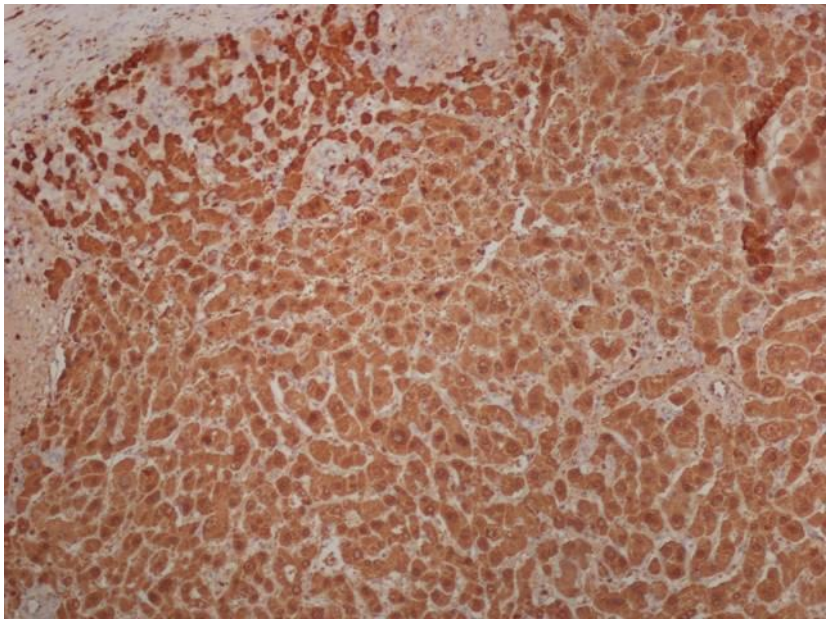

Figure 8 Strong and diffuse GS staining pattern in a βHCA, suggesting an exon 3 mutation of the β-catenin gene, 100x.

2.5 *Nontumoral liver*

Non tumoral liver examination showed a normal parenchyma in eight patients (66.7%) and steatosis in four (33.3%). The degree of steatosis was grade II (6-33%) in two cases and grade III (34-66%) in the other two. Macroscopically, one specimen had characteristics compatible with fatty liver disease. One patient with steatosis also presented septal fibrosis (stage 3).

No microadenomas (<1cm; ≥1mm), additional FNH or hemangiomas were found in the resected specimens.

All the patients who presented abnormalities in the nontumoral parenchyma turned out to have IHCA but this did not reach statistical significance.

Table 3 Immunohistochemical features of IHCA (n=9) and βHCA (n=3).

Immunohistochemical Marker	**IHCA** n (%)	**βHCA** n (%)
GS		
- *Normal staining*	6 (66.7%)	0
- *Diffuse and strong*	2 (22.2%)	3 (100%)
- *Heterogeneous and faint*	1 (11.1%)	0
Nuclear β-catenin		
- *Negative*	8 (88.9%)	3 (100%)
- *Positive*	1 (11.1%)	0
LFABP		
- *Negative*	0	0
- *Positive*	9 (100%)	3 (100%)
CRP		
- *Negative*	0	3 (100%)

- *Positive*	9 (100%)	0
SAA		
- *Negative*	7 (77.8%)	3 (100%)
- *Positive*	2 (22.2%)	0
Iron		
- *Negative*	6 (66.7%)	2 (66.7%)
- *Positive*	3 (33.3%)	1 (33.3%)
Copper		
- *Negative*	8 (88.9%)	3 (100%)
- *Positive*	1 (11.1%)	0
Lipofuscin		
- *Negative*	8 (88.9%)	1 (33.3%)
- *Positive*	1 (11.1%)	2 (66.7%)
Melanin		
- *Negative*	9 (100%)	3 (100%)
- *Positive*	0	0
CK 7		
- *Negative*	8 (88.9%)	3 (100%)
- *Positive*	1 (11.1%)	0
Glypican – 3		
- *Negative*	9 (100%)	3 (100%)
- *Positive*	0	0
HSP 70		
- *Negative*	9 (100%)	3 (100%)
- *Positive*	0	0

3. Clinical-pathological correlations

In our population we did not find any statistical significant correlations between HCA subtype and clinical variables, namely age, sex, BMI, OC use and comorbidities. However, there was a trend for an increased association between diabetes mellitus and βHCA (p=0.127).

Discussion

HCAs are rare and benign liver neoplasms. Current management, according to the European Association for the Study of the Liver Guidelines, [15] depends on size, pattern of progression and gender. Male gender, large tumours (≥5cm) and the presence of *CTNNB1* mutations in exon 3 [3,5,7] are risk factors for bleeding and malignant transformation and are thus indications for surgical resection. Women with HCAs are advised to stop oral contraception and to lose weight, with reassessment 6 months later. If the HCA is greater than 5cm or if it increases in size despite OC discontinuation, a surgical resection should be performed. Otherwise, the follow-up should be annual with MRI. Some patients present multiple HCAs in the radiological exams. In these cases, the management is determined according to the characteristics of the largest HCA. [3]

The new molecular classification proposed by Nault et al. [5] (Figure 1) defined subtypes of "at-risk HCA" that benefit from surgical treatment. Furthermore, they describe the new sonic-hedgehog subtype, associated with both histologic and symptomatic bleeding.

By allowing the stratification of patients according to the risk, subtyping of HCAs could modify patient care. Considering male gender is a risk factor *per se*, this classification is not as useful in the male population. However, in females presenting HCAs it is important to identify the subtypes which pose a greater risk for complications (b^{ex3}HCA, b^{ex3}IHCA and shHCA). Thus, we can manage patients more accurately and avoid overtreatment of low risk

HCAs, [5,16] which is particularly important considering a hepatectomy is an invasive procedure that can cause postoperative morbidity, as occurred in two patients in our study. Nevertheless, the great advances in liver surgery over the past decades, especially with the advent of laparoscopy, have enhanced the overall safety of these resections, with lower morbidity. [3]

Another important feature of this new classification is the dissection of the β-catenin activated subtype according to the exome location of the mutation. Recent data revealed that only mutations in exon 3 of the *CTNNB1* gene, including in the S45 hotspot are associated with a higher risk of HCC development, while mutations in exons 7 and 8 do not pose this risk. [3,7,16] These different mutations also imply distinctive GS staining patterns on immunohistochemical analysis. While a strong homogeneous cytoplasmic expression of GS and nuclear positivity of β-catenin suggest a mutation in exon 3, a mild heterogeneous GS staining pattern especially around the hepatic veins and usually associated with no nuclear expression of β-catenin, is more characteristic of mutations in exons 7 and 8. [7] Nevertheless, in our study population, the presence of a β-catenin mutation was inferred by a diffuse GS staining, observed in five cases (41.7%), considering that only one HCA had a positive nuclear β-catenin. As a matter of fact, this phenomenon has been reported in 15% of βHCA and might be related to low levels of nuclear β-catenin untraceable by immunohistochemistry or a deregulation in other elements of the Wnt signalling pathway. [17,18] This shows the importance of using an immunohistochemical panel, with a combination of nuclear β-catenin and GS staining, for the correct subtyping of βHCA. Despite all this, the GS staining pattern may be doubtful in some cases, in which a molecular analysis for mutation screening should be performed. [7]

Overall, this classification is relevant to clinical practice in a perspective of a personalized medicine, in which the management of the HCA would depend on its subtype defined by MRI

or percutaneous biopsy, since the panel of immunohistochemical markers used in the surgical specimen can also be applied to a biopsy fragment.

In fact, a retrospective study conducted by Ba-Ssalamah et al. [19] suggested that Gadoxetic Acid-enhancing MRI could be useful in differentiating subtypes of HCA. However, several other studies describe MRI as an excellent technique to identify only typical HHCA and IHCA. [3,7,20]

The increasing knowledge of this kind of tumour and its molecular characterization have allowed a better understanding of the specific deregulated pathways that contribute to clonal hepatocyte proliferation, as in HNF1A inactivation in HHCA, activation of IL6/JAK/STAT pathway in IHCA, β-catenin in βHCA and sonic-hedgehog (Shh) in the new shHCA.

The hedgehog (Hh) pathway is usually dormant in healthy liver cells but it can be reactivated in the presence of an acute or chronic damage to the liver, posing as a critical component of the hepatic regeneration. In fact, subsequently to a liver injury, an increased production of Hh ligands (such as Shh) by different cells and a decrease in Hh inhibitors, promote Hh pathway activation, which will contribute to the reconstruction of healthy liver tissue. However, this activation must be transitory since maturation of the proliferative immature cells, aiming to replace the dying/injured hepatocytes, is inhibited by high levels of activity of this pathway. In truth, when persistently activated, Hh signalling is associated with scar formation and consequent cirrhosis, ultimately leading to HCC. [21]

However, in other organs, the sonic hedgehog pathway is mostly associated with benign tumours and its activation in shHCA reinforces its role in benign tumorigenesis. [5] Further research is obviously needed regarding the impact of this pathway on liver tumours.

In what concerns the Wnt/β-catenin pathway, research has proved its important role in defining the zonal pattern characteristic of liver acinus. The definition of three zones

(periportal, intermediary and perivenous zone) is due to the existence of an oxygen gradient (ranging from 60-65 mmHg in the periportal blood to 30-35 mmHg in the perivenous blood) as well as gradients of morphogens like Wnt, hedgehog, hormones and growth factors. There is a liaison between β-catenin activation and oxygen levels. In lower oxygenated areas, such as the perivenous hepatocytes, the β-catenin is stabilized and transported to the nucleus, where it induces DNA replication, while the oxygen enriched periportal zone expresses high levels of its negative regulator - adenoma polyposis coli (APC). [22] As a future challenge, it would be interesting to assess if the development of βHCA (and the risk of malignant transformation) is somehow under the influence of the hepatic zonation. In other words, if the *CTNNB1* mutation tends to occur mainly in the hepatocytes localized in hypoxic areas, which already present high activity of this pathway and therefore may be more prone to a deregulation of cell proliferation, or if it can also affect the periportal zone, since it has been described that if a genetic ablation of APC is performed and the β-catenin pathway activated, the periportal zone can acquire a perivenous phenotype. [22]

In our series, two IHCAs showed focal cytological atypia and one of these presumably had a β-catenin mutation. In fact, some HCAs show atypical morphologic features, like nuclear atypia, loss of reticulin or pseudoglandular structures resembling well-differentiated HCC and making the differential diagnosis harder. [18] Moreover, some HCAs occur in uncommon clinical contexts, as in male patients or females older than 50 years, which was also observed in our study population. Taking this into consideration, Bedossa et al. [23] suggested a new diagnostic category called *well-differentiated hepatocellular neoplasm of uncertain malignant potential* (HUMP) to better classify these entities, previously referred to as "atypical adenomas", and identify the cases where a more thorough study and close follow-up must be conducted. For example, screening for the presence of TERT promoter mutations will help to

assess if the malignant transformation has already occurred in these "borderline HCAs". [5,7,24]

All these discoveries contribute to a better understanding of the chronological sequence of molecular alterations in HCA and its transformation into HCC. We can draw an analogy between the accumulation of mutations implicated in the adenoma-carcinoma sequence in colorectal cancer, in which the Wnt/β-catenin role is well defined [25,26] and the development of HCC arising from HCAs, a process not yet fully understood (Figure 9).

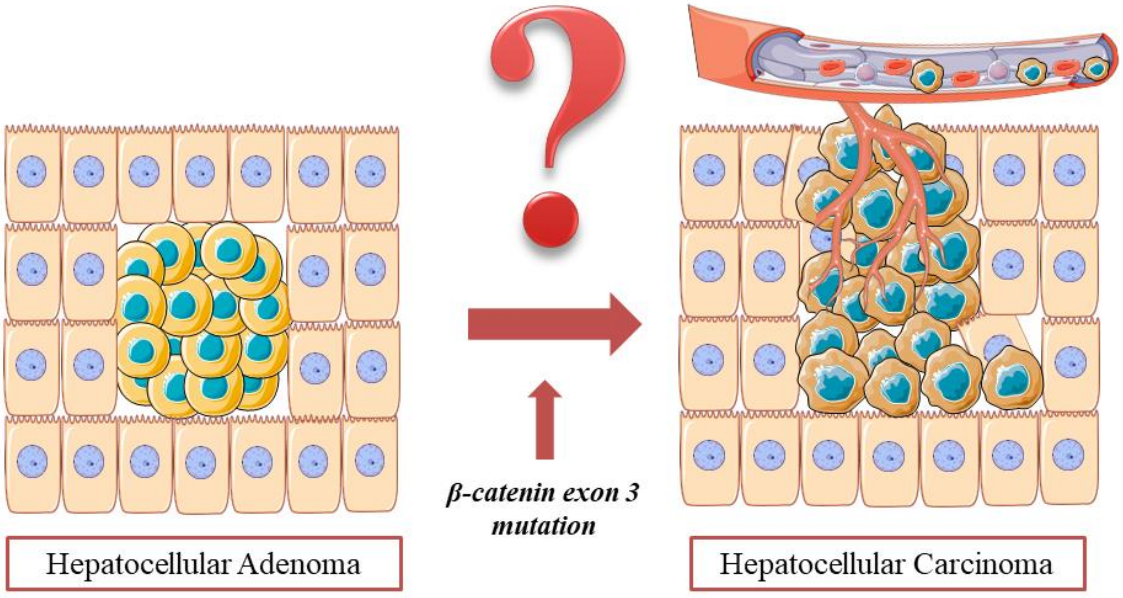

Figure 9 Schematic representation of the development of a hepatocellular carcinoma arising from an original hepatocellular adenoma in a non-cirrhotic liver (the role of the β-catenin exon 3 mutation and the still unknown additional genetic abnormalities).

Ultimately, the thorough study of these pathways may open the door to the development of specific therapeutic agents which could target key elements and thus inhibit the hepatocyte proliferation and development of HCC from HCAs. [3,5] Some studies have already described the potential role of specific agents, like ruxolutinib and dasatinib that could suppress the IL6/JAK/STAT pathway activation. In the future and perhaps more importantly,

considering the risk of malignant transformation, would be to find an efficient drug that could inhibit *CTNNB1* mutations, which has not yet been discovered. [3,5]

Although HCAs may lead to malignant transformation to HCC, [1-6] the third leading cause of cancer-related death worldwide, [27] the exact extent of HCA contribution for the global incidence of HCC is unknown. [28] As a future challenge it would be interesting to assess if, in a population of HCC arising from HCA, the HCA subtype from which the HCC arises (βHCA or βIHCA) somehow influences HCC's clinical behaviour. This is particularly relevant given the increasing incidence of HCC arising in non-cirrhotic liver, whereby a distinct carcinogenic pathway could be involved. [28] Further studies are obviously needed.

Our series revealed a preponderance of the inflammatory subtype (75%) as well as a high prevalence of obesity and metabolic syndrome, which is in accordance with previous studies. [3,5,7]. Furthermore, although not statistically significant, four of these patients also presented steatosis in the non-tumoral liver, reflecting the underlying metabolic disorder.

However, our study has some limitations, mainly in what concerns the size of the sample, the retrospective nature of the study and the unavoidable bias associated with a surgical series. This could explain the lack of association between HCA subtype and clinical variables, since the cases do not represent the whole HCA population. Lastly, considering this study was already in progress when the molecular classification was revised, and due to time constraints, we were unable to screen either the exon 3 mutation of the *CTNNB1* gene in the βHCA or the sonic hedgehog. However, we intend to do this in the near future.

Regardless, it is important to emphasize that this was the first time a full morpho-phenotypical characterization was performed in a Portuguese cohort of patients undergoing hepatectomy for HCA.

Conclusion

Great developments have been made in the field of HCA subtyping, especially in what concerns genetic analysis with exome-specific mutation screening and identification of deregulated signalling pathways in the different subtypes. Considering the increasing clinical relevance of this classification, we are moving in a direction of a more specific, subtype-oriented management of these neoplasms, which might change the current guidelines for surgical resection, especially in the female population and therefore allow the practice of a more personalized medicine.

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1. **Comunicação Oral:** "Adenoma Hepatocelular – Classificação Morfo-Fenotípica: Caracterização de uma População de Doentes submetidos a Hepatectomia" – Congresso Português de Hepatologia 2018, 21ª Reunião Anual da Associação Portuguesa para o Estudo do Fígado (APEF).

   Andreia Santos, Rui Caetano Oliveira, Henrique Alexandrino, José Guilherme Tralhão, Francisco Castro e Sousa, Júlio Soares Leite, Maria Augusta Cipriano

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