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Avaliação da heterogeneidade inter-nodular, nos níveis morfológico e molecular, em casos de carcinomas hepatocelular

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Patologia

Orientador: Prof. Dr. Venâncio Avancini Ferreira Alves

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Assessment of morpho-molecular inter-nodular heterogeneity in the primary and metastatic disease of patients with hepatocellular carcinoma

Thesis presented to the Faculdade de Medicina, Universidade de Sao Paulo to obtain to degree of Doctor in Science

Pathology Program

Advisor: Prof. Dr. Venâncio Avancini Ferreira Alves

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.”

José de Alencar

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Abreviaturas dos títulos dos periódicos de acordo com List of Journals Indexed in Index Medicus.

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List of abbreviations and initials

AFP	Alpha-fetoprotein
BCLC	Barcelona Clinic Liver Cancer
CHC	“Carcinoma hepatocelular”
CT	Computed tomography
CTC	Circulating tumor cell
ctDNA	Circulating tumor DNA
DATASUS	“Departamento de informática do Sistema Único de Saúde”
E&S	Edmondson & Steiner
EMT	Epithelial-mesenchyme transition
et al	and others
FFPE	Formalin-fixed paraffin-embedded
FMUSP	“Faculdade de Medicina da Universidade de São Paulo”
H&E	Hematoxylin & eosin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HC-FMUSP	“Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo”
HCV	Hepatitis C virus
IHC	Immunohistochemistry/immunohistochemical
IHQ	“Imuno-histoquímica”
INCA	“Instituto Nacional do Câncer”
ISMMS	Icahn School of Medicine at Mount Sinai

K19	Keratin 19
MCA	Multiple correspondence analysis
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NI	Not informed

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Resumo

Martins-Filho SN. *Avaliação da heterogeneidade inter-nodular, nos níveis morfológico e molecular, em casos de carcinomas hepatocelular* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2019.

INTRODUÇÃO: Carcinoma hepatocelular (CHC) é um câncer de alta mortalidade e incidência crescente em países ocidentais. O diagnóstico de CHC é realizado através de exames de imagem e a grande maioria dos Centros de Tratamento de Câncer não recomendam biópsias incisionais para confirmação histopatológica do diagnóstico de CHC. Além disso, apenas os pacientes com CHC em estágios precoces são submetidos a tratamentos com intenção curativa como ressecção cirúrgica e transplante hepático. Dessa forma, o acesso a amostras teciduais em pacientes com CHC em estágios intermediários e avançados é bastante limitada, dificultando a avalição de achados morfológicos e eventos moleculares associados a progressão tumoral, e em especial relacionados a disseminação extra-hepática. A avalição de amostras de autópsia com adequada representação da doença primária e metastática pode superar tais limitações e sugerir mecanismos e padrões de disseminação a distância em CHC. **MÉTODOS:** O presente estudo incluiu 88 autópsias em pacientes com CHC, abrangendo 20 pacientes com metástase à distância. Micro matrizes teciduais (TMA) foram construídas com 194 nódulos hepáticos e 36 nódulos extra-hepáticos desses pacientes. A avaliação dos nódulos incluiu múltiplos critérios histológicos como diferenciação tumoral; graus nuclear, nucleolar e arquitetural, e celularidade. Imuno-histoquímica (IHQ) foi realizada para marcadores de diferenciação hepatocitária (HepPar1, Arginase e CD10), status de mutação de *CTNNB1* (β-catenina e Glutamina Sintetase), propriedades biológicas de células progenitoras em CHC (Queratina 19, CD44 e EpCam), e marcadores de transição epitélio-mesênquima (Vimentina e Claudina 1). A heterogeneidade fenotípica na doença primaria foi avaliada em 50 pacientes com múltiplos nódulos hepáticos e, na doença metastática, em 12 pacientes com múltiplos nódulos extra-hepáticos. Mutações na região promotora de *TERT* foram avaliadas em seis pacientes com doença multi-nodular primária e metastática. **RESULTADOS:** Foram observadas metástases para os pulmões (16/20, 80%), peritônio (4/20, 20%), linfonodos (4/20, 20%) e glândula adrenal (3/20, 15%). Metástases subclínicas, não detectadas em exames de imagem e avaliação macroscópica, foram identificadas em 30% dos pacientes com comprometimento pulmonar. Concentração sérica de alfa-feto-proteína ≥ 100 ng/mL, nódulo dominante ≥ 5.0 cm, multi-nodularidade, invasão macrovascular,

alto grau histológico, nuclear e arquitetural, celularidade e expressão IHQ de Queratina 19 e EpCam na doença primária mostraram associação com a presença de metástases em CHC. Todos os nódulos metastáticos reproduziram os achados histológicos e IHQ da doença primária correspondente. Heterogeneidade fenotípica inter-nodular foi detectada em 27/50 (54%) pacientes com múltiplos nódulos hepáticos. A heterogeneidade extra-hepática foi menos expressiva, presente em apenas 2/12 (17%) pacientes com múltiplos nódulos metastáticos. Também houve limitada heterogeneidade para mutações na região promotora de *TERT* na doença metastática comparada à doença primária. **CONCLUSÕES:** CHC tem forte tropismo hematogênico e predileção por metástases pulmonares. O CHC metastático possui alta prevalência de altos graus histológicos e de marcadores de células progenitoras. A restrita heterogeneidade fenotípica da doença metastática comparada à doença primária sugere restrições evolutivas e seleção de clones tumorais na disseminação extra-hepática de CHC.

DESCRITORES: carcinoma hepatocelular; autópsia; imuno-histoquímica; metástase neoplásica; micrometástase de neoplasia; heterogeneidade tumoral.

Abstract

Martins-Filho SN. *Assessment of morpho-molecular inter-nodular heterogeneity in the primary and metastatic disease of patients with hepatocellular carcinoma* [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2019.

INTRODUCTION: Hepatocellular carcinoma (HCC) is a deadly cancer with increasing incidence in western countries. Diagnosis of HCC is based on imaging exams and most Cancer Centers do not recommend core-biopsies for definitive histopathological confirmation. Moreover, only patients with early disease stage are eligible to curative-intent treatments including surgical resection and liver transplantation. Hence, access to tissue samples in HCC patients with intermediate and advanced disease is limited, which further precluded the evaluation of morphological features and molecular drivers of HCC dissemination, particularly in relation to metastatic spread. Evaluation of autopsy specimens with adequate representation of primary and metastatic disease can overcome such limitations and provide insights on the mechanisms and patterns of distant dissemination in HCC. **METHODS:** This study included 88 autopsy specimens from patients with HCC, including 20 with distant metastases. Tissue microarrays (TMA) were generated from 194 hepatic and 36 extra-hepatic nodules histologically available from these patients. All nodules were assessed for multiple histological features including degree of differentiation, nuclear, nucleolar and architectural grades, and cellular crowding. Immunohistochemistry (IHC) was performed for markers of hepatocyte differentiation (HepPar1, Arginase and CD10), *CTNNB1* mutation status (β-catenin and Glutamine Synthetase), HCC stem-like features (Keratin 19, CD44 and EpCam), and epithelial-mesenchyme transition (Vimentin and Claudin 1). Phenotypic heterogeneity in the primary disease was assessed in 50 patients with multiple hepatic nodules and heterogeneity in the metastatic disease was evaluated in 12 patients with multiple extra-hepatic nodules. Mutations in the *TERT*-promoter region was evaluated in six patients with multi-nodular primary and metastatic disease. **RESULTS:** Metastatic sites included lungs (16/20, 80%), peritoneum (4/20, 20%) lymph nodes (4/20, 20%) and adrenal gland (3/20, 15%). Subclinical micro-metastases, undetected in imaging and macroscopic examination, were identified in 30% of the patients with disseminated disease to the lung. AFP serum concentration ≥ 100 ng/mL, dominant nodule ≥ 5.0 cm, multi-nodularity, macrovascular invasion, high histological, nuclear and architectural grades, cellular-crowding, and expression of Keratin 19 and EpCam in the primary disease were associated with the presence of distant metastases in HCC. Histological and IHC analyses showed that all HCC metastatic nodules could be

traced back to the primary disease. Phenotypic inter-nodular heterogeneity was detected in 27/50 (54%) patients with multinodular hepatic disease. Heterogeneity was less pronounced in extra-hepatic nodules, being present in only 2/12 (17%) patients with multiple metastatic tumors. These results were further validated by the limited mutation heterogeneity of the *TERT*-promoter region in metastatic compared to primary nodules. **CONCLUSIONS:** HCC shows a strong hematogenous tropism and predilection for lung dissemination. Metastatic HCC nodules are enriched in histological features of aggressive behavior and in markers of stem-like properties. The limited phenotypic inter-nodular heterogeneity within the primary compared to metastatic nodules suggests evolutionary constrains in HCC extra-hepatic dissemination.

DESCRIPTORS: carcinoma, hepatocellular; autopsy; immunohistochemistry; neoplasm metastasis; neoplasm micrometastasis; tumor heterogeneity.

1. INTRODUCTION

1.1 Liver cancer: epidemiology and risk factors

Primary liver cancer is the second leading cause of cancer-related mortality worldwide. In 2015, it is estimated that 788,000 people died from liver cancer around the globe[1]. Data from the Surveillance, Epidemiology, and End Result (SEER) database indicate that the death rate by liver cancer had a steep increase of 2.5% each year over 2006–2015 in the United States. Conversely, the death rate for cancer of any site decreased 1.5% each year over the same period in that country[2]. According to the Brazilian National Cancer Institute ("Instituto Nacional do Câncer", INCA), liver cancer is not among the ten most common cancers in Brazil[3], although its real incidence is not clear, likely due to a lack of appropriate population-based studies[4]. Nevertheless, reports from the informatics department of the Brazilian public health system (Departamento de informatica do sistema unico de saude, DATASUS) indicate that 9,786 people died from liver cancer in the country in 2016, which represents an increase in mortality of 134% compared to 1996 [5] (**Figure 1**).

The major histological subtype – accounting for 85% to 90% – of primary liver cancer is hepatocellular carcinoma (HCC). This is a malignant tumor of hepatocellular differentiation that usually arises in a setting of chronic inflammation with hepatocyte damage and repair, and liver fibrosis. Sustained liver injury and progression of fibrosis lead to cirrhosis, which dramatically increases the risk of HCC development. Indeed, cirrhosis of any etiology is the

most relevant risk factor for HCC development, being present in up to 90% of all HCC cases[1,6].

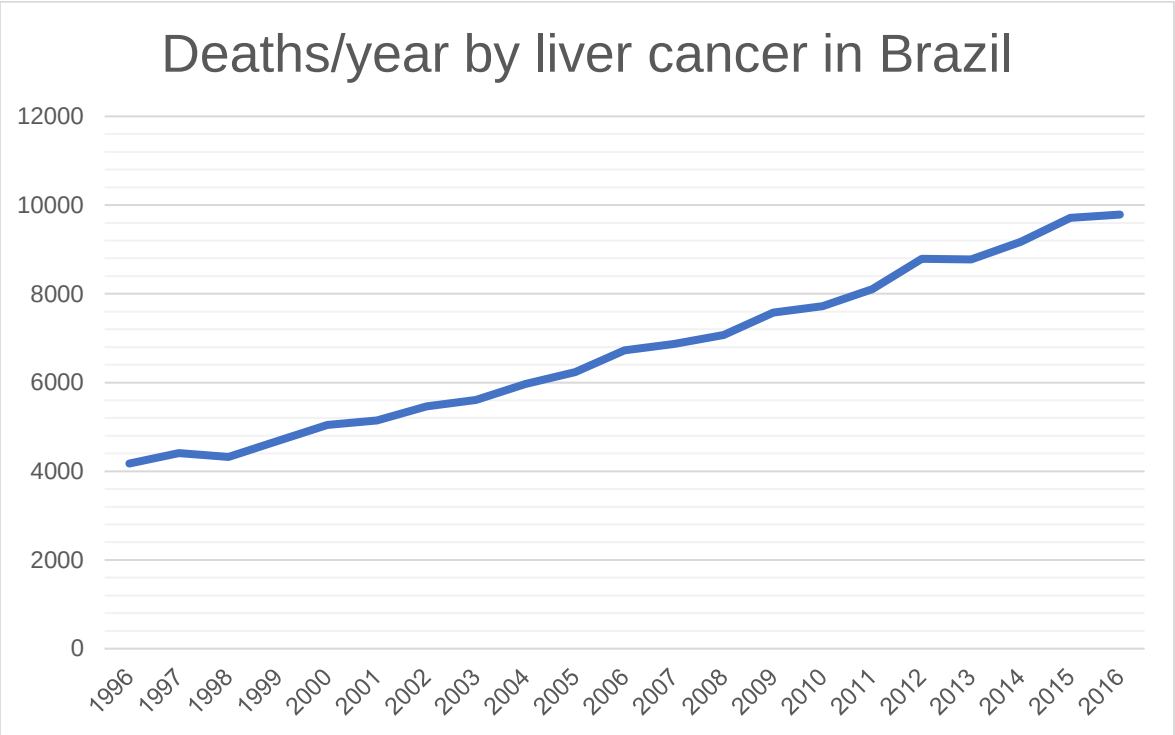

Figure 1 – Deaths by liver cancer in Brazil per year. This graph was generated with information collected from the DATASUS-TABNET[5].

The geographic distribution of HCC shows marked global variation: close to 80% of all cases are diagnosed in southeast Asia or sub-Saharan Africa. This can mainly be ascribed to the high incidence of hepatitis B virus (HBV) infection in these regions[7]. In fact, HBV infection currently accounts for half of all HCC cases. Multiple mechanisms have been implicated in HBV-induced HCC, including hepatocyte damage related to inflammation and cirrhosis, oxidative stress, damage from viral proteins HBx and HBs and insertional mutagenesis associated to the integration of the viral DNA to the host genome[8,9]. These mechanisms can be summarized into two major disease pathways: inflammation-

fibrosis-cirrhosis HCC induction or direct HBV carcinogenic effect. This latter pathway can even bypass chronic inflammation and liver fibrosis to induce HCC in a non-cirrhotic setting[10,11].

In the past years, there has also been a dramatic increase in HCC incidence in western countries, mostly due to hepatitis C virus (HCV) infection and, more recently, non-alcoholic fatty liver disease (NAFLD)[12]. The increase in HCV prevalence can be ascribed to intravenous drug abuse and to unscreened blood transfusions, most notably from the 1960s to the 1980s. Although this latter method of HCV infection is no longer frequent due to mandatory blood screening prior to transfusion, HCV-induced HCC is at its peak incidence due to the hiatus of 20 to 40 years between HCV infection and cancer development[13]. The mechanisms of HCV-induced carcinogenesis are associated to chronic inflammation and development of cirrhosis secondary to the viral infection[14].

The increase in NAFLD prevalence, including its most aggressive type, the non-alcoholic steatohepatitis (NASH), parallels the obesity and diabetes epidemics in western countries. Close to 90% of all obese and 70% of all diabetic patients develop NAFLD, and up to a third of those patients have NASH[15,16]. The impact of poor lifestyle choices (e.g. poor diet) in liver injury is so evident that animal models exposed to fast food western diet (high-fat, high-fructose and high-cholesterol) develop hepatocyte ballooning and “chicken-wire” fibrosis, mimicking human NASH. As in humans, continuous exposure to such environmental injuries leads to the development of HCC[17].

Chronic alcohol abuse (>80g/day) is another major risk factor for liver cancer, associated with a fivefold increase in HCC development compared to the general population. Alcohol consumption increases oxidative stress, which can

promote hepatocyte damage. Chronic exposure then leads to liver injury (similar to NASH), and eventually to cancer development[18,19]. Other rarer risk factors for HCC include hemochromatosis[20], auto-immune hepatitis[21], α1-antitrypsin deficiency[22] and, less frequently, Wilson's disease[23].

In summary, HCC is an aggressive cancer with increasing incidence in western countries including Brazil. It usually develops in a setting of chronic inflammation and cirrhosis, although some clinical conditions – most notably HBV infection – can develop HCC in the absence of cirrhosis.

1.2 Hepatocellular carcinoma: screening, diagnosis and treatment

Surveillance programs offer the opportunity of early HCC diagnosis and are usually associated with improved survival[1,6]. In a large surveillance program in Brazil, as many as 8.1% of all cirrhotic patients developed HCC and 79.2% of those were diagnosed at early disease stages[4]. These results indicate the need of a country-wide screening program for patients at high risk of developing HCC. In fact, current clinical guidelines recommend HCC screening for patients with cirrhosis of any etiology as well as for patients with HBV irrespective of cirrhosis. The standard test for screening of this cancer is ultrasonography (US) every 6 months. Serologic exams, most notably α-fetoprotein, show unsatisfactory accuracy rates and are typically not recommended as standalone tests. Finally, coupling US to α-fetoprotein increases HCC detection rates but at the expense of higher false-positive results and increased costs[1,24].

Patients with nodules larger than 1 cm at the screening US should be submitted to contrast-enhanced computed tomography (CT) or magnetic

resonance imaging (MRI) for definitive clinical diagnosis. Nodules with contrast uptake in the arterial phase followed by washout in the venous phase of either CT or MRI can be confidently diagnosed as HCC. Core-needle biopsies are recommended only if the imaging results are inconclusive. This represents a huge discrepancy to other frequent cancers, where biopsies and histopathological evaluation remain the standard practice for diagnosis prior to treatment.

Following diagnosis, patients with HCC are staged according to the Barcelona Clinic Liver Cancer (BCLC) algorithm (**Figure 2**). In brief, only very-early and early stage patients are eligible for curative-intent treatments, including tumor resection and liver transplantation. Unfortunately, these represent less than half of the patients at diagnosis. Patients at intermediate and advanced stages are eligible for chemoembolization and systemic therapy (e.g. sorafenib) that improve overall survival, but rarely achieve disease remission. Currently, there are no targeted therapies approved for HCC treatment[6,25].

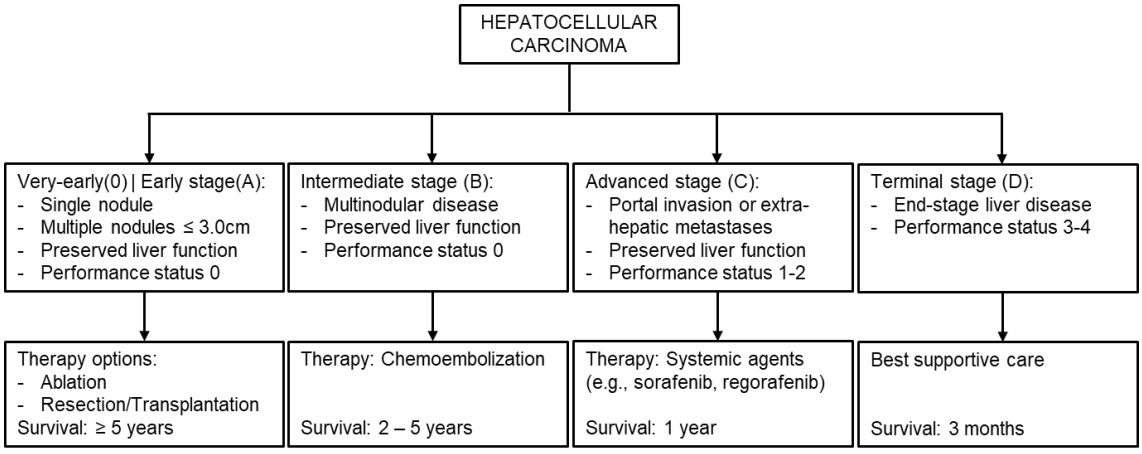

Figure 2 – Simplified BCLC algorithm.

1.3 Hepatocellular carcinoma: pathology

Pathological evaluation of HCC – as any other cancer – starts with the macroscopic characterization of the tumor specimen[26]. This includes essential gross features such as tumor focality, size of nodule(s) and its (their) relationship to the nontumoral parenchyma (e.g. tumor borders and macrovascular invasion). In surgically resected tumors, Eggel* apud Ishak *et al* [27] recommends stratifying HCC in 1) nodular form: sharply demarcated single or multiple nodules; 2) massive form: massive tumor mass, not well-demarcated, involving multiple liver segments or the whole left or right lobe. Smaller satellite nodules are common; and 3) diffuse form: numerous small nodules scattered in the whole liver that mimic the pseudo-lobules of cirrhosis (**Figure 3**). Finally, macroscopic reports should document solitary nodules inferior to 2 cm as “small HCC” [26,28].

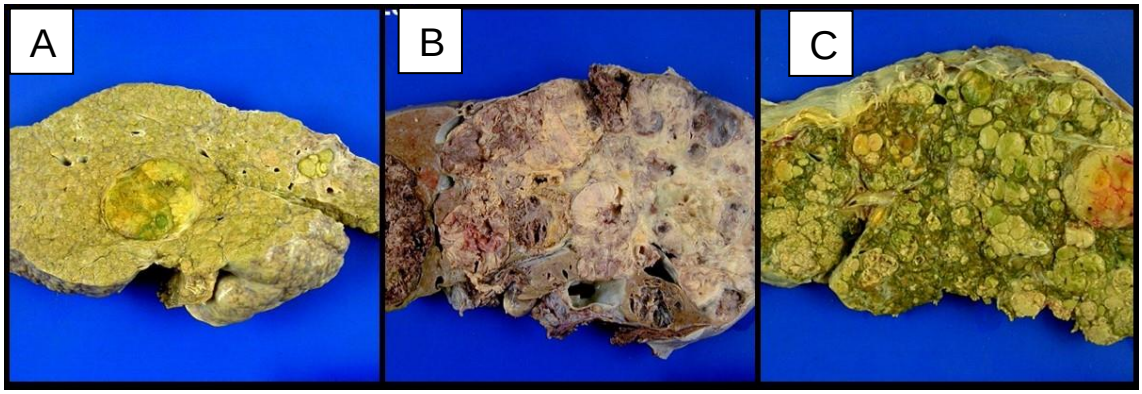

Figure 3 – Macroscopic classification of HCC according to Eggel: nodular (A), massive (B) and diffuse (C) forms.

Histological diagnosis of HCC is often straightforward: tumors constantly show architectural changes compared to normal liver including trabecular thickening, pseudoglandular formation and/or diffuse organization of the

hepatocytes[29]. Also, vascular invasion is a common finding in this cancer[30,31], which further indicates the malignant nature of the specimen under investigation. Cellular, nuclear and nucleolar pleomorphism, and presence of atypical mitosis can also aid the pathologist in the diagnosis of HCC. Nevertheless, histological diagnosis might be challenging in small vaguely nodular tumors with mild cytoarchitectural changes as well as in large, undifferentiated tumors with highly pleomorphic cells[32,33]. In the former situation, the pathologist should be vigilant for subtle HCC-features such as increase in cellular density compared to the adjacent liver, foci of small pseudoglands, presence of multiple unpaired arteries and diffuse fatty changes[32]. In the latter, a broad immunostaining panel and clinical/imaging exclusion of other cancer types might be crucial for a definitive diagnosis[33–35].

Beyond diagnostic confirmation, histological reports of HCC specimens should always include tumor differentiation (histological grade) and status of lymphovascular invasion. The most traditional grading system for HCC is that of Edmondson & Steiner (E&S), published in 1954[36]. These authors classified HCC in four different grades:

- “Grade I: reserved for tumors where the difference between the tumor cells and hyperplastic liver cells is so minor that a diagnosis of carcinoma rests upon the demonstration of more aggressive growths in other parts of the neoplasm.” Hence, diagnosis of pure grade I HCC should be rare, and the possibility of hepatic adenoma or dysplastic tumors should be discarded.

- “Grade II: cells show marked resemblance to normal hepatic cells.” Acini (pseudoglands) are frequent and their lumen are often filled with bile and/or protein precipitate. Cells are more hyperchromatic than normal hepatocytes, with granular and eosinophilic cytoplasm. Nuclei are usually large.
- “Grade III: nuclei are larger and more hyperchromatic than in grade II cells.” There is high nuclear to cytoplasmic ratio. Cytoplasm is still granular and eosinophilic, but less than in grade II tumors. Pseudoglands are also less frequent and presence of bile or protein precipitate is rare. Vascular invasion is frequent.
- “Grade IV: The growth pattern is medullary in character, trabeculae are difficult to find, and cell masses seem to lie loosely without cohesion in vascular channels. Only rare acini are seen.” Nuclear to cytoplasmic ratio is higher than in other grades: nuclei are large and intensely hyperchromatic, and cytoplasm is often scanty, with few granules.

Different degrees of vascular invasion have been characterized in HCC. Roayaie *et al* proposed a four-tier classification for vascular invasion in this cancer, with impact on prognosis. These authors showed that tumors with histological invasion of a small vessel distant from the tumor or of a muscular-wall vessel (grade 3) or macroscopic evidence of vascular invasion (grade 4) have higher recurrence rates and worse outcome[37]. Albeit elegant, this classification is not easily reproducible and thus not adopted in the clinical practice. Instead, pathology guidelines simply recommend the evaluation of microvascular (histologic) and macrovascular (macroscopic) invasion[28] (**Figure 4**).

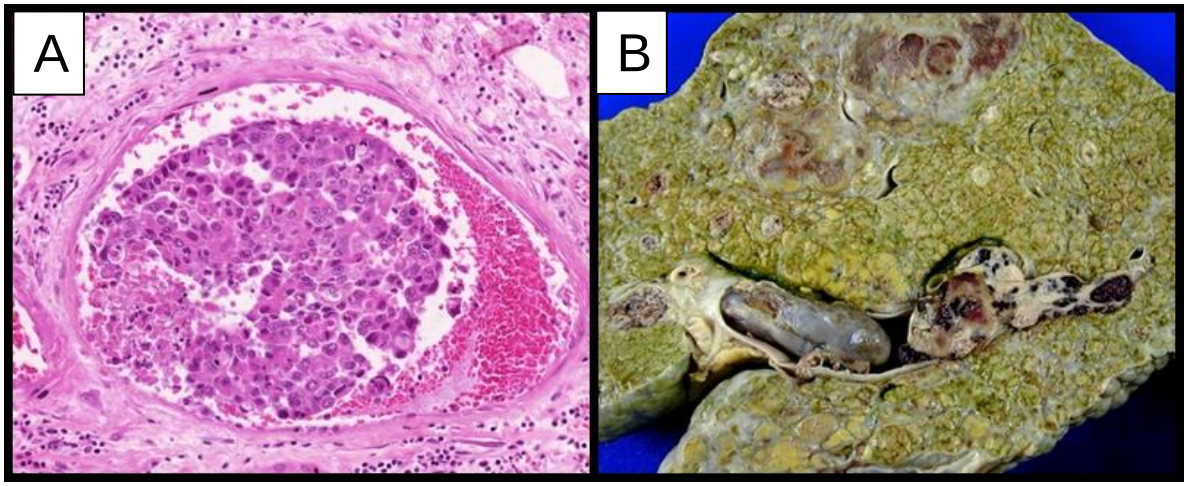

Figure 4 – Examples of HCC microvascular (A) and macrovascular (B) invasion.

In up to 30% of the cases, HCC samples also show histological features that allow them to be classified into specific histological subtypes[38], including:

- Steatohepatitic HCC: diagnosis is based on the presence of steatohepatitic features such as ballooning and steatosis in more than 50% of the tumor cell[39]. This variant comprises up to 35% of all HCC and is common in patients with metabolic syndrome and NASH. Prognosis is usually better or similar to classical HCC[40,41].
- Sarcomatoid HCC: defined as tumors enriched for spindle cells, although the minimum cutoff for diagnosis is yet to be established. This variant is rare (<1% of all HCC) and prognosis is worse than classical HCC[42].
- Clear-cell HCC: tumors show clear cell changes due to cytoplasmic accumulation of glycogen or lipids; the suggested cutoff for diagnosis is presence of clear cytoplasm in more than 50% of the tumor cells. Renal cell carcinoma is an obvious differential diagnosis, but clear-cell HCC

tends to retain expression of markers of hepatocellular lineage (e.g., HepPar1 and Arginase). The approximate frequency of this variant is 5-7% and its prognosis is unclear[43,44].

- Scirrhous HCC: defined as HCC with intratumor fibrosis in more than 50% of the tumor area. Fibrolamellar carcinoma is an important differential diagnosis, but it usually occurs in younger patients with no background liver injury[45,46]. Conversely, scirrhous HCC is prevalent in older patients, often develop in a background of liver injury and show high expression of Keratin 19[47]. The approximate frequency of this variant is 3% and prognosis is similar to classical HCC but worse than fibrolamellar carcinoma[47,48].
- Lymphocyte-rich HCC: defined as well-to-moderately differentiated HCC with diffuse lymphocytic infiltration, particularly in the trabeculae. Data is limited, but this variant seems to have favorable prognosis[49].
- Lymphoepithelioma-like HCC: defined as poorly-differentiated tumors with syncytial sheets of epithelioid cells amongst lymphocytes. Immunohistochemistry is required for confirmation of hepatocellular lineage. This variant is rare (<1%) and prognosis is unclear[50].

Despite the clinical and biological relevance of macroscopic and histological features for the characterization of HCC, there are relevant limitations to the pathological analysis of this cancer. First, as aforementioned, needle-biopsies and histopathological analysis are not required for definitive diagnosis and are not the standard practice in most services[1,6]. There is a compelling argument that needle biopsies add an unnecessary risk of clinical complications and could altogether be avoided given the high confidence of imaging exams for HCC

diagnosis. These risks include chronic pain and severe bleeding which, in a context of chronic liver injury, are even more severe[51]. There is also increased risk of tumor seeding in the needle trajectory and disease expansion[52]. However, as procedures get safer, these complications tend to be rarer and better handled. In fact, in a large service in which liver biopsy for HCC diagnosis is the standard practice, the surgical department at Toronto General Hospital demonstrated that the risk of complication is low and does not impact survival. Moreover, histopathological evaluation of needle biopsies provides valuable information about disease aggressiveness. For instance, the Toronto group uses histological differentiation to expand the criteria for liver transplantation in patients with HCC, with fantastic results[53,54]. Other groups also showed that expression of the progenitor marker Keratin 19 (K19) in biopsy specimens help predict disease response to sorafenib in patients with advanced HCC[55,56]. Recently, clinician-scientists have also vouched for HCC biopsies, since they offer important histological evidences for the differential diagnosis and also allow for biomarker-oriented research and improved patient allocation in biology-driven clinical trials[57,58].

Second, only patients diagnosed with very-early or early stage HCC are eligible for surgical therapies[6]. In other words, pathology specimens of tumors at intermediate and advanced stages – including metastatic sites – are not common. It is thus possible that morphological features that are prevalent in the advanced disease – and likely associated to worse outcome – are overlooked and deemed as less prevalent or important than they really are. While this issue could be mitigated if needle biopsies were the standard practice for this cancer's diagnosis, autopsy studies also present a good opportunity to understand and describe

morphological details of HCC in different disease stages, most notably in metastatic sites. In fact, Felipe-Silva *et al* showed, in a cohort of 80 autopsy patients, that expression of K19 is more prevalent in metastatic rather than primary HCC[59].

Third, there is little consensus on the histopathological evaluation of HCC, including key features such as the degree of differentiation (histological grade). This was ratified by a recent systematic review by Martins-Filho & Paiva *et al* (**Appendix A-1**) that highlighted inconsistencies in HCC histological grading in the literature (**Figure 5**). The authors showed that multiple publications adopt the E&S histological grading for HCC while others use the World Health Organization (WHO), the Union for International Cancer Control system or the Liver Cancer Study Group of Japan systems, among others. Despite this lack of consensus in the literature, Martins-Filho & Paiva *et al* showed that histological grading is relevant for risk stratification in HCC. Led by the precedent set by breast cancer, the authors also proposed a new grading system based on the scoring of multiple objective criteria (e.g. nuclear grade, architectural grade and cellular crowding) to minimize interpretation biases across institutions and contribute to a more uniform histological evaluation of HCC[60].

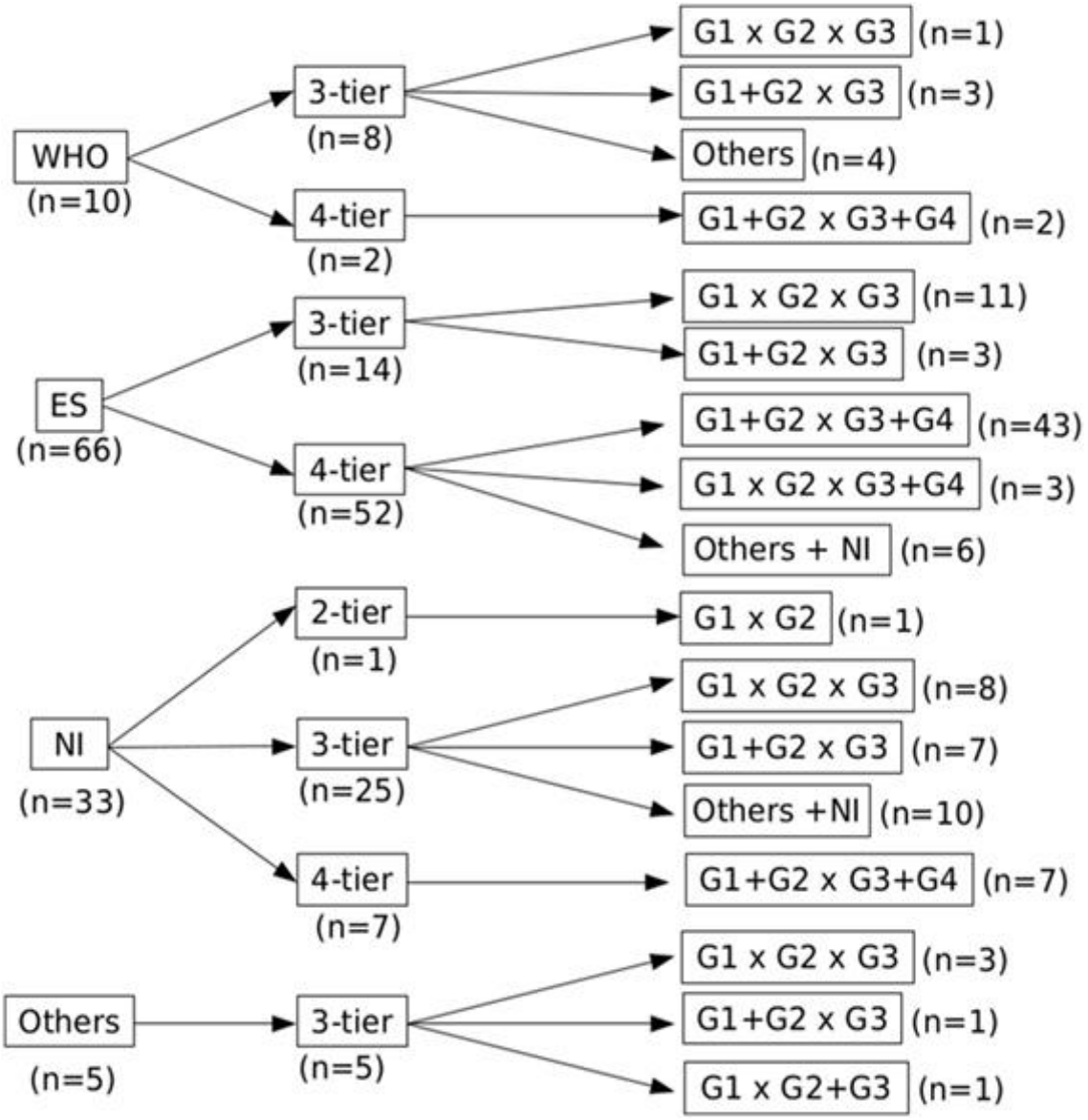

Figure 5 – There are huge discrepancies in HCC histological grading in the literature. Although most publications adopted the classic Edmondson & Steiner classification, some studies used different grading systems (or did not inform the grading system adopted). The number of tiers and how grades were organized prior to data analyses also varied greatly among the publications. Abbreviations: E&S: Edmondson & Steiner; G – grade; NI – not informed; WHO – World Health Organization. SOURCE: Martins-Filho and Paiva *et al.* Frontiers of Medicine;2017[60].

1.4 Hepatocellular carcinoma: molecular pathology

Immunohistochemistry (IHC), a major precursor of molecular pathology and, nowadays, an excellent tool for integrative studies between histology and genomics[61–63], is useful for the confirmation of hepatocellular lineage in routine

samples and for testing biomarkers of prognostic impact in HCC. Markers of hepatocellular differentiation include HepPar1 (expressed in 80-90% of HCC), Arginase (90-95%) and CD 10 (60-80%)[35,64–67]. Poorly-differentiated HCC tends to show lower expression of these markers, although reports in the literature describe occasional cases of well-differentiated HCC negative even to Arginase[68]. Hence, a combination of hepatocellular markers might be important for confirmation of hepatocyte lineage and HCC diagnosis. Relevant biomarkers with prognostic impact in HCC are summarized in the **Table 1**. Few of these markers, with the notable exception of K19, have been validated by multiple cohorts and are adopted in the clinical practice.

Table 1 – Immunohistochemical markers of prognostic impact in HCC

Markers	Frequency (literature)	Prognostic impact
Keratin 19	15-20%	Intermediate filament normally expressed in the pancreatobiliary epithelium[69]; considered a marker of stem-like properties and associated with poor outcome in HCC[55,70]. Tumors with ≥5% expression of Keratin 19 show higher prevalence of vascular invasion, metastases and even resistance to treatment with sorafenib[55,71–73].

(continued)

Markers	Frequency (literature)	Prognostic impact
EpCAM	10-30%	Epithelial cell adhesion molecule associated with cell proliferation and differentiation; considered a marker of stem-like properties and associated with aggressive behavior in HCC[74,75].
CD 44	25-40%	Cell surface molecule responsible for cell-to-cell interactions and cell migration. Considered a marker of stem-like (although eventually epithelial-mesenchyme) properties associated with tumor growth, poor differentiation and unfavorable outcome in HCC[76–78].
CD 117	2-48%	Receptor tyrosine kinase encoded by the *KIT* gene. Expression of this markers was reported in hepatic progenitor cells and staining in HCC was associated with aggressive behavior[79,80]. However, another large cohort showed no significant expression of this marker in this cancer[81].

(continued)

Markers	Frequency (literature)	Prognostic impact
CD 133	15-40%	Trans-membrane glycoprotein associated to cell regeneration. Expression was reported in HCC cell lines and correlates to stem-cell properties in this cancer[82–84].
β-catenin and Glutamine synthetase	20-40%	Markers associated with Wnt signaling activation and *CTNNB1* mutations[85]. Tumors usually show low histological grades and favorable outcome[86].
Vimentin	1-5%	Intermediate filament of mesenchymal cells commonly implicated in epithelial-mesenchyme transition in carcinomas[87]. In HCC, this marker is prevalent in tumors with sarcomatoid phenotype and is associated with high histological grades and poor outcome[42,88,89].
Claudin 1	75-90%	Transmembrane protein associated to tight junctions in epithelial cells. Absence of Claudin 1 expression in carcinomas is associated with epithelial-mesenchyme properties[90]. HCC with no expression of this marker usually show high histological grades and poor outcome[91].

Multiple studies have explored genomic alterations in HCC including oncogenic mutations, transcriptomic signatures, methylation profiles and microRNA changes[92–94]. The Cancer Genome Atlas (TCGA) Research Network has integrated these different molecular features/platforms and published a comprehensive analysis summarizing the main genomic alterations in this cancer. The TCGA consortium reported 26 significantly mutated genes in HCC and confirmed previous publications on main cancer drivers such as *p53* (31%) and *CTNNB1* (27%). *TERT*-promoter mutations were found in 44% of the cases and thus validated as the most frequent somatic mutations in HCC. Clustering analysis of DNA copy number, DNA methylation, mRNA, miRNA and protein array data generated three HCC molecular subtypes associated to specific demographic, pathologic and mutation data. The first cluster (iClust1) was enriched for tumors with high grade and macrovascular invasion and showed overexpression of proliferation markers (such as *MYBL2*, *PLK1* and *MKI67*) and low prevalence of *CTNNB1* and *TERT*-promoter mutations. Patients were usually younger, female and Asians. Conversely, iClust2 and iClust3 showed higher prevalence of *TERT*-promoter and *CTNNB1* mutations. They were also enriched for *CDKN2* silencing by hypermethylation and mutations in *HNF1A*. Tumors in iClust2 showed lower grades and lower prevalence of microvascular invasion while tumors in iClust3 depicted higher frequency of *TP53* mutations, deletions in 17p and hypomethylation of CpG sites[95].

Further associations between molecular and clinicopathologic features were explored by Calderaro *et al*. Those authors showed that HCC histological subtypes are associated to specific underlying molecular features. For instance, scirrhous HCC is associated with TSC1/TSC2 mutations and epithelial-

mesenchyme transition (EMT) properties, whereas steatohepatitic HCC shows frequent JAK/STAT activation and are *TP53*, *CTNNB1* and *TERT*-promoter wild type[96]. Findings from the TCGA consortium study and Calderaro *et al* also supported the conception of a new HCC histological subtype, the macrotrabecular massive (architectural feature), characterized by high frequency of vascular invasion and *TP53* mutation. Finally, both studies validated previous findings on *CTNNB1*-driven HCC being usually well-differentiated tumors, with microtrabecular or pseudoglandular architecture[95,96].

Although much progress has been made in the genomics characterization of HCC in the recent years, molecular biology studies have similar limitations as pathologic analyses: limited number of biopsy samples and lack of specimens from patients with intermediate and advanced/metastatic disease. Hence, IHC, mutation and gene expression analyses in this cancer are biased towards early-stage tumors submitted to surgical intervention. Again, this lack of comparisons between samples from different tumor stages precludes a more confident evaluation of the molecular mechanisms of HCC progression and distant dissemination. Liquid biopsy could potentially address some of these biological questions. Isolation and analysis of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) from patients with HCC is feasible and serial blood collections from a given patient might identify molecular changes associated to disease relapse and extra-hepatic spread[97]. Liquid biopsy might even help monitor HCC intratumor heterogeneity, as suggested by a recent publication that described the transcriptomic profile of CTCs detected from two patients with advanced-stage HCC (BCLC C). By combining flow cytometry and high-density single cell RNA sequencing, the authors showed transcriptomic heterogeneity (including

differential expression of IGF2) among the CTCs collected from one of the patients[98].

A major limitation of liquid biopsy, however, is the lack of methodological consensus. Many different protocols for sample collection and storage, and DNA/cell isolation and sequencing have been described in the literature, but few have been standardized and approved for clinical use. Validation of pre-analytical and analytical protocols is essential for a safer and more widespread adoption of liquid biopsy in the clinical practice[99,100]. Furthermore, diagnostic accuracy and specific applications of liquid biopsy are better outlined in cancer types with easy access and thorough phenotypic and molecular characterization of tissue samples. For example, the first clinically validated liquid biopsy assay, the Cobas EGFR Mutation Test v2, was specifically approved for testing *EGFR* mutations (focus on T790M) that might confer resistance to first generation *EGFR* inhibitors in *EGFR*-mutated lung adenocarcinomas. Extensive sampling and analysis of multiple tissue samples from lung cancer was an essential reference for validating this liquid biopsy assay[101]. As mentioned before, tissue samples are scarce in HCC, particularly in the advanced disease. Therefore, potential roles for liquid biopsy might be overlooked due to the lack of adequate phenotypic and molecular characterization in this cancer.

1.5 Hepatocellular carcinoma: heterogeneity and autopsy analyses

Autopsy specimens allow for sampling of advanced-stage HCC and offer a valuable opportunity to study heterogeneity and patterns of HCC dissemination. Indeed, autopsies allow for extensive sampling of primary and metastatic nodules

from any advanced/aggressive tumor. Recent studies have used autopsy specimens to provide insights on cancer progression in pancreatic[102] and prostatic[103] cancer. These data have improved the understanding of the cancer clonal composition and metastatic behavior of those cancer types. In one of the few studies including autopsy samples from patients with HCC, Felipe-Silva *et al* identified a higher prevalence of K19 and p53 protein expression in advanced tumors[59]. At that study, analyses mostly focused on a single tumor area, which limited the assessment of tumor heterogeneity (and its impact on distant dissemination) and precluded paired primary-metastatic comparisons on an individual basis.

Here, we expand on the previous publication by Felipe-Silva *et al* to provide a detailed report on phenotypic correlates between primary and metastatic HCC using multiple samples collected from autopsies. We used stringent criteria to focus the analysis on patients with sampling of at least two tumor areas, nontumoral liver and common metastatic sites (regardless of macroscopic evidence of disease). Our main objective was to evaluate patterns of extra-hepatic dissemination and assess inter-nodular heterogeneity in patients with intermediate and advanced HCC.

2. OBJECTIVES

2.1 General objective

To assess the morphological and molecular inter-nodular heterogeneity in primary and metastatic hepatocellular carcinoma (HCC) and infer patterns of extra-hepatic dissemination in a large cohort of autopsy patients with HCC.

2.2 Specific objectives

2.2.1 Evaluate the distribution of the metastatic disease (e.g. lungs, adrenal gland and lymph nodes) in patients with HCC submitted to autopsy.

2.2.2 Assess clinical and pathological features in the primary tumor that are associated with extra-hepatic dissemination in HCC.

2.2.3 Analyze major cytological and histological criteria in HCC and assess their distribution in the primary and metastatic disease.

2.2.4 Investigate the immunohistochemical expression of markers of hepatocyte differentiation, epithelial-mesenchyme transition, progenitor-cell features and *CTNNB1* mutation status in primary and metastatic HCC.

2.2.5 Assess the distribution of mutations in the *TERT* promoter region in a subset of HCC patients with metastatic disease.

2.2.6 Explore associations between histological and immunohistochemical features to improve the assessment of high-grade versus low-grade HCC.

2.2.7 Investigate the histological, immunohistochemical and *TERT*-promoter mutational heterogeneity within the primary and metastatic disease in patients with HCC to infer patterns of extra-hepatic dissemination.

3. METHODS

3.1 Study design and ethics

This is a retrospective observational study conducted at the Department of Pathology at the University of Sao Paulo School of Medicine (Faculdade de Medicina da Universidade de Sao Paulo, FMUSP) in collaboration with the Division of Liver Diseases at the Icahn School of Medicine at Mount Sinai (ISMMS, New York, USA).

Ethical approval was issued by the Institutional Review Board from the Hospital das Clínicas da FMUSP (HC-FMUSP): approval number 1.297.918; date: 25/AUG/2015 (**Appendix B**). All the patient samples were assigned a study ID and remained de-identified for the entire duration of this study. Furthermore, no personal health identifier/information was used to conduct data analyses.

3.2 Study population

This study was conducted in autopsy samples from HC-FMUSP. From January 2000 to December 2015, a total of 13,238 routine autopsies were performed at the Division of Anatomic Pathology / Pathology Department at HC-FMUSP. Among them, we identified 219 performed on patients with HCC. Formalin-fixed paraffin-embedded (FFPE) blocks from 41 patients had been previously retrieved for other studies and were unavailable at the Pathology Archive. Samples from 178 patients were retrieved and reviewed for the eligibility criteria:

Inclusion criteria:

- Adequate information on the number of tumors and the size of the dominant nodule;
- Specimens from liver resection or transplantation, if the surgical procedure was performed within one month from the autopsy;
- Histological representation of non-tumoral liver;
- Histological representation of at least two distinct tumor regions;
- Histological representation of vital organs, particularly common metastatic sites (e.g., lung), regardless of imaging and/or macroscopic findings. The objective was to identify precursor lesions in these common metastatic sites.

Exclusion criteria:

- Advanced tissue autolysis, as defined by endothelial cells detachment and/or nuclear clumping;
- Post-mortem interval higher than 24 hours, regardless of tissue viability;
- Tumors with mixed phenotype (e.g., combined hepatocellular-cholangiocarcinoma).
- Patients diagnosed with fibrolamellar carcinoma.

Eighty-eight (88) patients met all the criteria and had their clinical records reviewed for relevant information: age, gender, risk factors, date of diagnosis, and alpha-fetoprotein (AFP) concentration. Only AFP results issued within five days from the autopsy were included in the analysis.

Imaging exams and autopsy reports (including macroscopic pictures) were re-examined and all histological slides from the 88 patients were reviewed to assess additional pathological information including macrovascular invasion, microvascular invasion and cirrhosis. All HCC nodules identified from the 88 patients were classified according to current criteria into specific histological subtypes: NOS (not otherwise specified), steato-hepatitic, sarcomatoid, lymphocyte-rich, clear cell and scirrhous. Finally, the panoramic analysis of all histological slides was used to select the most appropriate areas (details below) for tissue microarray (TMA) confection.

3.3 Pathological analyses

TMAs were generated for the evaluation of additional histological features and for the immunohistochemical (IHC) analyses. TMA blocks and IHC reactions were performed at the Laboratory of Liver Diseases (LIM 14) at FMUSP.

3.3.1 Tissue microarrays

One-millimeter tissue cores were transferred from their original FFPE to recipient TMA blocks with the MTA1 tissue arrayer (Beecher Instruments, Inc). Forty-five Fisher® charged slides were sequentially cut (5µm) and stored at a –20°C freezer for upcoming IHC reactions. Additional four slides were cut – every 15 charged slides – and stained for hematoxylin & eosin (H&E) for the morphological analyses.

TMA sampling included all the nodules histologically available from each patient. Furthermore, nodules that were represented in multiple FFPE blocks, nodules with intra-tumor fibrosis or with a nodule-in-nodule pattern had each different tumor region adequately sampled (**Figure 6**).

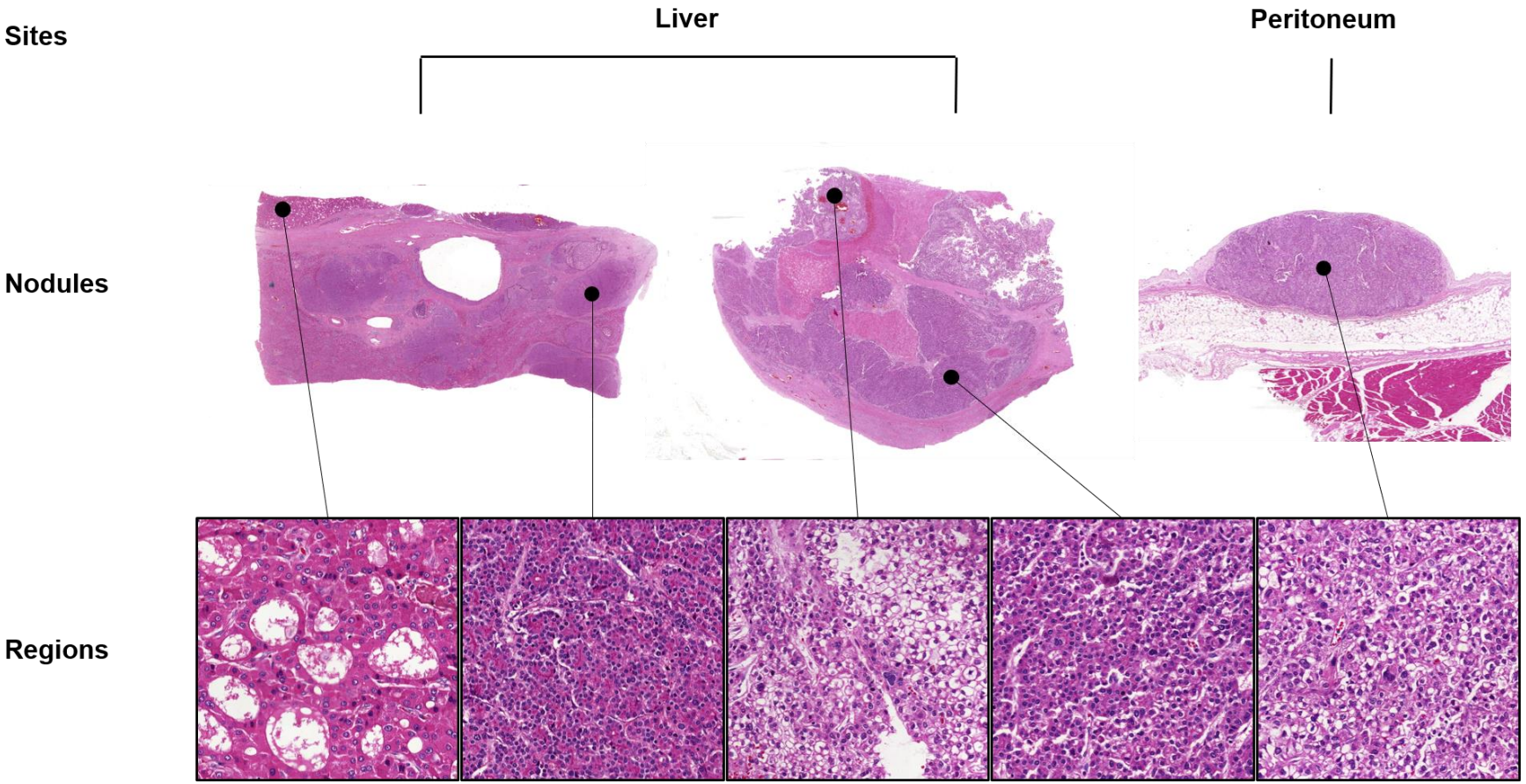

Figure 6 – Schematics of the multi-region tissue microarray (TMA) spotting. All the nodules histologically available were sampled in the TMA blocks. Furthermore, nodules with intra-tumor fibrosis or with distinctive histological patterns (such as nodule-in-nodule) had each different area adequately sampled.

After accounting for the tissue loss associated with the TMA technique, a total of 230 nodules from the 88 patients were included for the evaluation of histological grades and IHC studies. This included 194 hepatic and 36 extra-hepatic nodules (**Figure 7**).

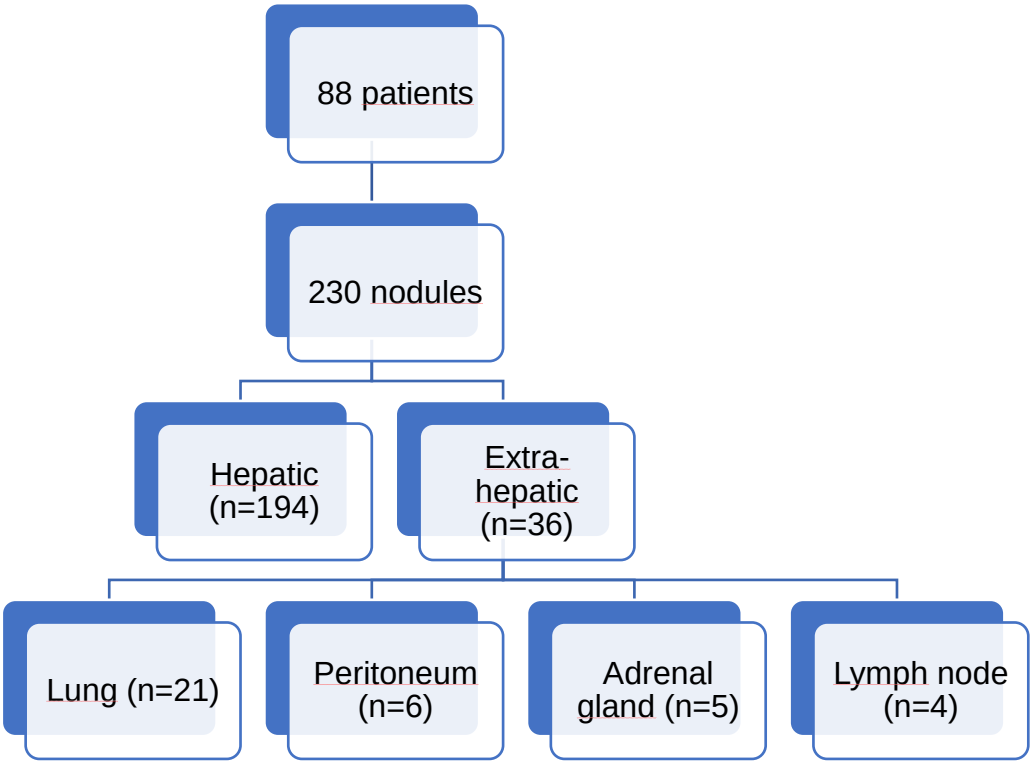

Figure 7 – Distribution of the hepatic and extra-hepatic nodules in the tissue microarray (TMA) blocks.

3.3.2 Evaluation of histological grades

Beyond the histological analysis in the panoramic sections, additional histological criteria was evaluated in the TMA slides, including degree of differentiation (histological grade) according to Edmondson & Steiner and relevant cytoarchitectural features – as recently proposed by the Martins-Filho & Paiva *et al* [60] – including cellular crowding and nuclear, nucleolar and architectural

grades. All histological criteria were individually assessed in a 4-tier classification, and later dichotomized in high and low grade (**Table 2, Figures 8–12**). The histological analyses were performed in two consecutive H&E slides from the TMA blocks. Each HCC nodule was classified according to the highest grade identified in its TMA spots. Accordingly, patients were classified according to the highest grades observed in their nodules.

Table 2 – Histological Characterization of the Samples

Histological Features		Grading Criteria
Histological Grade*	low-grade	Grade 1 - diagnosis rest upon the demonstration of more aggressive patterns in the neoplasm. Distinction from hyperplastic conditions is difficult.
		Grade 2 - marked resemblance to normal hepatic cells. Cell borders are sharp and clear cut, acini are frequent, and bile is often present.
	high-grade	Grade 3 - large nuclei, breakup or distortion of the trabecular pattern. Bile is less frequent. Tumor giant cells are numerous.
		Grade 4 - scanty cytoplasm, medullary pattern of growth. Lack of cohesiveness. Occasional spindle or short plump cells.
Architecture	low-grade	Grade 1 - well-defined trabecular plates (2-3 cells wide).
		Grade 2 - pseudoglandular and irregular patterns.
	high-grade	Grade 3 - midtrabecular (4-10 cells wide) and solid patterns.
		Grade 4 - macrotrabecular (>10 cells wide). (continued)

Histological Features		Grading Criteria
Cellularity	low-grade	Grade 1 - more than 1 nucleus fit between 2 adjacent nuclei.
		Grade 2 - 1 nucleus fits between 2 adjacent nuclei.
	high-grade	Grade 3 - less than 1 nucleus fits between 2 adjacent nuclei.
		Grade 4 - nuclear overlapping.
Nuclear Grade**	low-grade	Grade 1 - homogeneous, near-normal nuclei.
		Grade 2 - mildly pleomorphic nuclei, with some degree of membrane irregularity.
	high-grade	Grade 3 - pleomorphic nuclei, with major changes in shape.
		Grade 4 - marked pleomorphism, anaplastic giant cells.
Nucleolar Grade***	low-grade	Grade 1 - inconspicuous nucleoli (barely seen at 400x).
		Grade 2 - small but evident nucleoli (seen at 100-200x).
	high-grade	Grade 3 - large nucleoli (clear at 100x).
		Grade 4 - prominent nucleoli at low-magnifications (40x).

*Exempts from Edmondson & Steiner's (E&S) grading classification of HCC[36]. **Adapted from the Grading Classification proposed by Goodman and Ishak[27]. ***Transposed from Fuhrman's grading classification of renal cell carcinomas[104]. A combination of cellular crowding, nuclear, nucleolar and architectural grades were proposed by the authors as a novel potential approach for a new grading classification of HCC[60].

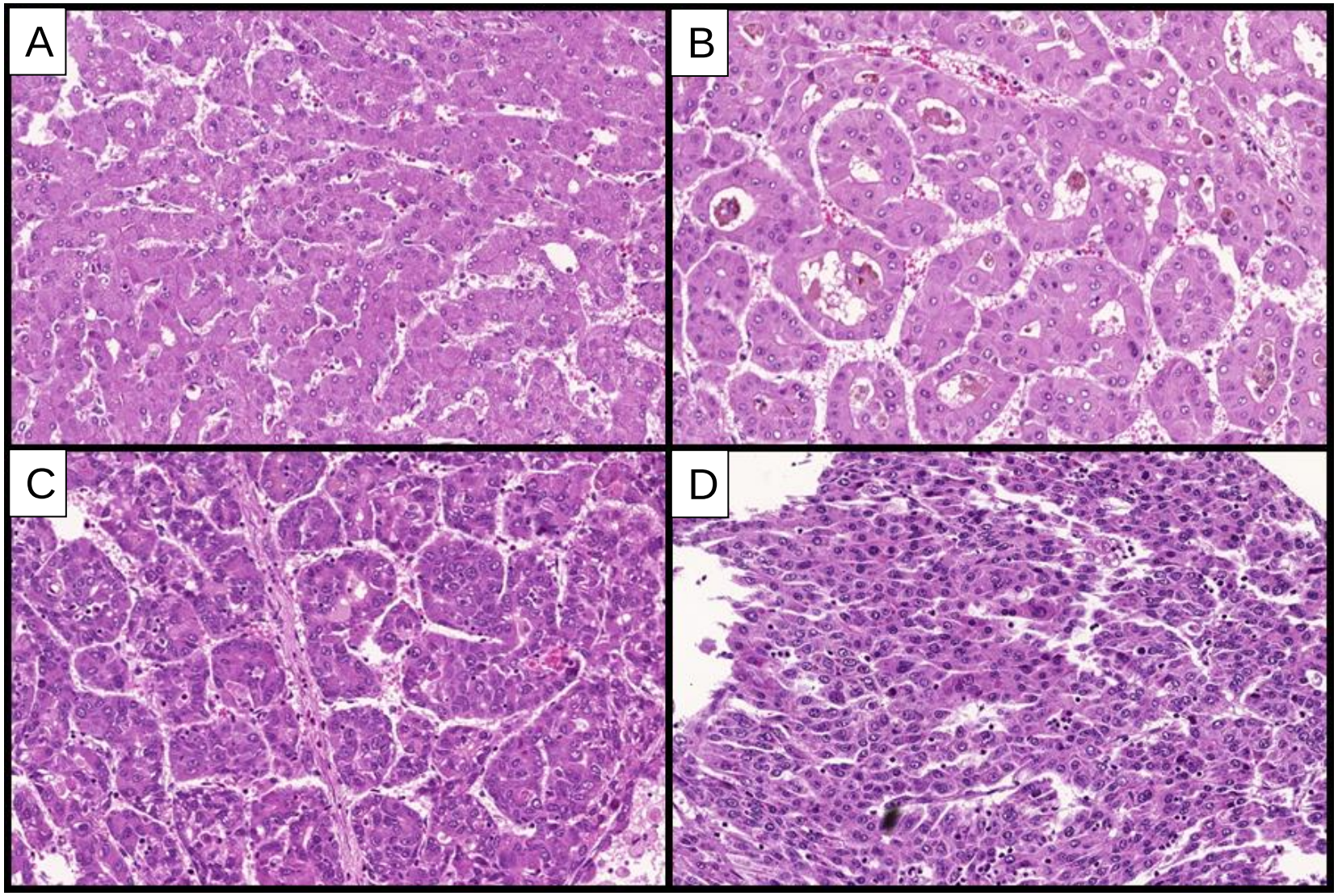

Figure 8 – Histological grade according to Edmondson-Steiner (A: grade 1, B: grade 2, C: grade 3, D: grade 4). H&E, 200x.

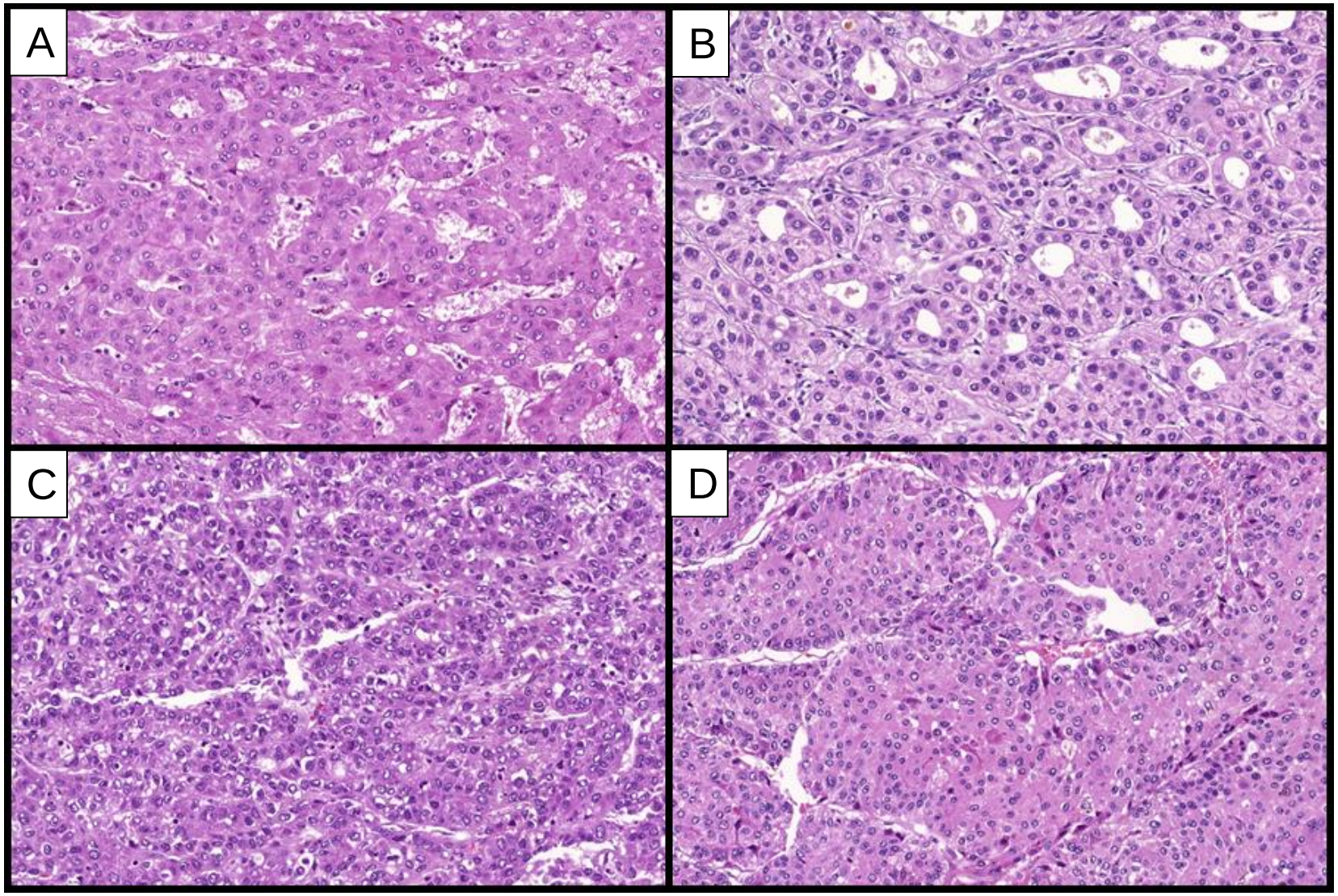

Figure 9 – Architectural grade (A: grade 1, B: grade 2, C: grade 3, D: grade 4) H&E, 200x.

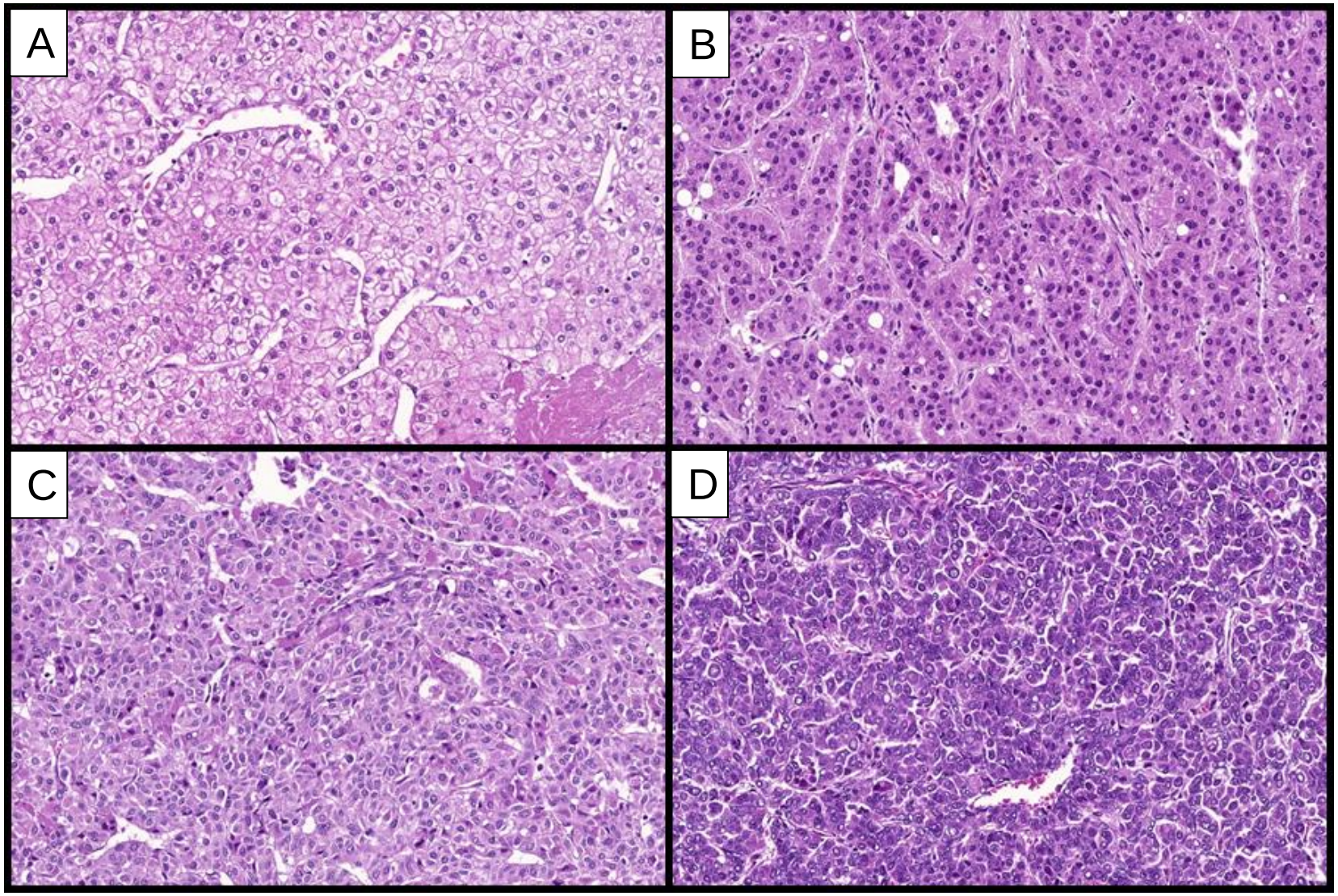

Figure 10 – Cellular crowding (A: grade 1, B: grade 2, C: grade 3, D: grade 4). H&E, 200x.

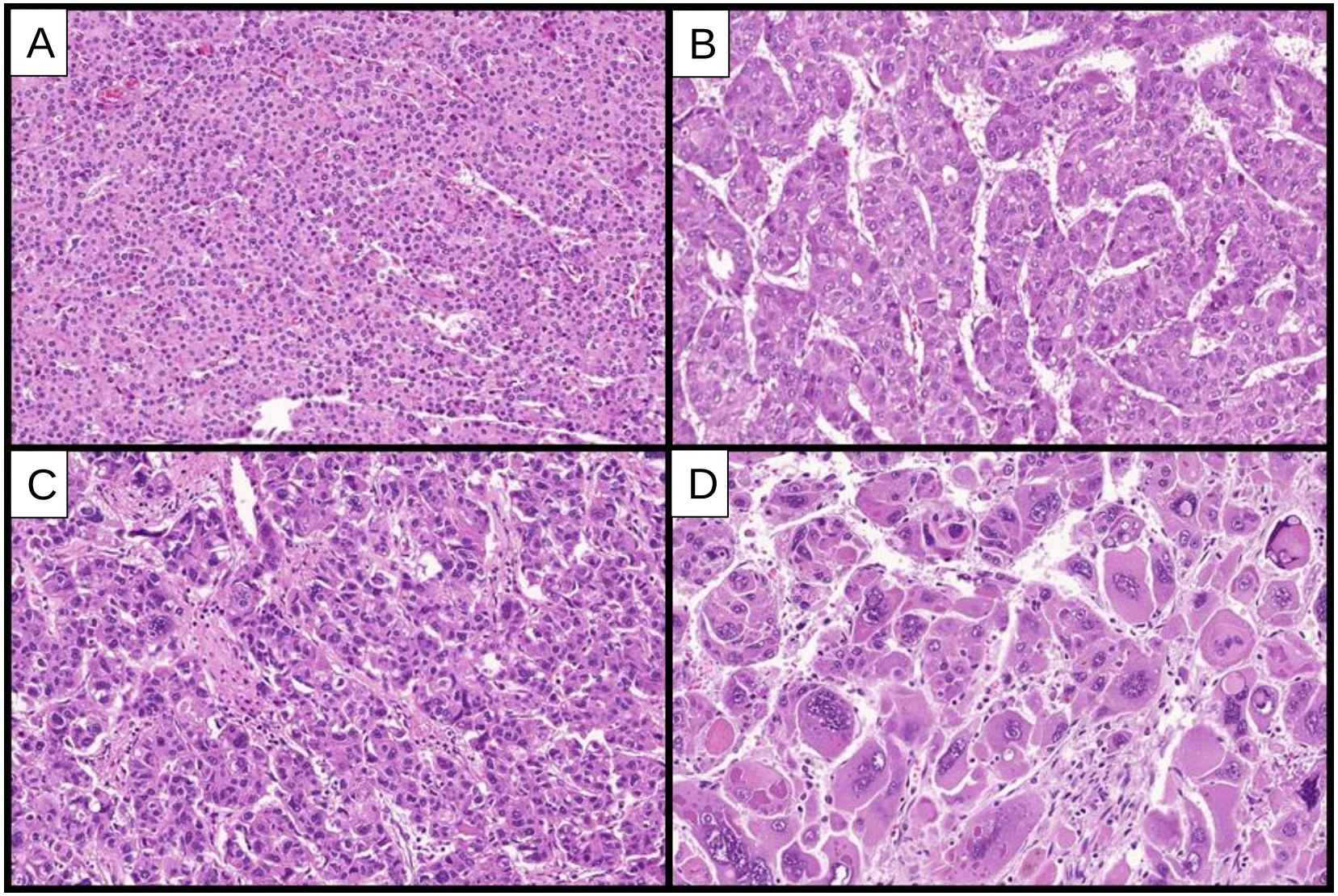

Figure 11 – Nuclear grade: adapted from the Goodman and Ishak HCC grading (A: grade 1, B: grade 2, C: grade 3, D: grade 4). H&E, 200x.

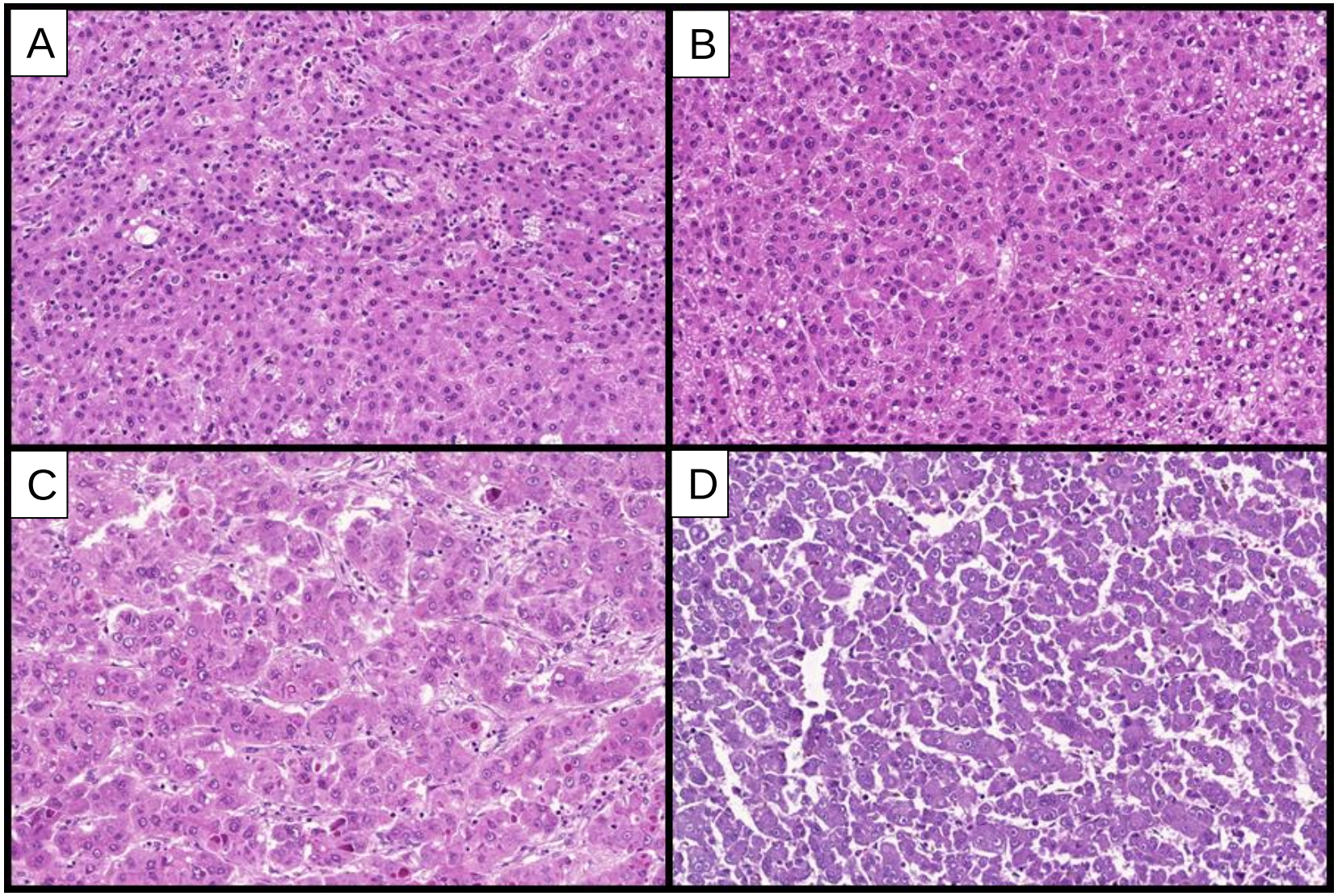

Figure 12 – Nucleolar grade: adapted from the Fuhrman's grading system (A: grade 1, B: grade 2, C: grade 3, D: grade 4) H&E, 200x.

3.3.3 Immunohistochemistry

IHC was performed for markers of hepatocyte differentiation (HepPar1, Arginase and CD 10), *CTNNB1* mutation status (β-catenin and Glutamine Synthetase), HCC stem-like features (Keratin 19, CD 44, CD 117, CD 133 and EpCam), and epithelial-mesenchyme transition (Claudin 1 and Vimentin). Results for β-catenin and Glutamine Synthetase were combined and disclosed as the status of the Wnt signaling pathway[105]. All IHC reactions were performed in the charged slides (5µm) obtained from the TMA blocks. The HCC nodules were classified – based on a combination of their TMA spots – in positive or negative for each marker according to specific criteria:

- HepPar1: granular cytoplasmic positivity in ≥ 5% of the sample;
- Arginase: granular cytoplasmic positivity in ≥ 5% of the sample;
- CD 10: membrane positivity in ≥ 5% of the sample;
- β-catenin: cytoplasmic and/or nuclear positivity in ≥ 5% of the sample;
- Glutamine Synthetase: strong and diffuse cytoplasmic positivity;
- Keratin 19: cytoplasmic positivity in ≥ 5% of the sample;
- CD 44: membrane positivity in ≥ 5% of the sample;
- CD 117: membrane positivity in ≥ 5% of the sample;
- CD 133: membrane positivity in ≥ 5% of the sample;
- EpCam: membrane or cytoplasmic positivity in ≥ 5% of the sample;
- Loss of Claudin 1: to address epithelial-mesenchyme properties, evaluation aimed at identifying cases with loss or very low expression of this marker (< 1% of cell membranes stained);

- Vimentin: cytoplasmic positivity in ≥ 5% of the sample;

The 88 patients were then classified according to their nodules: positive expression for a marker in a nodule was also interpreted as positive expression in the patient level. In other words, in patients with multinodular disease, if one nodule was positive for a marker, the patient was also considered positive for that marker.

IHC reactions were performed as per institutional protocols (LIM 14, HC-FMUSP). In brief, tissue sections were initially dewaxed and rehydrated. Following adequate antigen retrieval, endogenous peroxidase activity was blocked with a 6% hydrogen peroxide on methanol solution for 10 minutes (3 times). Non-specific protein-protein reactions were suppressed with CASBlock™ (Invitrogen/Zymed, USA) for 10 minutes at 37°C. Incubation with primary antibodies was performed at 37°C for 30 minutes, followed by overnight incubation at 4°C. To avoid biotin-tyramide and avidin-biotin interactions, signal amplification was performed with Novolink Polymer (Vision Biosystems™, UK) for 30 minutes at 37°C. Development was achieved with 100 mg% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, catalog D5637, USA), 1% dimethyl sulfoxide (Sigma, catalog D5879, USA) and 0.06% hydrogen peroxide in PBS for 5 minutes at 37°C. Slides were counterstained with Harris' hematoxylin, dehydrated and mounted with Entellan (Merck, catalog 1.07961, Germany). Titers, clones and the interpretation criteria for each antigen are summarized in the **Table 3**.

Table 3 – Immunohistochemical Antigens and Reaction Conditions

Antigen	Clone	Antigen Retrieval	Titers	Interpretation (Positive Reaction)
HepPar1	**OCH1E5** (ROCHE)	Steamer, TRIS-EDTA, pH9	1/50	Granular cytoplasmic positivity in ≥5% of the sample
Arginase	**Polyclonal**†† (Sigma Life Sciences)	Steamer, Citrate, pH6	1/1000	Granular cytoplasmic positivity in ≥5% of the sample
CD 10	**56C6** (DAKO)	Steamer, TRIS-EDTA, pH9	1/200	Membrane positivity in ≥ 5% of the sample
CD 117	**YR145** (Cell Marquee)	Steamer, TRIS-EDTA, pH9	1/400	Membrane positivity in ≥ 5% of the sample
CD 133	**Polyclonal**†††† **(Abcam)**	Steamer, TRIS-EDTA, pH9	1/100	Membrane positivity in ≥ 5% of the sample
CD 44	**DF1485** (DAKO)	Steamer, Citrate, pH6	1/100	Strong membrane and/or cytoplasmic positivity in ≥ 5% of the sample

(continued)

Antigen	Clone	Antigen Retrieval	Titers	Interpretation (Positive Reaction)
Keratin 19	**B170** (Leica)	Steamer, TRIS-EDTA, pH9	1/200	Cytoplasmic positivity in ≥ 5% of the sample
EpCAM	**BerEP4** (Cell Marque)	No antigen retrieval	1/50	Membrane or cytoplasmic positivity in ≥ 5% of the sample
Vimentin	**V9** (Cell Marque)	Steamer, TRIS-EDTA, pH9	1/300	Cytoplasmic positivity in ≥ 5% of the sample
Loss of Claudin 1	**Polyclonal**† (Zymed)	Steamer, Citrate, pH6	1/200	Loss of membrane staining (< 1% of the sample)
β-catenin*	**14** (BD Transd. Laboratories)	Steamer, TRIS-EDTA, pH9	1/400	Cytoplasmic and/or nuclei positivity in ≥ 5% of the sample
Glutamine Synthetase*	**GS6** (ROCHE)	Steamer, Citrate, pH6	1/8000	Strong and diffuse cytoplasmic positivity in ≥ 5% of the sample

† Rabbit polyclonal (code 18-7362). †† Rabbit polyclonal (code 003595). ††† Rabbit polyclonal (code RP003). †††† Rabbit polyclonal (code ab19898).
* These markers were evaluated together and disclosed as the status of *Wnt* signaling pathway.

3.4 Molecular analyses

Patients with multiple hepatic and/or extra-hepatic nodules, residual tissue following TMA spotting and low post-mortem interval (< 12 hours) were included in the molecular analyses (n=8). DNA extraction and sequencing were performed at the Division of Liver Diseases at ISMMS.

3.4.1 DNA extraction

Two different methods were implemented to extract tissue from the FFPE blocks, accordingly:

- Punch needle: tissue cores were collected directly from the FFPE block with disposable biopsy punch needles (Miltex, CE) (**Figure 13**).
- Laser microdissection: tissue was dissected from membrane slides (15µm) with the Leica Microsystems LMD6500.

DNA was extracted with the QIAamp® DNA FFPE Tissue Kit (QIAGEN) following the manufacturer's instructions. The purified DNA was run on a 2100 Bioanalyzer Instrument (Agilent) for size estimation, and its concentration was measured by fluorometric quantitation using Qubit (ThermoFisher). Samples with low DNA yield (<50 ng) and/or highly degraded (mean fragment size <100 base-pairs) were excluded.

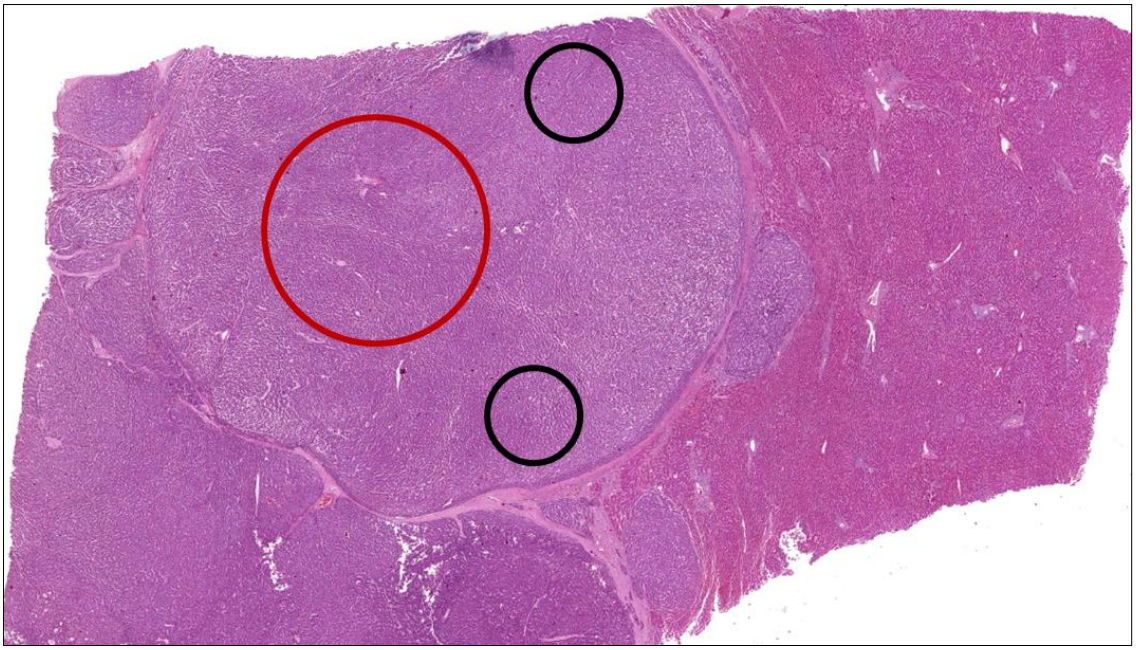

Figure 13 – Histological representation of an HCC-adjacent liver interface. Punch needle for DNA extractions (red circle) privileged areas close to the TMA spots (black spots).

3.4.2 High-throughput DNA amplicon sequencing

Samples with DNA yield > 500ng were submitted to DNA targeted sequencing at the Genomics Core of ISMMS. Library enrichment was performed with the TruSeq Custom Amplicon from Illumina. Probes were arranged through DesignStudio®, as previously reported[97], to cover the main exonic as well as relevant intronic regions of frequently mutated genes in HCC. Sequencing was performed with the Illumina MiSeq platform (read depth: 101x).

3.4.3 PCR amplifications and *TERT* promoter mutation status

Samples with DNA yield < 500ng and samples with residual DNA that was not used for the high-throughput targeted analysis were used for direct

sequencing of the *TERT* promoter region. PCR amplification of the duplicate segment of the *TERT* promoter region was conducted with the following primers: CAGCGCTGCCTGAAACTC and GTCCTGCCCCTTCACCTT. Each PCR product was assessed on a 2.0% agarose gel, and later sequenced in both directions with the BigDye Terminator Cycle-Sequencing Kit (Macrogen) reactions and loaded on an ABI PRISM 3730xl DNA analyzer. Sequences were assessed with the SnapGene® Viewer 3.3.4 software.

Given the high number of mutations introduced by FFPE tissue processing[106], we focused the evaluation on the two most prevalent *TERT* promoter mutations (C228T and C250T)[107].

3.5 Heterogeneity analyses

Inter-tumor heterogeneity was assessed in patients with multinodular hepatic and/or extra-hepatic disease. Histological heterogeneity focused on differences among tumor grades (low versus high) within the nodules from the same patient. IHC heterogeneity was characterized by the different protein status (positive versus negative) among nodules from the same patient. Finally, significant heterogeneity was defined as the presence of both histological and IHC heterogeneity. To further validate the histological and IHC heterogeneity analysis, mutation status of the *TERT* promoter region was compared within primary and metastatic disease on a patient-by-patient basis.

3.6 Statistical analysis

Chi-square and Fisher exact tests were used to evaluate association amongst the histological and IHC variables, to seek for associations between the clinicopathological variables and the presence of distant metastases and to assess which pathological feature was enriched in the metastatic nodules compared to the primary ones. The phi coefficient score was used to calculate the strength of the association/correlations between the histological and IHC variables. Multiple correspondence analysis (MCA) was performed to seek for patterns of sample distribution according to the tumor's histological grades. MCA is useful for clustering samples with similar features, and further illustrated the associations between the histological variables. Finally, a binomial logistic regression model was used to investigate independent correlates of extra-hepatic dissemination. A p-value inferior to 0.05 was considered significant. All analyses were conducted by the author (SNMF) in the SPSS 22.0 software (SPSS Inc, Chicago, USA) and R studio with appropriate plugins and packages.

4. RESULTS

The major results of the study were recently published (**Appendix A-2**)[108]. The outline of the results section is shown in **Figure 14**. The clinicopathological features of the cohort were described, followed by a detailed characterization of the metastases, and the correlative and heterogeneity analyses.

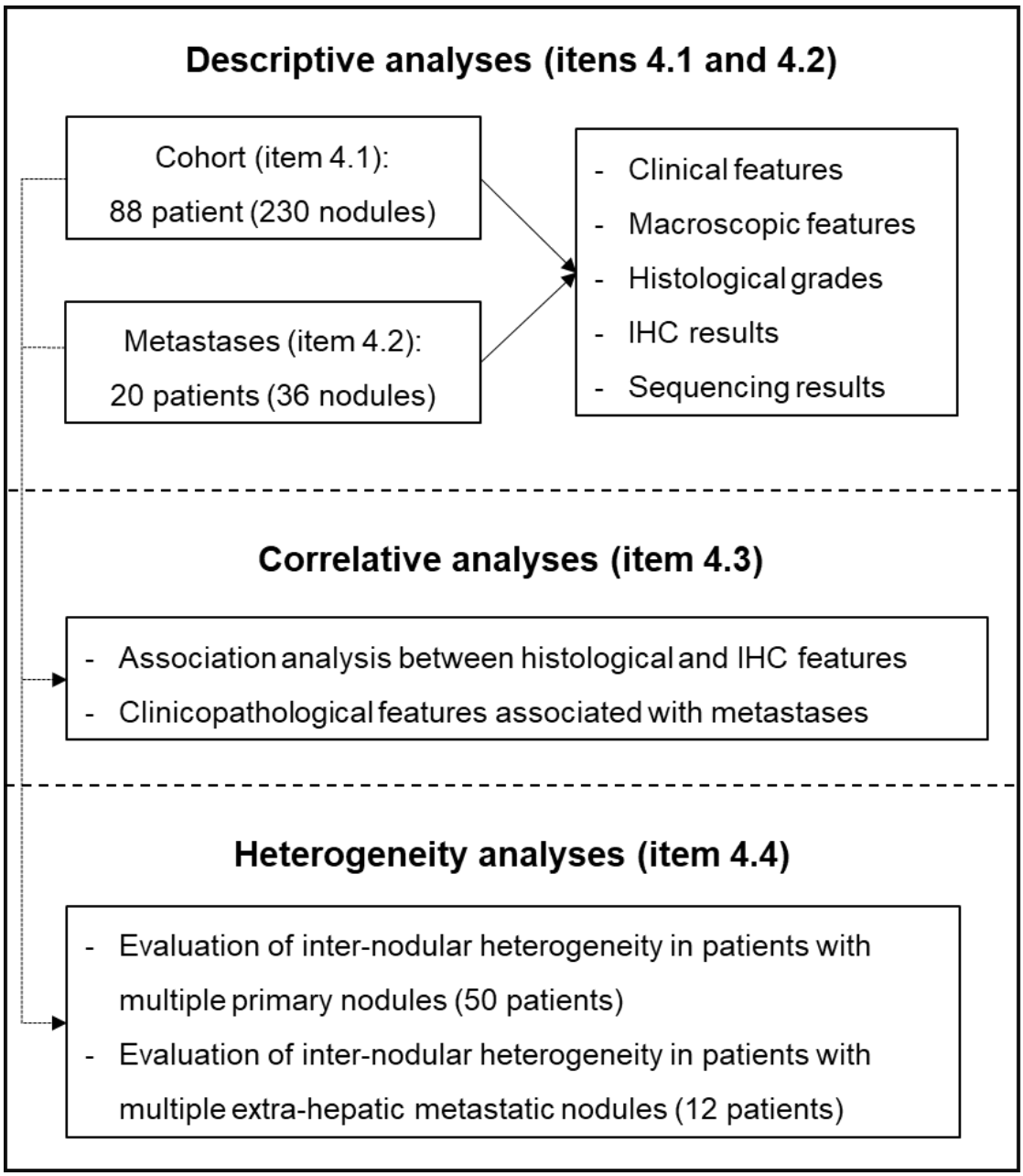

Figure 14 – Flow-chart of the results section. Abbreviation: IHC – immunohistochemistry.

4.1 Descriptive analysis of the cohort

4.1.1 Baseline clinical and pathological features

The baseline clinical and pathological information from the 88 patients is summarized in **Table 4**. In accordance with HCC epidemiology in Western countries[12], most patients were male (62/88, 70%), with background cirrhosis (79/88, 90%) associated to HCV infection (47/88, 53%). Of the nine patients without cirrhosis, one had HBV infection with no fibrosis. The remaining eight patients had mild or intermediate fibrosis due to HBV infection (3/8), HCV infection (1/8) or non-specific reactive hepatitis (4/8). The BCLC stage was not widely available (and therefore not collected) because several patients were diagnosed at the emergency room, did not undergo imaging exams and were only referred to supportive care (n=49, 56%). Instead, tumors were classified according to the pathological TNM staging system (AJCC 7th edition)[109].

Multinodular HCC was identified in 55/88 (63%) patients. HCV infection remained the most common etiology in these patients (26/55, 47%). Multinodular HCC can be due to 1) Multicentric origin (synchronous tumors): each HCC nodule arises independently, or 2) Intrahepatic metastases: nodules arise from a primary HCC. Distinction between synchronous HCC and intrahepatic metastases is not straightforward and usually requires careful integration of macroscopic, microscopic and molecular features[110–112]. Unfortunately, such distinction was not possible in the current study mainly because macroscopic reports greatly varied among the autopsies.

Extra-hepatic disease was identified in 20/88 (23%) patients. HBV infection was the most frequent disease in patients with metastasis (HBV: 7/20, 35% vs. HCV: 4/20, 20%). Extra-hepatic metastatic sites included lungs (16/20, 80%), lymph nodes (4/20, 20%), peritoneum (4/20, 20%), adrenal glands (3/20, 15%) and bones (2/20, 10%). Bone metastases had not been collected to avoid visible deformities or were insufficient for the analyses proposed here. In 6/16 (37.5%) patients with disseminated disease to the lungs, the metastatic foci were incidental findings. This included HCC cell-clusters entrapped in the lung vasculature, previously undetected in imaging studies and/or gross examination of the lungs (**Figure 15**). These disseminated cell-clusters showed unequivocal signs of viability and proliferation (i.e. mitotic figures) and were recognized as true metastatic precursors. For simplicity, they will be henceforth referred as “lung microvascular metastases”.

Table 4 – Baseline clinical and pathological features

Variable	N (%)	Variable	N (%)
Gender		**Pathological Stage**	
Male	62 (70)	I + II	49 (56)
Female	26 (30)	III	19 (22)
Age		IV	20 (23)
< 40	4 (5)	**Number of nodules**	
40 – 64	54 (61)	Single nodule	33 (38)
≥ 65	30 (34)	Multiple nodules	55 (62)
Primary disease*		**Size (dominant nodule)**	
HCV	47 (53)	< 5 cm	51 (58)
HBV	15 (17)	≥ 5 cm	37 (42)
Alcohol	18 (20)	**Microvascular invasion**	
NASH	4 (5)	Presence	79 (90)
Non-specific hepatitis	6 (7)	Absence	9 (10)
Others	6 (7)	**Macrovascular invasion**	
Cirrhosis		Presence	23 (26)
Presence	79 (90)	Absence	49 (56)
Absence	9 (10)	Not available	16 (18)
AFP concentration		**Distant Metastases**	
< 100 ng/mL	46 (52)	Presence	20 (23)
≥ 100 ng/mL	22 (25)	Absence	68 (77)

* Some patients had an overlap of primary diseases. Abbreviations: HCV - hepatitis C virus; HBV - hepatitis B virus; NASH - non-alcoholic steatohepatitis; AFP – alpha-fetoprotein.

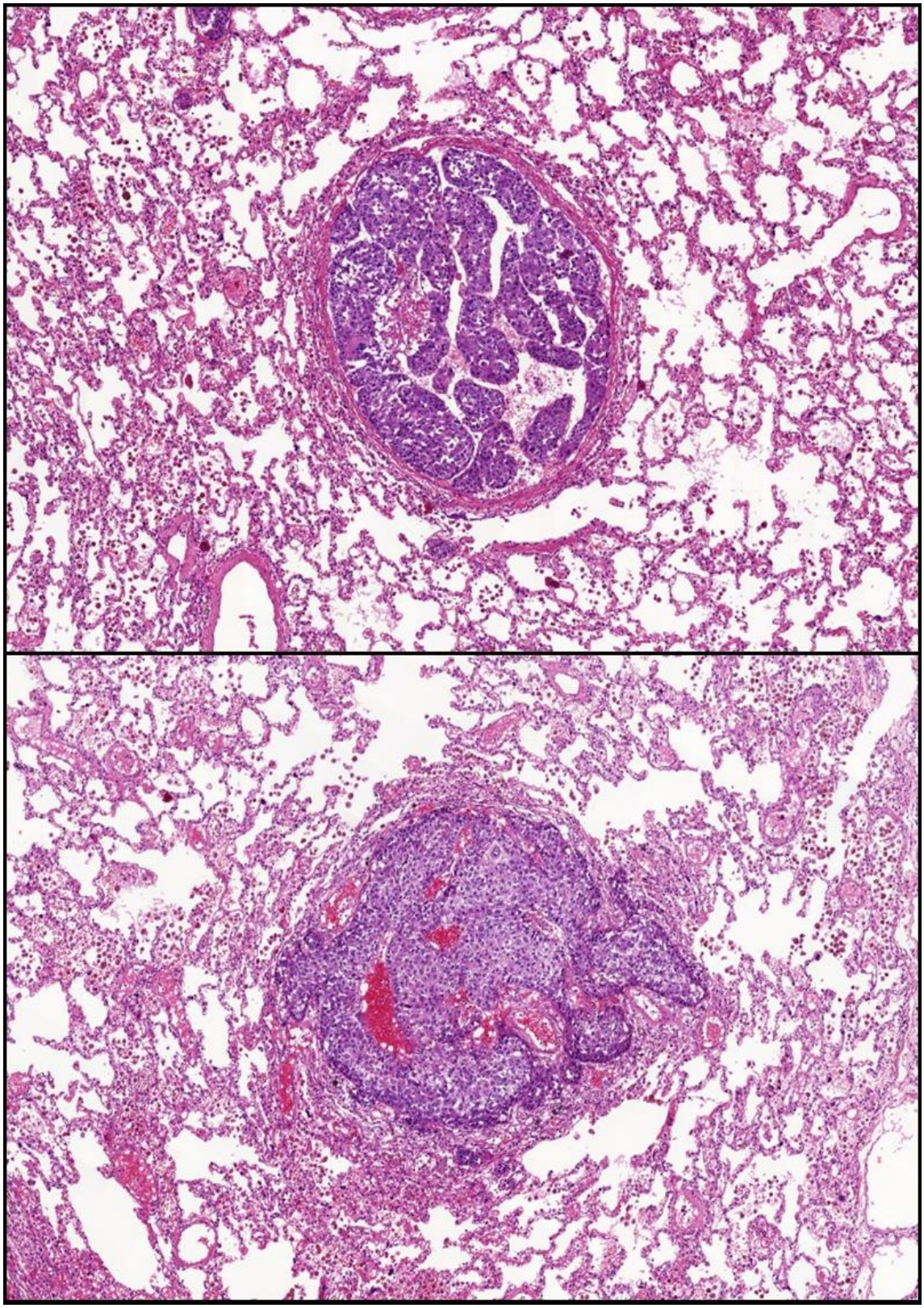

Figure 15 – Clusters of tumor cells within the lung microvasculature. These cells first accommodate and distend the lumen of the vessel (A), and then emit extensions towards the organ's parenchyma (B). There were no signs of autolysis, necrosis or apoptosis, but evidence of proliferation (mitosis) in these microvascular metastases. HE (50x).

4.1.2 Histological results

The histological findings from the 88 patients and 230 nodules are summarized in **Tables 5 and 6**, respectively. HCC histological subtypes were detected in 28/230 (12%) nodules from 15/88 (17%) patients, including:

- Steato-hepatitic HCC: identified in 14 out of 20 nodules from 11 patients. Most patients had stage I or stage II cancer (8/11, 73%) associated to HCV infection (4/11, 36%) or non-specific reactive hepatitis (3/11, 27%). Type 2 diabetes was a common comorbidity reported in the medical records from these patients (5/11, 45%). Most of the nodules with this phenotype showed low E&S histological grade (9/14, 64%);
- Sarcomatoid HCC: six out of seven nodules from one patient. This patient had stage IV cancer associated to HCV infection. Macrovascular invasion was also detected. All nodules were of high E&S histological grade;
- Clear cell HCC: all five nodules from a single patient. This patient had stage IV cancer associated to phospholipid syndrome. Four nodules were of high E&S histological grade;
- Scirrhous HCC: Two nodules from a single patient, who had stage III cancer associated to HCV infection. Both nodules were of high E&S histological grade.
- Lymphocyte-rich HCC: one out of two nodules from a patient who had stage II cancer associated to HCV infection. This nodule was of low E&S histological grade.

Table 5 – Histological results (Patients, n = 88)

Histological Feature	High Grade	Low Grade
Histological Grade (E&S)	51 (58%)	37 (42%)
Nuclear Grade	42 (48%)	46 (52%)
Nucleolar Grade	62 (70%)	26 (30%)
Architectural Grade	49 (57%)	39 (43%)
Cellular Crowding	51 (58%)	37 (42%)

Table 6 – Histological results (Nodules, n = 230)

Histological Feature	High grade	Low Grade
Histological Grade (E&S)	142 (62%)	88 (38%)
Nuclear Grade	92 (40%)	138 (60%)
Nucleolar Grade	129 (56%)	101 (44%)
Architectural Grade*	116 (52%)	108 (48%)
Cellular Crowding	127 (55%)	103 (45%)

* Architectural grade could not be adequately assessed in 6 nodules due to tissue fragmentation in the TMA blocks.

4.1.3 Immunohistochemical results

The results for the IHC analyses in the 88 patients and 230 nodules are summarized in **Tables 7 and 8**, respectively. There was no expression of CD 133 in the samples, and the expression of CD 117 was restricted to two nodules from a single patient. These markers were excluded from further analyses. Conversely, there was a high expression of Claudin 1, Arginase and HepPar1,

which suggests that most HCC samples retained their epithelial properties and, to some extent, their hepatocellular differentiation. Of note, the six nodules with Vimentin expression also showed loss of Claudin 1 and sarcomatoid histology. These results recapitulate previous reports[42,88] and show how histological features – specifically spindle cell morphology – can suggest EMT properties in HCC samples.

Table 7 – Immunohistochemical results (Patients, n = 88)

Marker	Positive	Negative
Wnt signaling markers	23 (26%)	65 (74%)
HepPar1	80 (91%)	8 (9%)
Arginase	87 (99%)	1 (1%)
CD 10	76 (86%)	12 (14%)
CD 117	1 (1%)	87 (99%)
CD 133	0	88 (100%)
Keratin 19	18 (20%)	70 (80%)
CD 44	25 (28%)	63 (72%)
EpCam	4 (5%)	84 (95%)
Vimentin	1 (1%)	87 (99%)
Loss of Claudin 1	4 (5%)	84 (95%)

Table 8 – Immunohistochemical results (Nodules, n = 230)

Marker*	Positive	Negative
Wnt signaling markers	33 (15%)	192 (85%)
HepPar1	185 (82%)	40 (18%)
Arginase	202 (90%)	23 (10%)
CD 10	142 (63%)	84 (37%)
CD 117	2 (1%)	223 (99%)
CD 133	0	225 (100%)
CD 44	45 (20%)	181 (80%)
Keratin 19	42 (19%)	182 (81%)
EpCam	10 (4%)	215 (96%)
Vimentin	6 (3%)	217 (97%)
Loss of Claudin 1	15 (7%)	210 (93%)

* There were minor physical losses associated with the TMA technique, thus affecting the total number of nodules assessed by IHC.

4.1.4 DNA sequencing results

The outline of the sequencing experiments is depicted in **Figure 16**. Eight patients with multiple hepatic and extra-hepatic nodules, residual tissue following TMA spotting and low post-mortem interval (< 12 hours) were included for DNA sequencing analyses. Fifty-five samples from these eight patients were selected for DNA extraction including 31 primary nodules, 16 metastatic nodules and eight samples from non-tumoral tissue (used to call somatic mutations).

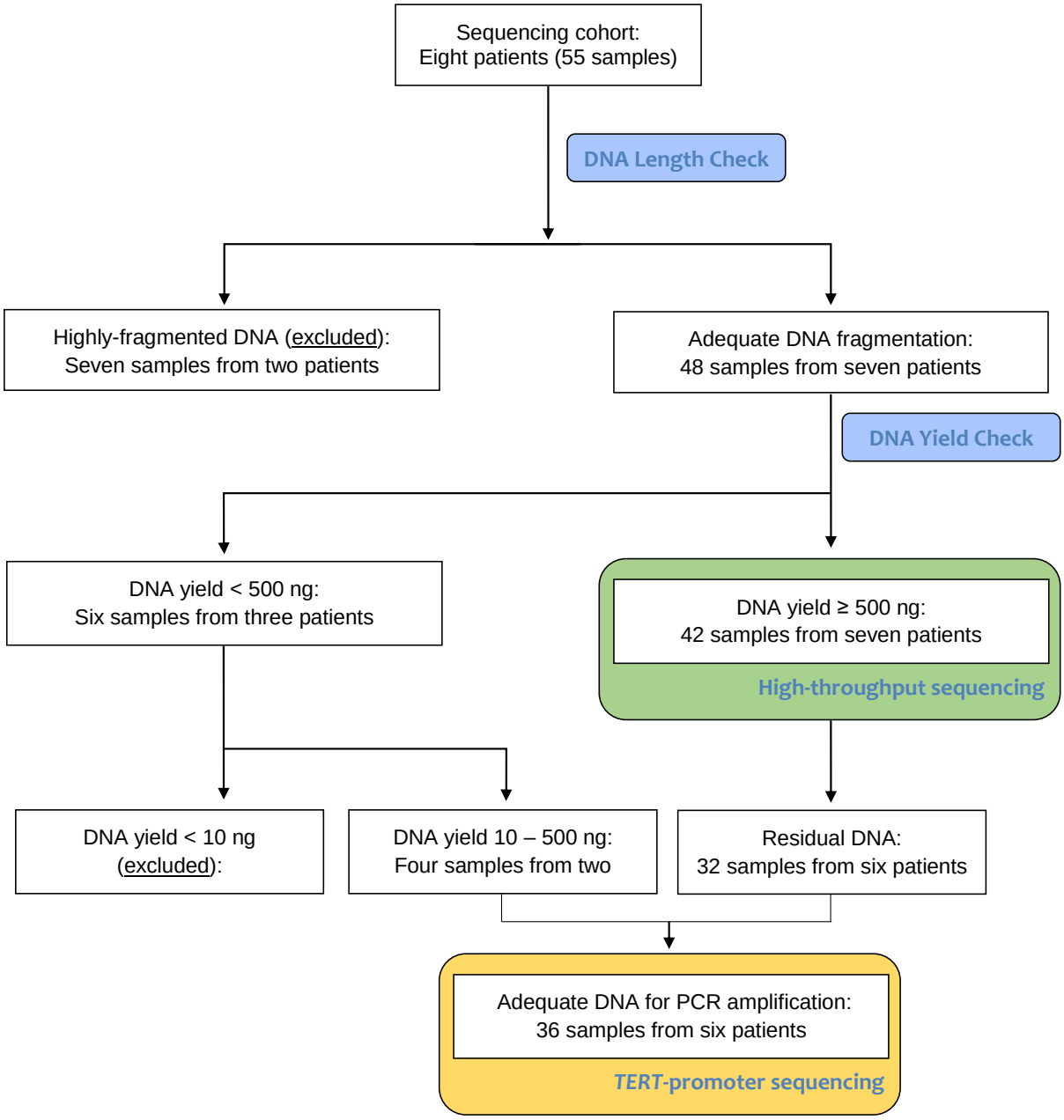

Figure 16 – Flowchart of the sequencing experiments

Seven samples presented with high DNA degradation and were considered not suitable for sequencing (**Figure 17A**). Additionally, two samples had very low DNA content (total DNA yield < 10 ng after 2 consecutive extractions) and were also excluded. Notably, 6/9 inappropriate samples came from the same patient.

The remaining 46 samples with adequate DNA yield showed DNA fragmentation consistent with formalin-fixation (**Figure 17B**) and were included in the sequencing experiments.

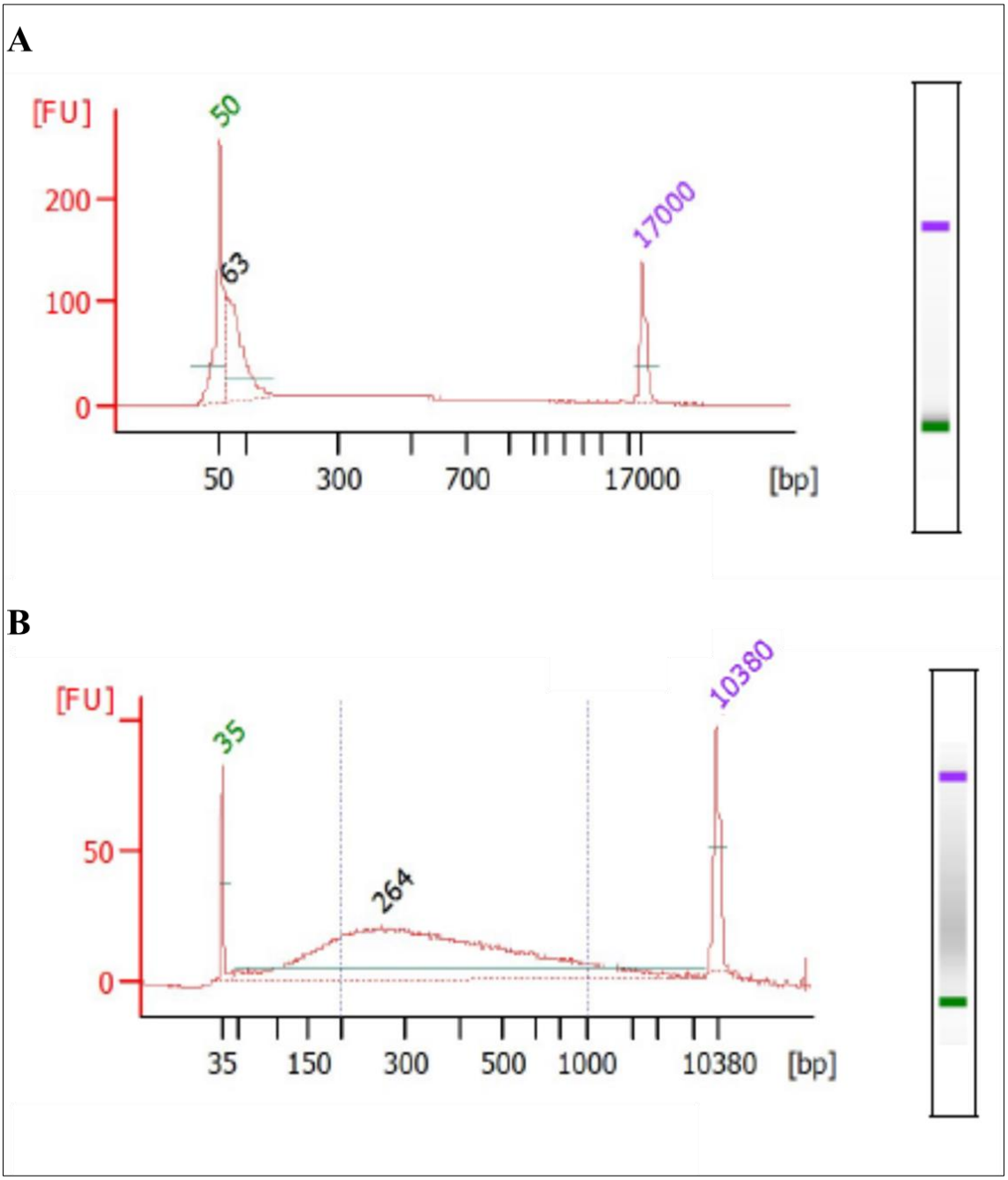

Figure 17 – A: Sample with very high DNA degradation, with average DNA fragment of 63 base-pairs. B: Typical FFPE sample, with average DNA fragment of 264 base-pairs.

Forty-two samples from seven patients had DNA yield > 500 ng and were submitted to library enrichment and high-throughput DNA sequencing at the Genomics Core of ISMMS. These included 20 primary nodules, 15 metastatic nodules and seven samples from non-tumoral tissue. Most of the samples failed to yield adequate sequencing libraries and the experiment was discontinued. Some technical missteps were identified during library enrichment (most notably excessive DNA shearing) that might have been implicated in these results. Alternatively, DNA-DNA or DNA-protein crosslinking due to formalin overexposure could have precluded adequate DNA denaturation, which is an important analytical step that precedes DNA amplification in samples submitted for sequencing[106,113].

Thirty-six samples from six patients – including 32 submitted to targeted amplicon sequencing – were also submitted to PCR amplification of the *TERT*-promoter region followed by Sanger sequencing. These included 17 primary nodules, 13 metastatic nodules and 6 samples from non-tumoral tissue. Six tumor samples did not meet quality criteria for confident mutation calling. Mutations in the two reported hotspots for *TERT*-promoter mutations were detected in 8/24 (33%) nodules from 3/6 (50%) patients. Wild-type sequences were identified in 16/24 (67%) nodules and in the non-tumoral tissue from all the patients (**Figure 18**). There were no significant associations between *TERT*-promoter mutation status and histological or IHC features, likely due to the small sample size.

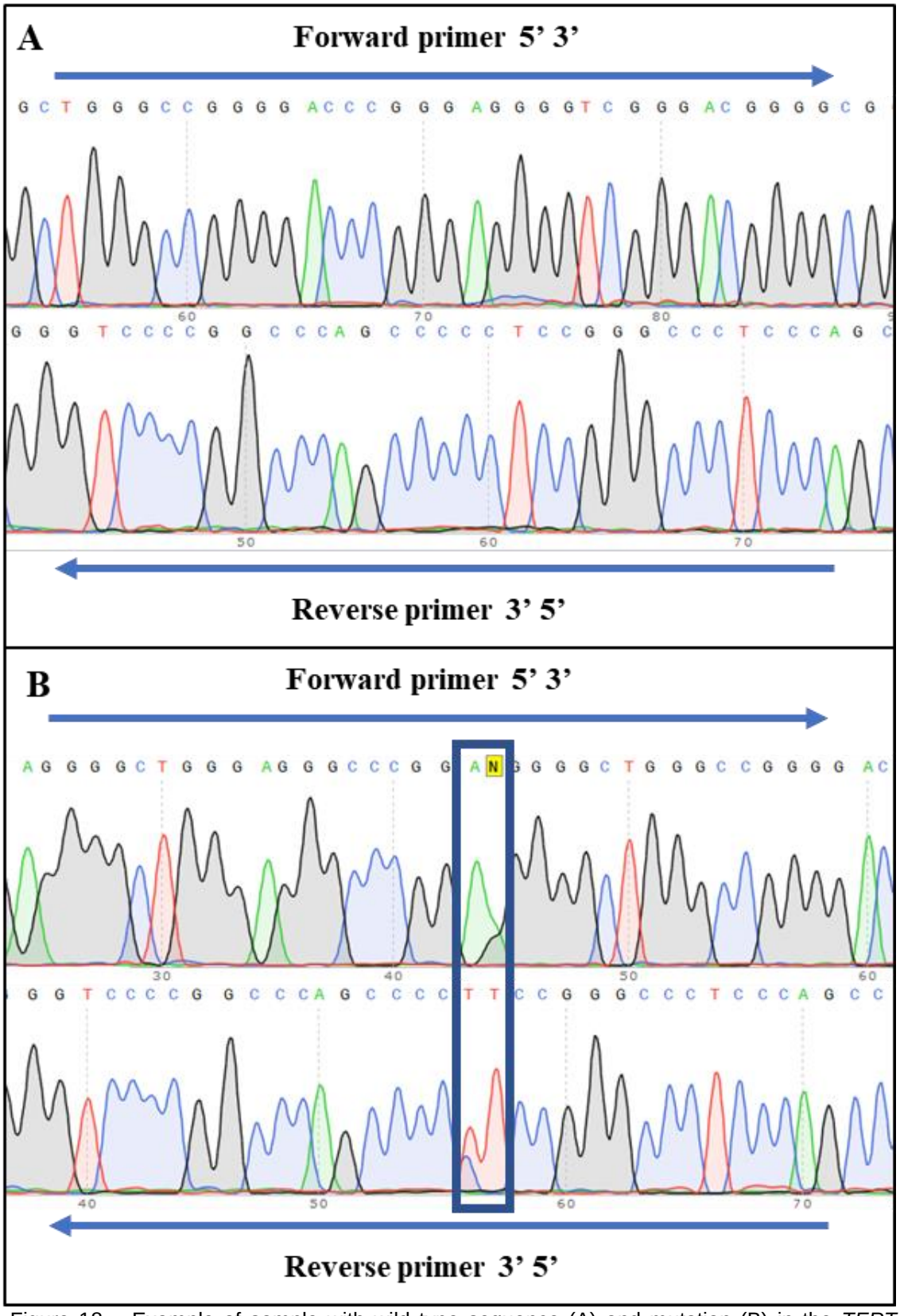

Figure 18 – Example of sample with wild type sequence (A) and mutation (B) in the *TERT* promoter region.

4.2 Descriptive analyses of the extra-hepatic metastases

4.2.1 Histological findings

The histological features of the extra-hepatic metastases are summarized in **Table 9**. Metastases usually showed high histological grades: 32/36 (89%) nodules were grades III or IV according to E&S. Exceptions included patients A006, A024 and A025, who presented with at least one low grade metastatic nodule (**Figure 19**). HCC histological subtypes were identified in 4/36 (11%) extra-hepatic nodules, including sarcomatoid HCC in the lung and lymph node metastasis from one and clear cell HCC in both lung nodules from another patient. Lymphocyte-rich, scirrhous and steato-hepatitic HCC were not identified in the metastatic disease.

Table 9 – Histological results in the extra-hepatic nodules (n = 36)

Histological Feature	High grade	Low Grade
Histological Grade (E&S)	32 (89%)	4 (11%)
Nuclear Grade	20 (56%)	16 (44%)
Nucleolar Grade	21 (58%)	15 (42%)
Architectural Grade*	21 (68%)	10 (32%)
Cellular Crowding	22 (61%)	14 (39%)

* Architectural grade could not be adequately assessed in five nodules due to tissue fragmentation in the TMA blocks.

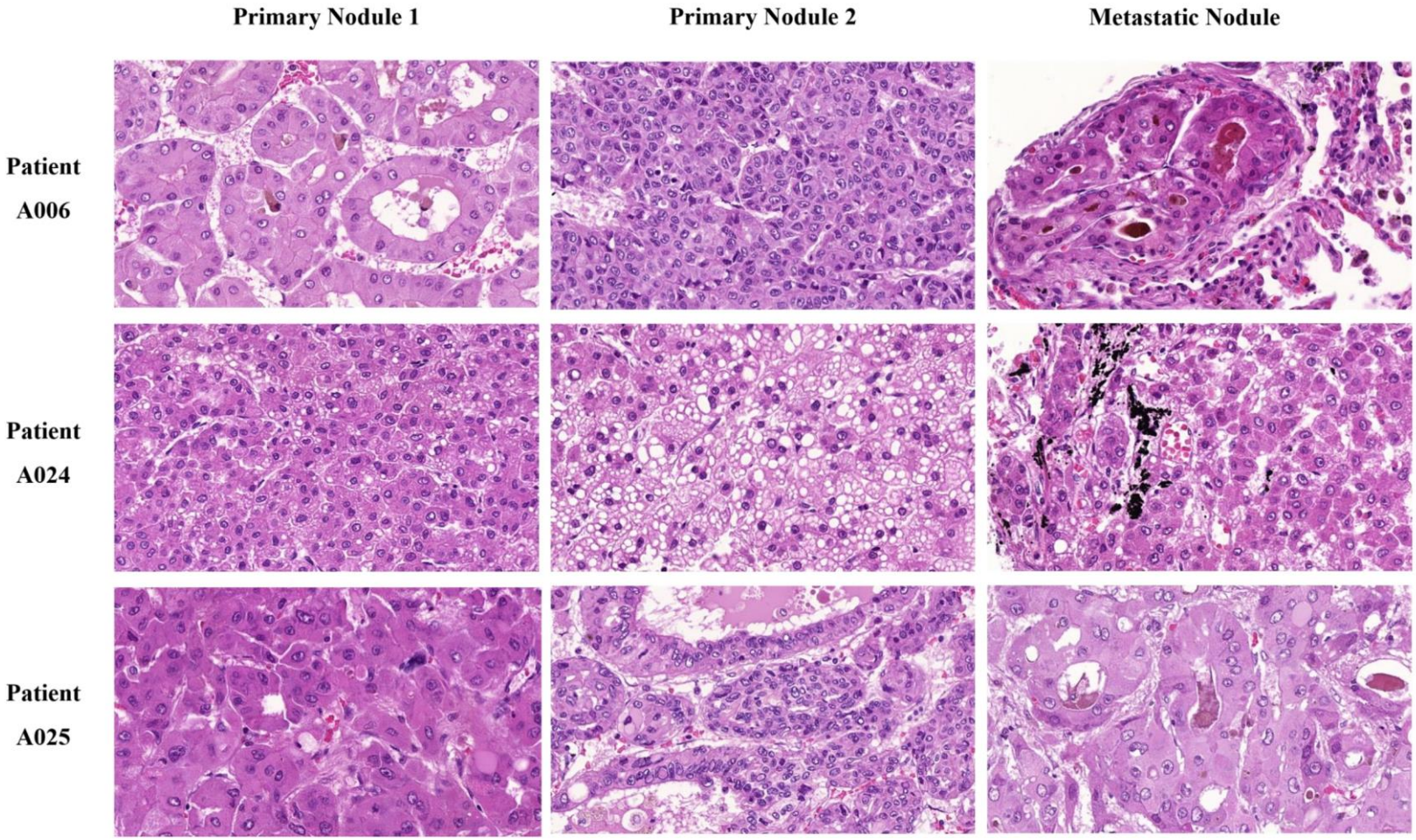

Figure 19 – Histological representation of primary and metastatic nodules from patients A006, A024 and A025. These patients showed low grade metastatic disease. Interestingly, patients A006 and A025 had both low- and high-grade nodules in the primary disease. SOURCE: Martins-Filho *et al.* Histopathology;2019[108].

4.2.2 IHC findings

The IHC results in the extra-hepatic nodules are summarized in **Table 10**. The expression of markers of hepatocyte differentiation was high in the cohort (225/230 nodules, 98%), including the metastatic disease. Indeed, the expression of either HepPar1, Arginase or CD 10 was observed in all 36 extra-hepatic nodules, suggesting that this combination of markers of hepatocyte differentiation is useful for defining the tissue of origin in challenging situations (e.g. aggressive disease with widespread dissemination). Markers of active Wnt signaling pathway showed similar distribution within primary and metastatic tumors (4/34, 12% vs. 29/191, 15%, p=1). Although not statistically significant, expression of stem-like markers was higher in metastatic rather than in primary nodules. In fact, the expression of either K19, EpCam or CD 44 was observed in 16/34 (44%) metastatic nodules and in 60/194 (31%) primary nodules (p=0.08). Markers of EMT were not frequent in the cohort. Vimentin expression was observed in 2/33 (6%) metastatic and in 4/190 (2%) primary nodules. Loss of Claudin 1 expression was noted in 3/34 (9%) metastatic and in 12/190 (6%) primary nodules. Notably, all nodules with positive IHC staining for Vimentin also had loss of Claudin 1 expression.

Table 10 – IHC expression in extra-hepatic HCC nodules (n = 36)

Marker*	Positive	Negative
Hepatocyte differentiation		
HepPar1	26 (76%)	8 (24%)
Arginase	30 (86%)	5 (14%)
CD 10	18 (55%)	15 (45%)
Any hepatocyte marker	36 (100%)	0
Wnt signaling pathway		
β-catenin and Glutamine Synthetase	4 (12%)	30 (88%)
Progenitor cell properties		
Keratin 19	14 (41%)	20 (59%)
EpCam	4 (12%)	30 (88%)
CD 44	6 (17%)	29 (83%)
Any progenitor marker	16 (46%)	19 (54%)
Epithelial-mesenchyme transition		
Vimentin	2 (6%)	31 (94%)
Loss of Claudin 1	3 (9%)	31 (91%)

*There were minor physical losses associated with the TMA technique, thus affecting the total number of nodules assessed by IHC.

4.2.3 *TERT*-promoter sequencing results

TERT-promoter mutations were identified in four metastatic nodules to the lungs in two patients. Eight metastatic nodules from four patients were wild type for the *TERT*-promoter region.

4.2.4 Phenotypic comparisons between primary-metastatic nodules

All the metastatic nodules in the cohort shared the histological, IHC, and TERT promoter sequencing results with at least one nodule from its corresponding primary disease. In other words, all metastases could be traced back to the primary tumor. **Figure 20** illustrates this finding, with emphasis on histological grade (E&S), K19 and EpCam. Although most metastases reproduced the highest grade found at the primary hepatic nodules, patients A006 and A025 presented with low grade metastases despite at least one nodule from their primary disease being of high grade. As mentioned before, patient A024 also showed low grade metastasis, but all primary nodules from this patient were also of low histological grade. The histological and IHC results were further validated with the TERT-promoter sequencing analysis. The two patients with TERT-promoter mutations in the metastatic disease also presented with mutations in the primary disease. Conversely, the four patients with wild-type metastatic disease had at least one wild type primary nodule.

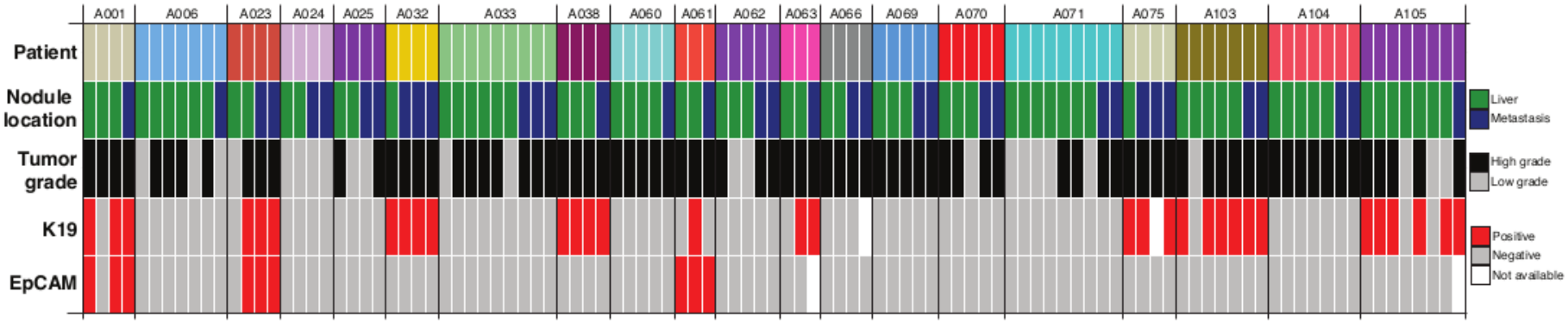

Figure 20 – Metastatic nodules recapitulated at least one nodule from the primary disease. Highlighted here are the phenotypic criteria associated with the presence of metastases in this cohort (Histological grade, Keratin 19 and EpCam). SOURCE: Martins-Filho *et al.* Histopathology;2019[108].

4.3 Correlative analyses: association studies

4.3.1 Clinical and pathological criteria associated with distant metastases

AFP serum concentration ≥ 100 ng/mL ($p=0.002$), dominant nodule ≥ 5.0 cm ($p<0.001$), multi-nodularity ($p=0.001$), macrovascular invasion ($p<0.001$), high histological ($p<0.001$), nuclear ($p=0.002$) and architectural ($p<0.001$) grades, cellular-crowding ($p=0.002$), and expression of K19 ($p=0.004$) and EpCam ($p=0.035$) in the primary disease were associated with the presence of distant metastases in the cohort. CD44 and markers of Wnt signaling activation did not show association with extra-hepatic dissemination, but were identified in the metastatic disease from four and three patients, respectively. Similarly, Vimentin expression was observed in the lung and lymph node metastases (as well as four primary nodules) from a single patient.

A binomial logistic regression model was designed to further describe the patterns of metastasis in these patients. Macrovascular invasion and the biochemical markers were not included in this model because data was not available for more than 10% of the samples. Tumor size ($p=0.003$), multi-nodularity ($p=0.05$) and, to a lesser extent, high tumor grade ($p=0.06$) were independently associated to the presence of metastasis in our model. When focusing on histological predictors of the presence of lung microvascular metastases, K19 staining ($p=0.02$), and high E&S grades ($p=0.01$) and architectural ($p=0.01$) grades showed significant association.

Metastatic nodules, when compared to the primary ones, showed a higher prevalence of high histological (55% vs. 83%, $p=0.0015$) and nuclear (33% vs.

53%, p=0.036) grades. There were no significant differences between these groups for architectural grade (p=0.075) and cellular crowding (p=0.72). Among the IHC markers tested, metastatic nodules were enriched for Keratin 19 (38% vs. 15%, p=0.009) and EpCam (12% vs. 3%, p=0.04).

4.3.2 Association amongst histological features

An MCA plot was designed to cluster the HCC nodules according to the histological grades and to illustrate associations between these histological features (**Figure 21**). First, it shows that the two major sample clusters correspond to nodules that were ubiquitously high grade (far left) or low grade (far right) to all the histological grades evaluated. In fact, 45/230 (20%) nodules were high grade and 37/230 (16%) nodules were low grade according to all histological criteria. There was also a notable prevalence of nodules that were low grade to all histological variables but nucleolar grade (20/230, 9%) and nodules that were high grade to all histological variables but cellular crowding (16/230, 7%). Also, the MCA plot suggests that E&S and architectural grades were the variables with the strongest association (smallest angle within vectors). This was ratified by the high phi coefficient score between these variables (φ=0.78, p <0.001). Conversely, nucleolar grade and cellular crowding showed a much weaker association (φ = 0.12, p =0.07).

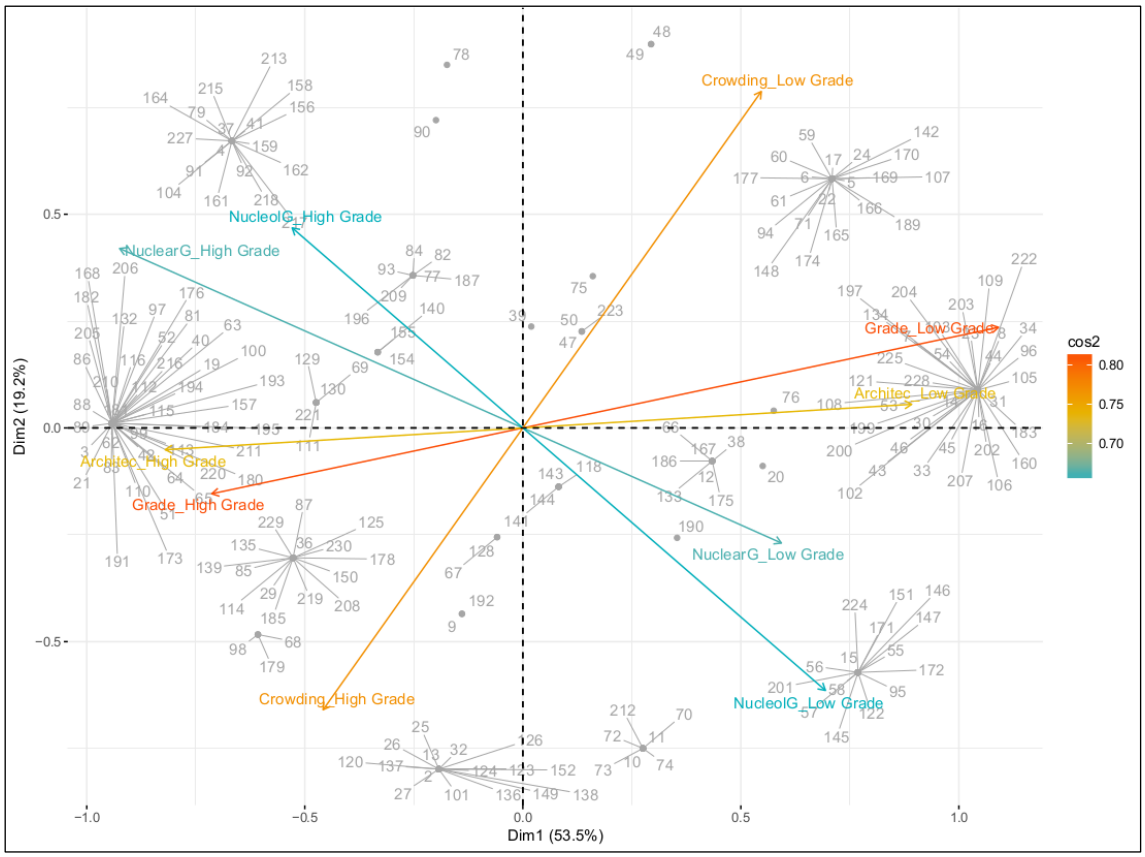

Figure 21 – MCA plot depicting the distribution of the nodules according to the histological variables. Each number represents an individual nodule (mapped to a dot). The association between two variables is defined by the angle between their vectors (arrows).

4.3.3 Association amongst IHC markers

Positive associations between IHC markers are depicted in **Table 11**. Co-expression of markers from the same biological category were common. Also, progenitor-cell markers and markers of EMT properties were often associated. Surprisingly, CD 44 (progenitor-cell) and CD 10 (hepatocyte differentiation) showed strong positive association. In fact, 35/224 (16%) nodules from 20/88 (23%) patients showed expression of both markers. These nodules also showed a higher prevalence of high nucleolar grade (86% vs. 51%, p<0.001), high architectural grade (69% vs. 49%, p=0.042) and low cellular crowding (63% vs.

42%, p=0.026) when compared to the remaining nodules in the cohort. There was no ubiquitous clinical feature in these nodules/patients.

Table 11 – Association amongst IHC markers (co-expression)

Co-expressed IHC Markers	Degree of association
Vimentin x Loss of Claudin 1	$p < 0.001$
CD 44 x Loss of Claudin 1	$p < 0.001$
Keratin 19 x Vimentin	$p < 0.001$
Keratin 19 x EpCam	$p < 0.001$
CD 44 x Vimentin	$p < 0.001$
Keratin 19 x Loss of Claudin 1	$p < 0.001$
HepPar 1 x Arginase	$p < 0.001$
CD 44 x Keratin 19	$p = 0.009$
CD 44 x CD 10	$p = 0.04$
CD 10 x HepPar 1	$p = 0.045$

Only statistically significant associations are presented.

The association analyses also identified mutually exclusive IHC markers (**Table 12**). Most commonly, markers of hepatocyte differentiation were negatively associated to progenitor-cell markers (except for CD 10 and CD 44) and markers of EMT properties. Keratin 19 and markers of active Wnt signaling pathway were also mutually exclusive: within the 42 K19 positive nodules and the 33 Wnt positive nodules, only one expressed both markers.

Table 12 – Association amongst IHC markers (mutual exclusivity)

Mutually exclusive IHC Markers	Degree of association
Arginase x Loss of Claudin 1	$p < 0.001$
Arginase x Vimentin	$p < 0.001$
Keratin 19 x Arginase	$p < 0.001$
EpCam x CD 10	$p < 0.001$
HepPar 1 x Loss of Claudin 1	$p = 0.006$
Wnt signaling x Keratin 19	$p = 0.008$
Keratin 19 x CD 10	$p = 0.009$
Keratin 19 x HepPar 1	$p = 0.011$

Only statistically significant associations are presented.

4.3.4 Association between IHC biological categories and histological features

4.3.4.1 Hepatocyte differentiation and histological features

The association between markers of hepatocyte differentiation and the histological grades is depicted in **Table 13**. Expression of HepPar1 and Arginase were strongly associated with low histological, architectural and nuclear grades, and weakly associated with low nucleolar grade and cellular crowding. CD 10 staining showed strong association with low E&S grade, low cellular crowding, weak association with low nuclear grade, and no association with nucleolar and architectural grades.

Table 13 – Association between markers of hepatocyte differentiation and histological features (p values)

Marker	Histological Grade (low)	Architectural Grade (low)	Nuclear Grade (low)	Nucleolar Grade (low)	Crowding (low)
HepPar1	**0.001**	**0.002**	**0.007**	0.16	0.164
Arginase	**0.04**	**0.03**	**0.04**	0.19	0.18
CD 10	**0.04**	0.89	0.07	0.27	**<0.001**

Significant associations were highlighted (bold)

4.3.4.2 Active Wnt signaling pathway and histological features

The expression of markers of active Wnt signaling pathway did not show strong association with any histological feature. There was, however, a trend between the expression of these markers and high tumor grade (p=0.083) and cellular crowding (p=0.089).

4.3.4.3 Progenitor cell properties and histological features

The expression of K19, EpCAM or, to a lesser extent, CD 44 was associated with multiple histological features of aggressive behavior (**Table 14**). Notably, all three markers were strongly associated with high architectural and nuclear grades.

Table 14 – Association between progenitor-cell markers and histological features (p values)

Marker	Histological Grade (high)	Architectural Grade (high)	Nuclear Grade (high)	Nucleolar Grade (high)	Crowding (high)
Keratin 19	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.06
EpCam	**0.008**	**0.003**	**0.02**	0.19	0.36
CD 44	0.49	**0.01**	**0.04**	**<0.001**	0.07

Significant associations were highlighted (bold)

4.3.4.4 Expression of markers of epithelial-mesenchyme transition

The associations between EMT properties and histological features are presented in **Table 15**. Although not statistically significant, all nodules with Vimentin expression showed high E&S grades.

Table 15 – Association between EMT markers and histological features (p values)

Marker	Histological Grade (high)	Architectural Grade (high)	Nuclear Grade (high)	Nucleolar Grade (high)	Crowding (high)
Vimentin	0.08	**0.03**	0.22	**0.04**	0.23
Loss of Claudin 1	**<0.001**	**<0.001**	**0.002**	0.1	0.06

Significant associations were highlighted (bold)

4.4 Heterogeneity analyses in the primary and metastatic disease

Fifty-five patients in the cohort presented with at least two hepatic nodules according to imaging exams or pathological analyses. In three of these patients, histological representation was restricted to the main HCC tumor, i.e., there was no FFPE block available from the secondary nodule(s). Furthermore, in two patients, there was tissue loss associated with the TMA technique. Hence, 50 patients were included for the internodular heterogeneity analysis. Among those, 12 patients also presented with multiple extra-hepatic nodules.

Heterogeneity for histological grades was detected in 42/50 (84%) patients with multinodular hepatic disease. IHC heterogeneity was less pronounced, being detected in 27/50 (54%) of the cases. Furthermore, IHC heterogeneity was usually conditioned to histological heterogeneity in the cohort, i.e., samples that were heterogenous for at least one IHC marker were also heterogeneous for any of the histological grades. The only exception was patient A035, who presented with two high grade HCC nodules, but only one showed focal expression (5%) of CD 44.

Significant heterogeneity – combined histological and IHC heterogeneity – was detected in 26/50 (52%) of the cases. Notably, heterogeneity was less pronounced in the extra-hepatic nodules, being observed in only 2/12 (17%) patients with multiple metastatic samples: patients A033 and A025. Patient A033 showed multiple metastatic nodules to the adrenal glands, with different histological grades and patterns of HepPar1 expression; one of the metastatic nodules even displayed a metastasis-to-metastasis phenotype (**Figure 22**).

Patient A025 showed expression of markers of active Wnt signaling in the lymph nodes, but not in the lung metastases (**Figure 23**).

These results were further validated by the evaluation of TERT-promoter mutations. Mutational heterogeneity was detected in the primary disease of 2/6 patients. The remaining four patients were either wild-type (n=3) or had TERT-promoter mutation (n=1) in all the hepatic nodules. However, there was no mutational heterogeneity within the metastatic disease. In other words, the TERT-promoter mutation status was the same in all metastatic sites from a given patient (**Figure 24**).

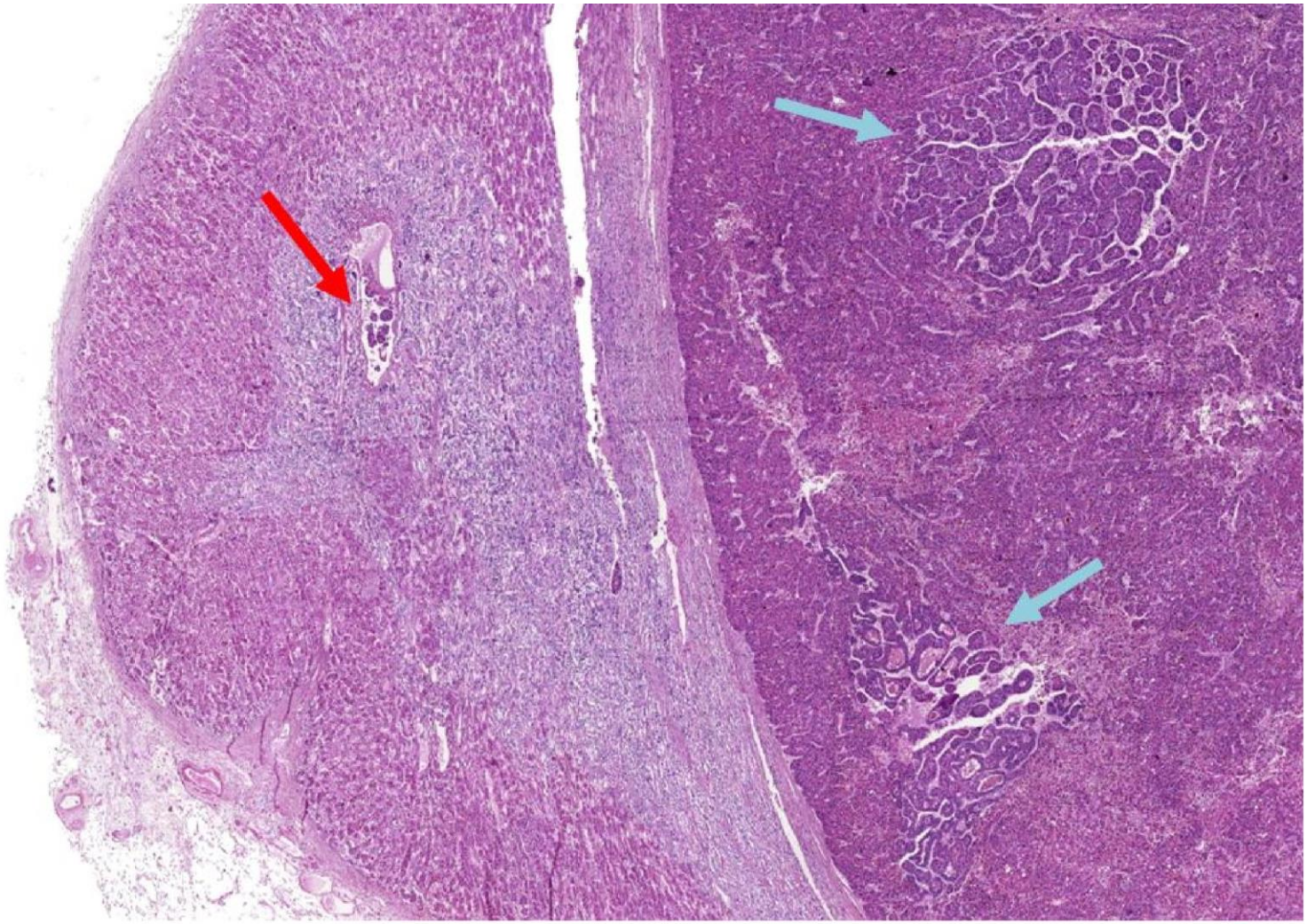

Figure 22 – Histological representation of an adrenal metastasis with two distinct histological patterns. Interestingly, a tumor with higher cellular crowding and number of mitosis (blue arrows) seems to be colonizing the previously established metastasis. We also found multiple emboli in the primary and metastatic disease (red arrow) with clusters of cells from the “colonizing” tumor. H&E staining – 20x. SOURCE: Martins-Filho *et al.* Histopathology;2019[108].

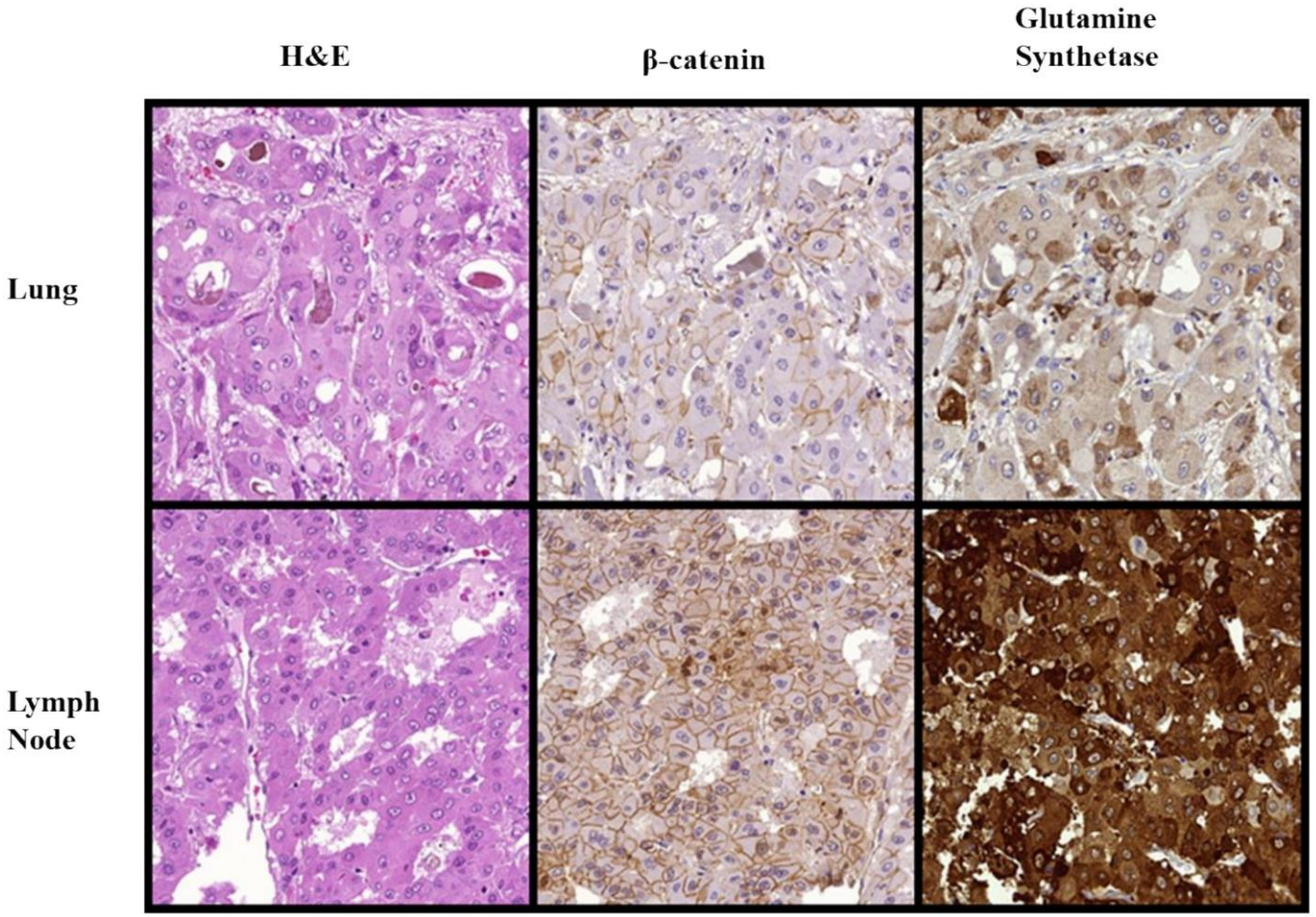

Figure 23 – Differential expression of Wnt markers between the lung and lymph node metastasis. The lung metastases showed lower cellular crowding and some bile production. It was negative for the markers associated with Wnt activation. On the other hand, the lymph node metastasis showed higher cellular crowding, no bile production (but pseudocysts with red blood cells) and was positive for markers of Wnt activation. The primary nodules reproduced the heterogeneity observed in the metastases. H&E staining – 200x. SOURCE: Martins-Filho *et al.* Histopathology;2019[108].

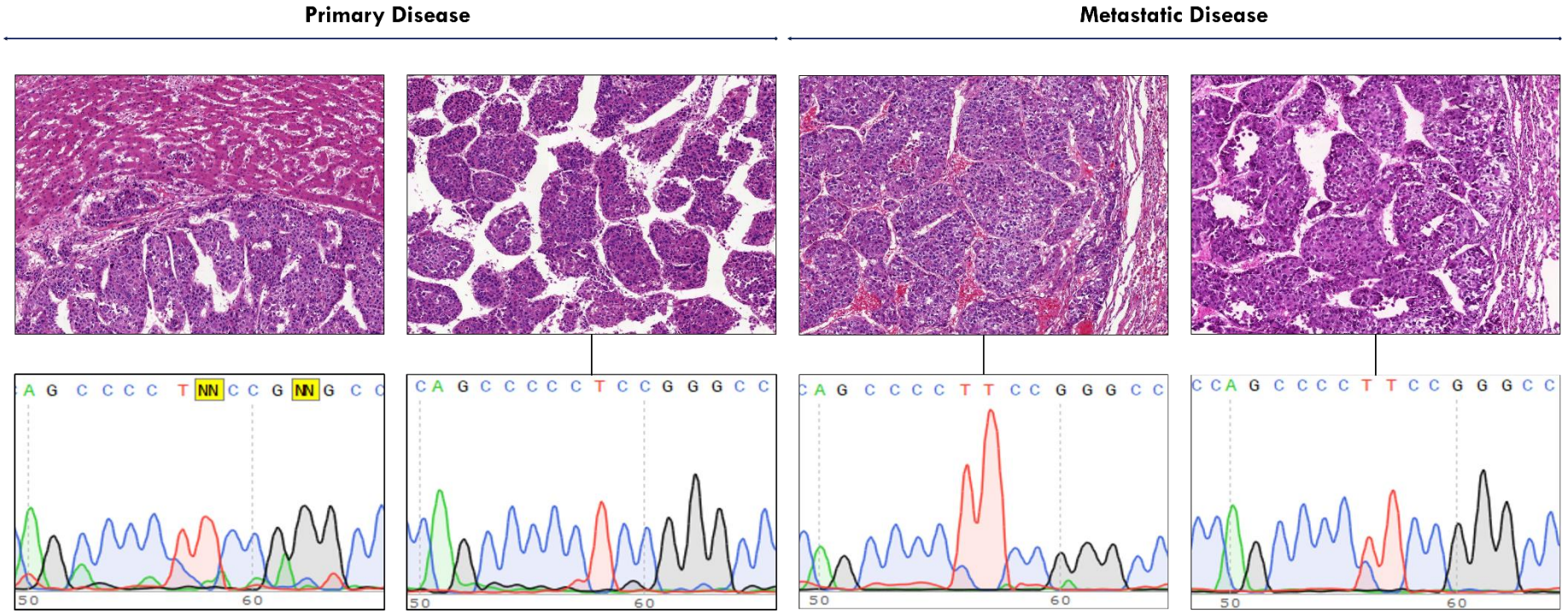

Figure 24 – Tumor heterogeneity was attenuated within the metastatic disease. This patient presented with multiple liver and lung nodules. One primary nodule depicted a hotspot *TERT*-promoter mutation while another didn't. Two metastatic nodules from different lung lobes were homogeneous at the morphological and molecular levels: they both presented with the same hotspot TERT-promoter mutation observed in one of the liver nodules. H&E staining – 100x. SOURCE: Martins-Filho *et al.* Histopathology;2019[108].

5. DISCUSSION

This study provides a comprehensive phenotypic evaluation of 230 nodules collected from 88 autopsy specimens from patients with HCC. Fifty-five (55/88, 63%) patients had multi-nodular disease and, from those, 50 had adequate histological representation of multiple hepatic nodules, supporting inter-nodular heterogeneity analyses. Metastases were identified in 20/88 (23%) autopsy specimens, from which paired primary-metastatic comparisons were derived. In 12/20 (60%) autopsies from patients with metastatic disease, multiple metastatic nodules were adequately sampled, allowing for an unprecedented analysis of phenotypic inter-nodular heterogeneity of HCC extra-hepatic sites.

The low incidence of metastases in the cohort suggests that few patients with HCC die due to extra-hepatic dissemination. A retrospective analysis of CT-scans from 402 patients with HCC revealed that only 37% developed metastases. Furthermore, most of these patients had advanced liver tumors when the metastatic disease was detected[114]. These autopsy and imaging data indicate that HCC opposes other solid malignancies, where metastatic spread is a major event in tumor progression and is responsible for more than 90% of cancer-related deaths[115]. In fact, metastases are a common mechanism of progression or resistance to therapy in breast, pancreatic, colorectal and lung cancer, guiding the management and follow-up of patients under treatment[103,116,117]. Conversely, the major causes of death in HCC include growth of the primary tumor and complications of background cirrhosis (e.g., gastrointestinal bleeding, sepsis). The emergence of new therapies for viral hepatitis, cirrhosis and HCC (e.g., immune checkpoint inhibitors) might change this panorama: improved control of

the hepatic disease might increase the role of metastases as a mechanism of resistance and disease progression in this cancer. It is thus crucial to identify clinical cues that predict for HCC dissemination, report the topography of metastases and describe the phenotypic and molecular composition of the extra-hepatic disease.

HCC has strong hematogenous tropism and predilection for lung dissemination. Imaging analysis shows that 55% of HCC patients with metastatic disease have lung involvement, followed by abdominal lymph nodes (41%) and bones (28%)[114]. The major limitation of such studies is the lack of morphological assessment of the metastases, particularly to confirm the tumor's hepatocellular lineage. Cancer progression is usually followed by phenotypic changes and early-stage HCC can often develop into aggressive transitional tumors with morphologic and genetic features of mixed hepatocellular-biliary differentiation[118]. Hence, autopsy specimens are necessary for describing the patterns of evolution in "pure" HCC: they allow for extensive sampling and lineage confirmation in primary and metastatic sites. In the present study, focused on pure HCC, 21/36 (58%) extra-hepatic nodules came from the lungs, followed by 6/36 (17%) from the peritoneum, 5/36 (14%) from the adrenal glands and only 4/36 (11%) from lymph nodes; bone metastases were not available or collected. Notably, the overall low prevalence of lymph node metastases underlines the weak lymphatic tropism of pure HCC; there were no distinctive pathological features in the tumor specimens from patients with lymph node metastases. In contrast to HCC, Addeo *et al* among others described the high prevalence and prognostic value of lymph node metastases in cholangiocarcinoma[119–121]. Additionally, De Vito *et al* elegantly suggested a dichotomous hematogenous-

lymphatic tropism in a case series of combined hepatocellular-cholangiocarcinoma[122]. Autopsy studies investigating the patterns of dissemination, particularly in tumors with mixed phenotype, should follow.

Autopsy specimens also offer the unique opportunity to assess origin and development of metastases. For instance, in this cohort, lung microvascular metastases were detected in 30% of the HCC patients with distant dissemination. These metastatic niches were previously undetected by imaging or upon macroscopic examination, which further endorses the importance of the strict autopsy protocol at HC-FMUSP, particularly sampling of all vital organs (e.g, brain, heart, lung) regardless of any evidence of disease. The clinical significance of these lung microvascular metastases is not fully clear since their actual prevalence is unknown. However, from a biological perspective, these disseminated tumor cells entrapped in a distant organ's vasculature are the first step prior to parenchymal invasion and clinically identifiable metastases[123]. Genome-wide clonal evaluation of the primary disease followed by analysis of the microvascular and full-blown lung metastases could provide insights on the molecular determinants of distant dissemination and even predict the timing of lung metastatic development in HCC. Unfortunately, the high-throughput sequencing experiments proposed here did not yield adequate results. Additional investments in the autopsy service at HC-FMUSP will be important to enable a prospective collection of cryopreserved samples or simply to enhance the quality and timing of formalin fixation, and thus improve the quality of nucleic acids extracted from autopsy samples. Nevertheless, since histological structures and most cell/tissue proteins are remarkably resistant to fixation artifacts, the detailed histopathologic evaluation of the cohort identified high E&S and architectural

grades and K19 staining in the primary disease as strong and independent predictors of the presence of these lung microvascular metastases. In other words, patients with HCC co-harboring these three pathological findings should be followed closely for the development of lung metastases.

Other clinicopathologic features enriched in patients with extra-hepatic disease of any site compared to patients with liver-restricted disease included AFP serum concentration ≥ 100 ng/mL, dominant nodule ≥ 5.0 cm, multi-nodularity, macrovascular invasion, high E&S grade, and expression of K19 and EpCam. These variables have also been associated with recurrence and survival in patients with early-stage HCC, although only few reports, including the present study, have assessed their prevalence in patients with extra-hepatic dissemination. In the logistic regression model, E&S grade showed a near-significant role (p=0.06) for predicting metastases within specimens with multinodular disease and dominant nodule ≥ 5.0 cm. Similarly, Shindoh *et al* showed the importance of tumor differentiation for predicting overall survival in patients with large HCC[124]. Also, the Toronto General Hospital group described the importance of histological grading in selecting patients for liver transplantation beyond Milan Criteria[53,54]. Altogether, these clinical and autopsy data indicate an important and independent role for histological evaluation in predicting outcome (survival or metastatic dissemination) even in large HCC.

Amongst the pathologic features proposed by Martins-Filho & Paiva *et al* for the evaluation of HCC, cellular crowding and high nuclear and architectural grades showed association with distant dissemination. Nucleolar grade showed a trend, although not statistically significant. The use of these cytologic and histologic variables in the study was motivated by the lack of consensus on HCC

histological grading in the literature[60]. This lack of consensus is a consequence of the subjective nature of the major classifications currently adopted, the E&S and WHO grading systems. The use of more objective criteria should lessen interpretation biases, reduce inter-observer (and inter-institutional) variability and finally consolidate the role of histological grading in HCC. Beyond their association with metastatic dissemination, these histological variables correlated with multiple IHC markers of hepatocyte differentiation and stem-like properties. Notably, high nuclear, nucleolar and architectural grades correlated with expression of K19. Conversely, low cellular crowding showed strong association with expression of CD 10. These results are exciting, as they suggest that these histological features – individually or in combination – translate innate biological properties of these tumors. Conceivably, they might even correlate with HCC molecular subclasses. Nevertheless, survival analyses are ultimately required to validate the clinical applicability of these variables. Finally, inter-observer studies should follow, aimed at assessing their reproducibility, particularly compared to current histological grading standards.

The present study also consolidated an extended sampling strategy for TMA construction. TMAs were originally designed as a population-screening tool, where sampling focuses on one (few) representative area(s) from the whole tumor specimen[125,126]. The primary goal of a TMA is to allow high-throughput histological and IHC analyses of samples from multiple patients. Furthermore, IHC reactions are performed in fewer slides, reducing costs and susceptibility to pre-analytical errors. Over the last fifteen years, the laboratory of liver diseases at HC-FMUSP (LIM 14) specialized in the manufacture of TMA blocks, with more than 450 arrays constructed for studies in liver cancer[59,127] as well as other cancer

types[128,129]. In these previous studies, the laboratory has followed the sampling standards for TMA: one-to-five TMA spots were selected to represent the patient specimen. Here, this traditional approach was expanded to include multiple spots from all the nodules histologically available and even distinctive areas within each nodule (see Methods for details). As a result, associations between histological and IHC features were derived from 230 tumor nodules instead of 88 patient specimens, greatly increasing statistical power. Moreover, this strategy still reduced the number of slides stained while allowing for inter-nodular heterogeneity as well as paired primary-metastatic analyses.

Association studies in all 230 nodules further stressed the biological dichotomy between hepatocyte differentiation and stem-like properties in HCC. Expression of HepPar1, Arginase and CD 10 correlated with low tumor grades and showed no association with metastatic spread whereas expression of K19, EpCam and CD 44 correlated with high tumor grades and were enriched in the metastatic disease. These biological properties are intrinsically related to the development of mature hepatocytes from hepatic progenitor cells. As mentioned before, phenotypic changes usually accompany tumor progression, and cellular dedifferentiation and transdifferentiation are well-characterized phenomena in cancer[130,131]. Briefly, cell dedifferentiation is a cellular process in which a mature cell reverts to an earlier developmental stage, i.e., a mature cell transforms into its evolutionary precursor or even into its progenitor cell. Cell transdifferentiation, on the other hand, refers to lineage reprograming of a mature cell, i.e., a mature cell converts into another mature cell without reverting to an earlier developmental stage. In the specific case of the liver, non-tumoral mature hepatocytes show high mRNA and protein expression of markers of

hepatocellular differentiation and low mRNA and protein expression of markers of stem-like properties. Molecular alterations in these cells can induce malignant transformation into HCC, and these tumors tend to initially retain, to the extent of lineage markers, the transcriptomic and proteomic profile of a mature hepatocyte[132]. They are usually better differentiated histologically and show more favorable outcome. Alternatively, hepatic injury and molecular abnormalities can induce transdifferentiation of mature hepatocytes into biliary-tree cells, with further propensity to develop cholangiocarcinoma[133], or promote dedifferentiation of mature hepatocytes into hepatocyte precursors or even hepatic progenitor cells[118]. As these processes unveil, there is reduction in the expression of hepatocyte markers and increase in the expression of biliary and stem-like markers including K19. Of note, hepatocyte precursors and hepatic progenitor cells that reside in the normal liver are also susceptible to molecular alterations and *de novo* malignant transformation[134]. In summary, HCC with expression of hepatocellular markers usually arise from malignant transformation of mature hepatocytes. Conversely, HCC with stem-like markers might arise from *de novo* malignant transformation of hepatocyte precursors or hepatic progenitor cells, or from cellular dedifferentiation of mature-hepatocyte-derived HCC. Nevertheless, these molecular events translate into phenotypic features (most notably tumor differentiation) that can be exposed by morphological and IHC analyses.

Beyond lineage-tracing, IHC studies can sometimes suggest the mutational status of a gene in a tumor. Bioulac-Sage *et al* showed, by IHC, that a combination of nuclear β-catenin and overexpression of glutamine synthetase had an 85% sensitivity and 100% specificity in predicting *CTNNB1*-mutated hepatic adenomas[85]. Similar results were described in HCC[135]. Essentially,

oncogenic *CTNNB1* mutations lead to activation of the canonical Wnt pathway, which then leads to accumulation of cytoplasmic β-catenin and its translocation into the nuclei. In the liver, activation of the Wnt pathway is also associated with up-regulation of genes involved in the glutamine metabolism[136,137], most notably glutamine synthetase. *CTNNB1*-mutated HCC are usually less aggressive tumors that retain hepatocyte-differentiation and show low expression of stem-like markers. These results were further confirmed here by the mutual-exclusivity of Wnt markers and K19 (p=0.008). However, the trend between the expression of Wnt markers and high E&S grade (p=0.08) and high cellular crowding (p=0.09) was a surprising result. Previously, Felipe-Silva *et al* also reported a strong association between the expression of nuclear β-catenin and high Ki-67 labeling index (p<0.0005)[59]. Albeit intriguing, these results might be explained by the higher prevalence of advanced-stage HCC in autopsy cohorts. Conversely, most studies assessing *CTNNB1* mutation status and β-catenin/glutamine synthetase expression were performed in earlier stage tumors. Conceivably, early-stage Wnt-activated HCCs, as they progress, could acquire novel molecular events and evolve into highly-cellular and poorly-differentiated tumors. Interestingly, this trend between Wnt markers and aggressive morphology did not translate into a higher prevalence of metastases. Indeed, the expression of Wnt markers was observed in 12% of the extra-hepatic nodules, close to the 18% prevalence in the hepatic tumors (overall expression: 15%).

Morphological, functional and genetic tumor heterogeneity have long been reported in HCC. In fact, Edmondson and Steiner, in their pivotal histological grading publication from 1954, have already described “extreme variation in maturity of cells” and the contiguous presence of different tumor grades in the

same tumor specimen[36]. In 1987, Kenmochi *et al* objectively quantified those differences and reported intratumor heterogeneity for E&S grades in 31/65 (47.7%) surgically resected HCC[138]. In 1979/1980, the liver pathology group at ISMMS assessed, in back-to-back publications, the expression of liver-related serum antigens and cell-function enzymes and suggested, by co-staining patterns, that HCC cells are functionally heterogeneous. Also, studies from the late 1980s and the 1990s investigated DNA ploidy heterogeneity in HCC, with conflicting results[139–141]. These publications advanced the characterization of HCC and provided insights into the mechanisms of hepatocarcinogenesis and tumor progression, but had, at that time, limited clinical application for patient management.

More recently, advent of novel chemotherapeutic agents, multi-kinase inhibitors and targeted therapies among others has resurfaced the scientific interest in understanding intratumor heterogeneity and how it can affect therapy response. In fact, intratumor heterogeneity is considered a major determinant for failure in phase III clinical trials following promising results in preclinical models. Furthermore, improvements and ease of access to microarray and sequencing technologies allowed for cheaper and higher throughput genomic analyses of multiple tumor regions and provided novel details about the impact of tumor heterogeneity in cancer progression, development of metastases and resistance to drug therapies. In an elegant publication, Zhai *et al* performed whole-genome and exome sequencing of multiple tumor regions from 11 HCC specimens, including two from recurrent disease. The authors showed different degrees of genetic variability across the HCC specimens, i.e., some tumors showed high while others low genetic diversity. They also suggested that minimal genetic

adaptations are required for the development of intra-hepatic metastases in the form of recurrent disease. However, genetic intratumor heterogeneity was enhanced within these intra-hepatic metastases compared to their corresponding primary tumors[142]. Hence, response to therapy could be less predictable in intra-hepatic metastases compared to their primary counterparts. Lin *et al* investigated both genomic and epigenomic heterogeneity in HCC by whole exome sequencing of 52 tumor regions from 11 specimens and methylation-array analyses of 22 samples from five specimens. Those authors reported high epigenomic heterogeneity in HCC, although the biological and clinical implications of those findings are still largely unknown. They also described branched distribution of 10/34 (29%) putative driver mutations in HCC, i.e., these mutations were not present in all regions of a tumor specimen. However, mutations in major cancer drivers such as *TP53, KIT, SYK* and *PIK3CA* were all truncal, i.e., ubiquitous to all tumor regions, and should be favored in drug screening experiments[143]. Unfortunately, both studies overlooked the degree of histological heterogeneity in these different tumor regions and did not evaluate extra-hepatic sites.

In fact, few studies have performed integrative analyses of histological, immunohistochemical and genomic heterogeneity in HCC. In a cohort of small HCC, An *et al* identified heterogeneity for E&S grades in 14/41 surgical specimens. In 11 of those samples, the authors assessed the mutation status of *TP53* exons 5 and 8 and *CTNNB1* exon 3, and performed IHC for Ki67, p53 and β-catenin in serial tumor sections. Interestingly, genetic heterogeneity was observed in the patient with *CTNNB1* mutation: the poorly-differentiated but not the well-differentiated tumor region had a point mutation in this gene. Nuclear expression of β-catenin was also present in this specimen and was higher in the

mutated tumor region. Nuclear β-catenin was also detected in 1/10 *CTNNB1* wild-type specimen, but mutations in other *CTNNB1* loci could not be ruled out. *TP53* mutation was detected in one specimen and was ubiquitous to all tumor regions; p53 expression was also present in this sample. However, 7/10 *TP53* wild-type specimens showed p53 expression, suggesting low correlation between mutation status and protein expression in this gene. Nevertheless, the authors showed an important correlation between overexpression of p53 and high ki67 indexes and poor tumor differentiation[144]. Alves *et al* described similar results, suggesting that protein expression of p53 – regardless of *TP53* mutation status – is of prognostic value in HCC[145]. Friemel *et al* assessed 120 tumor areas from 23 early-stage HCC nodules and showed histological heterogeneity for tumor grades and/or architectural patterns in 87% of the cases. Furthermore, the authors showed that IHC heterogeneity for relevant HCC-markers was less pronounced (39% of cases) and usually conditioned to the histological heterogeneity. They also showed that mutation heterogeneity to *TP53* and *CTNNB1* was present in 5/23 cases (22%), which were also heterogeneous histologically and immunohistochemically. These results suggest that mutation heterogeneity for relevant HCC driver genes can be inferred by morphological features. Finally, Craig *et al* performed RNA sequencing of 38 tumor regions from 10 HCC specimens: transcriptomic intratumor heterogeneity was detected in 4/10 tumors and highly correlated with histological findings[146].

The remarkable morpho-molecular heterogeneity studies by An *et al*, Friemel *et al* and Craig *et al* were conducted in surgically resected, early-stage HCC. Therefore, to advance on knowledge currently available, the present study focused on assessing inter-nodular instead of intratumor heterogeneity in HCC.

In other words, emphasis was placed on comparisons between the different nodules in multinodular specimens, on a patient-by-patient basis. Once again, it is important to highlight that these analyses are only attainable in autopsy cohorts, given that access to surgical samples in advanced and metastatic HCC is limited. Evaluation of inter-nodular heterogeneity in the primary disease was conducted in 156 nodules from 50 patients (median of three nodules per patient): significant heterogeneity was detected in 52% of those cases. Mutation status for the *TERT*-promoter region was assessed in six patients: three (50%) had mutations and three were wild-type, in accordance with prevalence reported in the literature[95]. Mutation heterogeneity for *TERT*-promoter within hepatic nodules was detected in 2/3 specimens. These results further confirm the high phenotypic and molecular heterogeneity of HCC primary tumors. Most interestingly, significant inter-nodular heterogeneity in distant metastases was restricted to only 2/12 (17%) cases with multiple extra-hepatic nodules. Moreover, *TERT*-promoter mutation heterogeneity was not detected in the metastatic disease. Although it might have been ideal to evaluate additional mutations or copy number changes, the current findings indicate limited heterogeneity in metastatic compared to primary HCC, suggestive of evolutionary constrains during HCC dissemination. There is no other study addressing morphological and/or genomic heterogeneity in metastatic HCC, but data from Reiter *et al* indeed suggest that heterogeneity of functional driver mutations is limited among untreated metastases in other cancer types[147].

Finally, the evaluation of 101 nodules – 65 hepatic and 36 extra-hepatic – from the 20 patients with metastatic disease showed high histological and IHC concordance between the primary and metastatic nodules within each patient

(**Figure 20**). In fact, all the metastases in the cohort shared the histological, IHC and, when available, *TERT*-promoter mutation status with at least one nodule from the primary disease. These results, in combination with the low phenotypic heterogeneity of metastatic sites and the high prevalence of advanced hepatic disease in patients with extra-hepatic dissemination, suggest that the development of metastases is a late event in HCC. Hence, extra-hepatic tumors might have had little time to develop unique mutations and transcriptomic signatures that could alter the expression of key proteins and, ultimately, the tumor's phenotype.

6. CONCLUSIONS

6.1. Metastatic dissemination is not a common cause of death in HCC, being present in only 20/88 (23%) of the autopsy specimens reported here. Instead, advanced intra-hepatic disease and complications of liver cirrhosis account for most deaths in HCC patients.

6.2. Multi-regional sampling of cancers including common metastatic sites that are not macroscopically affected should be considered as part of an academic autopsy protocol.

6.3. HCC has a strong predilection for hematogenous dissemination, and the lung is the most common metastatic site in this cancer. A systematic evaluation of lung lobes in autopsy specimens also shows that this organ is constantly affected by microvascular metastases, previously undetected by imaging and/or macroscopic examination.

6.4. Lymph node metastases are not common in pure HCC. The possibility of phenotypic changes and acquisition of mixed/intermediate hepatocellular-biliary morphology should be considered in liver cancer with dissemination to lymph nodes.

6.5. AFP serum concentration ≥ 100 ng/mL, dominant nodule ≥ 5.0 cm, multi-nodularity, macrovascular invasion, high histological, nuclear and architectural grades, cellular-crowding, and expression of K19 and EpCam in the primary disease were associated with the presence of distant metastases in HCC. Histological grading shows an important role for predicting metastatic dissemination (and clinical outcome) even in large HCC tumors.

6.6. Morphological grading of HCC based on nuclear, nucleolar and architectural features and cellular crowding correlates with expression of immunohistochemical markers of biological importance in this cancer, thus encouraging future studies aimed at assessing the inter-observer and inter-institutional variability of such histological features or even their association with mutations and transcriptomic signatures.

6.7. The extended method for TMA manufacture including multiple spots from all nodules histologically available in a cancer specimen allow for the investigation of inter-nodular heterogeneity and patterns of cancer evolution and might better recapitulate the full scope of the disease when compared to the standard single-region TMAs.

6.8. Wnt-activated HCC, during cancer progression, might acquire new molecular events that promote tumor deterioration and changes in biological behavior, as suggested by the near-significant associations between Wnt-related markers and high histological grade and cellular crowding in autopsy specimens.

6.9. Extra-hepatic HCC nodules share the histological, immunohistochemical and *TERT*-promoter mutation status with at least one nodule from the primary disease, which suggests that metastatic dissemination might be late event in this cancer. Moreover, the limited phenotypic inter-nodular heterogeneity in metastatic compared to primary HCC suggests evolutionary constrains during extra-hepatic dissemination.

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Appendixes

Appendix A1: Martins-Filho SN, Paiva C, Azevedo RS, Alves VAF. Histological Grading of Hepatocellular Carcinoma-A Systematic Review of Literature. Front Med. 2017;4:193.

Appendix A2: Martins-Filho SN, Alves VAF, Wakamatsu A, Maeda M, Craig AJ, Assato AK, et al. A phenotypic map of disseminated hepatocellular carcinoma suggests clonal constraints in metastatic sites. Histopathology. (in press) 2019 Jan 12;

Appendix B: Research Ethics Committee approval letter (Portuguese and English)

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SYSTEMATIC REVIEW
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Histological Grading of Hepatocellular Carcinoma—A Systematic Review of Literature

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Background: Histological grading typically reflects the biological behavior of solid tumors, thus providing valuable prognostic information. This is also expected in hepatocellular carcinoma (HCC), although limited access to biopsy samples and a lack of standardization might hinder its full predictive value in this cancer.

Objectives: In order to better understand the current practices of histological grading in HCC, we examined the latest publications addressing its impact on the outcome of patients following surgical treatment.

Methods: We searched the PubMed (MEDLINE) database under the headings "hepatocellular carcinoma," "grade OR grading," and "prognosis." Qualitative and quantitative assessment of publications was performed according to the reference they used to grade their tumors (e.g., Edmondson–Steiner, World Health Organization).

Results: We reviewed a total of 216 articles: 114 enclosed adequate information and were included herein. Among these, we found divergences and inaccuracies in the histological grade assessment of this cancer, which might have led to a non-standardized grade distribution, with further impact on data analysis. Nevertheless, in most of them, poor tumor differentiation correlated with worse prognosis, expressed by lower overall and/or disease-free survival.

Conclusion: While histological grading of HCC has an important prognostic role, there is an unsatisfactory heterogeneity on the microscopic assessment of this tumor, urging for a movement toward standardization.

Keywords: hepatocellular carcinoma, histological grading, grading systems, Edmondson and Steiner, prognosis

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INTRODUCTION

Image-guided needle biopsies and histopathological evaluation are the gold standard for the diagnosis of most solid organ neoplasms. They also allow for tumor subtyping and pave the way for integrated studies in cellular and molecular biology that will ultimately improve the management of patients with cancer. However, considering the current clinical guidelines, needle biopsies are seldom required for hepatocellular carcinoma (HCC) diagnosis, being reserved for suspicious, but non-diagnostic lesions on imaging examinations (1, 2). This remarkable discrepancy to the general oncological practice restricts our ability to define and select subgroups of patients for new drugs and clinical trials, and might explain the scarcity of effective therapeutic strategies in this cancer (3–5).

On the other hand, the gross and histological evaluation of HCC specimens obtained by surgical resection has continuously allowed for the identification of histological subtypes including fibrolamellar (6, 7), lymphoepithelioma-like (8), and steatohepatitic HCC (9), as well as morphomolecular features such as the distinct patterns of vascular invasion (10) and the expression of stemness markers such as Keratin 19 (assessed by immunohistochemistry or by molecular pathology) (11, 12), well-established independent prognostic factors in HCC.

A major prognostic feature in solid tumors from virtually every organ, histologic grading is also expected to reflect the tumor's biological behavior in HCC. However, the classical and most commonly adopted grading system for this cancer is Edmondson–Steiner (ES), published in the far 1954 (13), which might need to be revalidated or even updated according to more contemporary histopathological approaches.

To better understand the current practices of histological grading in HCC, we examined the latest publications addressing their impact on survival and recurrence in patients following surgical treatment. Strikingly, we found a great divergence regarding histological grade assessment in this cancer. Herein, we present these findings, as we briefly review some of the grading systems for HCC and discuss a potential approach for a higher consonance on the microscopic assessment of this tumor.

METHODS

On August 3, 2016, we searched the PubMed (MEDLINE) database to raise potentially relevant articles. Keywords were "hepatocellular carcinoma," "grade OR grading," and "prognosis" appearing on the title or abstract. We selected all the publications from January 1, 2011, to August 3, 2016, and limited the search to include only those available in full text, in English, and with humans as the species under the study. We excluded the reviews, those with irrelevant content, repeated or inconsistent data, and those in which the final intervention was not liver resection (LR), nor liver transplantation (LT).

The information collected from each article included first author name, year of publication, interval of data collection, modality of surgical treatment, previous interventions, number of samples, histological grading system, and its impact on outcome (univariate and multivariate analyses, when available) (Table S1 in Supplementary Material).

The studies were initially classified based on the modality of surgical treatment employed: LT, LR, and LR + LT. Considering that the clinical management of patients varies considerably following LR and LT, we conducted the descriptive analysis separately for these groups, and excluded the articles that had dealt with both interventions.

We then screened for the reference (depicted on the methods or bibliography) each publication used to grade their tumors. Studies that referred to the ES 1954 publication were analyzed altogether (ES subgroup). Studies that have referred the World Health Organization (WHO) book on the "Classification of Tumours of the Digestive System" as their main reference were considered, in our analysis, a different subgroup (WHO subgroup). We also identified additional histological classification/references (aggregated as "OTHERS") and studies that did not inform which grading system they have used to analyze HCC (NI subgroup).

Finally, we selected the studies from the ES and WHO subgroups that have disclosed the univariate impact of the histological grade with a 95% confidence interval (CI), and organized forest plots to quantify the importance of histological grading in HCC. Different estimates of relative risk (odds ratio and hazard ratio) were combined, as previously described (14). Fixed and random effects meta-analyses and forest plot-based estimates for hazard ratios were calculated by inverse variance weighting using the R Project for Statistical Computing (R Core Team, 2016), with R Commander package (version 2.3, October 2016) and plugin EZR (version 1.33, September 2016). Eligible studies that performed their analyses in two different cohorts had both results included. Due to the limited number of articles that analyzed data following LT, we restrained our quantitative evaluation to the LR publications. Additional graphs were designed with the software package SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). In all situations, a $p < 0.05$ was considered significant.

RESULTS

Characterization of HCC Histological Grading in the Literature

We identified 216 articles in our online database search. After screening and assessing our eligibility criteria, 114 studies were selected and thoroughly analyzed. A summary of our study selection process is summarized in **Figure 1**.

Most of the studies included in our analysis belonged to the ES subgroup ($n = 66$) and prioritized a 4-tier histological grade distribution. In contrast, and most likely due to differences from the ES classification, WHO ($n = 10$) and OTHERS ($n = 5$) reference subgroups organized tumors in 3-tiers (**Figure 2**). In this latter subgroup, we identified four publications that used the histological classification proposed by the Union for International Cancer Control (*sic*) and one that used the classification from the Liver Cancer Study Group of Japan.

The number of tiers also showed some disparities: authors under the ES subgroup who organized tumors in 3-tiers had a significantly higher percentage of G1 and lower percentage of G3 tumors when compared to ES 4-tier, while a more identical distribution when compared to the other 3-tier subgroups (**Figure 3**).

The reference and particularly the number of tiers also played an important role on how the histological grades were organized prior to data analysis (**Figure 2**). While 3-tier studies tended to assess each grade individually (G1 × G2 × G3), 4-tier studies usually dichotomized them in low (G1 + G2) and high grades (G3 + G4); some of them even presented their results with different grades combined.

Interestingly, large-scale clinical and genomic data constantly support this latter approach, suggesting that the biological behavior of G2 HCC is closer to G1 than to G3 (15, 16). Once again, we analyzed the distribution of low grade (G1 and G2) and high grade (G3 and, when available, G4) HCC: overall, 38.0% of the

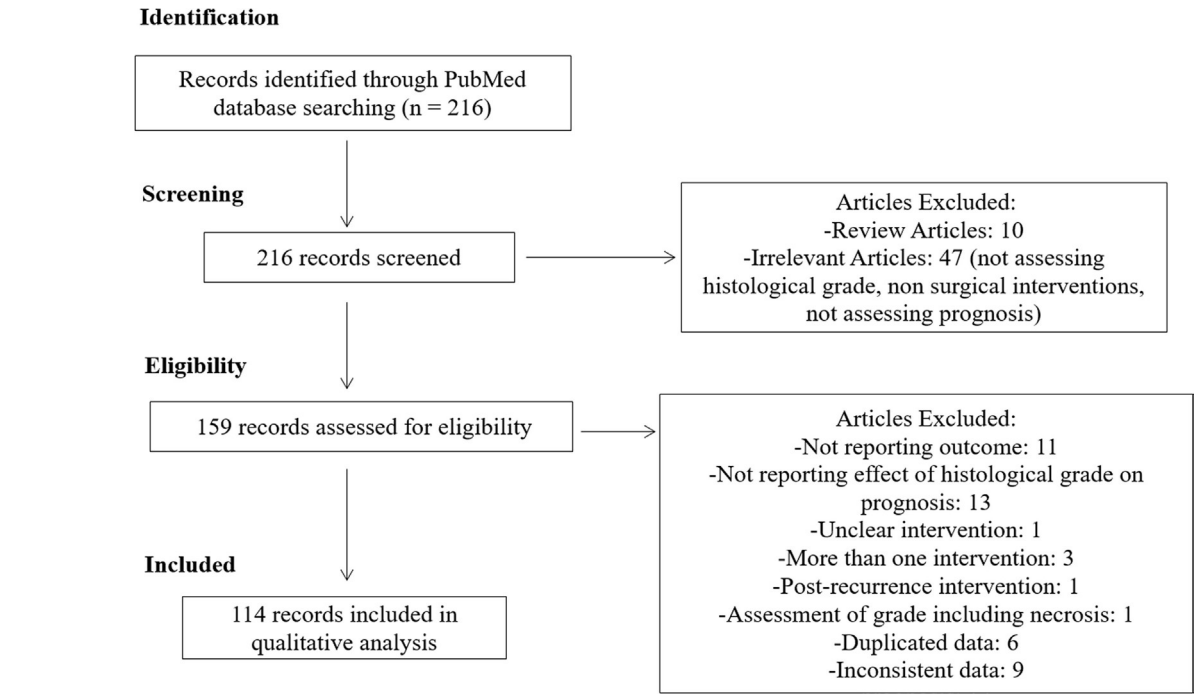


FIGURE 1 | Flow diagram of the study selection process.

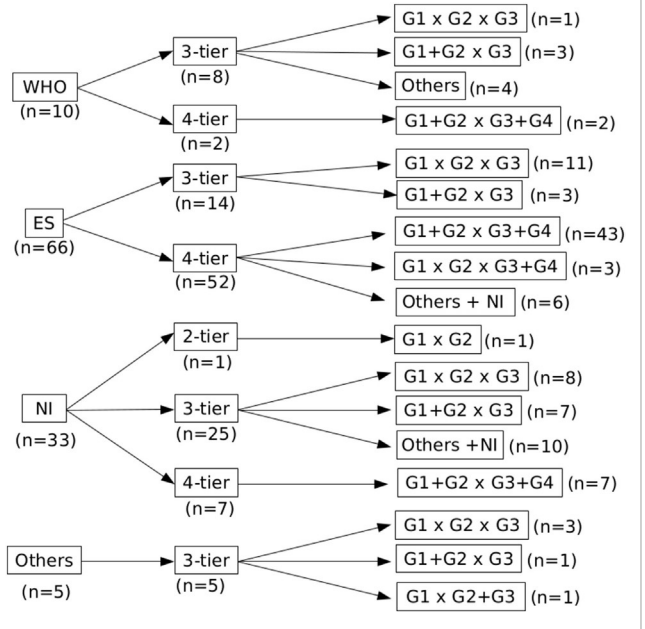


FIGURE 2 | Distribution of the studies according to the grading reference, number of tiers, and data analysis. There is a lack of consensus on the classification of hepatocellular carcinoma in the literature, illustrated by different grading systems and number of tiers.

tumors were considered high grade. However, there were only 27.8% of high-grade tumors in the WHO subgroup, as opposed to 38.8 and 39.8% in the ES and NI subgroups, respectively.

Impact of the Histological Grade in the Prognosis of HCC Patients Treated with LT

Twelve cohorts—distributed in 11 studies—evaluated the impact of the histological grade on the prognosis of HCC patients submitted to LT. Results for the univariate analysis were displayed in eight cohorts: correlation between higher grades and poor outcome was observed in 5 (62.5%) of them. Ten cohorts displayed the results for the multivariate analysis and 6 (60.0%) found correlation between outcome and grade.

Only four studies clearly described their grading classification: all belonged to the ES subgroup. The two studies that organized tumors in 4-tiers found correlation between grade and outcome in both univariate and multivariate analyses. The two remaining publications organized tumors in 3-tiers: one found correlation in the univariate, but not in the multivariate analysis, and the other showed only a trend between grade and outcome, although not statistically significant.

Impact of the Histological Grade in the Prognosis of HCC Patients Treated with LR

From the 103 studies based on LR, 86 had performed univariate analysis including histological grade. Overall, 56 (65.1%) of these showed a better outcome for patients with a lower histological grade. One study that divided patients into two categories—"AFP negative" (≤20 ng/ml) and "AFP positive" (>20 ng/ml)—found that grading was a significant predictor of survival only in this latter category (17). One article assessed patients with single small (<2 cm) and single large (>2 cm) HCC separately. Favorable

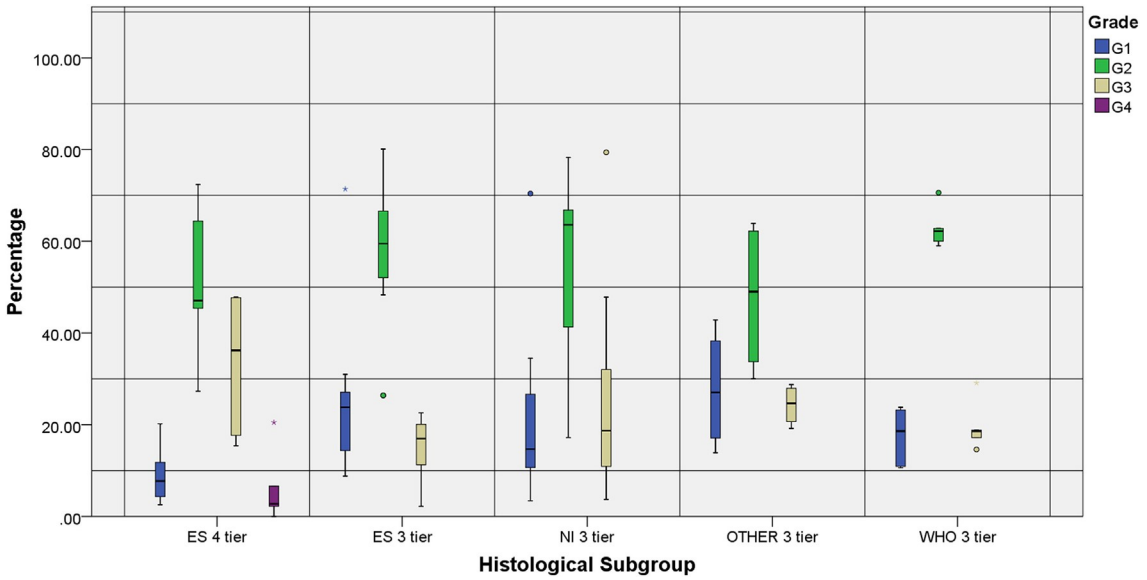


FIGURE 3 | Individual grade distribution according to the reference subgroup and number of tiers. All groups represented show a higher distribution of the intermediate grades compared to the marginal ones. Two studies that were not included in the chart—WHO 4-tier (G1: 8.9%, G2: 45.5%, G3: 42.7% and G4: 2.8%) and NI 4-tier (G1: 7%, G2: 59%, G3: 32% and G4: 2%)—reproduced the distribution observed in the ES 4-tier category.

overall survival for patients with better differentiated tumors was found for larger but not for smaller HCC (single large HCC G1 + G2: 71.63%, G3 + G4: 28.37%/single small HCC G1 + G2: 81.08%, G3 + G4: 18.92%) (18). Nonetheless, another study including only tumors ≤2 cm indicated significantly lower overall recurrence, advanced recurrence within 1 year, and advanced recurrence within 2 years in better differentiated HCC (19).

Among the 77 articles that declared the grading system used, 64 assessed prognostic significance of tumor grade in the univariate level. Forty three (67.2%) found significant correlation between grading and prognosis. For the most commonly adopted grading system (ES 4-tier) histological grade was a significant predictor of outcome in 32 of 42 (76.2%) articles. Among the eight studies that used ES as a 3-tier system, 5 (62.5%) showed significant correlation. In the WHO subgroup, lower grade was associated with better outcome in only 2 (25%) of the eight studies and 1 (50%) of the two studies when it was considered a 3- and 4-tier classification, respectively. Of note, one study within WHO 3-tier compared "non-poor" tumors (NP: containing only G1 and G2), "poorly containing" (PC: containing G3, predominant G1 or G2), and poorly differentiated (PD: predominant G3). Significantly better overall survival and recurrence-free survival were found for NP when compared to PC and to PD, whereas no significant difference was detected between PC and PD cases (20). In a similar fashion, an ES 4-tier study demonstrated that tumors with focal areas of G3 have worse outcome when compared to homogeneous G2 tumors (15). For the subgroup "OTHERS," univariate analysis of the impact of grading in prognosis showed correlation in 3 (75%) of the four studies.

When results are pooled, histological grade shows correlation with survival for both ES and WHO subgroups. In the former, however, we observed a high heterogeneity ($I^2 > 60\%$, $p < 0.01$), either when evaluating the impact of grade on overall or disease-free survival (**Figures 4A,B**). Interestingly, there was a higher consistency in the results from the WHO subgroup (**Figure 4C**). In this latter subgroup, however, analysis was restrained to overall survival due to the limited number of publications evaluating its impact on disease-free survival.

DISCUSSION

This comprehensive review of recent international literature on HCC grading demonstrates that, still now, the most broadly adopted reference for histological grading in HCC is the ES system, published in *Cancer* in 1954 (13). The WHO, on its prestigious "blue book" series, displays an adaptation of the original reference on its "WHO Classification of Tumours of the Digestive System," whose latest edition date from 2010 (21). Despite being published more than 60 years ago, the original classification is transcribed and still recommended by the College of American Pathologists on its protocol for examination of HCC, updated in 2011 (22).

Both the 1954 publication and the WHO 2010 book share a lot of similarities in their characterizations, but do not completely overlap, especially if the morphologist strictly follows their descriptions. Among these similarities are the facts that both recognize four different grades for HCC, and consider a combination of structural and cellular features for defining the final grade. Some differences encompass the mild cytological atypia and acinar architecture, which can accompany the thin trabecular tumors that falls under WHO's grade I (well-differentiated) HCC, but can also be described by the "marked resemblance to normal hepatic cells" and frequent acini, now in ES's grade II (Figure S1 in

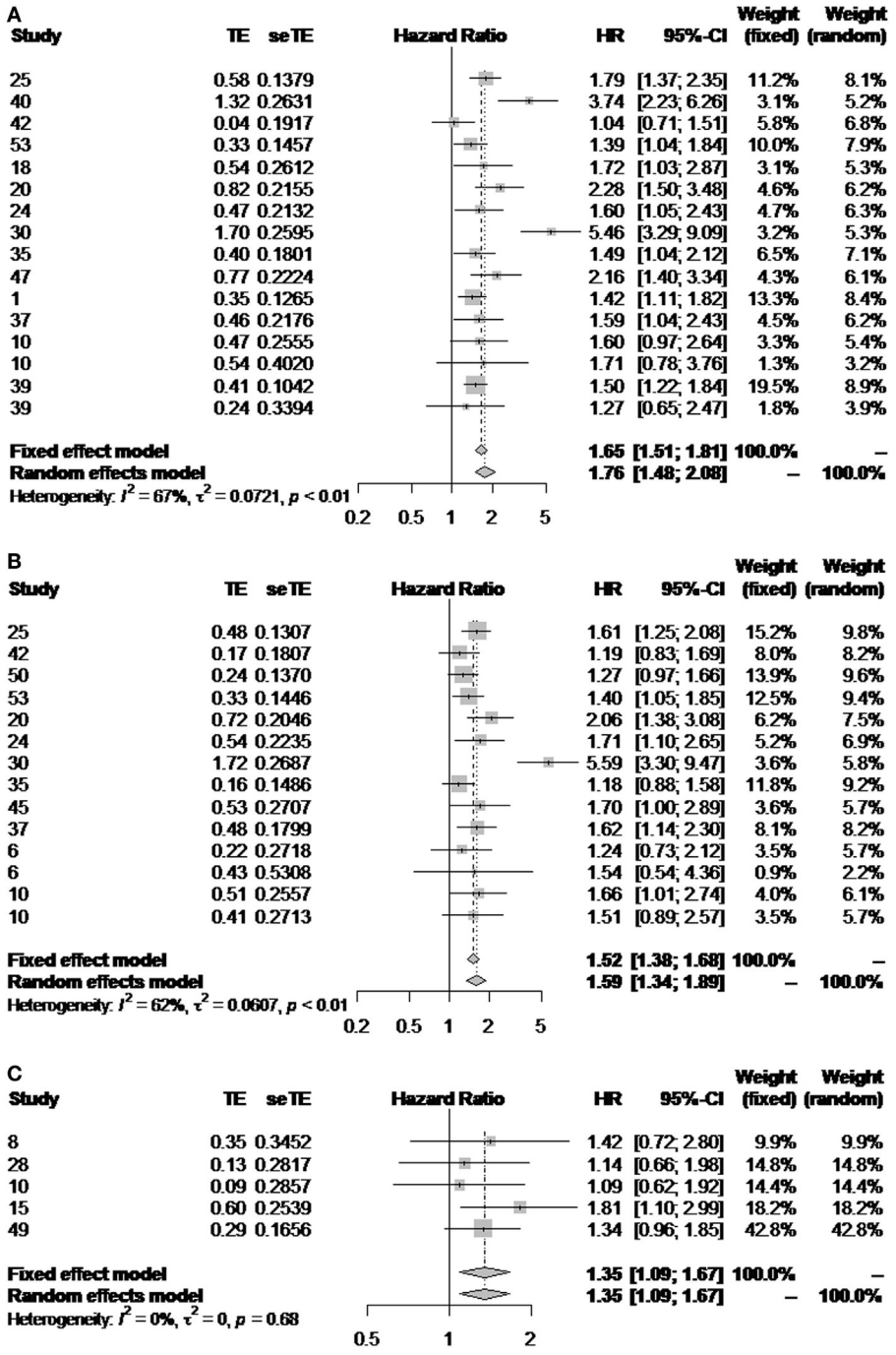


FIGURE 4 | Impact of the histological grade in outcome. Forest plot diagrams illustrating the impact of the ES classification on overall **(A)** and disease-free survival **(B)**, and the impact of the WHO classification on overall survival **(C)**.

Supplementary Material). In fact, Edmondson and Steiner even state that "Grade I is best reserved for those areas in Grade-II carcinomas where the difference between the tumor cells and hyperplastic liver cells is so minor that diagnosis of carcinoma rest upon the demonstration of more aggressive growth in other parts of the neoplasm." This description seems more illustrative of HCC in which the differentiation from dysplastic nodules or adenomas is challenging and relies on the evaluation of other areas of the tumor.

Furthermore, defining WHO's worst grade as undifferentiated is potentially misleading, as this pathological terminology is reserved for anaplastic tumors in which the embryonal lineage is yet to be established. To avoid further confusion, we defend the use of "undifferentiated HCC" for PD tumors with focal

anaplastic areas and the use of "undifferentiated carcinoma" for homogenously anaplastic cancer following immunohistochemical demonstration of epithelial markers, yet no characterization of hepatocellular lineage. Additional differences and exempts from WHO and ES can be found in **Table 1**.

Besides subtle, these nuances seem to have induced several authors to classify HCC in 3-tiers when referring to the WHO (**Figure 2**), and provide the basis for the lower percentage of high-grade tumors when comparing WHO (24.6%) and ES (37.0%). This, in turn, might account for the differences regarding outcome in these subgroups, since the distinction between G2 and G3 seems to be the cornerstone for histological grade impact on outcome. In fact, while 73.5% of the publications in the ES subgroup following LR found a statistically significant correlation between grade and outcome, only 30% in the WHO subgroup did so.

On the other hand, authors who use the WHO as their reference tend to grade their tumors more homogeneously, thus presenting less-conflicting results (**Figures 2** and **4C**). For instance, the percentage of G1 tumors ranges from 8.9 to 23.8% when the WHO is the reference, and goes from 2.38 to 78% in the ES subgroup (including ES 3-tier and 4-tier). These results are intriguing, especially considering that the original classification restrains the diagnosis of G1 tumors and recommends HCC to be classified according to the worst area. Variability in tumor characteristics between the centers could explain such divergences, though it is legitimate to raise the concern that this is partially induced by different interpretations of the ES classification, thus affecting the way of grading.

On top of those reproducibility issues, the ES grading classification was proposed on an autopsy cohort (further limiting its predictive value) and, at that time, could not incorporate the distinct HCC histological patterns and clinical and molecular advances. It is important to acknowledge that long-standing and iconic classifications of neoplasms from other organs such as the Gleason System for prostate carcinomas (pivotal for its visual guide and for the assessment of phenotypical tumor heterogeneity) and the SBR classification for breast carcinomas (clearly defining specific criteria—architecture, nuclear atypia, and mitoses) have been challenged by the molecular characterization of these tumors. The remarkable advances achieved in the organs where pathology has remained the core of medical approach for the diagnosis have yielded a new paradigm of "morpho-molecular classifications," leading to fantastic improvements on the clinical management of these tumors (23).

Attempts for improving grade assessment of HCC were made. For instance, Goodman and Ishak, in the second edition of the AFIP Liver Fascicles, proposed a modified ES grading system, placing bigger emphasis on nuclear pleomorphism. While pure grade I tumors were still unlikely and undistinguishable from adenomas, differences would encompass the following grades (24). Noticeably, giant cell carcinomas were shifted from G3 in the original classification to G4 in this modified version (Figure S2 in Supplementary Material).

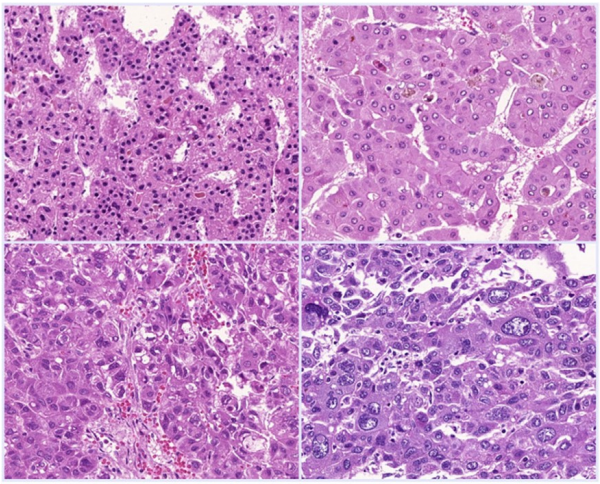
Similarly, Lauwers et al. described a histologic predictive index for HCC, combining nuclear features and microvascular invasion to stratify tumors in fair and poor prognosis (25). Although promising, these classifications were not validated by other groups/bigger cohorts and are not recommended by current protocols.

There is thus a fertile ground for an update or even a new grading classification for HCC. Considering the aforementioned examples on prostate and breast carcinoma, HCC should also be classified according to more objective criteria, acquiescent to outcome, histological patterns, and molecular subclasses. A potential approach would be to individually classify different histological parameters (such as architecture, cellularity, nuclear and nucleolar pleomorphism, and, perhaps, mitoses/proliferation index), which, desirably, should be scored to yield the stratification of tumors in low or high grade (**Figure 5**).

Even with great variability and lack of consensus, grading appears to be a relevant prognostic factor in HCC: in most of

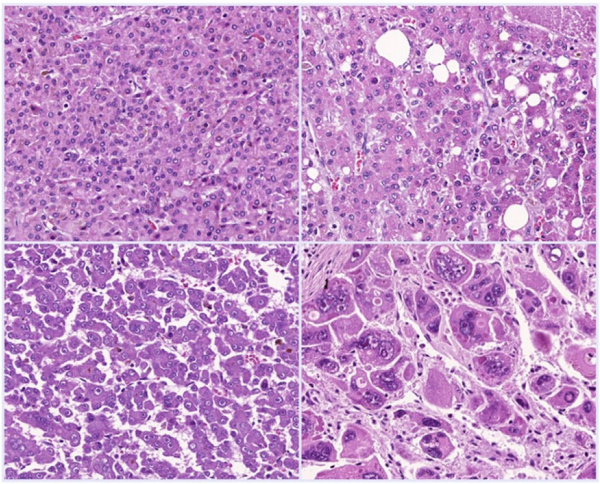
TABLE 1 | Histological features from Edmondson and Steiner (ES) publication and WHO book.

Reference	Grades	Architecture	Cytology	Other features
World Health Organization (21)	Well differentiated	Thin trabecular, frequent acinar structures	Minimal atypia	Fatty change is frequent
	Moderately differentiated	Trabecular (3 or more cells in thickness) and acinar	Abundant eosinophilic cytoplasm, round nuclei with distinct nucleoli	Bile or proteinaceous fluid within acini
	Poorly differentiated	Solid	Moderate to marked pleomorphism	Absence of sinusoid-like blood spaces
	Undifferentiated	Solid	Little cytoplasm, spindle, or round-shaped cells	—
Edmondson and Steiner (13)	Grade I	—	—	Areas of carcinoma where distinction from hyperplastic liver is difficult
	Grade II	Trabecular, frequent acini (lumen varying from tiny canaliculi to large thyroid-like spaces)	Resemblance to normal hepatic cells; larger nuclei; abundant acidophilic cytoplasm	Cell borders sharp and clear cut; acini containing bile or protein precipitate
	Grade III	Distortion of trabecular structure, acini less frequent than grade II	Larger, more hyperchromatic nuclei, granular but less acidophilic cytoplasm	Acini are less frequent; tumor giant cells may be numerous
	Grade IV	Medullary, less trabeculae, rare acini	Highly hyperchromatic nuclei, scanty cytoplasm, with fewer granules	Loss of cell cohesiveness; giant, spindle or short-plump cells can be found



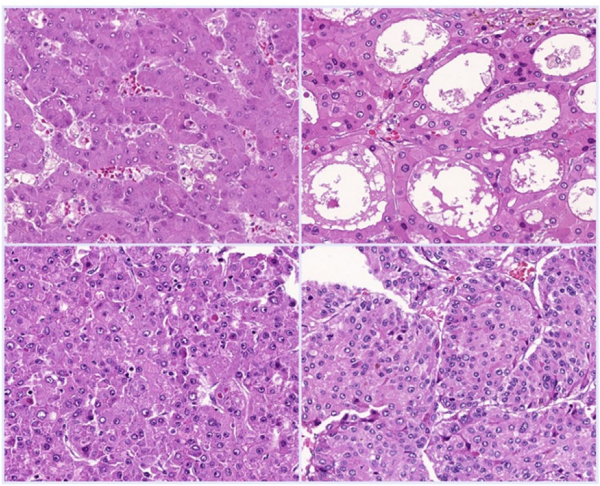
Nuclear Grade:

- Grade I: Homogeneous, near-normal nuclei;
- Grade II: mild pleomorphism;
- Grade III: moderate pleomorphism, irregular distribution of chromatin;
- Grade IV: marked pleomorphism, bizarre nuclei.



Nucleolar Grade (similar to Fuhrman's):

- Grade I: nucleoli barely seen at 400x;
- Grade II: evidente nucleoli at 100-200x;
- Grade III: large nucleoli, visible at 100x;
- Grade IV: prominent nucleoli at 40x.



Architectural Grade:

- Grade I: trabecular, 2-3 cells wide;
- Grade II: pseudoglandular pattern;
- Grade III: mid-trabecular (4-10 cells wide);
- Grade IV: macrotrabecular (> 10 cells wide) or solid/bizarre patterns.

FIGURE 5 | Potential approach for a new grading classification on HCC. Tumors would be classified into four grades for each histological feature and, depending on the combined scored, stratified in low or high grade. Each feature or their combination would then be cross-examined with the patterns of vascular invasion (micro and macrovascular), the expression of stem-like markers (e.g., Keratin 19) and even with the HCC molecular subclasses. Exemplified here are nuclear, nucleolar, and architectural grade, but other histological variables such as cellularity and even mitotic index could also be explored.

the studies, poor tumor differentiation correlated with worse prognosis, expressed by lower overall and/or disease-free survival.

While we could not address regional differences, we acknowledge them as possible confounders to our results. Nevertheless, for the main objective of assessing histological grade in HCC outcome, we tried to overcome that limitation by pooling different studies together. Also, it would have been ideal to compare the differences between the ES and WHO references in the same cohort to better understand how their divergences really affect histological grading and its impact in outcome. However, and considering that those divergences are subtle, a blinded approach and a huge cohort would be required.

CONCLUSION

The present comprehensive review of the literature from 2011 to 2016 clearly shows that histological grading of HCC can still be considered a relevant prognostic marker. Our view is that, in the constant search for predictors of a favorable response to potentially curative treatments, histological grade might be an important variable, to which other biomarkers can sum and help determine prognosis. However, to be able to assess its authentic value, better definitions and greater uniformity are needed.

AUTHOR CONTRIBUTIONS

SNMF and CP contributed equally to this work. SNMF worked on data analysis and interpretation, and on the drafting of the article. CP worked on data collection and on the drafting of the article. RSA helped with data analysis. VAFA performed a critical review of the article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fmed.2017.00193/full#supplementary-material.

FIGURE S1 | Histological representation of a low-grade hepatocellular carcinoma. Hepatocellular carcinoma with trabecular architecture (three to four cell thickness) and mild cytological atypia. Some acini can be observed, filled with proteinaceous fluid. This tumor is classified as well differentiated according to the WHO criteria, but is better aligned under ES's grade II tumors.

FIGURE S2 | Histological representation of a high-grade hepatocellular carcinoma. This tumor would be classified as G3 according to ES, but as G4 according to the modified histologic classification proposed by Goodman and Ishak. Despite the bizarre-looking cells, this tumor retained the immunohistochemical expression of HepPar 1.

TABLE S1 | HCC-related articles evaluating the impact of histological grading on outcome.

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A phenotypical map of disseminated hepatocellular carcinoma suggests clonal constraints in metastatic sites

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Aims: Access to tissue in patients with hepatocellular carcinoma (HCC) is limited compared to other malignancies, particularly at advanced stages. This has precluded a thorough characterisation of molecular drivers of HCC dissemination, particularly in relation to distant metastases. Biomarker assessment is restricted to early stages, and paired primary–metastatic comparisons between samples from the same patient are difficult.

Methods and results: We report the evaluation of 88 patients with HCC who underwent autopsy, including multiregional sampling of primary and metastatic sites totalling 230 nodules analysed. The study included morphological assessment, immunohistochemistry and mutation status of the *TERT* promoter, the most frequently mutated gene in HCC. We confirm a strong predilection of HCC for lung dissemination, including subclinical micrometastases (unrecognised during imaging and macroscopic examinations) in 30% of patients with disseminated disease. Size of dominant tumour nodule; multinodularity; macrovascular invasion; high histological, nuclear and architectural grades; and cellular crowding were associated with the presence of extrahepatic metastasis. Among the immunohistochemistry markers tested, metastatic nodules had significantly higher K19 and EpCAM expression than primary liver tumours. Morphological and immunohistochemical features showed that metastatic HCC could be traced back to the primary tumour, sometimes to a specific hepatic nodule.

Conclusions: This study suggests limited heterogeneity in metastatic sites compared to primary tumour sites.

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Introduction

Primary liver cancer is the second leading cause of cancer-related mortality worldwide.[1] With 750 000 new cases every year (33 000 in the United States), it has become the fastest-growing malignancy in the United States, both in terms of incidence and mortality.[2] Recent projections estimate that liver cancer will be the third leading cause of cancer death in the United States by 2030, surpassing breast, prostate and colorectal malignancies.[3] Major risk factors for hepatocellular carcinoma (HCC), the most frequent form of liver cancer, are hepatitis B (HBV) or C (HCV) viral infection, alcohol abuse and non-alcoholic fatty liver disease (NAFLD). Survival rates are dismal, particularly in patients diagnosed at advanced stages, of whom only 18% of patients will be alive 5 years after the diagnosis.[2]

Access to tissue samples for patients at intermediate and advanced HCC stages is challenging. Currently, surgical interventions are only recommended for tumours at early stage. Also, according to clinical practice guidelines, HCC can be diagnosed with imaging techniques [i.e. magnetic resonance imaging (MRI) or computerised tomography (CT) scan], restricting needle biopsies to a few specific indications.[4] A direct consequence is that most molecular biomarker studies in HCC have been conducted on resection specimens from patients at early stages,[5,6] where tissue is accessible. Only a few reports have provided a comprehensive analysis of dominant molecular or phenotypical alterations in HCC patients at advanced stages, and even more rarely in metastatic tumours.

Cancer progression results from the accumulation of DNA mutations and epigenetic events, as demonstrated by numerous studies using multiregional sequencing.[7–9] Histopathological and cytological features are also valuable to trace peculiarities associated with tumour evolution. For instance, the dissemination trajectory of well-differentiated thyroid carcinomas can be confidently predicted based on cytoarchitectural features, which has clinical implications. Papillary carcinomas have a strong lymphatic tropism, as opposed to the haematogenous tropism of follicular carcinomas. They often present with clinical involvement of regional lymph nodes. Therefore, it is recommended that surgical intervention includes lymph-node dissection in papillary carcinomas.[10] These morphological changes translate intrinsic differences in the biological behaviour of thyroid cancer cells. Similarly, some distinct HCC histological variants show association with prognosis, e.g. sarcomatoid HCC are usually aggressive tumours, whereas lymphocyte-rich HCC tend to have a better outcome. Furthermore, there are histological phenotypes in HCC tightly associated with gene mutations and transcriptomic classes.[11] For example, *CTNNB1* mutated HCCs tend to have microtrabecular and pseudoglandular patterns, and generally lack a strong inflammatory component. Indeed, the immune exclusion of *CTNNB1* tumours has been further confirmed in other studies.[12] This contrasts with *TP53* mutated HCCs, which tend to have a solid pattern with frequent pleomorphic cells.

HCC has a strong haematogenous tropism. This is illustrated by the high prevalence of vascular invasion, even in relatively small tumours.[13] Common distant metastatic sites include the lungs, bones and adrenal glands, but the morphological and molecular composition of HCC metastases remain elusive. In one of the few studies including autopsy HCC samples, we identified phenotypical features associated with disease dissemination.[14] However, sampling was mostly restricted to a single tumour area, which limited the assessment of tumour heterogeneity (and its impact on distant dissemination) and precluded paired primary–metastatic comparisons on an individual basis. Herein, we provide a detailed report on phenotypical correlates between primary and metastatic HCC using multiple samples from a cohort of HCC autopsies. We used stringent criteria to focus the analysis on patients with adequate sampling of paired primary–metastatic disease.

Recently, the analysis of autopsy specimens has re-emerged as a valuable tool to understand cancer heterogeneity and patterns of evolution.[15,16] The major advantages are: (1) it provides a large quantity of samples, (2) it allows extensive sampling of different tumour nodules and metastatic sites and (3) it provides tissue from advanced/aggressive tumours. Indeed, recent studies using autopsy samples have provided novel insights into cancer progression in pancreatic[16] and prostate[17] cancer. These data have improved the understanding of cancer clonal composition and metastatic behaviour of these tumour types. In the present study, we report the analysis of autopsy specimens from 88 HCC patients, including

multiregional sampling of primary and metastatic sites totalling 230 nodules. We performed a thorough morphological assessment, investigated a panel of antibodies for immunohistochemistry and also checked for the mutation status of *TERT* promoter, the most frequently mutated gene in HCC. Integrative analysis of histological features such as tumour grade, progenitor cell[18] and epithelial mesenchymal transition (EMT)[19,20] markers and *TERT* promoter mutations suggest dominant evolutionary constraints in HCC metastatic sites.

Methods

PATIENTS

Between 2000 and 2015, a total of 13 238 autopsies were performed routinely at the Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP, Brazil). Among the autopsy cases, 219 were conducted on patients with HCC. To obtain a homogeneous cohort, we excluded patients with mixed phenotype [e.g. HCC-cholangiocarcinoma (CC)], advanced autolysis (defined by significant detachment of endothelial cells and nuclear clumping) or post-mortem interval higher than 24 h regardless of tissue viability. Combined HCC-CC was excluded based on morphology and/or immunohistochemistry, i.e. classic biliary gland formation and/or diffuse keratin 7 or K19 staining. We only included patients with at least two samples from different tumour sites within the liver and/or distant organs (i.e. lungs, adrenal gland). The autopsy protocol at HC-FMUSP includes sampling of all vital organs (e.g. lung, brain, heart), regardless of any macroscopic evidence of disease. Eighty-eight patients, including 21 from our previous cohort,[14] fulfilled the eligibility criteria and were included (Figure 1). Clinical records, autopsy reports, imaging examinations and histological slides were reviewed for relevant clinicopathological information. AFP plasma levels were collected within 5 days of the autopsy. The study was approved by the Institutional Review Board from HC-FMUSP (approval number 1297918, 26/OCT/2015).

HISTOLOGICAL EVALUATION AND IMMUNOHISTOCHEMISTRY

Tissue microarrays (TMA) were originally designed as a population-screening tool, primarily aimed at comparisons across samples from different patients.[21,22] In this study, we expanded the traditional approach by sampling all the nodules histologically available from a given patient. Nodules that were represented in multiple tissue blocks, nodules with intratumoural fibrosis or with a nodule-in-nodule pattern had different areas adequately sampled (Figure S1). TMA microdissection was performed manually: 1-mm tissue cores were removed from their original paraffin blocks and inserted into new recipient ones with the MTA1 tissue arrayer (Beecher Instruments, Inc., Sun Prairie, WI, USA). A total of 194 liver and 36 extrahepatic nodules from 88 patients were represented in seven TMA blocks. All nodules were assessed for multiple histological features, including degree of differentiation (histological grade), cellular crowding and nuclear, nucleolar and architectural grades. Each morphological feature was organised in a four-tier classification, and later dichotomised as high- and low-grade (Table S1). The nodules were also classified according to major HCC histological variants, namely steatohepatitic, clear cell, scirrhous, sarcomatoid and lymphocyte-rich.[23,24]

Immunohistochemistry (IHC) was performed for markers of hepatocyte differentiation (HepPar1 and arginase), WNT pathway (β-catenin and glutamine synthetase), HCC progenitor cell features (K19, CD44 and EpCAM), markers of EMT (vimentin and claudin-1) and cell proliferation (Ki67). Results for β-catenin and glutamine synthetase IHC were combined as a proxy for WNT signalling pathway activation.[25] All the reactions were performed on paraffin-embedded sections (5 μm) from the TMA blocks. When appropriate, antigen retrieval was conducted using either a pH 6.0 citrate buffer (Spring Bioscience PMB1-250; Spring Bioscience, Pleasanton, CA, USA) or pH 9.0 Tris-ethylenediamine tetraacetic acid (EDTA) (Spring Bioscience PMB4-250) buffer solutions for 40 min in a steamer at 95°C. Endogenous peroxidase activity was blocked three times with a 6% hydrogen peroxide on methanol (v/v) solution for 10 min. Non-specific protein–protein reactions were suppressed with CASBlock™ (Invitrogen/Zymed, San Francisco, CA, USA) for 10 min at 37°C. Incubation with primary antibodies was performed at 37°C for 30 min, followed by overnight incubation at 4°C. Signal amplification was performed with Novolink Polymer (Vision Biosystems™, Newcastle, UK) for 30 min at 37°C. Development was achieved with 100 mg % 3,3′-diaminobenzidine tetrahydrochloride (catalogue no. D5637; Sigma, St Louis, MO, USA), 1% dimethyl sulphoxide (catalogue no. D5879; Sigma) and 0.06% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min at 37°C. Slides were counterstained with Harris's haematoxylin, dehydrated and mounted with Entellan (catalogue no. 107961; Merck, Darmstadt,

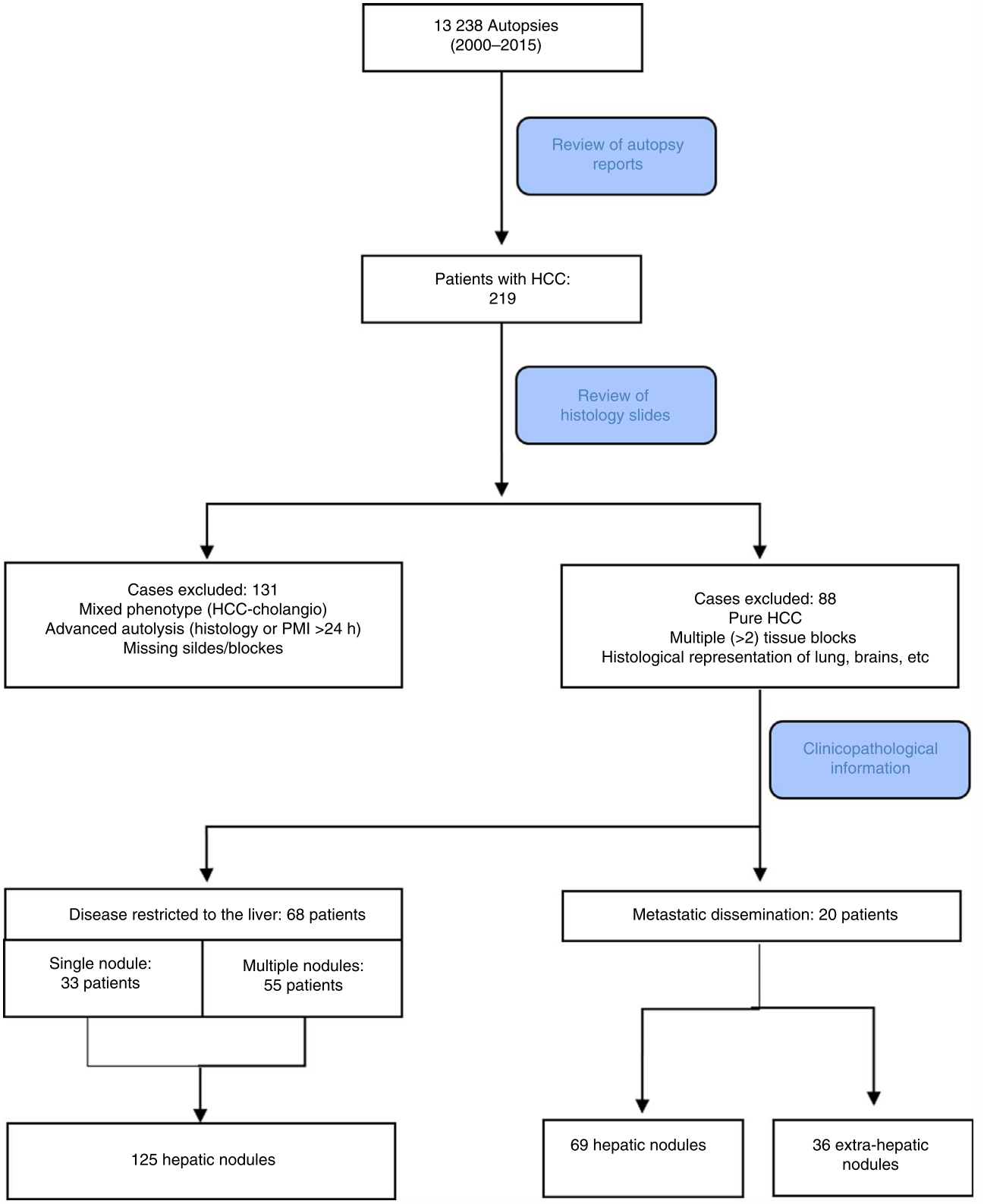

Figure 1. Summary flowchart of sample selection.

Germany). Adequate positive and negative controls were selected for each primary antibody. Titres, clones and the interpretation criteria[18,25–29] for each antigen are summarised in Table 1.

IMMUNOFLUORESCENCE

We used immunofluorescence (IF) analysis of NR2F1 and p27 as markers of cancer dormancy. As shown in other solid tumours, such as breast cancer,[30] cancer cell dormancy frequently precedes metastasis. We conducted IF on 5 μm full slides from patients with lung micrometastasis. Antigen retrieval was achieved with Target Retrieval Solution (S1699; Dako, Carpenteria, CA, USA) for 30 min in a pressure cooker. Blocking was conducted for 1 h at room temperature with a solution including ×1 Tris-buffered saline (TBS), 10% bovine serum albumin (BSA) and 0.3% Triton-X. Incubation with primary antibodies against NR2F1 (anti-COUP TF1–EPR10841; 1 : 100 dilution) and p27 (anti-p27 KIP 1; 1 : 50 dilution) co-stained with HepPar1 (to confirm hepatocellular lineage) was performed overnight at 4°C. This step was followed by incubation with AlexaFluor® 488 (Invitrogen A21121; Carlsbad, CA, USA) and AlexaFluor® 594 (Invitrogen A11037) secondary fluorescent antibodies for 1 h at room temperature. Nuclei were labelled with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen D1306) diluted in PBS (1 : 10), and slides were mounted with Fluoromount-G® (SouthernBiotech, Birmingham, AL, USA). All IF slides were analysed with a Nikon Eclipse NI microscope with compatible filters. We chose IF for this analysis because the antibodies used to test for dormancy were optimised for this assay.

DNA EXTRACTION AND *TERT* PROMOTER MUTATION STATUS

DNA was extracted from tissue cores collected from the formalin-fixed paraffin-embedded (FFPE) blocks with disposable biopsy punch needles (Miltex, CE, Plainsboro, NJ, USA). In one patient with lung metastasis, material was collected using the Leica Microsystems LMD6500 Laser Microdissection as previously described.[31] DNA was extracted using the QIAamp® DNA FFPE tissue kit following the manufacturer's instructions. The purified DNA was run on a 2100 Bioanalyzer Instrument (Agilent, Santa Clara, CA, USA) for size estimation, and its concentration was measured by fluorometric quantitation using Qubit (ThermoFisher, Fremont, CA, USA). Samples with low DNA yield (<50 ng) and/or highly degraded [mean fragment size <100 base pairs (bp)] were excluded.

Table 1. Immunohistochemical antigens and reaction conditions

Antigen	Clone	Retrieval method	Titres	Interpretation (positive reaction)
HepPar1	OCH1E5 (Roche)	Steamer, Tris-EDTA, pH9	1/50	Granular cytoplasmic positivity in ≥5% of the sample
Arginase	Polyclonal* (Sigma Life Sciences)	Steamer, citrate, pH6	1/1000	Granular cytoplasmic positivity in ≥5% of the sample
β-catenin	14 (BD Transd. Laboratories)	Steamer, Tris-EDTA, pH9	1/400	Any nuclear positivity in the sample
Glutamine synthetase	GS6 (Roche)	Steamer, citrate, pH6	1/8000	Strong and diffuse cytoplasmic positivity
Keratin 19	B170 (Leica)	Steamer, Tris-EDTA, pH9	1/200	Patchy cytoplasmic positivity in ≥5% of the sample
CD 44	DF1485 (Dako)	Steamer, citrate, pH6	1/100	Membrane positivity in ≥5% of the sample
EpCAM	BerEP4 (Cell Marque)	No retrieval	1/50	Membrane and/or cytoplasmic positivity in ≥5% of the sample
Vimentin	V9 (Cell Marque)	Steamer, Tris-EDTA, pH9	1/300	Cytoplasmic positivity in ≥5% of the sample
Claudin-1	Polyclonal† (Zymed)	Steamer, citrate, pH6	1/200	Membrane positivity in ≥1% of the sample
Ki67	MIB1 (Dako)	Steamer, citrate, pH6	1/80	Nuclear positivity: 10% intervals

EDTA, Ethylenediamine tetraacetic acid.
*Rabbit polyclonal (code 003595).
†Rabbit polyclonal (code 18-7362).

Non-tumoral tissue from each patient was further collected to determine somatic *TERT* promoter mutations.

The targeted PCR amplification of the duplicate segment of the *TERT* promoter region was performed with the following primers: CAGCGCTGCCTGAAACTC and GTCCTGCCCCTTCACCTT. Each PCR product was assessed on a 2.0% agarose gel, and sequenced later in both directions using the BigDye Terminator Cycle-Sequencing Kit (Macrogen, Chapel Hill, NC, USA) reactions and loaded onto an ABI PRISM 3730xl DNA analyser. Sequences were analysed using the SnapGene® Viewer 3.3.4 software. Due to the high number of mutations introduced by FFPE tissue processing,[32] we focused the evaluation on the two most prevalent *TERT* promoter mutations (C228T and C250T).[33]

ASSESSMENT OF CLONAL COMPOSITION AND EVALUATION OF INTERTUMOUR HETEROGENEITY

Each nodule was classified according to morphological features, IHC staining patterns and, when applicable, *TERT* promoter mutation status. The integration of these variables allowed for a comprehensive comparison between the primary and metastatic disease on a patient-by-patient basis. Furthermore, we evaluated the intertumour heterogeneity in patients with multinodular disease. After accounting for the physical losses associated to the TMA technique, we identified 50 patients with primary and 12 patients with metastatic multinodular disease. Extrahepatic nodules for different organs were identified in six patients. Considering the high morphological heterogeneity in HCC,[26] a sample was only considered to harbour significant heterogeneity if it presented variations on both morphological and molecular (IHC or *TERT* promoter mutation status) levels.

STATISTICAL ANALYSES

χ^2 and Fisher's exact tests were used to evaluate the association between the categorical variables with the presence of metastases, as well as to assess which pathological feature was enriched in the metastatic nodules compared to the primary ones. We used the non-parametric Wilcoxon's rank sum test to compare continuous variables with dichotomous categorical variables. To identify independent predictors of the presence of metastasis, we used binary logistic regression. A *P*-value inferior to 0.05 was considered significant. All analyses were conducted with SPSS version 22.0 software (SPSS, Inc., Chicago, IL, USA) and the statistical package R (R Development Core Team 2008).

Results

INCIDENTAL LUNG MICROMETASTASES IN HCC

The analysis included 230 nodules (194 liver nodules and 36 extra-hepatic metastasis) from 88 patients with HCC (Figure 1). We included a median of two nodules per patient [interquartile range (IQR) = 1–4]. As expected, most of the patients were male (62 of 88, 70%), with background liver cirrhosis (79 of 88, 90%) due to hepatitis C virus (HCV) infection (47 of 88, 53%). Of the nine patients without cirrhosis, one had HBV infection with no fibrosis. The remaining eight patients had either mild or intermediate fibrosis (portal expansions and occasional porto–portal bridging) due to HBV infection (three of eight), HCV infection (one of eight) or cryptogenic hepatitis (four of eight). Median tumour size for the dominant nodule was 40 mm (IQR = 20–66). Table 2 summarises the main clinical and pathological characteristics of the cohort. Multinodular and extrahepatic disease were identified in 55 (62.5%) and 20 (22.7%) of the 88 patients, respectively. Metastatic sites included lungs (16 of 20, 80%), lymph nodes (4 of 20, 20%), peritoneum (4 of 20, 20%) and adrenal glands (3 of 20, 15%). Bone metastases were suggested by imaging examinations in two patients, but tissue was either not collected to avoid visible deformities or was insufficient for adequate histological analyses. In 6 of 20 (30%) patients with disseminated disease, lung metastatic foci were an incidental finding upon autopsy. This included metastatic HCC cells restricted to the lung microvasculature (Figure 2), with bona fide liver origin as confirmed by HepPar1 staining. These lung micrometastases were undetected in earlier imaging studies and/or gross examinations of the lungs. We speculated that these micrometastases could harbour dormant cells, and to test this we performed immunofluorescence for known markers of dormancy in other malignancies such as NR2F1 and p27.[33,34] There was no expression of these markers in the stained sections. Therefore, we could not find definitive evidence of cancer cell dormancy in any of the six patients with lung micrometastasis.

PROGENITOR CELL MARKERS AND TUMOUR GRADE IN HCC METASTASIS

We first evaluated the association between different phenotypical features in the primary tumour and the

Table 2. Clinical and pathological variables

Variable	*n* (%)	Variable	*n* (%)
Gender		AFP concentration	
Male	62 (70)	<100 ng/ml	46 (52)
Female	26 (30)	≥100 ng/ml	22 (25)
Age (years)		Not available	20 (23)
<65	58 (66)	Number of nodules	
≥65	30 (34)	Single nodule	33 (38)
Underlying liver disease*		Multiple nodules	55 (62)
HCV	47 (53)	Size (dominant nodule)	
HBV	15 (17)	<5.0 cm	51 (58)
Alcohol	18 (20)	≥5.0 cm	37 (42)
NASH	4 (5)	Vascular invasion	
Cryptogenic	6 (7)	Microvascular	79 (90)
Haemochromatosis	4 (5)	Macrovascular†	23 (32)
Others	2 (2)	Distant metastases	
Cirrhosis		Presence: single organ‡	14 (16)
Presence	79 (90)	Presence: different organs	6 (7)
Absence	9 (10)	Absence	68 (77)

HCV, Hepatitis C virus; HBV, Hepatitis B virus; NASH, Non-alcoholic steatohepatitis; AFP, Alpha-fetoprotein.
*Some patients had an overlap of primary diseases.
†Data not available in 16 patients.
‡Four patients had multiple metastatic nodules for the same organ.

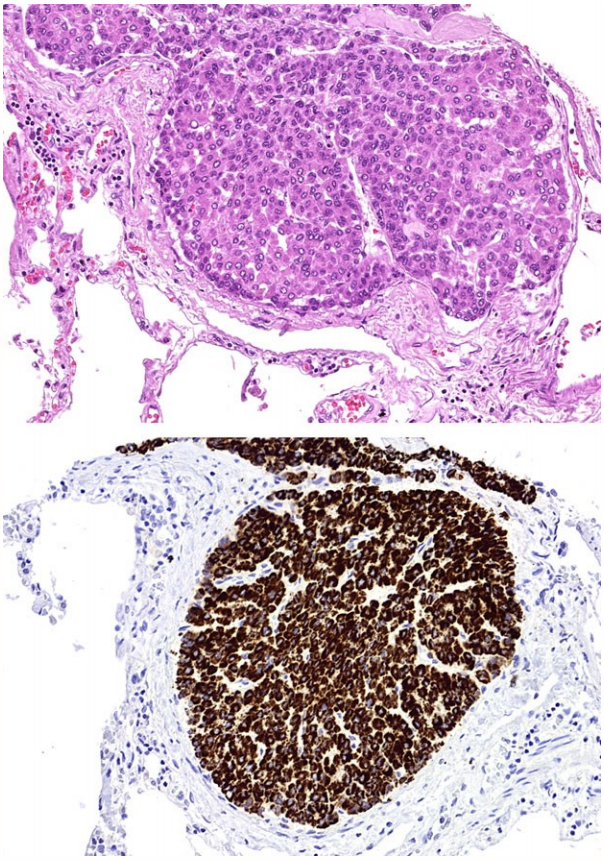

Figure 2. Clusters of tumour cells within the lung microvasculature. These cells accommodated and distended the vessel lumen. There were no signs of autolysis, necrosis or apoptosis, but some evidence of proliferation (mitosis) was present in these microvascular metastases.

presence of metastasis on autopsy examination. Size of the dominant tumour nodule ($P < 0.001$), multinodularity ($P = 0.004$), macrovascular invasion ($P = 0.003$), high histological ($P < 0.001$), nuclear ($P = 0.01$) and architectural ($P = 0.005$) grades and cellular crowding ($P = 0.03$) were significantly associated with the presence of extrahepatic metastasis. Among the IHC markers tested, extrahepatic nodules had significantly higher K19 (38% versus 15%, $P = 0.003$) and EpCAM (12% versus 3%, $P = 0.04$) positive staining than liver tumours. As predicted, both markers tended to be co-expressed in the same nodules. Sarcomatoid features and vimentin expression were observed in the liver and extrahepatic nodules from only a single patient, which probably explains the weak association of these features with the presence of distant metastases in this cohort. Clear cell HCC was also detected in the liver and lung nodules from a single patient. Steatohepatitic phenotype was identified in nodules from 11 of 88 (12.5%) patients. None of these patients presented with extrahepatic metastases. Finally, lymphocyte-rich and schirrous HCC were identified in one patient each, none with distant dissemination.

We next developed a binary logistic regression model to describe patterns of metastasis in HCC patients based on the variables described above. We did not include macrovascular invasion in this model, as the number of missing values for this variable was higher than 10%. Tumour size ($P = 0.003$), multinodularity ($P = 0.05$) and, to a lesser extent, high tumour grade ($P = 0.06$) were independently associated with the presence of metastasis in this model. Next, we focused on histological predictors of the presence of lung micrometastases and found a significant association with K19 staining ($P = 0.02$), and

high tumour ($P = 0.01$) and architectural ($P = 0.01$) grade.

We next sought to evaluate how concordant were histological features and progenitor cell markers between primary and metastatic nodules on a patient-by-patient basis (Figure 3). As expected, all the metastases in the cohort shared morphological and IHC features with at least one region in the primary tumour. Metastatic sites were of high tumour histological grade in all patients except three of 20 (15%) (i.e. A006, A024, A025, Figure 4). Strikingly, patients A006 and A025 had low-grade metastases despite at least one region of their primary tumours being of high grade. This discrepancy promoted us to evaluate whether these low-grade metastases had additional features suggestive of an aggressive tumour not captured with morphology. Thus, we assessed the Ki67 labelling index (LI) using immunohistochemistry as a proxy for proliferative activity in tumour cells. We analysed three patients who showed low-grade metastases and seven patients with high-grade metastases (Figure 4). The Ki67 LI was consistent with degree of differentiation, with low-grade metastases showing lower LI than high-grade metastases. This reinforces the notion that metastasis is a complex process not only determined by the

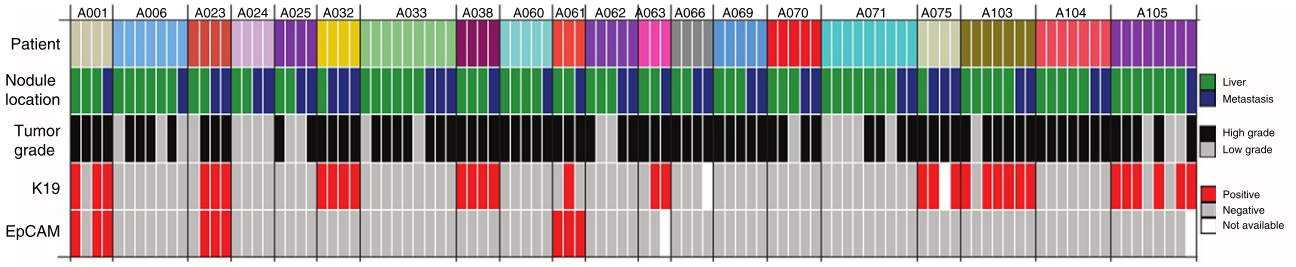

Figure 3. Distribution of histological grade and stem-like markers in 101 nodules from 20 patients with paired primary-metastatic HCC.

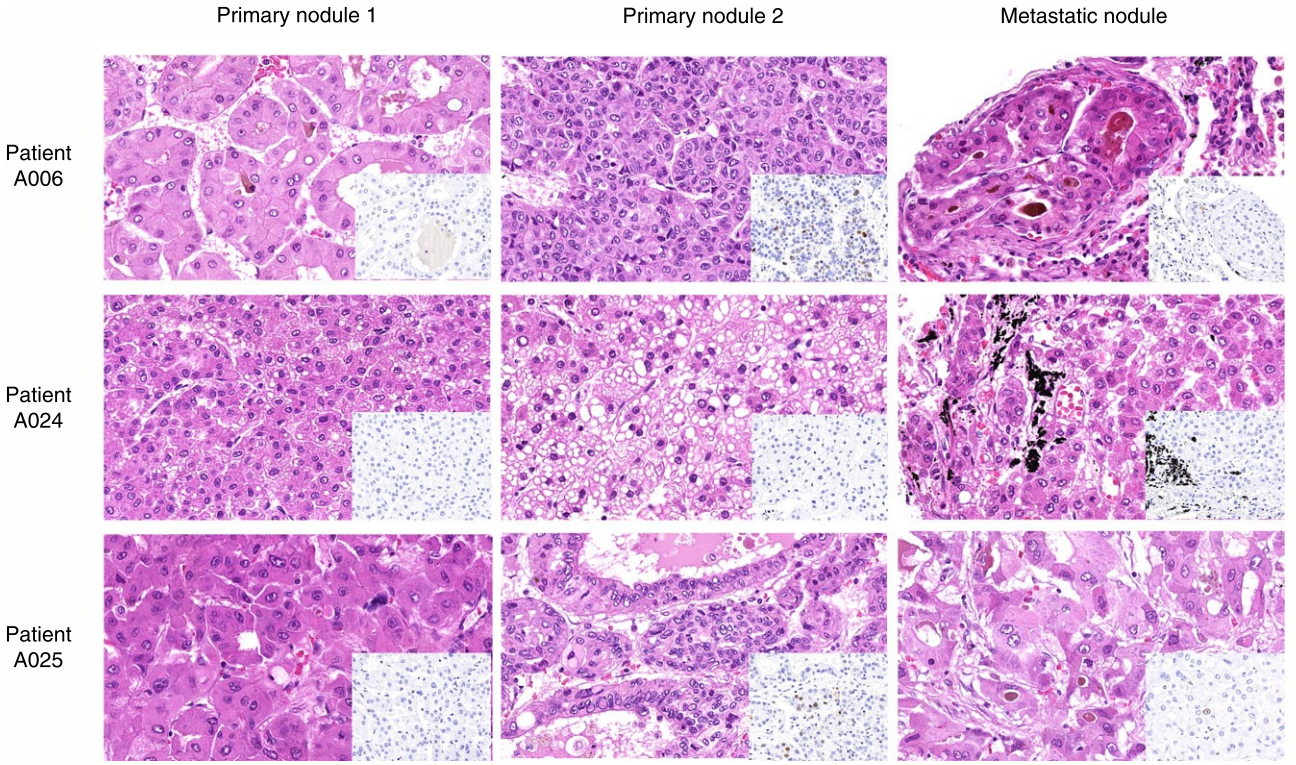

Figure 4. Histological representation of patients with low histological-grade metastases. Counterintuitively, two patients with (at least one) high tumour grade primary nodule showed a low-grade metastatic tumour. The Ki67 labelling index was consistent with tumour grade in these tumours.

aggressiveness of the tumour cells but by a permissive local microenvironment.

LIMITED PHENOTYPICAL HETEROGENEITY IN METASTATIC HCC

To understand more clearly tumour clonal distribution in patients with advanced HCC, we compared the degree of histological (i.e. tumour and nuclear grade) and phenotypical heterogeneity (IHC for progenitor cell and EMT markers) between primary liver tumours and their metastasis. In addition, we checked for *TERT* promoter mutations in six patients with multiple primary tumour nodules and metastatic sites. *TERT* promoter mutations are the most frequent single nucleotide variant present in approximately 60% of patients with HCC.[5] The goal of analysing the mutation status of *TERT* promoter between different tumour nodules of the same patient was to evaluate genetic heterogeneity and differences in clonal composition within patients. As this analysis only evaluated one mutation, it was not possible to construct phylogenetic trees to trace clonal composition, but it could provide pilot data on internodular genetic heterogeneity in patients with metastatic HCC. Within these six patients, we extracted DNA from 30 nodules and from six samples from non-tumoural tissue, which we used as germline to call somatic *TERT* promoter mutations. Of the 30 nodules, 17 were from primary tumour (i.e. at least two different nodules from each of the six patients) and 13 were from metastasis (at least two nodules from each of the patients). We extracted DNA from the 30 nodules and six of them did not meet the quality criteria for either sequencing or confident mutation calling. From the remaining 24 nodules, eight had *TERT* promoter somatic mutations in one of the two well-known hot-spots,[35] while 16 were wild-type.

For this study, we defined phenotypical heterogeneity as the presence of two different regions depicting different tumour grade (low versus high) and differential IHC status for any of the protein markers assessed. When focusing on intrahepatic tumour nodules, we observed phenotypical heterogeneity in 26 of 50 (52%) of the patients for whom multiple samples were available. Interestingly, intertumour heterogeneity within the metastases was much more limited, being observed only in 2 of 12 (17%) of the patients with multiple sampling from metastatic sites. One of these two patients had multiple adrenal metastasis, with two different histological patterns and HepPar1 expression, sometimes displaying a metastasis-to-metastasis configuration (Figure 5). The second patient showed positive staining for markers of active WNT signalling in the infiltrated lymph node, but not in the lung metastasis (Figure 6). Regarding *TERT* promoter mutation results, we observed mutational heterogeneity between primary liver tumour nodules of two of six patients (i.e. there were discordant mutation results in different tumour nodules from the same individual in two of six patients). The remaining four patients were either wild-type (n = 3) or had *TERT* mutation (n = 1) in all the hepatic nodules. However, there was no mutational heterogeneity within the metastatic disease in any of these patients. In other words, the *TERT* promoter mutation status was the same in all metastatic sites from all patients analysed (Figure 7).

Discussion

This study reports on a comprehensive evaluation of morphological and phenotypical features of multiple tumour regions from 88 autopsy specimens from patients with HCC, including 20 with extrahepatic metastatic disease. Our goal was to understand how tumour grade and key phenotypical features (i.e. expression of progenitor cell and EMT markers) were distributed in different tumour sites in patients with advanced disease. As tissue biopsy is not standard practice in HCC patients and samples from surgical and transplant specimens are mainly from early stages of HCC, the use of autopsy material is crucial to understand more clearly the distribution of these

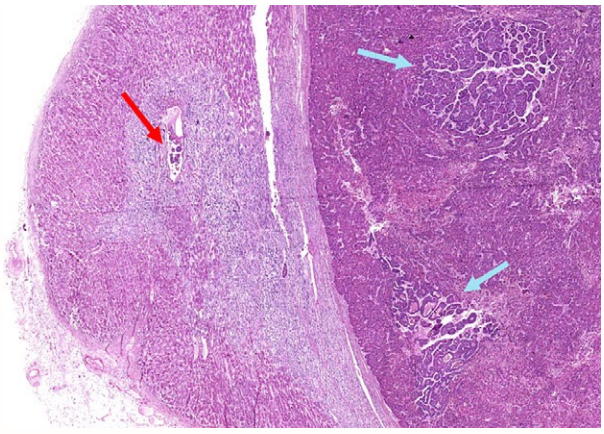

Figure 5. Histological representation of an adrenal metastasis with two distinct histological patterns. Interestingly, a tumour with higher cellular crowding and number of mitosis (blue arrows) seems to be arising within the established metastasis. We also found multiple emboli in the primary and metastatic disease (red arrow) with clusters of tumour cells from the less differentiated tumour.

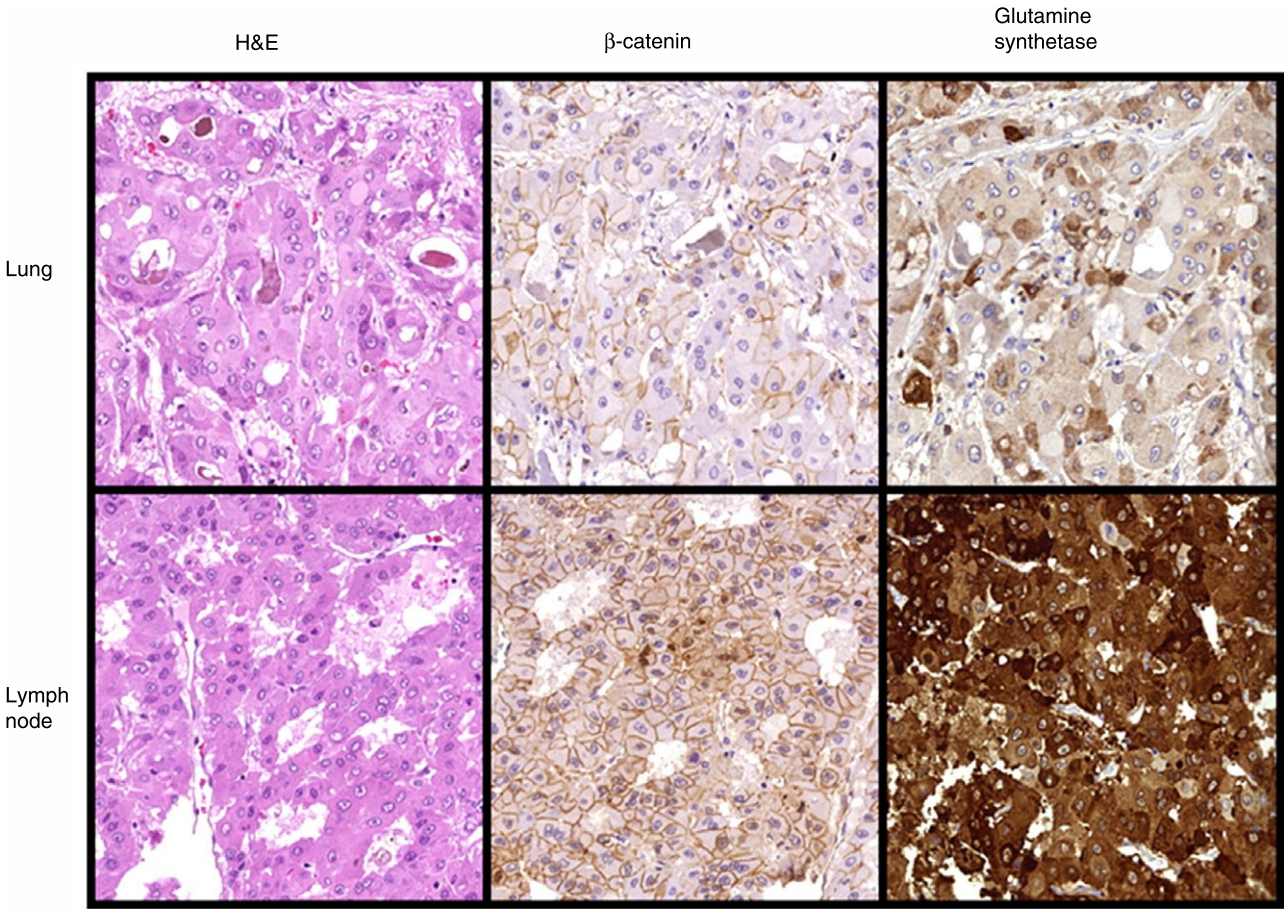

Figure 6. Differential expression of WNT markers between the lung and lymph node metastasis. The lung metastases showed lower cellular crowding and some bile production. It was negative for the markers associated with WNT activation. Conversely, the lymph node metastasis showed higher cellular crowding, no bile production (but pseudocysts with red blood cells) and was positive for markers of WNT activation. The primary nodules reproduced the heterogeneity observed in the metastases.

features in patients with disseminated disease. We describe how metastatic HCC sites recapitulate the morphological and phenotypical features found in at least one region of the corresponding primary tumours. Notably, in 30% of the patients with disseminated tumours, we observed micrometastasis to the lung microvasculature, previously unnoticed on imaging or upon gross examination. The clinical significance of this is unclear, as the actual prevalence of lung micrometastases is unknown. Prior studies have suggested that these disseminated tumour cells entrapped in the microvasculature of distant organs are the first step prior to parenchymal invasion and clinically identifiable metastases.[36] However, the mechanisms that determine how these early metastatic niches progress to full-blown macroscopic metastasis in HCC are unclear. Also, the time-lapse of this process is unknown, but data from pancreatic cancer[16] suggests that the period of early subclinical tumour dissemination may be longer than initially thought. We failed to find markers of cell dormancy in these micrometastases, but as these markers were derived from other tumour types, we cannot exclude an HCC-specific transcriptional programme governing cell dormancy in lung micrometastases.

The degree of cell differentiation is usually associated with prognosis in HCC, despite the lack of consensus on HCC histological grading in the literature.[37] In this study, we found that high histological grades in the primary tumour were associated with the presence of distant metastases. Also, distant metastases were mainly constituted by poorly differentiated tumours, further suggesting the role of histological grading as a morphological proxy for HCC aggressiveness. In fact, large-scale pathological studies have suggested that even focal areas of poor differentiation in the primary tumour predicts poor survival.[38–40] Indeed, some hospitals included

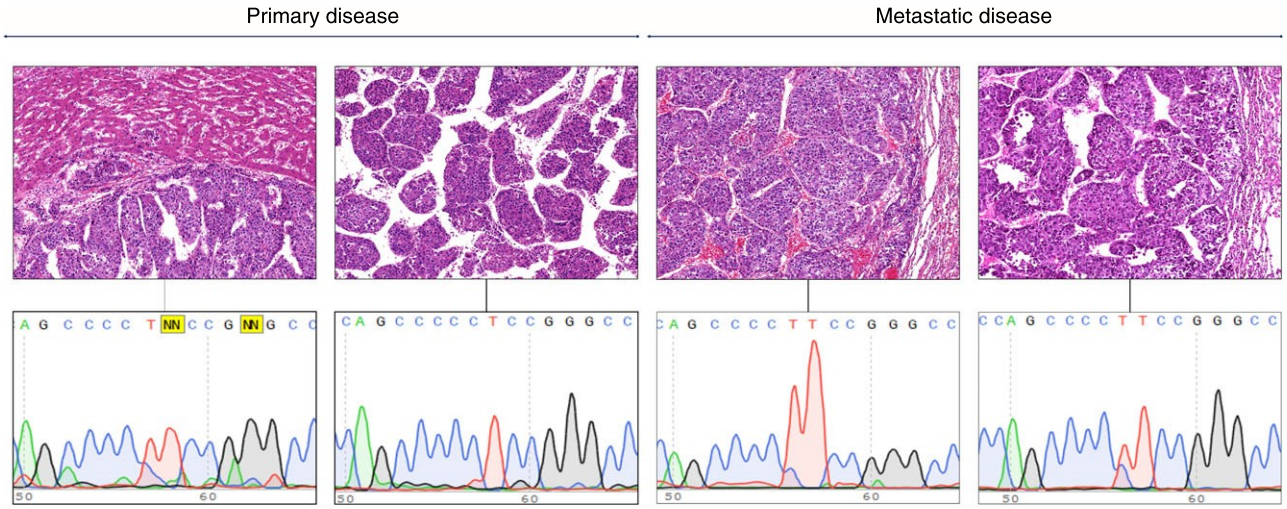

Figure 7. Tumour heterogeneity was attenuated within the metastatic disease. This patient presented with multiple liver and lung nodules. One primary nodule depicted a hot-spot *TERT* promoter mutation while another did not have it. Two metastatic nodules from different lung lobes were homogeneous at the morphological and molecular levels: they both presented with the same hot-spot *TERT* promoter mutation observed in one of the liver nodules.

histological grading among the eligibility criteria for liver transplantation: patients with low-grade tumours had significantly better outcome even when tumours are large, beyond the Milan Criteria.[41,42] Notably, in the present study, poor differentiation was not ubiquitous within metastatic HCC. Two patients showed moderately differentiated tumours in their lung metastases, whereas we detected areas of poor differentiation in their paired liver tumours. Despite being uncommon, this finding poses new questions on how early cells acquire metastatic potential, at least in a subset of HCC. This finding is in accordance with a recent study that described early metastatic dissemination in breast cancer.[43] Although HCC histological subtypes were not common in the present series, 11 of 88 (12.5%) patients presented with steatohepatitic HCC. Interestingly, none of these patients had extrahepatic metastases.

This study also found a significant association between expression of progenitor cell markers in the primary tumour, particularly K19, and the presence of metastatic disease. We noted expression of this intermediate filament in 20% of the patients, aligned with the reported prevalence of 10–18%.[44,45] The fact that a significant number of the patients have metastatic disease explains the high K19 expression in our cohort. Expression of K19 has been frequently considered as a poor prognostic factor in HCC, being associated with vascular invasion, worse overall and disease-specific survival and resistance to sorafenib.[18,44–47] Genomic studies have also suggested that patients with tumours with progenitor cell features have worse survival.[48] We confirm our previous finding of high K19 staining in metastatic HCC cells,[14] thus suggesting that K19 staining identifies the cells primarily involved in HCC dissemination in a subset of patients.

Different studies demonstrate the value of analysing intratumour heterogeneity to understand cancer progression.[7–9] The analysis of whole exome sequencing data in multiple regions of clear cell renal cell carcinomas helped to identify seven evolutionary subtypes later segregated into two categories based on proliferation rate, with further implications in metastatic potential.[49] Immunohistochemical patterns of oestrogen receptor (ER) expression allowed estimation of intratumour heterogeneity in breast cancer, which correlated with survival.[50] In the present study, we also used histological and phenotypical data to trace clonal composition and tumour evolution in HCC. Previous studies in other malignancies utilised autopsy specimens to trace cancer clonal evolution using DNA sequencing data.[16] We found that tumour grade and K19 staining were heterogeneously distributed among the different primary HCC nodules in the same patient. However, we did not find such heterogeneity in metastatic sites in those patients with multiple metastases. This was further suggested in the subset of patients for which *TERT* promoter mutation analysis was available (i.e. mutation status was heterogeneous among the primary tumour sites, but not in the metastatic nodules). These data suggest that metastatic cells may be

subjected to stronger evolutionary constraints compared to primary tumours. Alternatively, this limited heterogeneity could be due to a time bias, as metastases developed later during tumour evolution, having less time to evolve compared to primary tumours. A limitation of our study is the relatively small sample size of metastatic cases. Also, we do not have genome-wide DNA sequencing data to provide a thorough evaluation of clonal composition between the different tumour sites and confirm clonal constraints in metastatic sites.

In summary, this study provides insights on the distribution of phenotypical heterogeneity in advanced HCC, particularly to the extent that they help to delineate evolutionary constraints in 20 patients with metastatic disease. The demonstration of histological lung micrometastases and low-grade differentiation in some of the metastases opens new hypotheses on the timing and patterns of dissemination of HCC cells. Such findings also illustrate how autopsies can go beyond their primary objective of defining the cause of death and help address biological questions. Multiregional sampling of large cancers including common metastatic sites that were not macroscopically affected should be considered as part of an academic autopsy protocol. As high-throughput sequencing technologies become more readily available in challenging specimens (such as FFPE samples), comprehensive molecular analysis of paired primary and metastatic sites collected from autopsies will be crucial to understand the mechanisms that govern cancer dissemination.

Acknowledgements

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Conflicts of interest

A.V. has received consulting fees from Guidepoint and Fujifilm; advisory board fees from Exact Sciences, Nucleix and NGM; and lecture fees from Exelixis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Histological characterization of the samples.

Figure S1. Schematics of multi-region tissue microarray (TMA) spotting. All the nodules histologically available were sampled for the TMA blocks. Furthermore, nodules with intra-tumor fibrosis or distinctive histological patterns (such as nodule-in-nodule) had these different areas adequately sampled. Histological and immunohistochemical results were defined per nodule (based on the combination of all the areas spotted).

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da heterogeneidade intratumoral, nos níveis morfológico e molecular, em casos de carcinoma hepatocelular.

Pesquisador: VENANCIO AVANCINI FERREIRA ALVES

Área Temática:

Versão: 2

CAAE: 49042015.3.0000.0068

Instituição Proponente: HOSPITAL DAS CLINICAS DA FACULDADE DE MEDICINA DA U S P

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.297.918

Apresentação do Projeto:

Trata-se de um estudo para avaliar a heterogeneidade intratumoral da Universidade de São Paulo,com a colaboração de Universidade de Icahn/ Hospital Mount Sinai (Nova Iorque, EUA).Serão realizadas avaliações morfológicas e moleculares em casos de carcinoma hepatocelular.O projeto está claro e seus resultados poderão contribuir para apoio diagnóstico nesta enfermidade.No entanto, apesar do estudo ser retrospectivo amostras de tumor de pacientes que evoluíram a óbito por carcinoma hepatocelular serão armazenadas em outro centro.

Objetivo da Pesquisa:

Os pesquisadores vão correlacionar achados morfológicos–através de revisão histológica de reconhecido ou potencial impacto prognóstico com achados moleculares, obtidos através de sequenciamento de material genético, para avaliação de mutações específicas.O projeto tem relevância na área médica e seus resultados poderão ser extremamente úteis na prática clínica.

Avaliação dos Riscos e Benefícios:

O desenvolvimento do protocolo de pesquisa não trará riscos para o sujeito da pesquisa e os benefícios serão refletidos em maior reconhecimento dos tumores e no diagnóstico desta enfermidade

Comentários e Considerações sobre a Pesquisa:

A coordenação da pesquisa é brasileira, no entanto terá a cooperação estrangeira prevista na RES 466/15.

Considerações sobre os Termos de apresentação obrigatória:

A pesquisa dispensa TCLE e os termos de apresentação obrigatória estão dentro das normas desta comissão.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Sem pendências éticas.

Considerações Finais a critério do CEP:

Em conformidade com a Resolução CNS nº 466/12 – cabe ao pesquisador: a) desenvolver o projeto conforme delineado; b) elaborar e apresentar relatórios parciais e final; c)apresentar dados solicitados pelo CEP, a qualquer momento; d) manter em arquivo sob sua guarda, por 5 anos da pesquisa, contendo fichas individuais e todos os demais documentos recomendados pelo CEP; e) encaminhar os resultados para publicação, com os devidos créditos aos pesquisadores associados e ao pessoal técnico participante do projeto; f) justificar perante ao CEP interrupção do projeto ou a não publicação dos resultados.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_563568.pdf	26/10/2015 15:06:07		Aceito
Outros	pendencia_CEP.pdf	26/10/2015 15:02:18	Sebastião Nunes Martins Filho	Aceito
Declaração de Instituição e Infraestrutura	Carta_convite_MountSinai.pdf	09/09/2015 12:41:07	Sebastião Nunes Martins Filho	Aceito
Declaração de Instituição e Infraestrutura	Aprovacao_DAPHC.pdf	04/09/2015 09:24:41	Sebastião Nunes Martins Filho	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	IsencaoTCLE.pdf	04/09/2015 09:19:44	Sebastião Nunes Martins Filho	Aceito
Outros	CadastroOnlineCapPesq.pdf	04/09/2015 09:19:19	Sebastião Nunes Martins Filho	Aceito

Folha de Rosto	FolhadeRostoPlataformaBrasil.pdf	01/09/2015 12:07:50	Sebastião Nunes Martins Filho	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_detalhado_Plataforma_Brasil.pdf	25/08/2015 15:45:44	Sebastião Nunes Martins Filho	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 27 de Outubro de 2015

Assinado por:
ALFREDO JOSE MANSUR
(Coordenador)

REPÚBLICA FEDERATIVA DO BRASIL
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LIVRO/BOOK Nº 92 TRADUÇÃO/TRANSLATION Nº 10586 FOLHA/SHEET Nº 53

I, MARIO MIGUEL FERNANDEZ ESCALEIRA, Public Sworn Translator for the PORTUGUESE, ENGLISH, FRENCH and SPANISH languages, in and for the State of São Paulo, Brazil, certify that on this 29th day of February 2016, in this city of São Paulo, was submitted to me a text written in the PORTUGUESE language, which I hereby translate into the ENGLISH language, word for word, to the best of my knowledge and ability, as follows:

Hospital das Clínicas da FMUSP
Research Ethics Board for the Analysis of Research Projects - CAPPesq
RESEARCH PROJECT

Title: ASSESSMENT OF INTRATUMOR HETEROGENEITY AT THE MORPHOLOGICAL AND MOLECULAR LEVELS, IN CASES OF HEPATOCELLULAR CARCINOMA.
Chief Researcher: Venancio Avancini Ferreira Alves
Version: 2
Researcher: Sebastião Nunes Martins Filho CAAE: 49042015.3.0000.0068
Academic Purposes: Ph. D. Degree
Advisor: Venancio Avancini Ferreira Alves
Institution: HCFMUSP
Department: PATHOLOGY

CEP CONSUBSTANTIATED OPINION

On-line record: 14042
Opinion number: 1.297.918
Report Date: 10/27/2015 - ad-referendum
Project Submission: The purpose of the study is to assess intratumor heterogeneity at Universidade de São Paulo, with the collaboration of Icahn University/Mount Sinai Hospital (New York, USA). The study shall involve morphological and molecular assessments in cases of hepatocellular carcinoma. The project is clear and its results may contribute for a diagnosis support in this disease. However, although the study is retrospective, samples of tumor of patients which evolved to decease due to hepatocellular carcinoma shall be stored in another center.
Research Goals: The researchers shall correlate morphological findings - through histological review with a recognized or potential prognostic impact and molecular findings, obtained through the sequencing of genetic material, for assessment of the specific changes. The project is relevant in the medical domain and its results may be extremely useful in clinical practice.
Assessment of the Risks and Benefits: The development of the research protocol shall not cause risks to the research subject and the benefits shall be reflected in a greater recognition of tumors and in the diagnosis of this disease.
Comments and Considerations about the Research: The Brazilian parties shall coordinate the research, although the foreign cooperation is provided for as per RES 466/15.
Considerations about the Mandatory Submission Terms: The research dispenses with TCLE and the mandatory submission terms comply with the rules of this Board.
Conclusions or Pending Matters and List of Inadequacies: No ethical pending matters verified.
Opinion Status: Approved
CONEP's Approval Needed: No
Final Considerations at the discretion of CEP: Pursuant to Resolution CNS nº 466/12 - the researcher shall: a) develop the project as designed; b) prepare and submit partial and final reports; c) submit the data requested by the CEP, at any time; d) keep custody of the research file under his/her responsibility for 5 years, with individual files and all other documents as recommended by the CEP; e) to forward the results for publication, with the corresponding credits to associate researchers and the technical team that has participated in the project; f) to justify before the CEP any interruption of the project or the non-publication of the results.
São Paulo, October 27, 2015.
<signature> Prof Dr. Alfredo José Mansur Coordinator of the Research Ethics Board for the Analysis of Research Projects - CAPPesq
<at the bottom of both pages, it reads as follows:>
Rua Dr. Ovídio Pires de Campos, 225 - Prédio da Administração - 5º andar
ZIP 05403-010 - São Paulo - SP
Phone 55 11 2661- 7585 - 55 11 2661-6442 - Extensions: 16, 17, 18 / cappesq.adm@hc.fm.usp.br

Further Naught. I certify that the preceding is a true, faithful and unabridged rendering into English of the original Portuguese version. In witness whereof, I set my hand and seal, on the date and in the city first mentioned.

São Paulo, 29 de fevereiro de 2016 Recibo 7041 Emolumentos: R$ 205,00

MARIO MIGUEL FERNANDEZ ESCALEIRA
TRADUTOR JURAMENTADO