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Non-Alcoholic Fatty Liver Disease-Related Hepatocellular Carcinoma: Immunohistochemical Assessment of Markers of Cancer Cell Metabolism

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Keywords
Hepatocellular carcinoma · Non-alcoholic fatty liver disease · Monocarboxylate transporters · Warburg effect

Abstract
Introduction: Hepatocellular carcinoma (HCC) has been associated to non-alcoholic fatty liver disease (NAFLD). We sought to investigate the immunoexpression of several glycolytic metabolism-associated markers in patients with HCC associated to NAFLD and associate these factors to their clinical-pathological characteristics. ***Methods:*** We evaluated 35 HCC specimens from 21 patients diagnosed with non-alcoholic steatohepatitis (NASH) undergoing liver resection (12 patients), liver transplantation (8 patients), or both (1 patient). Histological features, clinical aspects, demographic and biochemical data, as well as the immunohistochemical reactivity for monocarboxylate transporters 1, 2, and 4; their chaperone CD147; carbonic anhydrase IX; and glucose transporter-1 (GLUT1) were assessed. ***Results:*** Metabolic-associated cirrhosis was present in 12 of the 21 patients (8 child A and 4 child B scores). From 9 patients without cirrhosis, 3 presented NASH F3 and 6 NASH F2. Sixteen (76%) had diabetes mellitus, 17 (81%) arterial hypertension, and 19 (90%) body mass index above 25 kg/m^2; 8 (38%) had dyslipidemia. From 35 nodules, steatosis was found in 26, ballooning in 31 nodules, 25 of them diagnosed as steatohepatitic subtype of HCC. MCT4 immunoexpression was associated with extensive intratumoral fibrosis, advanced clinical stages, and shorter overall survival. GLUT1 was noticeable in nodules with extensive intratumoral steatosis, higher intratumoral fibrosis, and advanced clinical stages. Immunohistochemical expression of the metabolic biomarkers MCT4 and GLUT1 was higher in patients with Barcelona-clinic liver cancer B or C. GLUT1 correlated with higher degree of steatosis, marked ballooning, intratumoral fibrosis, and higher parenchymal necroinflammatory activity. ***Conclusion:*** Our data indicate that the expression of the glycolytic phenotype of metabolic markers, especially GLUT1 and MCT4, correlates with a more severe course of HCC occurring in NASH patients.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) has been associated to several etiological factors including insulin resistance, type 2 diabetes, obesity, and drug toxicity [1]. NAFLD, a highly prevalent chronic liver disease globally, can evolve to a non-alcoholic steatohepatitis (NASH), a potentially aggressive condition, that in turn can progress to cirrhosis and to hepatocellular carcinoma (HCC). It is mostly associated with genetic factors such as expression of Patatin-like phospholipase domain-containing 3 and to environmental factors [2]. The increase in HCC incidence is attributed to an augmented incidence of metabolic alterations related to obesity, diabetes, and NAFLD [3], as well as to chronic C hepatitis, still an international public health problem despite the success of direct antiviral therapy. Since the differential diagnosis, grading, and architectural ("fibrosis") staging of NASH include the findings of steatosis, ballooning, and necroinflammation, potentially leading to fibrosis and even to cirrhosis, liver biopsy assessment is mandatory for its diagnosis. Additionally, the combination of NASH and HCC has been associated to the increased index of cardiovascular morbidity and mortality [4, 5].

Reprogramming of the energy metabolism has been associated with aggressiveness of many cancers. For decades, the so-called Warburg Effect has been a target of oncology investigation. The scientific rescue of the centenary Warburg premises postulated that cancer cells have an increase in the glycolytic metabolism, with increased glucose uptake and glycolysis rates, linked to the production of lactate (lactate fermentation) as the end product, independently of oxygen concentrations [6, 7]. To prevent intracellular acidity and glycolysis inhibition by accumulation of the end product, lactate, cells need to upregulate specific proteins involved in lactate transport and pH regulation, namely, monocarboxylate transporters (MCTs) and carbonic anhydrase IX (CAIX) [8, 9]. The hyperglycolytic and acid-resistant phenotype of malignant neoplasms contribute to cancer progression through angiogenic stimulation and invasion, among other factors. Thus, potential therapeutic strategies based on blocking lactate efflux from cancer cells into the tumour microenvironment have been studied in a variety of solid tumours [10–12]. The MCT isoforms 1, 2, and 4 are the most well-described isoforms responsible for proton-coupled lactate transport across the plasma membrane [13]. Importantly, these transporters require the co-expression of an ancillary protein for their plasma membrane location and transport activity. The principal chaperone of MCT1 and MCT4 is CD147, while MCT2 is mainly associated with gp70 [14]. We have recently demonstrated that MCT1, 2, and 4 play a fundamental role in the maintenance of this glycolytic phenotype, by mediating lactate efflux from many types of cancer cells, and influence the prognostic of cancer patients [9]. We have also studied the expression of CD147, glucose transporter-1 (GLUT1), an essential glucose transporter, and lactate dehydrogenase-A, in cirrhosis and HCC cases. Previous studies from our group demonstrated that MCT4 and GLUT1 showed progressively higher expression from non-neoplastic to primary HCC and to metastases, while MCT2 expression is lost during tumour progression. In opposition to MCT4, MCT2 expression was associated with better prognosis [15]. These results provided evidence for the role of MCT4 and GLUT1 in HCC development. Based on the results of our previous studies, including also our recent clinical-pathological assessment of HCC occurring in a cohort of NASH cases clinically followed [16] and on the potential usefulness of markers of glycolytic phenotype to understand HCC biology, the present study aimed to investigate the expression of GLUT1, MCTs, CD147, and CAIX in a series of HCC clinically associated with NASH.

Materials and Methods

Human Tumoural Samples

Thirty-five hepatic nodules from 21 adult patients (≥18 years) with HCC secondary to well-established NASH examined and treated at the Liver Transplant Division or Liver Surgery and Biliary Tract Division of the University of Sao Paulo School of Medicine Hospital (HC-FMUSP) were collected from 2005 to 2015. Detailed clinical and histopathological data have been previously published [16]. NASH was diagnosed when consistent present or past histological features of steatosis + ballooning + lobular inflammation, and all other known causes of liver disease were excluded. Architectural staging of NASH (fibrosis, 0–4) followed the criteria presented by Kleiner et al. [17]. Metabolic syndrome was defined according to the National Cholesterol Education Program Adult Treatment Plan III guidelines (ATP-III) [18]. Diagnosis of HCC was based on the international guidelines of the American Association for the Study of Liver Diseases [19] and European Association for the Study of the Liver [20]. All cases had histopathological diagnosis performed on surgical/explant specimens and confirmed in a thorough review by an experienced liver pathologist (VAFA) according to the criteria defined by the International Consensus Group for Hepatocellular Neoplasia [21]. The histological diagnosis of "steatohepatitic" subtype of HCC (SH-HCC) followed the criteria defined by Salomão et al. [22].

This retrospective series is composed of 35 formalin-fixed paraffin-embedded nodules, which were organized into tissue microarrays (TMAs) with 1 mm diameter cores. Each nodule was represented in the TMA by multiple cores representing different areas

Table 1. Detailed aspects for each antibody used in immunohistochemistry

Protein	Antigen retrieval	Antibody	Antibody dilution and incubation time
MCT1	Citrate buffer (0.01 M, pH = 6), 98°C, 20′	AB3538P; Chemicon International	1:400, overnight
MCT2	Citrate buffer (0.01 M, pH = 6), 98°C, 20′	sc-50322; Santa Cruz Biotechnology	1:200, 2 h
MCT4	Citrate buffer (0.01 M, pH = 6), 98°C, 20′	sc-50329; Santa Cruz Biotechnology	1:500, 2 h
CD147	EDTA (1 mM, pH = 8), 98°C, 20′	sc-71038; Santa Cruz Biotechnology	1:400, overnight
GLUT1	Citrate buffer (0.01 M, pH = 6), 98°C, 20′	ab15309-500; AbCam	1:500, 2 h
CAIX	Citrate buffer (0.01 M, pH = 6), 98°C, 20′	ab15086; AbCam	1:2,000, 2 h

GLUT1, glucose transporter-1; CAIX, carbonic anhydrase IX.

of the tumour, selected by an experimented pathologist (SNMF) – the tumour centre, the invasion front of the tumour – as well as of non-tumoural liver tissue – samples from liver tissue adjacent to the tumour and non-tumoural tissue as far as possible from the tumour. Control samples were also included for TMA orientation, serving also as positive and negative controls for the immunohistochemical studies.

The following clinical and laboratory data were obtained retrospectively via review of medical records, which was performed by a single researcher (PBC), selecting data obtained at the time of HCC diagnosis: gender, age, weight, height, and alanine aminotransferase; aspartate aminotransferase; activity of prothrombin; bilirubin, albumin, platelet count, and Eastern Cooperative Oncology Group-Performance Status. Hepatic function was assessed using the Child-Pugh score. Body mass index (BMI) was subgrouped as BMI ≤ 25 and BMI > 25, encompassing overweight and obese patients, respectively. Also, clinicopathological data including the Barcelona-clinic liver cancer (BCLC) staging system, large portal or hepatic vein invasion, and treatment (resection or transplant) [23] were registered [24].

Immunohistochemistry

MCT1 and CD147 immunostaining were performed with a polymer system (UltraVision ONE Detection System; HRP Polymer Lab Vision Corporation, Fremont, CA, USA) as previously described [25]. Immunohistochemistry for MCT2, MCT4, GLUT1, and CAIX was performed according to the streptavidin-biotin-peroxidase complex principle (Ultravision Detection System Antipolyvalent, HRP; Lab Vision Corporation), as previously described [25]. Negative controls were performed by the use of appropriate serum controls for the primary antibodies (N1698 and N1699; Dako, Carpinteria, CA, USA). Colon carcinoma tissue was used as positive control for MCT1, MCT2, MCT4, and CD147; normal stomach for CAIX; and breast cancer for GLUT1. Tissue sections were counterstained with haematoxylin and permanently mounted. Detailed features for each primary antibody are displayed in Table 1.

Immunohistochemical Evaluation

The expression of lactate transporters – MCTs –(MCT1, MCT2, and MCT4), MCT chaperone – CD147, GLUT1, and the hypoxia marker – CAIX was studied in different compartments of each nodule. Thus, for each nodule, centre and the "invasive front" of the tumour were individually assessed for each marker, whereas the non-tumoural area was represented by the tissue adjacent to the tumour and by distant parenchyma. Two independent observers performed immunohistochemical evaluation, and discordant results were discussed in a double-head microscope to determine the final score.

Sections were scored semi-quantitatively for extension of staining as follows: 0: no immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5–50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Intensity of staining was semi-quantitated as follows: 0: negative, 1: weak, 2: intermediate, and 3: strong. The final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (scores 0 and 2) or positive (scores 3, 4, 5, and 6). Based on the concept of "hotspot," the spot which yielded the highest score achieved in whichever region from each nodule was selected as the most representative.

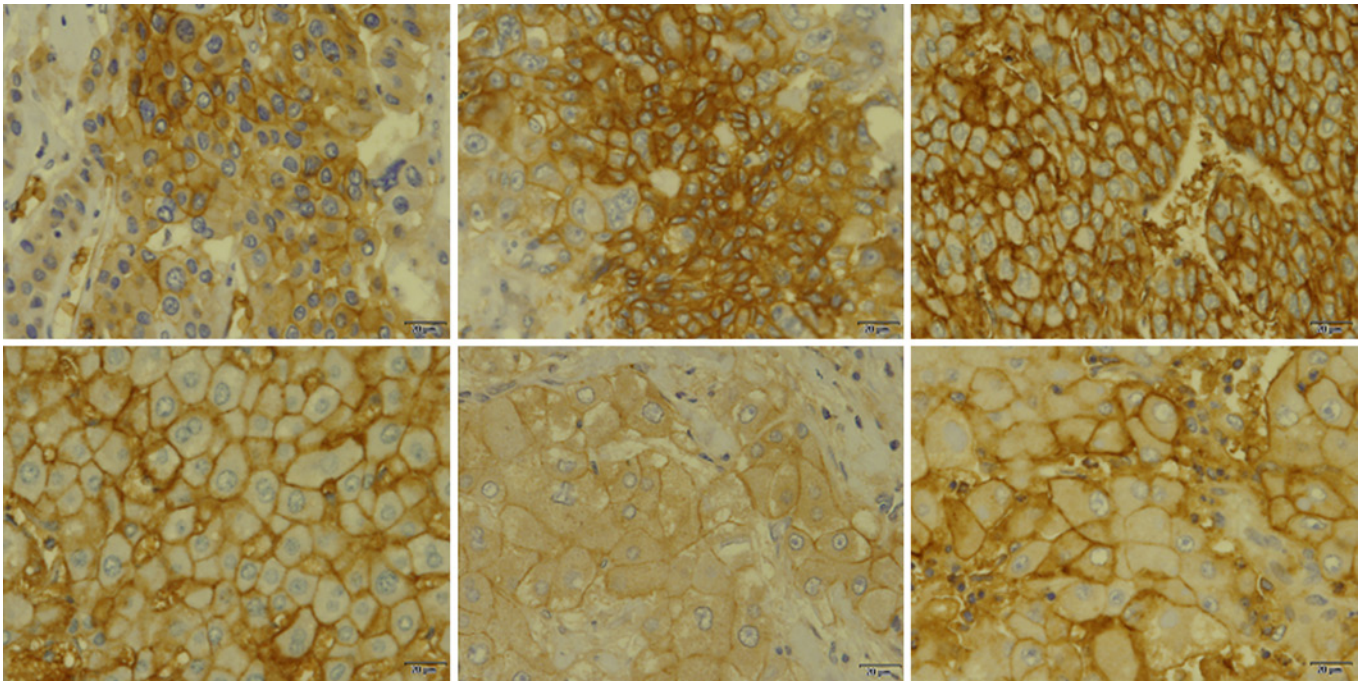
Beyond the overall result (positive vs. negative), protein localization was also individually assessed; whenever a sample presented staining of the membrane positive, the reaction was classified as "membranous pattern," even if cytoplasmic staining was concomitantly found. When plasma membrane expression was absent, the reaction was considered negative.

Statistical Analysis

Data were stored and analyzed using the IBM SPSS Statistics software (version 23; IBM Co., Armonk, NY, USA). All comparisons were examined for statistical significance using Pearson's χ^2 test (χ^2) or Fisher's exact test, according to sample characteristic. Overall survival was defined as the time between the date of first consultation and date of last information or patient death. Overall survival curves were estimated by the method of Kaplan-Meier, and data were compared using the Breslow test. The threshold for significance was $p \leq 0.05$. All the reported p values are 2-sided.

Results

A total of 35 nodules of HCC from 21 patients were included in this study. Cirrhosis was present in 12 patients: 8 patients child A and 4 patients child B scores. Among the 9 patients who did not present cirrhosis, 6



Color version available online

Fig. 1. Immunohistochemical expression of MCT1, MCT2, MCT4, CD147, GLUT1, and CAIX in HCC secondary to NAFLD samples. All the proteins were more importantly found in the plasma membrane of cells.

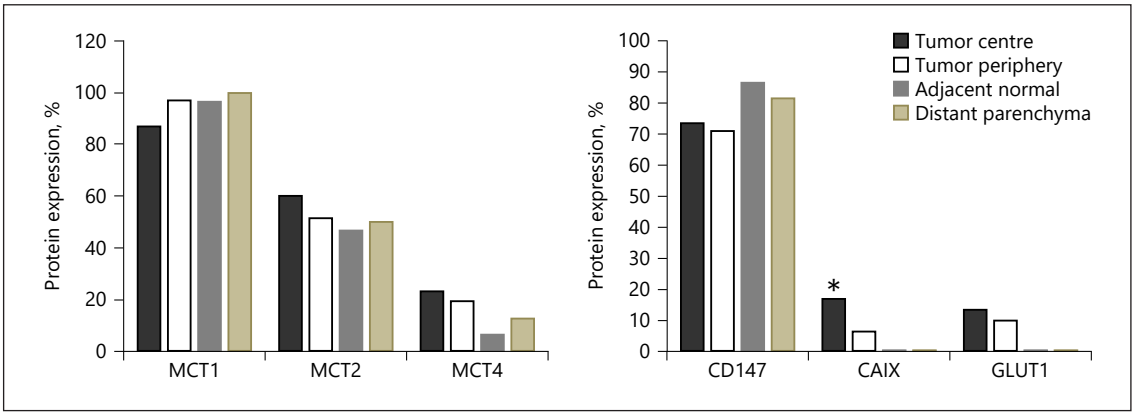


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Fig. 2. Frequency of the expression of MCT1, MCT2, MCT4, CD147, GLUT1, and CAIX in the different regions of the nodules. The positive immunohistochemical reactions localized in the plasma membrane. Pearson's χ^2 test was used to assess differences of expression frequency ($p < 0.05$): * versus normal adjacent.

patients had NASH stage F2 and 3 patients had NASH F3. The patients' ages ranged from 50 to 77 years, and 16 patients were male (76%). Sixteen patients (76%) had diabetes mellitus, 17 patients (81%) had arterial hypertension, and 19 patients (90%) had BMI above 25 kg/m^2. Only 8 patients (38%) had dyslipidemia. The alpha-fetoprotein level was normal in 13 patients. BCLC classification and staging of HCC in cirrhotic patients revealed 9 patients staged as BCLC A, 1 patient as BCLC B, and 2 cases as BCLC C. Regarding the 35 nodules, steatosis was found in 26 nodules, ballooning in 31 nodules, and 25 of them were diagnosed as SH-HCC (Fig. 1, 2).

Immunohistochemical Expression of the Markers in Histological Compartments

All proteins presented a membranous pattern of expression (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000521034). For MCT1, MCT2, and MCT4, no differences between normal and tumour cells were observed (Table 3; online suppl. Fig. 2). MCT1 was the most frequently expressed isoform in the tumour. Importantly, CAIX and GLUT1 plasma membrane positivity were only detected in the centre of the tumour.

Pathological Significance of Cell Metabolism Markers

Table 2 describes the histopathological data of 35 nodules from 21 patients with HCC secondary to NASH. Table 3 depicts the frequencies of protein expression of MCTs, CD147 GLUT1, and CAIX in the centre of the tumours. Higher MCT1 expression in the centre of the tumour was significantly associated with a higher architectural NASH stage ($p = 0.026$, Table 4). In opposition, MCT1 expression in the centre of the tumour was negatively associated to "periportal necroinflammatory activity" ($p = 0.026$) and to intratumoral steatosis ($p = 0.005$). Regarding MCT2 (Table 5), the expression in the centre of the tumour was significantly correlated with the absence of lobular inflammation ($p = 0.035$), whereas in the invasion front of the tumour, it was associated with a high architectural stage of NASH ($p = 0.002$).

As depicted in Table 6, CAIX expression in the centre of the tumour was significantly associated with no structural alterations, with a high architectural NASH stage and with a BCLC high stage, whereas GLUT1 expression was only associated with a BCLC high stage. GLUT1 expression in the invasion front of the tumour was correlated with intratumoral fibrosis. The expression of GLUT1 in the centre of the tumour was significantly associated with peritumoural inflammation, whereas absence of CAIX was significantly associated with BCLC stage 0 and with NASH structural stage. Also relevant, the absence of GLUT1 in the invasion front of the tumour was significantly associated with several variables: NASH structural stage, no parenchyma activity, absence of ballooning, absence of intratumoral fibrosis, and BCLC stage 0.

Table 2. Histopathological data of 35 nodules from 21 patients with HCC secondary to NAFLD

Variable	*N*	%
Structural alterations (*n* = 35)		
Negative/minimal	10	28.57
Major	25	71.43
Portal infiltration (*n* = 35)		
Absent/low	32	91.43
High	3	8.57
Periportal activity (*n* = 35)		
Absent/low	21	60.00
High	14	40.00
Parenchymal activity (*n* = 35)		
Absent/low	23	65.71
High	12	34.29
Cirrhosis staging, Laennec (*n* = 23)		
3 or 4A or 4AR	11	47.83
4B or 4C	12	52.17
Hepatocellular ballooning (*n* = 35)		
Absent/low	29	82.86
High	6	17.14
Steatosis (*n* = 35)		
Absent/low	28	80.00
High	7	20.00
Lobular inflammation (*n* = 35)		
Absent/low	21	60.00
High	14	40.00
Mallory-Denk bodies (*n* = 35)		
Absent/low	30	85.71
High	5	14.29
Tumoral grade – architecture (*n* = 35)		
Absent/low	17	48.57
High	18	51.43
Nuclear grade (*n* = 35)		
Absent/low	15	42.85
High	20	57.15
Intratumoral steatosis (*n* = 35)		
Absent/low	24	68.57
High	11	31.43
Intratumoral ballooning (*n* = 35)		
Absent/low	13	37.14
High	22	62.86
Intratumoral inflammation (*n* = 35)		
Absent/low	18	51.43
High	17	48.57
Intratumoral fibrosis (*n* = 35)		
Absent/low	24	68.57
High	11	31.43
Vascular infiltration (*n* = 35)		
Absent/low	19	54.29
High	16	45.71
Peritumoral inflammation (*n* = 35)		
Absent/low	13	37.14
High	22	62.86
Peritumoral fibrosis (*n* = 35)		
Absent/low	24	68.57
High	11	31.43

NAFLD, non-alcoholic fatty liver disease; HCC, hepatocellular carcinoma.

Discussion

Following the recent trend to approach HCC according to specific causes and, whenever possible, to histological subtypes [26], the present study is one of the pio-

Table 3. Frequencies of protein expression of MCTs, CD147 GLUT1, and CAIX

	N	*MCT1 MP* positive, *n* (%)	*MCT2 MP* positive, *n* (%)	*MCT4 MP* positive, *n* (%)	*CD147 MP* positive, *n* (%)	*GLUT1 MP* positive, *n* (%)	*CAIX MP* positive, *n* (%)
T_centre	30	26 (86.7)	18 (60.0)	7 (23.3)	22 (73.3)	4 (13.3)	5 (16.7)
T_periphery	31	30 (96.8)	16 (51.6)	6 (19.4)	22 (67.7)	3 (9.7)	2 (6.5)
Adjacent_NT	30	29 (96.8)	14 (46.6)	2 (6.7)	27 (86.7)	0 (0.0)	0 (0.0)
Distant_NT	16	16 (100.0)	8 (50.0)	2 (12.5)	13 (81.2)	0 (0.0)	0 (0.0)

T_centre, centre of the tumour; T_periphery, periphery of the tumour; Adjacent_NT, adjacent normal tissue; Distant_NT, distant normal tissue; GLUT1, glucose transporter-1; CAIX, carbonic anhydrase IX; MCTs, monocarboxylate transporters.

Table 4. Association of MCT1 expression with the pathological parameters

Tumour centre	*N*	*MCT1 MP* positive, *n* (%)	*p* value
Periportal activity			
Absent/low	17	17 (100.0)	**0.026**
High	13	9 (69.2)	
Steatosis intratumoral			
Absent/low	21	21 (100.0)	**0.005**
High	9	5 (55.56)	
Architectural grade			
Absent/low	13	9 (69.23)	**0.026**
High	17	17 (100.0)	

Table 5. Association of MCT2 expression with the pathological parameters

	N	*MCT2 MP* positive, *n* (%)	*p* value
Tumour centre			
Lobular inflammation			
Absent/low	17	13 (76.5)	**0.035**
High	13	5 (38.5)	
Tumour periphery			
Architectural grade			
Absent/low	13	4 (30.1)	**0.002**
High	18	12 (66.7)	

Table 6. Association of GLUT1 and CAIX expression with the pathological parameters

Tumour centre	*N*	*CAIX MP* positive, *n* (%)	*p* value	*GLUT1 MP* positive, *n* (%)	*p* value
Structural alterations					
Negative/minimal	6	3 (50.0)	**0.041**		
Major	24	2 (8.3)			
Architectural grade					
Absent/low	13	0 (0.0)	**0.052**		
High	17	5 (29.4)			
BCLC					
BCLC 0 or A	19	1 (5.3)	**0.047**	2 (10.5)	**0.002**
BCLC B or C	11	4 (36.4)		7 (63.6)	

GLUT1, glucose transporter-1; CAIX, carbonic anhydrase IX; BCLC, Barcelona-clinic liver cancer.

neers on assessing a subpopulation of HCC secondary to metabolic syndrome/obesity and diabetes, as related to histological markers of the "SH-HCC" as well as to immunohistochemical markers of hypoxia cell metabolism-related to metabolic reprogramming of MCTs, CAIX, and GLUT1. The immunohistochemical assessment was performed in different areas of the neoplasms and of non-neoplastic liver tissue. MCT1 and MCT2, and also CD147 decorate quite similarly the different portions of the samples including the non-neoplastic areas of the liver. These

findings show that these metabolic markers in carcinoma cells have ubiquitous distribution. However, when tumour was compared with non-neoplastic liver, there was a different and significant expression of CD147, CAIX, and GLUT1, which corroborate the premise that cancer cells expressed more frequently and intensively these markers [25, 27]. MCT plasma membrane positivity is highly associated to the cancer cell activity but not with non-malignant cells [28]. We observed that MCT1, MCT2, and MCT4 plasma membrane expression positive reactions were observed in all liver nodular compartments, but no differences between normal and tumour cells were observed. Conversely, CAIX and GLUT1 positivity was exclusively detected in the neoplastic compartment, reinforcing previous reports from our group in other neoplasms [8]. This important finding further demonstrates that hepatocarcinogenesis related to NAFLD is strongly related to metabolic shifts.

Several studies have shown MCT1 and MCT4 overexpression in neoplastic tissues compared with non-neoplastic parenchyma in different malignancies [29]. We know that the acid-resistant phenotype is an essential condition for the survival and aggressiveness of tumour cells, associated with increased production of lactate, which is exported to the extracellular environment, contributing to the acidic microenvironment. In this context, MCTs play an important role in maintaining this acid-resistant hyperglycolytic phenotype, allowing the maintenance of high glycolytic rates through lactate efflux and pH regulation by the co-transport of protons [9].

A previous study from our group conducted by Alves et al. [15] in a series of 80 cases of HCC due to several causes (assessed by necropsy) demonstrated a progressive increase of MCT4 expression from non-tumour parenchyma to primary HCCs and to metastases. In the present series, we observed that plasma membrane expression of MCT4 at in the tumour front of invasion was associated with clinical-pathological markers of worse prognosis, such as higher grade of intratumoral fibrosis (grades 3 + 4), higher degree of peritumoural inflammation (degrees +3), and more advanced stages (BCLC B + C).

On the other hand, in a meta-analysis reported by Wang et al. [30], with 26 studies involving a total of 2,948 patients covering several types of solid tumours, GLUT1 overexpression correlated significantly with worse overall survival at 3 and 5 years ($p < 0.00001$). In subgroup analysis, the expression of elevated GLUT1 was associated with a worse prognosis in oral squamous cell carcinoma and breast cancer, but there was no significant correlation between GLUT1 overexpression and overall survival in colorectal, lung, cervical, and pancreas cancers. In the present series of HCC occurring in patients with NAFLD, GLUT1 overexpression at both the front of invasion and at the centre of the tumour was associated with mild structural alterations, and it was positive in HCCs converging in non-cirrhotic patients (67%). GLUT1 overexpression at the centre of the tumour correlated with more pronounced degree of peritumoural inflammation (grades 2 + 3) and also correlated with HCC with more advanced BCLC staging (BCLC B + C). Sun et al. [31] addressed the status of HCC metabolism by evaluating the expression of GLUT1 and the glutamine transporter ASCT2. A total of 192 HCC patients who underwent hepatic resection were submitted to immunohistochemical detection of GLUT1 and ASCT2, and both found a significant increase in tumour tissues compared to adjacent non-tumour tissues and both were positively associated with tumour size. In the present study, GLUT1 overexpression in the tumour periphery was found associated with higher degree of parenchymal activity (grades 2 + 3), intratumoral steatosis (grades 2 + 3), and peritumoural fibrosis (grades 3 + 4), and to more intense ballooning, which might represent the status of the tumour metabolism determined by GLUT1 expression. Further studies with clinical correlation should assess whether this might be a promising prognostic predictor for patients with HCC secondary to NAFLD. As in the central region, GLUT1 overexpression in the invasion front was also associated with more advanced HCC stage (BCLC B + C), which might become a prognostic factor as in other types of tumours [32]. Not surprisingly, increased GLUT1 seems to be closely related with HCC biological aggressiveness appearing as a potential candidate for target therapy for HCC [33]. Many recent studies have demonstrated the potential of cell metabolism in HCC development [34, 35]. To the best of our knowledge, the present study is pioneer in reporting the high expression of MCT4 and GLUT1, well-known markers of glycolytic metabolic phenotype in NAFLD-associated HCC, especially in tumours at stages BCLC B or C. Moreover, the expression of GLUT1 was further associated to higher degrees of steatosis, marked ballooning, intratumoral fibrosis, and higher parenchymal activity. Additionally, and also relevant, the positive results depicted from neoplastic and non-neoplastic areas showed that MCT4, CAIX, and GLUT 1 are more importantly expressed in the neoplastic areas. Previously, Ke et al. [36] have found that upregulation of MCT-4 (and also CD-147) was involved in glycolytic reprogramming to allow the viability of HCC under hypoxia. After all, we can then hypothesize that the strong

expression of these metabolic markers in the zones of high expression of glycolytic metabolism is somehow associated with the carcinogenesis of liver carcinoma related to NAFLD. Additionally, some questions are still open to be investigated in further studies, such as the role of HIF-1 in NAFLD patients. Han et al. [37] found that HIF1 is critically involved in the development of hepatic fibrosis in NAFLD patients, a topic that was not explored in this work. Importantly, HIF1-α-induced proteins improve the expression of enzymatic activities including lactate dehydrogenase and pyruvate dehydrogenase, which are well-recognized players related to Warburg effect, that support an appropriate microenvironment for cancer cell development and survival [38].

We do hope these findings may open an important venue of further investigation of metabolic, inflammation, and the molecular pathways in NAFLD-related HCC. It is exciting to hypothesize that future studies could reveal the potential for therapeutic target of these markers.

Statement of Ethics

This research project was approved by the Ethics Committee for Analysis of Research Projects (Cappesq) of the *Hospital das Clínicas* of Faculty of Medicine of São Paulo University, under the number: 385.602. Samples used in this study were obtained as part of routine medical care. Ethical approval for use of these samples for research purposes was not required for this study in accordance with local/national guidelines. Written informed consent from participants was not required in accordance with local/national guidelines.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by all authors. S.C.G., A.L.-F., V.A.F.A., and C.P.O. critically analyzed the results. The first draft of the manuscript was written by S.C.G. and A.L.-F., and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article [and/or] its online supplementary material. Further enquiries can be directed to the corresponding author.

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