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Measurement of circulating tumor cells determines treatment response and exitus in patients with hepatocellular carcinoma submitted to transarterial chemoembolization

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Simple Summary: Transarterial chemoembolization (TACE) is the standard of care for patients with hepatocellular carcinoma (HCC) at intermediate stage, according to Barcelona Clinic Liver Classification (BCLC) score system. TACE is considered a palliative treatment, and early biomarkers of treatment efficacy are welcome. The aim of this study was to evaluate if circulating tumor cells (CTCs) measured at baseline and 1-month after treatment might be relevant in predicting its effectiveness in patients at intermediate stage of HCC. The identification of CTCs as a reliable marker might recommend its utility for the recommendation of follow-up or reevaluate patients for alternative therapies.

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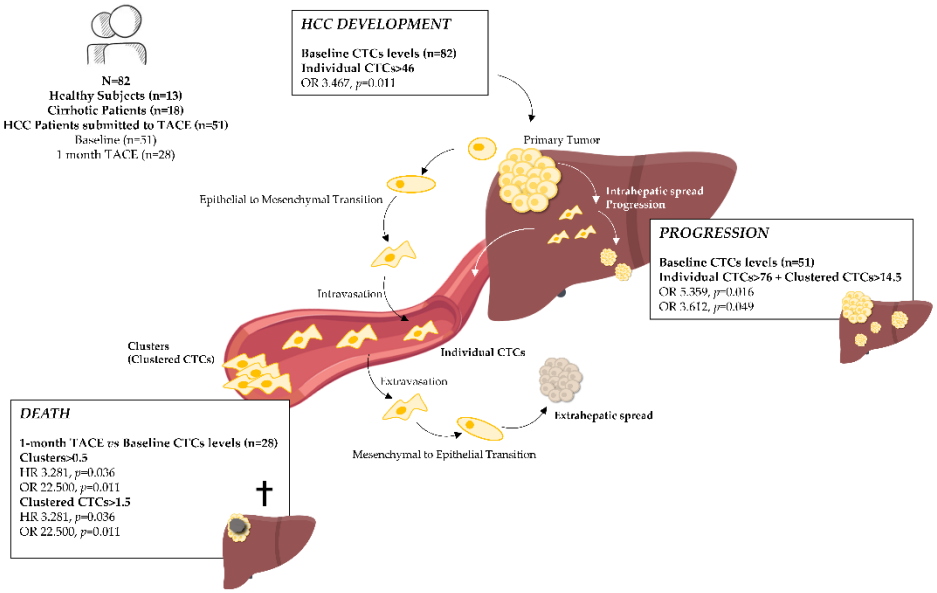
Abstract: Circulating tumor cells (CTCs) enumeration is a promising technique to predict cancer prognosis and treatment response. CTCs were evaluated in healthy subjects, cirrhotic controls and hepatocarcinoma (HCC) patients. CTCs were isolated using microfluidic system based on the expression of EpCAM, EGFR and three epithelial to mesenchymal transition (EMT) markers. Patients were stratified according to disease progression and exitus. Although counts of individual CTCs, clustered CTCs and α-fetoprotein (AFP) at basal level in patients with HCC were significantly increased compared with the values obtained in cirrhotic patients and control subjects, only individual CTCs (p=0.027), but not clustered CTCs (p=0.063) and AFP (p=0.072), were independent predictors of HCC development. The univariate regression model showed that basal levels of CTCs>46 were related to high risk of HCC (Odds Ratio 3.467, p=0.011). The stratification of our cohort according to disease progression and death showed that basal individual CTCs >76 (Hazard Ratio 5.131, p=0.004) were related to disease progression, as well as the difference of clustered CTCs between 1-month and baseline levels >1.5 were related to death (Hazard Ratio 10.204, p=0.036). In conclusion, the preoperative and 1-month measurements of CTCs in blood constitute useful markers to predict the outcome of patients under TACE treatment.



Keywords: Hepatocellular carcinoma, transcatheter arterial chemoembolization, circulating tumor cells, tumor progression, predictive marker

1. Introduction

Hepatocellular carcinoma (HCC) represents 80% of the primary hepatic neoplasms that appears mainly in the context of chronic liver cirrhosis [1]. Hepatitis B (HBV) and C (HCV), alcohol, aflatoxin B1, non-alcoholic steatohepatitis (NASH), tobacco, diabetes and congenital diseases are risk factors with variable geographic incidence that have been associated with induction and progression of HCC [2]. The number and size of tumors, degree of vascular invasion, presence of extrahepatic tumors, liver function and patient's functional status are used for the staging of patients with HCC, as well as

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for therapeutic recommendations according to the criteria of the Barcelona Clinic Liver Cancer (BCLC) [1]. The curative treatments (radiofrequency, liver resection, and orthotopic liver transplantation or OLT) are indicated during the early stages of the disease (BCLC 0-A), which show a high 5-years survival (50-80%), representing an average survival of more than 60 months [3]. However, nowadays, two third of patients diagnosed with HCC are in more advanced stages of the disease (BCLC B or C) [4]. Transarterial chemoembolization (TACE), which consists tumor arteria embolization with drug-eluting beads loaded with doxorubicin, is the recommended treatment for patients at the intermediate stages (BCLC B) [3]. The average survival of these patients is 26 months [3]. The intermediate stage appears to be a critical phase with high risk of evolving to more advanced stages of the disease (BCLC C) with poor prognostic factors such as vascular invasion, extrahepatic metastases and/or impaired hepatic function. Sorafenib is the standard of care for advanced HCC stage, as demonstrated in two large-scale trials such as the SHARP [5] and the Asia-Pacific [6] clinical trials. Nowadays, the average survival of patients in advanced stages treated with first and second line therapies ranges from 7 to 26 months [7].

Early screening of patients with HCC has been reported to improve the impact of therapeutic strategy and was an independent predictor of survival [8]. Therefore, to provide the best outcome of treatments, precise and effective biomarkers are urgently needed. Circulating tumor cells (CTCs) refer to cancer cells that are disengaged from the primary lesion, delivered to blood circulation, bone marrow, or lymphatic vessels and represent tumor progression and metastases [9]. The results of broad meta-analysis showed that the presence of CTC in blood was associated with poor recurrence free survival (RFS) and overall survival (OS), significantly increased risk of disease recurrence and death in patients with HCC [10]. Another metanalysis showed that CTCs positivity was significantly associated with relapse-free survival, overall survival and some clinical characteristics in patients with HCC [11].

The aim of the study was to correlate the levels of individual CTCs, clusters and clustered CTCs before and 1-month after TACE treatment in peripheral blood with clinical progression or effectiveness of treatment in patients with HCC at intermediate stage (BCLC B). Our data showed that the number of CTCs, clusters and clustered CTCs in blood at preoperative and 1-month post-treatment were predictive markers of disease progression and exitus in patients with HCC.

2. Results

2.1. CTCs showed higher prognostic value than AFP in the development of HCC

Table 1 shows demographic and epidemiological basal data of our cohort (n=82) from Hospital University "Virgen del Rocío" (Seville, Spain). The percentage of exitus was higher in HCC patients (35.3%, $p<0.001$). Liver function of patients with HCC measured by Child-Pugh staging system was better than that observed in cirrhotic patients ($p<0.001$). The basal values of glucose, α-fetoprotein (AFP) and individual CTCs were significantly higher in patients with HCC than cirrhosis ($p=0.006$, $p<0.001$ and $p=0.018$, respectively). (Table 1). An additional analysis was carried out to identify the increased power of CTCs compared with AFP measured at baseline in predicting the development

of HCC. Univariate regression models showed that individual CTCs but not AFP were associated with the presence of HCC ($p=0.027$ and $p=0.072$, respectively) (Table 2).

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Table 1. Demographic, epidemiological and clinical baseline data in control subjects (n=13), cirrhotic patients (n=18) and patients with HCC (n=51) (n=82). Data are expressed as mean ± SEM for quantitative variables, and as percentage (%, n) for categorical variables. Significant results (p<0.05) are shown in bold. Alanine aminotransferase, ALT; α-fetoprotein, AFP; Circulating tumor cells, CTCs.

ROC analysis showed areas under the curve (AUC) of 0.687 (p=0.005), 0.708 (p=0.002), 0.705 (p=0.002) and 0.797 (p<0.001) for individual CTCs, clusters, clustered CTCs and AFP, respectively (Figure 1a-1d). The cutoff values for each variable were identified in the corresponding ROC curves, 46 for individual CTCs, 2.5 for clusters, 6.5 for clustered CTCs and 7.25 AFP that defined HCC in our cohort of HCC patients (n=82) (Figure 1e). Next, analysis using the cut-off value of 46 individual CTCs further confirmed that this parameter was able to increase the probability of HCC (Odds Ratio=3.467, p=0.011) (Table 3).

	Total (n=82)	**Healthy Controls (n=13)**	**Cirrhotic Controls (n=18)**	**HCC Patients (n=51)**	***p* value**
Male, % (n)	84.1 (69)	69.2 (9)	77.8 (14)	90.2 (46)	0.128
Age (years)	64.2±1.3	51.5±3.4	58.9±1.2	68.3±2.1	**<0.001**
Exitus, % (n)	28.0 (23)	0 (0)	27.8 (5)	35.3 (18)	**<0.001**
Etiology, % (n)					
Alcohol	47.8 (33)	-	55.6 (10)	45.1 (23)	0.428
VHC	29.0 (20)	-	33.3 (6)	27.5 (14)	
EHNA	8.7 (6)	-	0 (0)	11.8 (6)	
Other	14.5 (10)	-	11.1 (2)	15.7 (8)	
Cirrhosis Stage, % (n)					
Child-Pugh Class A	51.2 (42)	0 (0)	16.7 (3)	76.5 (39)	**<0.001**
Child-Pugh Class B	17.1 (14)	0 (0)	55.6 (10)	7.8 (4)	
Child-Pugh Class C	6.1 (5)	0 (0)	27.8 (5)	0 (0)	
Non cirrhotic liver	25.6 (21)	100 (13)	0 (0)	15.7 (8)	
Glucose (mg/dL)	104.9±3.8	88.7±4.2	92.7±7.9	114.1±5.6	**0.006**
ALT (U/mL)	29.4±2.3	22.4±3.6	22.8±4.1	32.3±3.5	0.067
AFP (ng/mL)	1671.8±1095.7	2.3±0.8	9.85±2.7	2727.3±1779.2	**<0.001**
Individual CTCs	129.9±18.6	57.8±15.9	84.1±26.7	165.9±32.7	**0.018**
Clusters	11.2±3.3	0.3±0.2	7.0±2.5	16.8±6.3	**<0.001**
Clustered CTCs	50.9±25.5	0.3±0.2	18.1±5.7	85.4±50.6	**<0.001**

Table 2. Univariate regression models of quantitative CTCs and AFP measurements (n=82) for the risk of HCC development. Data were analyzed using logistic regression models (Odds Ratio, OR) with 95% Confidence Intervals (CI). Significant results (p<0.05) are shown in bold. Circulating tumor cells, CTCs.

Variable	**B**	**SE**	**p-value**	**OR**	**Low CI**	**High CI**

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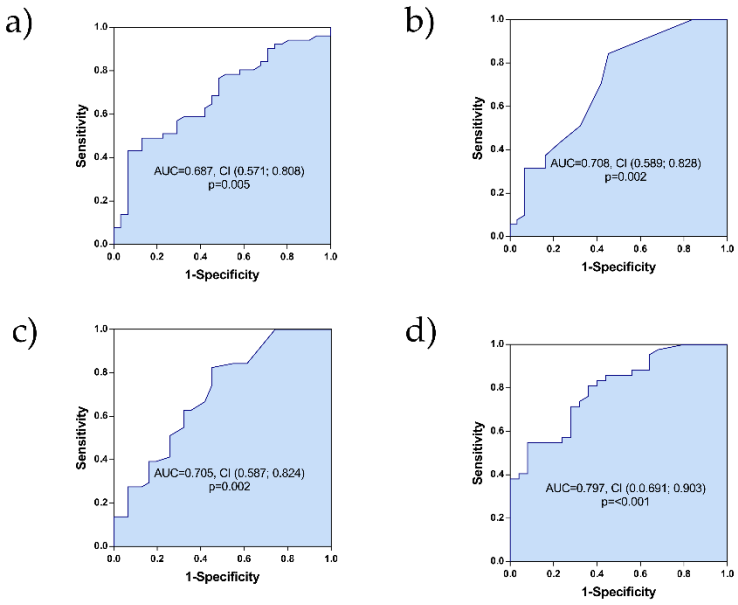
Individual CTCs	0.006	0.003	**0.027**	1.006	1.001	1.012
Constant	-0.155	0.339	0.646	0.856		
Clusters	0.054	0.030	0.075	1.056	0.955	1.120
Constant	0.118	0.286	0.680	1.125		
Clustered CTCs	0.021	0.011	0.063	1.021	0.999	1.043
Constant	0.086	0.283	0.763	1.089		
AFP	0.040	0.022	0.072	1.041	0.996	1.088
Constant	-0.309	0.354	0.383	0.743		



f)

Quantitative variable	Independent variable	Cut-off value	Sensibility (%)	Specificity (%)
Individual CTCs	HCC (ycs)	46	76.5	51.6
Clusters	HCC (yes)	2.5	70.6	58.1
Clustered CTCs	HCC (yes)	6.5	66.7	64.5
AFP	HCC (yes)	7.25	71.4	72

Figure 1. ROC curves of individual CTCs (a), clusters (b), clustered CTCs (c) and AFP (d) for the development of HCC including area under the curve (AUC), Confidence Intervals (CI) and significance, as well as the cut-off values from each variable with the respective sensibility and specificity values (e) from the studied cohort (n=82). Circulating tumor cells, CTCs.

Table 3. Univariate regression model for the risk of HCC development according to stablished cut-off values for individual CTCs measurements (n=82). Data were analyzed using logistic regression models (Odds Ratio, OR) with 95% Confidence Intervals (CI). Significant results ($p<0.05$) are shown in bold. Circulating tumor cells, CTCs.

Variable	B	SE	*p* value	OR	Low CI	High CI
Individual CTCs>46	1.243	0.488	**0.011**	3.467	1.332	9.022
Constant	-0.288	0.382	0.568	0.451		

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2. High baseline levels of individual CTCs were predictors of tumor progression after TACE

Next, we studied the relationship between baseline levels of CTCs and tumor progression during follow-up in patients with intermediate HCC (n=51). Therefore, we divided the overall cohort into progressors (n=21) and non-progressors (n=30) patients (Table 4). We found no statistical differences in several demographic or clinical parameters (sex, etiology, Child-Pugh cirrhosis stage, number, size or location of tumors, glucose, alanine transaminase or AFP) between both groups (Table 4). Time-to-progression (TTP), individual CTCs, number of clusters and clustered CTCs were significantly higher in patients with disease progression versus no progression (p=009, p=0.034, p=0.016 and p=0.025, respectively) (Table 4). As expected, individual CTCs showed moderate correlation with number of clusters and clustered CTCs (rho=0.829, rho=0.703, respectively, p<0.001) and these two variables showed a very strong correlation (rho=0.954, p<0.001) (Table 5).

Univariate and multivariate models were constructed based on CTCs quantitative data (Table 6). Cox univariate regression models considering the time to progression (TTP) showed that baseline individual CTCs, clusters and clustered CTCs increased the probability of progression after TACE (p=0.008, p=0.015 and p=0.016, respectively) (Table 6). Binary regression models showed that baseline individual CTCs and clusters were related to disease progression (p=0.028 and p=0.022, respectively) (Table 6). ROC analysis reinforced these results with AUC of 0.676 (p=0.034), 0.7000 (p=0.016) and 0.688 (p=0.025) for individual CTCs, clusters and clustered CTCs, respectively (Figure 2a-2c). The cutoff values for each variable were identified in the corresponding ROC curves, 76 for individual CTCs, 3.5 for clusters and 14.5 for clustered CTCs that defined disease progression in our cohort of HCC patients (Figure 2d). Kaplan-Meier curves were used to analyze progression according to these cut-off values (Figure 2e).

Cox univariate regression models considering stratification of patients according to baseline cut-off values gave positive results for individual CTCs, indicating that CTCs>76 were prognostic factors for progression over time (Hazard Ratio=5.131, p=0.004) (Table 7). On the other hand, univariate binary regression models gave significant results for the three dichotomized variables CTCs>76 (Odds Ratio =6.375, p=0.006), clusters>3.5 (Odds Ratio=4.318, p=0.017) and clustered CTCs>14.5 (Odds Ratio=4.469, p=0.014) (Table 7). The multivariate logistic regression analysis identified individual CTCs>76 (p=0.016; Odds Ratio: 5.359, Confidence Intervals 95%: 1.374-20.911) and clustered CTCs>14.5 (p=0.049; Odds Ratio: 3.612, Confidence Intervals 95%: 1.003-13.005) as independent predictors of progression after TACE (Table 7)

Table 4. Demographic, epidemiological and baseline clinical data in patients with HCC (n=51) categorized according to tumor progression during follow-up. Data are expressed as mean ± SEM for quantitative variables and as percentage (%, n) for categorical variables. Significant results (p<0.05) are shown in bold. Alanine aminotransferase, ALT; α-fetoprotein, AFP; Circulating tumor cells, CTCs.

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	Total (n=51)	No Progression (n=30)	Disease progression (n=21)	*p* value
Male, % (n)	90.2 (46)	86.7 (26)	95.2 (20)	0.391
Etiology, % (n)				
Alcohol	45.1 (23)	50.0 (15)	38.1 (8)	0.836
VHC	27.5 (14)	26.7 (8)	28.6 (6)	
EHNA	11.8 (6)	10.0 (3)	14.3 (3)	
Other	15.7 (8)	13.3 (4)	19.0 (4)	
Cirrhosis Stage, % (n)				
Child-Pugh Class A	76.5 (39)	76.7 (23)	76.2 (16)	0.708
Child-Pugh Class B	7.8 (4)	10.0 (3)	4.8 (1)	
Child-Pugh Class C	0 (0)	0 (0)	0 (0)	
Non cirrhotic liver	15.7 (8)	13.3 (4)	14.3 (3)	
Number of tumors, % (n)				
1	64.7 (33)	70.0 (21)	57.1 (12)	0.742
2	13.7 (7)	10.0 (3)	19.0 (4)	
3	9.8 (5)	10.0 (3)	9.5 (2)	
>3	11.8 (6)	10.0 (3)	14.3 (3)	
Size of tumors, % (n)				
≤ 3 cm	21.6 (11)	26.7 (8)	14.3 (3)	0.490
> 3 cm	78.4 (40)	73.3 (22)	85.7 (18)	
Location of tumors, % (n)				
Right Lobe	74.5 (38)	70.0 (21)	81.0 (17)	0.651
Left Lobe	7.8 (4)	10.0 (3)	4.8 (1)	
Both Lobes	17.6 (9)	20.0 (6)	14.3 (3)	
TTP (months)	11.9±1.6	18.1±2.7	6.2±1.3	**0.009**
Glucose (mg/dL)	112.5±5.0	119.6±8.8	110.3±7.4	0.440
ALT (U/mL)	32.5±3.1	32.5±3.1	31.3±4.7	0.917
AFP (ng/mL)	2662.4±1737.6	3498.7±3352.9	2262.9±1701.9	0.791
Individual CTCs	164.4±27.4	103.2±18.8	251.83±67.08	**0.034**
Clusters	15.3±5.1	6.5±1.8	30.5±13.8	**0.016**
Clustered CTCs	74.6±40.7	22.2±7.6	168.1±113.7	**0.025**

Table 5. Bivariate correlations (Spearman rho) of quantitative variables in the HCC cohort (n=51) at baseline. Significant results ($p<0.05$) are shown in bold. Circulating tumor cells, CTCs.

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Variable 1	Variable 2	Rho (Spearman)	*p* value
Individual CTCs	Clusters	0.829	**<0.001**
Individual CTCs	Clustered CTCs	0.703	**<0.001**
Clusters	Clustered CTCs	0.954	**<0.001**

Table 6. Univariate regression models of quantitative baseline CTCs measurements from patients with HCC (n=51). Data were analyzed using Cox regression models (Hazard Ratio, HR) according to Time-to-Progression (TTP), and logistic regression models (Odds Ratio, OR) with 95% Confidence Intervals (CI). Significant results ($p<0.05$) are shown in bold. Circulating tumor cells, CTCs.

Cox regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**HR**	**Low CI**	**High CI**
Univariate	**Individual CTCs**	0.002	0.001	**0.008**	1.002	1.001	1.004
	Clusters	0.010	0.004	**0.015**	1.010	1.002	1.019
	Clustered CTCs	0.001	0.001	**0.016**	1.001	1.000	1.002
Multivariate	**Individual CTCs**	0.005	0.002	0.039	1.005	1.000	1.010
	Clusters	-0.035	0.025	0.154	0.965	0.920	1.013
	Clustered CTCs	0.003	0.002	0.141	1.003	0.999	1.008
Logistic Regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**OR**	**Low-CI**	**High CI**
Univariate	**Individual CTCs**	0.005	0.002	**0.028**	1.005	1.001	1.010
	Constant	-1.187	0.458	0.010	0.305		
	Clusters	0.075	0.033	**0.022**	1.078	1.011	1.149
	Constant	-1.126	0.419	0.007	0.324		
	Clustered CTCs	0.015	0.008	0.053	1.050	1.000	1.031
	Constant	-0.893	0.373	0.017	0.410		
Multivariate	**Individual CTCs**	0.001	0.003	0.678	1.001	0.995	1.008
	Clusters	0.108	0.075	0.150	1.115	0.962	1.292
	Clustered CTCs	-0.100	0.011	0.346	0.990	0.968	1.011
	Constant	-1.301	0.489	0.008	0.272		

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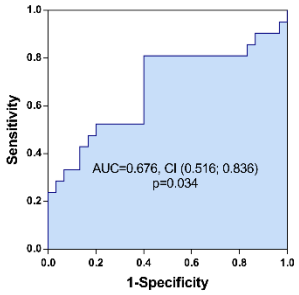

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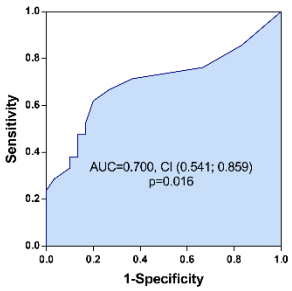

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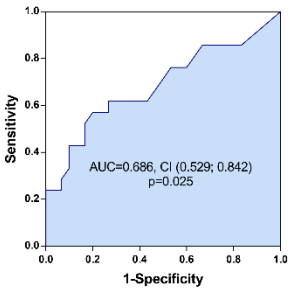

d)

Quantitative variable	Independent variable	Cut-off value	Sensibility (%)	Specificity (%)
Individual CTCs	Progression (yes)	76	81.0	60.0
Clusters	Progression (yes)	3.5	71.4	63.3
Clustered CTCs	Progression (yes)	14.5	61.9	73.3

e)

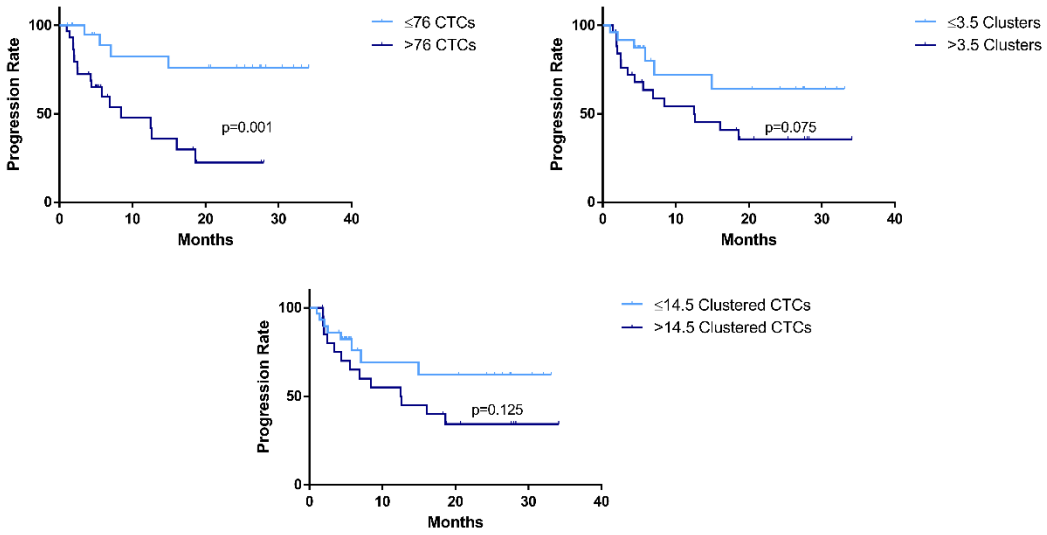

Figure 2. ROC curves of baseline individual CTCs (a), clusters (b) and clustered CTCs (c) for disease progression including area under the curve (AUC), Confidence Intervals (CI) and significance, as well as the cut-off values from each variable with the respective sensibility and specificity values (d) from HCC patients (n=51). Kaplan-Meier curves for analyzing disease progression were drawn according to these cut-off values (e). Circulating tumor cells, CTCs.

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Table 7. Univariate and multivariate regression models of quantitative baseline CTCs measurements from patients with HCC (n=51) according to stablished cut-off values. Data were analyzed using Cox regression models (Hazard Ratio, HR) according to Time-to-Progression (TTP), and logistic regression models (Odds Ratio, OR) with 95% Confidence Intervals (CI). Significant results (p<0.05) are shown in bold. Circulating tumor cells, CTCs.

Cox regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**HR**	**Low CI**	**High CI**
Univariate	**CTCs>76**	1.635	0.564	**0.004**	5.131	1.689	15.503
Logistic Regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**OR**	**Low CI**	**High CI**
Univariate	**CTCs>76**	1.852	0.669	**0.006**	6.375	1.718	23.661
	Constant	-1.504	0.553	**0.007**	0.222		
	Clusters >3.5	1.463	0.614	**0.017**	4.318	1.296	14.383
	Constant	-1.153	0.468	**0.014**	0.316		
	Clustered CTCs >14.5	1.497	0.610	**0.014**	4.469	1.351	14.778
	Constant	-1.012	0.413	**0.014**	0.364		
Multivariate	**CTCs>76**	1.679	0.695	**0.016**	5.359	1.374	20.911
	Clustered CTCs>14.5	1.284	0.654	**0.049**	3.612	1.003	13.005
	Constant	-1.960	0.638	**0.002**	0.141		

3.The increase of CTCs at 1-month after TACE versus baseline constituted a risk factor for patient death

We analysed the changes of CTCs levels 1 month after TACE versus baseline in a subpopulation of HCC patients (n=28). Table 8 shows demographic and clinical parameters of the cohort of patients who were died (n=6) and alive (n=22) at the time of evaluation. We found no statistical differences in several demographic or clinical parameters (sex, etiology, Child-Pugh, number, size or location of tumors, development of metastases, glucose, ALT and AFP) between both groups (Table 8). The changes in CTCs 1 month after TACE versus baseline were strongly associated with death (Table 8). In this regard, patients who died at any time during follow-up presented an increase in individual CTCs, clusters and clustered CTCs 1 month after TACE compared with baseline levels (p=0.046, p<0.001 and p<0.001, respectively). The increments of individual CTCs and clusters (rho=0.707, p<0.001), or individual CTCs and clustered CTCs (rho=0.733, p<0.001) were moderately correlated, while clusters and clustered CTCs (rho=0.943, p<0.001) were strongly correlated (Table 9).

Table 8. Demographic, epidemiological, clinical data with values of CTCs at baseline and the change of CTCs 1 month after TACE versus baseline (Δ). Data are expressed as mean ± SEM for quantitative variables and as percentage (%, n) for categorical variables. Significant results (p<0.05) are shown in bold. Alanine aminotransferase, ALT; α-fetoprotein, AFP; Circulating tumor cells, CTCs.

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	Total (n=28)	No Exitus (n=22)	Exitus (n=6)	*p* value
Male, % (n)	96.4 (27)	95.5 (21)	100 (6)	0.786
Etiology, % (n)				
Alcohol	35.7 (10)	31.8 (7)	50 (3)	0.472
VHC	25 (7)	27.3 (6)	16.7 (1)	
EHNA	17.9 (5)	22.7 (5)	0 (0)	
Other	21.4 (6)	18.2 (4)	33.3 (2)	
Cirrhosis Stage, % (n)				
Child-Pugh Class A	75.0 (21)	72.7 (16)	83.3 (5)	0.733
Child-Pugh Class B	7.1 (2)	9.1 (2)	0 (0)	
Child-Pugh Class C	0 (0)	0 (0)	0 (0)	
Non cirrhotic liver	17.9 (5)	18.2 (4)	16.7 (1)	
Number of tumors, % (n)				
1	60.7 (17)	59.1 (13)	66.7 (4)	0.722
2	17.9 (5)	18.2 (4)	16.7 (1)	
3	10.7 (3)	13.6 (3)	0 (0)	
>3	10.7 (3)	9.1 (2)	16.7 (1)	
Size of tumors, % (n)				
≤ 3 cm	21.4 (6)	22.7 (5)	16.7 (1)	0.617
> 3 cm	78.6 (22)	77.3 (17)	83.3 (5)	
Location of tumors, % (n)				
Right Lobe	75.0 (21)	68.2 (15)	100 (6)	0.144
Left Lobe	0 (0)	0 (0)	0 (0)	
Both Lobes	25.0 (7)	31.8 (7)	0 (0)	
Development of metastasis, % (n)				
No	82.1 (23)	81.8 (18)	83.3 (5)	0.715
Yes	17.9 (5)	18.2 (4)	16.7 (1)	
Glucose (mg/dL)	115.8±7.6	118.1±9.5	107.5±8.5	0.570
ALT (U/mL)	34.4±4.1	35.4±4.6	31.0±9.3	0.790
AFP (ng/mL),	3652.8±3061.3	141.9±75.9	130515.2±10962.1	0.059
Individual CTCs	152.8±30.9	154.5±37.2	146.8±7.3	0.978
Clusters	11.3±4.0	9.8±4.7	16.8±7.3	0.365
Clustered CTCs	38.0±13.1	29.5±13.4	69.2±36.1	0.365
ΔIndividual CTCs	-9.9±±40.1	-36.9±44.27	89.3±56.7	**0.046**
ΔClusters	1.1±5.4	-6.7±4.7	29.3±13.5	**<0.001**
ΔClustered CTCs	-3.3±14.1	-22.9±13.5	68.8±30.1	**<0.001**

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Table 9. Bivariate correlations (Spearman rho) of changes between CTCs 1 month after TACE versus baseline (n=28). Significant results (p<0.05) are shown in bold. Circulating tumor cells, CTCs.

Variable 1	Variable 2	Rho (Spearman)	*p* value
Individual CTCs	Clusters	0.707	**<0.001**
Individual CTCs	Clustered CTCs	0.733	**<0.001**
Clusters	Clustered CTCs	0.943	**<0.001**

Survival univariate and multivariate Cox regression models and binary regression models of quantitative variables of increments of CTCs are shown in Table 10. Cox univariate regression models considering survival showed that the change of clustered CTCs between 1-month after TACE versus baseline increased the probability of death (p=0.049) (Table 10). Binary regression models for changes of clusters after TACE showed increased risk of death (p=0.024) (Table 10). Then, ROC analysis was performed to obtain values of CTCs that could prognose exitus of patients (Figure 3a-3c). ROC analysis gave AUC of 0.732 (p=0.045), 0.939 (p=0.001) and 0.928 (p=0.002) for changes in individual CTCs, clusters and clustered CTCs measured 1-month after TACE versus baseline, respectively (Figure 3a-3c). Changes 1-month after TACE over 27 individual CTCs, 0.5 clusters and 1.5 clustered CTCs were identified as cut-off values for death (Figure 3d). Survival analysis was done according to these cut-offs and Kaplan-Meier curves were constructed (Figure 3e). Cox and binary regression models were also constructed for dichotomized variables (Table 11). Post-TACE changes of clusters>0.5 and clustered CTCs>1.5 increased the risk of death up to 2.323 times (Hazard Ratio=10.204, p=0.036) in survival models. Binary regression models indicated that changes in clusters>0.5 (Odds Ratio=22.500, p=0.011) and clustered CTCs>1.5 (Hazard Ratio=22.500, p=0.011) were important predictors of death at any time point in our cohort (Table 11). Multivariate analysis was not carried out due to the high dependence of variables.

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Table 10. Univariate and multivariate regression models of quantitative variables of changes in individual CTCs, clusters and clustered CTCs between 1-month TACE versus baseline from patients with HCC (n=28). Data were analyzed using Cox regression models (Hazard Ratio, HR) according to survival, and logistic regression models (Odds Ratio, OR) with 95% Confidence Intervals (CI). Significant results (p<0.05) are shown in bold. Circulating tumor cells, CTCs.

Cox regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**HR**	**Low CI**	**High CI**
Univariate	**Individual CTCs**	0.001	0.001	0.305	1.001	0.999	1.004
	Clusters	0.017	0.009	0.069	1.017	0.999	1.035
	Clustered CTCs	0.009	0.005	**0.049**	1.009	1.000	1.018
Multivariate	**Individual CTCs**	-0.006	0.005	0.185	0.994	0.984	1.003
	Clusters	0.036	0.044	0.410	1.037	0.952	1.129
	Clustered CTCs	0.008	0.012	0.494	1.008	0.985	1.031
Multivariate	**Individual CTCs**	-0.007	0.005	0.111	0.993	0.983	1.002
	Clusters	0.059	0.029	0.043	1.060	1.002	1.122
Multivariate	**Individual CTCs**	-0.003	0.003	0.230	0.997	0.991	1.002
	Clustered CTCs	0.016	0.007	0.028	1.016	1.002	1.030
Multivariate	**Clusters**	-0.012	0.026	0.627	0.988	0.939	1.039
	Clustered CTCs	-0.014	0.012	0.224	1.230	0.991	1.038
Logistic Regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**OR**	**Low CI**	**High CI**
Univariate	**Individual CTCs**	0.004	0.003	0.122	1.004	0.999	1.010
	Constant	-1.018	0.435	0.019	0.361		
	Clusters	0.193	0.085	**0.024**	1.213	1.026	1.433
	Constant	-2.348	0.810	0.004	0.096		
	Clustered CTCs	0.079	0.057	0.164	1.082	0.968	1.209
	Constant	1.939	0.687	0.005	0.144		
Multivariate	**Individual CTCs**	-0.003	0.007	0.693	0.997	0.984	1.011
	Clusters	0.153	0.133	0.249	1.165	0.899	1.511
	Clustered CTCs	0.034	0.095	0.721	1.034	0.859	1.245
	Constant	-2.271	0.803	0.005	0.103		

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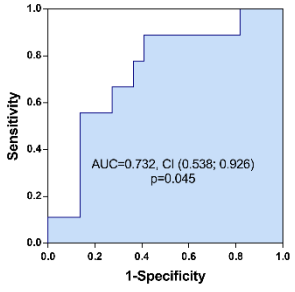
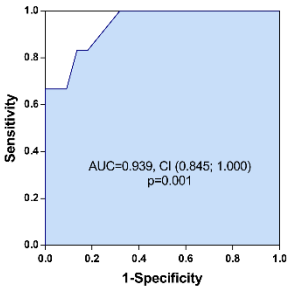
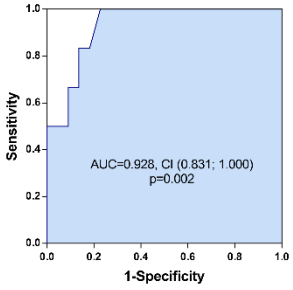
a)



b)



c)



d)

	Quantitative variable	Independent variable	Cut-off value	Sensibility (%)	Specificity (%)
Post-TACE Changes	Individual CTCs	Exitus (yes)	27	66.7	68.2
	Clusters	Exitus (yes)	0.5	83.3	81.8
	Clustered CTCs	Exitus (yes)	1.5	83.3	81.8

g)

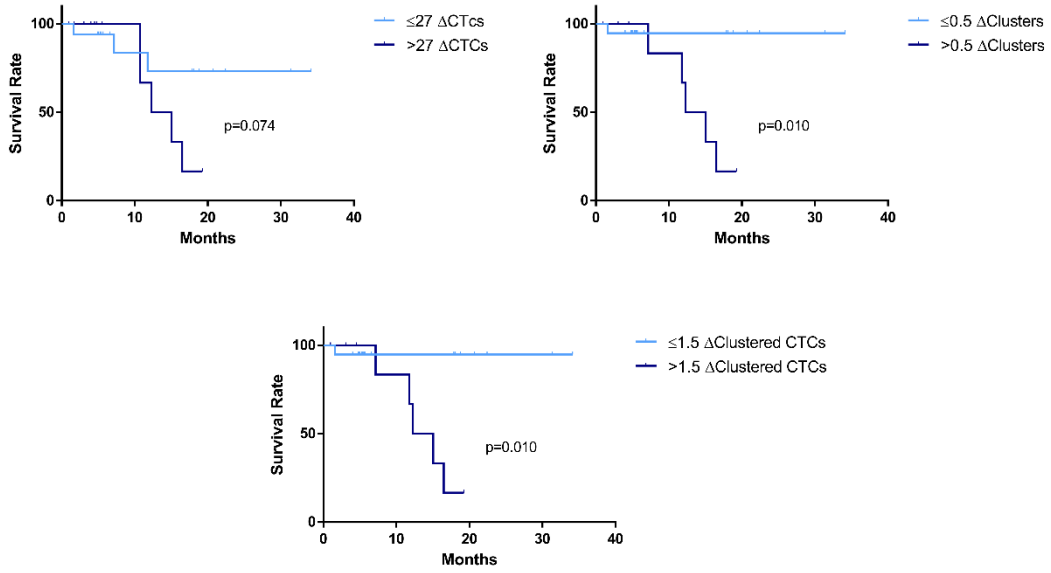


Figure 3. ROC curves of changes between 1-month TACE versus baseline in individual CTCs (a), clusters (b) and clustered CTCs (c) for death including area under the curve (AUC), Confidence Intervals (CI) and significance, as well as the cut-off values from each variable with the respective sensibility and specificity values (d) from HCC patients (n=28). Kaplan-Meier curves for analyzing survival were drawn according to these cut-off values (e). Circulating tumor cells, CTCs.

Table 11. Univariate regression models of clusters and clustered CTCs measurements according to stablished cut-off values from patients with HCC (n=28) after treatment with TACE. Data were analyzed using Cox regression models (Hazard Ratio, HR) according to survival, and logistic regression models (Odds Ratio, OR) with 95% Confidence Intervals (CI). Significant results (p<0.05) are shown in bold. Circulating tumor cells, CTCs.

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Cox regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**HR**	**Low CI**	**High CI**
Univariate	**Clusters>0.5**	2.323	1.106	**0.036**	10.204	1.168	89.108
	Clustered CTCs>1.5	2.323	1.106	**0.036**	10.204	1.168	89.108
Logistic Regression models							
Type of analysis	**Variable**	**B**	**SE**	***p* value**	**OR**	**Low CI**	**High CI**
Univariate	**Clusters>0.5**	3.114	1.227	**0.011**	22.500	2.031	249.239
	Constant	-2.890	1.027	0.005	0.056		
	Clustered CTCs>1.5	3.114	1.227	**0.011**	22.500	2.031	249.239
	Constant	-2.890	1.027	0.005	0.056		

3. Discussion

HCC represents 80% of the primary hepatic neoplasms that appears mainly in the context of chronic liver cirrhosis (1). TACE has shown a beneficial impact on patients with unresectable HCC by increasing long-term survival and TTP (12, 13). Although it remains advantageous, there is still a low to moderate degree of evidence of its effectiveness and many combinations with other therapies have recently emerged (14). Therefore, biomarkers of treatment response are needed. Several studies have identified radiological patterns that correlate with response after treatment. Preoperative magnetic resonance image (MRI) features based on irregular tumor margins and abnormal AFP levels in tumors >5cm could predict non-complete response after TACE coupled to high intensity focused ultrasound in an Asiatic cohort (15). Similarly, another study set up on MRI, has indicated that large tumors and intense arterial enhancement increase probabilities of incomplete response (16). Nevertheless, these studies have not identified predictors of complete disease remission. In addition, they are observative approaches that do not consider molecular characteristics of tumors.

EMT has been established as one of the main mechanisms in HCC progression. During these biological process, epithelial cells, which are usually attached to a basement membrane, lose their adhesion molecules and acquire mesenchymal characteristics that allow them to become migratory (17). Expression of cytoskeletal markers such as vimentin, β-catenin, α-SMA, extracellular matrix proteins like fibronectin or transcription factor Twist are typical hallmarks of EMT (18). Cell transdifferentiation is thought to play an important role in metastasis and tumor progression, contributing to generation of CTCs. In this multistep process, epithelial cancer cells detach from primary tumors and get access to general circulation, acquire anoikis resistance, and extravasate to generate secondary tumors at distance (19). Thus, CTCs with EMT markers might be considered relevant markers in liquid biopsies, which allow a minimally invasive access and that might be useful for monitoring the evolution of malignancies. According to their molecular phenotype, CTCs can be divided into epithelial, epithelial to mesenchymal or mesenchymal cells, having the second classification a stronger association with tumor dissemination (20).

Liquid biopsy has also been applied to study the prognosis and priority of patients with HCC in the waiting list for OLT. The procedure used in the present study, IsoFlux, has resulted more sensitive in detecting CTCs than CellSearch in patients in the waiting list for OLT (21). In addition, Ramírez et al. showed that CTCs measured by IsoFlux procedure were detected in 21 out of 24 patients, and ranged from 2 to 1768 counts per 10 mL of blood, and being associated with the time in the waiting

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list (22). A postoperative CTC count ≥3 appears to be a surrogate marker for the prediction of postoperative extrahepatic metastases after curative surgical resection of HCC (23). The CanPatrol CTC enrichment system based on cellular filtration using membranes with 8 μm diameter pores and tricolor RNA hybridization in situ (RNA-ISH) was not able to find any correlation between CTCs counts and tumor recurrence after OLT (24). In this line, other research using a CanPatrol-based approach has found no correlation between CTCs and recurrence in HCC (25). CTCs enriched by membrane filtration detected counts ranging between 0 and 31 that appeared to be predictors of clinical outcome in patients submitted to surgical intervention for HBV-related HCC (26). All these studies suggest that a great variability exists on data mainly due to technical issues and markers used to discriminate CTCs. We have demonstrated herein that our system has a great sensitivity in terms of detection, as CTCs were present in clinically relevant numbers in all blood samples. Regarding CTCs counts, IsoFlux provided higher CTCs counts than CellSearch, with established cutoffs of no more than 5 CTCs for bad prognosis (27-29). In particular, the use of EMT markers for the identification of CTCs appeared to be especially appropriate for the measurement of CTCs in intermediate HCC stages, when activation of shedding of tumor cells into bloodstream widely occurs (30).

The present study has determined the prognostic relevance of measurement of individual CTCs, number of clusters, clustered CTCs and AFP in control subjects, cirrhotic and HCC patients at intermediate stage. The cutoff values of baseline 46 individual CTCs, but not the cutoff of clusters, clustered CTCs and AFP, were associated with increased probability of HCC (Odds Ratio=3.467, p=0.011) (Figure 1, Table 3). The measurement of individual CTCs has been shown to be of value in determining the effectiveness of TACE. The preoperative positivity for the presence of CTCs in patients with HCC (73,8 % BCLC 0-A, 26,2% BCLC B-C) submitted to surgical intervention has been shown to be related to the effectiveness of adjuvant TACE reducing late recurrence (31). High EpCAM-positive CTC count predicts poor survival of patients with unresectable HCC treated with TACE (32). Our study further identified baseline individual CTCs>76 (p=0.016; Odds Ratio: 5.359, Confidence Intervals 95%: 1.374-20.911) and clustered CTCs>14.5 (p=0.049; Odds Ratio: 3.612, Confidence Intervals 95%: 1.003-13.005) as independent predictors of disease progression after TACE (Table 7). To our knowledge, there are few publications proving that CTCs shedding correlates and predicts TACE response. Fang et al. (33) have shown that CTCs isolated from peripheral or central blood by magnetic bead-labeled anti-human EpCAM monoclonal antibody using a magnetic cell separator were not associate to TTP after TACE. In contrast, serum levels of dickkopf-1, a negative regulator of the Wnt-beta signaling pathway, and the presence of CTCs detected by immunomagnetic beads combined with fluorescence *in situ* hybridization (FISH), have been connected to efficacy (34). These results support our findings although they are not fully comparable due to differences in CTCs enrichment procedures.

The effectiveness of treatment was addressed comparing changes in CTCs at 1-month after TACE treatment compared with baseline values. The increased changed levels of 27 individual CTCs, 0.5 clusters and 1.5 clustered CTCs were identified as cut-off values for death in our cohort of patients with HCC at intermediate stage (Figure 3). However, the Cox and binary regression models showed that post-TACE changes of clusters>0.5 and clustered CTCs>1.5 increased the risk of death up to 2.323 times (HR=10.204, p=0.036) in survival models. In particular, binary regression models indicated that changes in clusters>0.5 (OR=22.500, p=0.011) and clustered CTCs>1.5 (HR=22.500, p=0.011) were important predictors of death at any time point in our cohort (Table 11).

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Therefore, our results showed that increasing baseline levels of individual CTCs were able to predict disease progression. Furthermore, changes in clusters and clustered CTCs 1-month after TACE were foretold patient death. Our study suggests that those patients who fulfilled criteria of baseline CTCs>76 and clustered>14.5, and 1-month changes in clusters>0.5 or clustered CTCs>1.5 might be redirected to systemic therapy due to their intrinsic probabilities of bad prognosis. Thus, ideally, our variables aspire to be decisive checkpoints in algorithms for helping in clinical decision making and treatment allocation.

4. Materials and Methods

Patients: Patients with HCC at intermediate stage (BCLC B) (n=51) treated with TACE were recruited for the study (October 2017-August 2020). All patients were older than 18 years and provided written informed consent. CTCs were also obtained in a healthy population (n=13) and cirrhotic patients with no sight of radiologic malignancies (n=18) (December 2018-June 2019). CTCs were measured in blood samples obtained before (n=51) and 1 month after TACE (n=28) in patients with HCC at intermediate stage. Our cohort was stratified according to disease progression and death. Demographic information, epidemiological data and basal CTCs measurements from control subjects, cirrhotic patients and patients with HCC are shown in Table 1. Radiologic follow-up was maintained until the end of the study. All followed procedures were in accordance with the ethical standards of the responsible local committee (PI16/00090, Acta 13/2016) that additionally followed the guidelines included in the Helsinki Declaration of 1975, as revised in 2008.

Treatment efficacy evaluation: Patients were punctured through the right femoral artery and a catheter was delivered to the suprahepatic or the mesenteric artery. Angiography was used to localize target lesions with arterial enhancement. TACE routinely consisted in tumor irrigated vasculature were selectively catheterized and infused with microspheres precharged with doxorubicin until vessel complete embolization. TACE response was evaluated with computerized tomography (CT) scans by qualified radiologists following mRECIST criteria. Complete response was defined when target lesions did not show contrast enhancement nor washout, being tissue totally necrotized. Partial response was considered when there was still contrast enhancement but a reduction in size of the viable target lesions were evident, being tissue partially necrotized.

Enrichment of Circulating Tumor Cells: Isolation of CTCs was carried out using commercial Isoflux Epithelial to Mesenchymal Transition (EMT) Kit (910-0106, IsoFlux, Fluxion Biosciences, California, USA). This is based on the microfluidic separation of immunomagnetic beads coated with mouse monoclonal IgG antibodies against human epithelial EpCAM and epithelial growth factor receptor (EGFR), and mesenchymal (c-met, N-cadherin and vimentin) markers that capture CTCs from the mononuclear cell fraction of peripheral blood. Whole blood samples (6 ml) were collected in EDTA-K2 Vacutainer™ tubes (367873, Becton, Dickinson and Company, New Jersey, EEUU). Isolation of peripheral blood mononuclear cell (PBMC) was carried out with Ficoll-Paque™ Plus (17-1440-02, GE Healthcare, Illinois, EEUU) density gradient separation in frit coupled Leucosep™ tubes (227290, Greiner Bio-One, Kremsmünster, Austria). Tubes were filled with 15.2 mL of Ficoll and centrifuged 1000 x *g* 30 seconds. Phosphate buffered saline calcium and magnesium free (PBS-CMF) (5 ml) was added to the upper part of the frit and diluted 6 mL of blood were left to decant inside the tube. After centrifugation of samples at 800 x *g* 15 minutes without brake, PBMC fraction was obtained and pelleted at 280 x *g* 10 minutes. Commercial blocking solution was added to pellets, and

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incubated for 5 min on ice. Cell suspension was transferred to a microcentrifuge tube. Antibodies-coated beads were coupled to cell samples, and incubated for 2 hours at 4°C with rotation, and afterwards loaded into Isoflux microfluidic cartridges. CTCs were recovered with commercial Binding Buffer and transferred to a microcentrifuge tube. All samples were processed with a maximum delay of 4 hours.

Quantification of Circulating Tumor Cells: Upon CTCs enrichment, cells were fixed at room temperature for 20 minutes, and negatively selected using CD45 immunofluorescent labelling included in the commercial Isoflux™ CTC Enumeration Kit (910-0093, IsoFlux, Fluxion Biosciences, California, EEUU). Samples were previously blocked with normal donkey serum for 5 minutes. Cells were incubated with rabbit anti-CD45 primary antibody and donkey anti-rabbit-Cy™3-conjugated secondary antibody for 20 minutes. The positive selection was carried out upon permeabilization of cells with 0.2 % TX-100 and incubated with mouse monoclonal anti-cytokeratin-FITC-conjugated for 40 minutes. Cells were washed with Hoechst 0.02% to stain nuclei and placed on microscope slides with Mounting Media. The acquisition of images was done with a fluorescence direct Olympus BX-61 microscope (Shinjuku, Tokyo, Japan). CTCs were enumerated as intact nucleated cells with positive green fluorescence (Cytokeratin+) and negative red fluorescence (CD45-).

Efficiency of Isoflux in recovery cancer cells: HepG2 cells were labelled with CellTracker Green (Invitrogen Catalog Number C7025) at a concentration of 2μM in serum-free medium for 30 minutes at 37°C. Cells were harvested, and 1000 cells were added to 6 mL of blood from healthy subjects. Isoflux enrichment procedure was carried as described above. Tumor positive green fluorescence cells were determined by three independent evaluators. The percentage of recovery obtained was 82±7% (n=3).

Statistical Analysis: Numerical variables are summarized as mean ± SE and categorical variables as frequencies and percentages. It was assessed the homogeneity and the normal distribution of the variables by the Levene, and Kolmogorov-Smirnov test, respectively. The different comparison analysis was performed using student's *t* test or Mann-Whitney's U test according the positively or negative normality of the variables, respectively. To analyze the relationship between complete or partial response to treatment with the categorical variables (sex, etiology of HCC, cirrhosis stage, number, size and location of tumors, development of metastasis during post-TACE follow-up and new treatments), the Pearson chi-square or the Fisher's correction was used, applying Fisher's exact distribution or the Monte Carlo method when required. The analysis of quantitative variables such as age, glucose, alanine transaminase (ALT) and α-fetoprotein (AFP) in blood was carried out using the Mann-Whitney test.

Analysis of treatment efficacy was performed using basal CTCs (individual CTCs, cluster counts and clustered CTCs) and CTCs at 1-month after TACE (ΔCTCs, ΔCluster counts and ΔClustered CTCs). The association of CTCs profile and basal levels with clinical variables was conducted with the Kruskal-Wallis test and post-hoc analysis by Mann-Whitney tests when necessary. Quantitative CTCs variables were analyzed by Spearman bivariate correlations. Receiver operating characteristic (ROC) curve analysis was carried to find CTCs predictors of treatment response. CTCs profile was evaluated to find predictive values with the highest sensitivity and specificity.

Patients were distributed according to the treatment response or disease progression, and exitus. The univariate and multivariate binary logistic regression analysis identified different variables that were significantly associated with the progression of the disease or exitus in patients with intermediate HCC. We identified the cut-off values of the selected variables that give the best

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sensibility and specificity in the ROC curve for the prediction of disease progression or exitus. The presence of basal clusters >4.5 and basal CTCs in cluster>14.5 were modelled for the significant independent prediction of disease progression in the multivariate logistic regression analysis. The presence of 1-month after treatment clusters >0.5 and 1-month after treatment CTCs in cluster>1.5 were modelled for the significant independent prediction of exitus in the multivariate logistic regression analysis. A significance level of 5% was used throughout the variables studied. Statistical analyses were performed by SPSS statistical software v. 11.0 (SPSS Inc., Chicago, Illinois, USA).

5. Conclusions

The study correlated the baseline levels of CTCs with the presence of HCC and the disease progression after TACE. In addition, comparison of changes in the number of clusters and clustered CTCs at 1-month after TACE versus baseline identified patients with increased risk of death after TACE. The information might be relevant for clinical decision making in order to select patients that might not benefit from TACE, and consequently receiving systemic therapy at first might be alternative recommended.

Author Contributions: Conceptualization of the study, María del Carmen Garrido-Navas, María J. Serrano, José M. Álamo-Mártínez, Álvaro Giráldez-Gallego, María T. Ferrer-Ríos, Jordi Muntané; Methodology, Patricia de la Cruz-Ojeda, Elena Navarro-Villarán, Sandra Dios-Barbeito; Experimentation, María I. Gómez-Espejo, Inés M. Fernández-Luque, Concepción Meana, Manuela F. Domínguez-Borrero; Imaging and histological assessments, Carlos M. Gutiérrez-Martín and Francisco J. Castell-Monsalve; Formal analysis, Patricia de la Cruz-Ojeda, Juan M. Praena-Fernández; Writing—original draft preparation, Jordi Muntané; Writing—review and editing, Patricia de la Cruz-Ojeda, Juan M. Praena-Fernández, Jordi Muntané; Funding acquisition: José M. Álamo-Martínez, María J. Serrano, María T. Ferrer-Ríos, Jordi Muntané. All authors have read and agreed to the published version of the manuscript.

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