

# Macrophage profile in primary *versus* secondary liver tumors

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**Abstract:** Macrophages are important components of the tumor-associated infiltrate and are qualified as one of the major players of the cancer-related inflammation. It was shown that tumor cells can either stimulate or mediate apoptosis of tumor-associated macrophages (TAMs). To date, there is no general agreement regarding the influence of TAMs and their numbers on the progression of hepatocellular carcinoma (HCC) and hepatic metastases (HM). To analyze the presence of TAMs and compare their numbers in intratumoral (IT) and peritumoral (PT) areas with the clinical outcome of HCC and HM patients. Biopsies from 35 HCC and 39 HM cases were analyzed. Clinical and follow-up data was enrolled for each case; the colorectal cancer was the origin of 26 HM patients. TAMs were identified by immunohistochemistry using anti-CD68 monoclonal antibody. The quantitative assessment was performed by determining the mean number of CD68-positive cells in IT and PT areas in HCC and HM. Two threshold methods were applied: threshold 1 (T1) was calculated with the use of (-log) Cox method; threshold 2 (T2) was considered as 1/3 TAMs number of group's mean. For statistical analyses Mann-Whitney U-test, Spearman's correlation, Cox proportional hazard and Kaplan-Meier tests were applied. To date, 36.12% HCC and 27.78% HM patients were alive, median survival was 5 and 17 months for HCC and HM, respectively ( $P = 0.05$ ). We found significant two-fold decrease of TAMs numbers between IT vs. PT territories in both HCC and HM. A positive correlation between numbers of PT and IT TAMs was observed in HM group ( $r_s = 0.48$ ,  $P < 0.05$ ) but not in HCC. The number of TAMs was not associated with any studied clinical factor. Univariate Cox regression analysis showed that tumor stage  $\leq$  II ( $P = 0.01$ ) and increased number of PT TAMs ( $P = 0.06$ , only when T2 value was applied) were associated with favorable prognosis in HCC (HR = 2.614 and 2.457, respectively). Univariate and multivariate Cox analyses in HM revealed favorable prognosis for histological grade  $\leq$  G2 and one lobe tumors ( $P = 0.021$  and 0.045; HR = 0.395 and 0.438, respectively). Survival analysis retained the impact of increased TAMs numbers in peritumoral areas ( $P = 0.03$ ), tumor stages in HCC ( $P = 0.007$ ), lobes' number ( $P = 0.007$ ) and histological grade ( $P = 0.005$ ) on HM patients' outcome. In HCC and HM the low number of TAMs in intratumoral areas was related to the tumor cell microenvironment. The increased peritumoral TAMs number in primary liver tumors was associated with better prognosis. (*Folia Histochemica et Cytobiologica* 2014, Vol. 52, No. 2, 112–123)

**Key words:** macrophages; CD68; hepatocellular carcinoma; liver metastases; quantitative analysis; peritumoral; intratumoral; prognosis

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## Introduction

The hepatic tumor microenvironment expresses a unique cellular and molecular profile which may be characterized by cellular and non-cellular components [1] with interdependent functions and reciprocal signaling during their involvement in the process of carcinogenesis [2]. Macrophages, dendritic cells, mast cells, cytotoxic natural killer cells, and T cells of the hepatic microenvironment are responsible for the development of innate immune responses adapted to a tolerant immunological status [1, 3, 4] which is regarded as procarcinogenic. Moreover, liver extracellular matrix (ECM) contains a large diversity of molecules secreted by both parenchymal and non-parenchymal liver cells, such as growth factors, proteolytic enzymes with their specific inhibitors, and inflammatory cytokines [1, 2] which affect function of both immune and non-immune cells.

Macrophages are important components of the tumor-associated infiltration, being considered as major players of the cancer-related inflammation [5]. Tumor-associated macrophage (TAM) population is heterogeneous with two major subtypes described: classically activated (type I or M1) and alternatively activated macrophages (type II or M2) [6–8]. Referring the origin of the TAMs, it was hypothesized that microenvironmental factors induce the differentiation toward one specific phenotype [8] since supplementary and intermediary phenotypes were noticed by some studies [9]. Macrophages' heterogeneity, based on their functional plasticity [10], resulted in the comparison of the pro- or anti-tumor activity of TAMs to a "double-edged sword" [7, 11] or "Janus face" [12].

Recent studies sustain that TAMs represent an unfavorable prognostic factor in hepatocellular carcinoma (HCC), since the TAM-secreted molecules stimulate the growth and survival of HCC cells, the epithelial-mesenchymal transition (EMT), tumoral angiogenesis, ECM remodeling, and suppression of the anti-tumoral immunity [13, 14]. On the other hand, TAMs association to an improved postoperative survival and their role as positive prognostic factor in HCC were supported by other research groups [15, 16]. In the studies of TAMs involvement in the development of hepatic metastases (HM), the most numerous were related to the colorectal cancer (CRC)-associated metastases [17–21]. Similarly to HCC, macrophages in HM can act against metastatic cells in a direct cytotoxic way or by secretion of TNF and proteases [22], however, TAMs can also support survival and differentiation of malignant cells *via* secretion of growth factors [23] or metalloproteinases [24].

Within this context, the objective of our study was a qualitative and quantitative characterization

of TAMs in HCC *vs.* HM in intra- and peritumoral areas, followed by statistical assessment of TAMs as a possible prognostic factor.

## Material and methods

**Patients.** The retrospective study was approved by the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania, based on the patients' informed written consent for the usage of the biologic material for research. The study included 74 patients diagnosed with hepatocellular carcinoma (HCC, 35 cases — Group 1) and hepatic metastases of different origins (HM, 39 cases — Group 2) between January 2009 and December 2011, at the "Sf. Spiridon" University Hospital, Iași, Romania. The patients were surgically treated, without preoperative chemotherapy or radiotherapy. Within HM group the origin of the hepatic metastases was: colorectal cancer — 24 cases (18 colon cancer, 6 rectal cancer), stomach — 8 cases, pancreatic — 2 cases, gallbladder — 1 case, duodenal — 1 case, ovarian — 1 case, breast — 1 case, adrenal gland cancer — 1 case. The histopathological examinations were performed in the hospital's pathology laboratory, where the harvested tumor fragments were processed into paraffin blocks using classic histological technique. The patients' clinicopathological characteristics were summarized in Table 1 and Table 2. The median survival rates were 5 months for HCC and 17 months for HM cases. On 31<sup>st</sup> of January 2013 36.12% of HCC patients and 27.78% of HM patients were alive. All recorded patients' deaths were associated with diagnosed cancer disease.

**Immunohistochemistry.** For immunohistochemical (IHC) study formalin fixed, paraffin-embedded (FFPE) 4  $\mu$ m-thick tissue sections, corresponding to the areas previously examined by pathologist, were displayed on Superfrost Plus (Thermo Fischer Scientific, Fitchburg, WI, USA) slides. Sections were deparaffinized in two xylene baths 1<sup>st</sup> at 58°C for 30 min and 2<sup>nd</sup> at room temperature (RT) for 10 min. The rehydration was made in four following alcohol solutions (100%, 90%, 80%, and 70% EtOH) for 10 min each. For antigen retrieval, we used heat-induced epitope retrieval (HIER), by immersing the slides in a citrate buffer (pH 6.0) (Leica Biosystem, Newcastle Ltd, United Kingdom) at 98°C for 30 min. After cooling the slides at room temperature (RT) for 20 min the endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide solution (RT, for 10 min), followed by one wash with distilled water for 5 min. For the IHC identification of macrophages, slides were incubated with the primary antibody (1:1200, mouse anti-human CD68 monoclonal antibody, code: NCL-CD-68-KP1, Novocastra, Leica Biosystem), overnight at 4°C. The immunoreaction was developed with Novolink polymer kit (Novocastra, Leica Biosystem) as following: incubation with biotinylated secondary antibody and with the enzyme,

**Table 1.** Clinicopathological characteristics of patients — Group 1, hepatocellular carcinoma (HCC)

Variables	Variable description	Cases number	(%)
<b>Age</b>	Mean ± SD; median, range (years)	62.9 ± 10.7; 64, 23–83	
≤ 63		17	48.57
> 63		18	51.42
<b>Gender</b>			
Female		12	34.28
Male		23	65.71
<b>Tumor stage***</b>			
Stage I	T1N0M0	5	14.28
Stage II	T2N0M0	13	37.14
Stage IIIa	T3N0M0	16	45.17
Stage IV	TxNxM1	1	2.8
<b>Histological grade***</b>			
G1	Well differentiated	13	37.14
G2	Moderately differentiated	15	42.86
G3	Poorly differentiated	7	20.00

\*pTNM and histological divisions according to [43]; \*\*pTNM stages (IIIb, IIIc) and G4 were not diagnosed, therefore they were omitted

respectively, for 30 min each, at RT. Between each step of incubation (primary and secondary antibodies, enzyme), slides were washed in 3 baths of phosphate-buffer saline for 5 min each. For visualization, we used 3,3'-diaminobenzidine dihydrochloride (DAB, Novocastra, Leica Biosystem) for 3 min — the immunopositivity was confirmed through the presence of a brown product. The nuclei were then counterstained with hematoxylin and mounted in non-aqueous mounting medium. The negative control was made concomitantly by omitting the incubation with the primary antibody, and respecting all the other steps of the protocol. The positive control was made during the same run by using a tonsil tissue section.

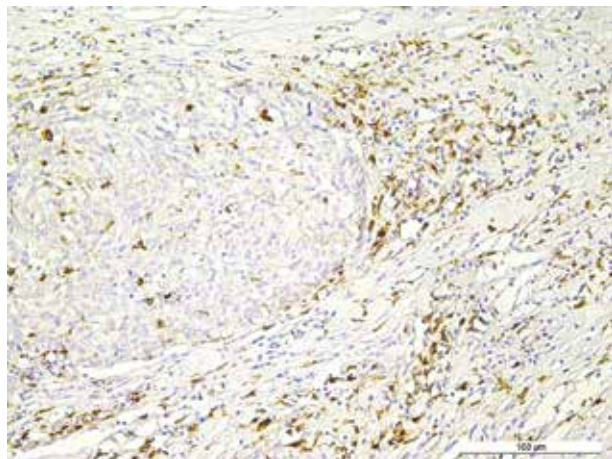
**Quantitative analysis.** The quantitative assessment was performed, for each case, by counting the number of CD68-positive cells in 10 microscopic fields with the highest density of macrophages, at 200 × magnification, separately for the intratumoral and peritumoral territories. Our method represented a modified variant of the semi-quantitative evaluation of macrophages proposed by Forssell and co-workers, in colon cancer [25]. The IHC evaluation was done independently by two histopathologists (E.R.A. and S.E.G.) trained in IHC interpretation, unaware of the clinical outcome. On the basis of the mean number of TAMs of each case, we have determined the mean number of macrophages for each studied group, separately for intratumoral (IT) and peritumoral (PT) territories.

**Table 2.** Clinicopathological characteristics of patients — Group 2, hepatic metastases (HM)

Variables	Variable description	Cases number	(%)
<b>Age</b>	Mean ± SD; median, range (years)	67.6 ± 11.9; 70, 35–86	
≤ 68		17	43.58
> 68		22	56.41
<b>Gender</b>			
Female		18	46.15
Male		21	53.84
<b>Tumor stage</b>			
Stage IV	TxNxM1	39	100
<b>Histological grade</b>			
G1	Well differentiated	3	7.69
G2	Moderately differentiated	20	51.28
G3	Poorly differentiated	14	35.89
G4	Undifferentiated	2	5.12
<b>Tumoral extension</b>			
One lobe		26	66.66
Many lobes		13	33.33

**Statistical analysis.** Statistical analysis has been performed using MedCalc software (MedCalc Software, Ostend, Belgium) and GraphPad Prism ver. 6 (GraphPad Software Inc., La Jolla, CA, USA). Patients' characteristics were given in absolute and relative numbers or as mean values with standard error of the mean (SEM). Mean macrophage scores for each subgroup were further checked with the use of D'Agostino normality test. Mann-Whitney U test was used for statistical comparison between two variables whereas Kruskal-Wallis ANOVA test was utilized when more than two independent variables were analyzed. For checking the associations between variables, Spearman's correlation was utilized.

**Estimation of thresholds.** Two thresholds were estimated: research and analytical. The first threshold (T1) was calculated for TAMs using univariate Cox regressions analyses (-log test). Therefore, cases with lower mean score than T1 for the respective division were considered as subgroup 1 (or ↓) whereas cases with higher mean macrophages score were treated as subgroup 2 (or ↑). The second threshold (T2) has been additionally used in order to verify if a lower value compared to the average value of the group might offer a superior refinement of the correlation with patients' survival. T2 was calculated as 1/3 of macrophages mean number for each group and territory divisions. Thus, specimens with lower macrophage number than T2 were marked to subgroup 3 (or ↓) whereas cases with higher number than



**Figure 1.** Pattern of macrophages distribution in HCC: numerous at the border of a tumoral nodule, very few dispersed within tumor (immunohistochemical, IHC, detection by anti-CD68 monoclonal antibody). Objective  $\times 20$

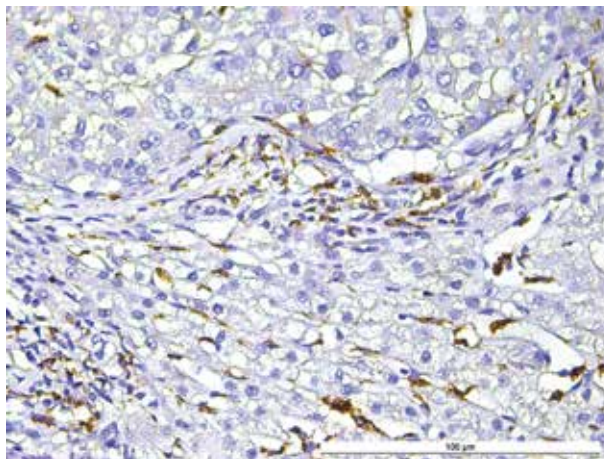
T2 were scored to subgroup 4 (or  $\uparrow$ ). Both T1 and T2 were further considered in survival analyses. Fisher  $2 \times 2$  test was utilized for the statistical comparison between subgroups based on T1/T2 classification.

**Survival analysis.** The Cox-Mantel proportional hazard regression model was used to evaluate the effect of explorative variables on survival of HCC and HM patients. First, univariate Cox regression analysis for every single variable was performed. Secondly, variables with a  $P$  value  $< 0.05$  were included into multivariate Cox regression analysis with a variable selection *via* backward elimination. All associations were presented as hazard ratios (HR) with their 95% confidence interval (CI) and  $P$  values. Kaplan–Meier estimations were performed to describe survival rates.

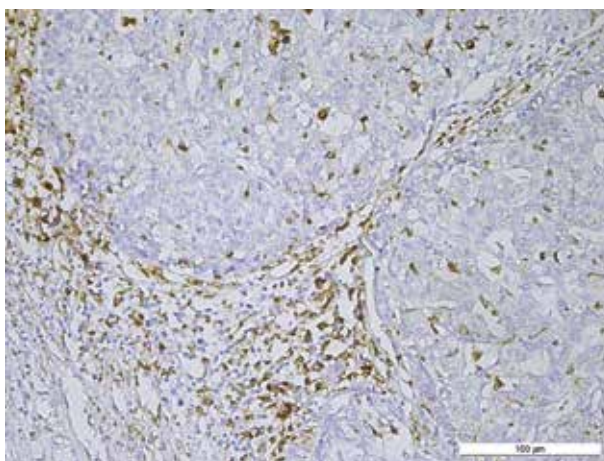
## Results

### Qualitative evaluation

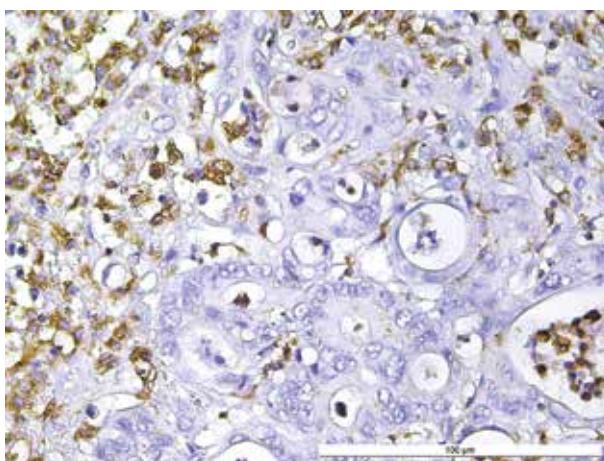
Microscopic examination has revealed both in Group 1 — HCC and in Group 2 — HM, CD68 immunoreactive (CD68<sup>+</sup>) dominant population in the periphery of tumoral islands or nodules, at the interface with the normal liver parenchyma (Figures 1–4). This territory could be identified in all cases, being considered as the tumor's invasion front. Macrophages were identified as either single isolated cells or in small compact groups, inside the tumor, being mainly disposed in the area corresponding to the most external 2–3 cell layers (Figures 1–4). The qualitative evaluation, based on a visual, subjective appreciation of macrophages' presence did not reveal differences between the distribution (location) patterns of these cells in the HCC vs. HM.



**Figure 2.** IHC detection of macrophages at the interface between HCC and normal liver parenchyma. Objective  $\times 40$



**Figure 3.** Distribution pattern of macrophages in HM: numerous at the border of the cancer proliferation, very few dispersed within tumor. IHC. Objective  $\times 20$



**Figure 4.** Macrophages disposed at the periphery of the metastatic nodule penetrating through the metastasized glands (colonic pseudocrypt). IHC. Objective  $\times 40$

**Table 3.** Patients' clinical data and numbers of macrophages in Group 1 — HCC

Case number	Clinical variables			Intratumoral macrophages			Peritumoral macrophages		
	Age	Tumor stage	Histological stage	Numerical profile (mean ± SD)	TAMs expression		Numerical profile (mean ± SD)	TAMs expression	
					T1*	T2**		T1&	T2&&
1	61	pT3Nx	G1	118.30 ± 23.22	↓	↑	303.90 ± 132.33	↑	↑
2	63	pT2Nx	G1	184.9 ± 73.40	↑	↑	317.80 ± 124.70	↑	↑
3	68	pT3Nx	G3	87.7 ± 44.71	↓	↑	50.20 ± 28.61	↓	↓
4	54	pT1Nx	G1	46.7 ± 29.90	↓	↑	172.00 ± 54.77	↑	↑
5	48	pT3Nx	G2	337.7 ± 50.78	↑	↑	344.80 ± 78.10	↑	↑
6	59	pT3Nx	G2	137.1 ± 68.82	↓	↑	289.20 ± 189.21	↑	↑
7	57	pT3Nx	G2	209.9 ± 81.09	↑	↑	59.30 ± 28.76	↓	↓
8	72	pT1Nx	G1	3.9 ± 2.46	↓	↓	32.00 ± 15.86	↓	↓
9	57	pT3Nx	G3	167.10 ± 98.92	↑	↑	227.70 ± 92.23	↑	↑
10	58	pT2Nx	G3	68.00 ± 25.15	↓	↑	272.60 ± 87.18	↑	↑
11	58	pT2Nx	G3	105.30 ± 97.32	↓	↑	156.10 ± 118.65	↑	↑
12	74	pT3Nx	G2	116.90 ± 50.35	↓	↑	248.30 ± 79.78	↑	↑
13	51	pT3Nx	G2	50.90 ± 25.28	↓	↑	170.90 ± 61.13	↑	↑
14	83	pT2Nx	G2	85.10 ± 50.34	↓	↑	376.50 ± 106.96	↑	↑
15	65	pT2Nx	G2	127.00 ± 57.42	↓	↑	0	↓	↓
16	74	pT2Nx	G1	0	↓	↓	253.40 ± 169.43	↑	↑
17	67	pT2Nx	G3	418.60 ± 137.75	↑	↑	578.50 ± 126.59	↑	↑
18	74	pT2Nx	G1	7.50 ± 7.97	↓	↓	76.20 ± 49.08	↓	↑
19	70	pT3Nx	G2	74.70 ± 25.56	↓	↑	318.40 ± 90.32	↑	↑
20	68	pT3Nx	G2	43.80 ± 12.56	↓	↑	58.00 ± 30.38	↓	↓
21	64	pT1Nx	G1	89.40 ± 16.22	↓	↑	134.00 ± 57.71	↓	↑
22	72	pT1Nx	G1	63.20 ± 33.15	↓	↑	195.10 ± 108.28	↑	↑
23	56	pT3Nx	G2	91.70 ± 11.84	↓	↑	247.70 ± 105.86	↑	↑
24	75	pT3Nx	G2	89.40 ± 23.15	↓	↑	52.10 ± 15.96	↓	↓
25	60	pT3Nx	G2	61.10 ± 20.61	↓	↑	329.00 ± 221.33	↑	↑
26	57	pT2Nx	G3	23.10 ± 13.21	↓	↓	226.70 ± 103.65	↑	↑
27	75	pT2Nx	G1	72.70 ± 23.36	↓	↑	613.20 ± 123.69	↑	↑
28	58	pT2Nx	G2	0	↓	↓	281.50 ± 136.75	↑	↑
29	58	pT1Nx	G1	133.50 ± 49.91	↓	↑	117.20 ± 50.83	↓	↑
30	71	p2NxM1#	G3	112.60 ± 64.33	↓	↑	358.60 ± 78.14	↑	↑
31	69	pT3Nx	G1	121.30 ± 28.14	↓	↑	248.60 ± 62.96	↑	↑
32	53	pT2Nx	G2	71.70 ± 38.12	↓	↑	484.70 ± 181.19	↑	↑
33	65	pT3Nx	G1	7.90 ± 4.40	↓	↓	148.20 ± 81.24	↑	↑
34	23	pT3Nx	G1	75.00 ± 38.97	↓	↑	83.60 ± 53.57	↓	↑
35	67	pT2Nx	G2	98.20 ± 65.55	↓	↑	151.30 ± 75.47	↑	↑
Mean ± SEM				100.05 ± 14.62			227.9 ± 24.81		
Median, range				87.70, 0–418.60			227.7, 0–613.2		
<i>P</i>									< 0.0001

\*IT Threshold 1 = 145.36; Subgroup 1 = ↓; Subgroup 2 = ↑

\*\*IT Threshold 2 = 33.33; Subgroup 1 = ↓; Subgroup 2 = ↑

\*PT Threshold 1 = 144.16; Subgroup 1 = ↓; Subgroup 2 = ↑

\*\*PT Threshold 2 = 75.96; Subgroup 1 = ↓; Subgroup 2 = ↑

#HCC metastases were diagnosed in bone

Table 4. Patients' clinical data and numbers of macrophages in Group 2 — HM

Case number	Clinical variables				Intratumoral TAMs			Peritumoral TAMs		
	Age	Tumor stage	Histological stage	Tumoral extension # of lobes	Numerical profile (mean ±SD)	TAMs expression		Numerical profile (mean ± SD)	TAMs expression	
						T1*	T2**		T1&	T2&&
1	58	pT3N2M1	G2	1	48.2 ± 17.68	↓	↑	381.2 ± 154.67	↑	↑
2	66	pT2NxM1	G3	1	56.2 ± 28.63	↓	↑	196.7 ± 126.22	↑	↑
3	63	pT4NxM1	G4	1	39.2 ± 24.96	↓	↓	47.8 ± 24.06	↓	↓
4	70	pT3N1M1	G3	1	79.3 ± 27.39	↓	↑	187.2 ± 37.67	↑	↑
5	69	pT3N2M1	G2	1	150.5 ± 143.92	↓	↑	200.7 ± 90.29	↑	↑
6	73	pT3N1M1	G2	1	233.2 ± 100.39	↑	↑	239.3 ± 114.79	↑	↑
7	80	pT3N2M1	G2	> 1	197.5 ± 72.31	↑	↑	289.7 ± 110.09	↑	↑
8	57	pT3N2M1	G2	1	180.6 ± 149.25	↑	↑	355.4 ± 151.00	↑	↑
9	78	pT3N2M1	G2	> 1	217.6 ± 76.95	↑	↑	226 ± 67.10	↑	↑
10	57	pT4N1M1	G2	1	49.2 ± 34.18	↓	↑	238.5 ± 83.78	↑	↑
11	79	pT3NxM1	G3	1	255.4 ± 143.89	↑	↑	623.8 ± 173.25	↑	↑
12	79	pT2bN3M1	G3	1	152.8 ± 40.97	↓	↑	185.1 ± 54.66	↑	↑
13	52	pT3N2M1	G2	> 1	145 ± 31.13	↓	↑	208.3 ± 96.77	↑	↑
14	78	pT3N3M1	G2	> 1	47.1 ± 19.54	↓	↑	91.3 ± 26.89	↓	↑
15	55	pT3N2M1	G3	1	200.9 ± 90.50	↑	↑	206.3 ± 110.10	↑	↑
16	62	pT3N1M1	G1	1	255.4 ± 143.89	↑	↑	623.8 ± 173.25	↑	↑
17	65	pT3NxM1	G1	> 1	176.1 ± 58.87	↑	↑	282.5 ± 128.09	↑	↑
18	82	pT3NxM1	G3	> 1	333.3 ± 95.73	↑	↑	397 ± 149.56	↑	↑
19	72	pT3N0M1	G2	1	0	↓	↓	179.5 ± 69.17	↑	↑
20	35	pT3N2M1	G2	1	190.7 ± 112.05	↑	↑	169.2 ± 72.91	↑	↑
21	86	pT3N0M1	G3	1	73.1 ± 39.58	↓	↑	217.7 ± 78.21	↑	↑
22	60	pT4N1M1	G1	1	35.2 ± 32.35	↓	↓	183 ± 81.01	↑	↑
23	83	pT4N2M1	G3	> 1	255.4 ± 143.89	↑	↑	623.8 ± 173.25	↑	↑
24	65	pT3N2M1	G2	1	188.8 ± 107.84	↑	↑	224.6 ± 107.04	↑	↑
25	78	pT3N2M1	G2	1	50.6 ± 24.67	↓	↑	84.2 ± 24.14	↓	↓
26	74	pT3N2M1	G2	1	47.3 ± 24.28	↓	↑	194.5 ± 81.33	↑	↑
27	35	pT4N1M1	G3	1	204.2 ± 107.80	↑	↑	242.9 ± 83.70	↑	↑
28	64	pT3NXM1	G3	> 1	128.9 ± 53.97	↓	↑	262.9 ± 57.96	↑	↑
29	48	pT3N1M1	G2	> 1	201.8 ± 116.67	↑	↑	410.2 ± 182.09	↑	↑
30	63	pT4N1M1	G2	1	67.1 ± 57.81	↓	↑	197 ± 62.54	↑	↑
31	72	pT3NxM1	G2	> 1	61.3 ± 30.65	↓	↑	398 ± 122.83	↑	↑
32	76	pT2N0M1	G3	1	152.8 ± 40.97	↓	↑	185.1 ± 54.66	↑	↑
33	61	pT3N3M1	G4	1	91.6 ± 18.31	↓	↑	100.5 ± 22.17	↓	↑
34	72	pT4N1M1	G2	1	138.7 ± 100.73	↓	↑	443.1 ± 180.71	↑	↑
35	68	pT4N2M1	G3	1	32.6 ± 16.16	↓	↓	205 ± 68.87	↑	↑
36	74	pT3NxM1	G3	1	17.7 ± 17.19	↓	↓	106.9 ± 49.52	↓	↑
37	82	pT3N1M1	G3	> 1	266.7 ± 159.46	↑	↑	51.7 ± 34.59	↓	↓
38	70	pT3NxM2	G2	> 1	29.7 ± 12.57	↓	↓	218 ± 57.14	↑	↑
39	75	p3N2M1	G2	> 1	150.5 ± 74.22	↓	↑	186.9 ± 55.94	↑	↑
Mean ± SEM					133.4 ± 13.75			253.0 ± 23.01		
Median, range					145.0, 0–333.3			208.3, 47.8–623.8		
P							< 0.0001			

\*IT Threshold 1 = 162.29; Subgroup 1 = ↓; Subgroup 2 = ↑

\*\*IT Threshold 2 = 44.46; Subgroup 1 = ↓; Subgroup 2 = ↑

\*PT Threshold 1 = 163.00; Subgroup 1 = ↓; Subgroup 2 = ↑

\*\*PT Threshold 2 = 84.33; Subgroup 1 = ↓; Subgroup 2 = ↑



### Quantitative evaluation

Table 3 and 4 present the numerical values of the macrophages quantification as well as clinical variables, in each individual case of both HCC and HM groups. Since TAMs scores or intratumoral subgroups did not pass D'Agostino normality tests, we used non-parametric tests for further statistical analyzes.

#### TAMs in hepatocellular carcinoma group

Morphometric analysis revealed more than 2-fold lower number of TAMs in IT vs. PT compartment in HCC tumors ( $P < 0.0001$ ; Table 3, Figure 5A). Threshold 1 (T1) values were calculated using Cox (-log) test as 145.36 for IT and 144.16 for PT macrophages, whereas Threshold 2 (T2) values were counted as 1/3 of mean values for subgroups as 33.33 for IT and 75.92 for PT groups (Table 3).

The analysis of IT macrophages by using threshold 1 revealed the presence of 30/35 (86%) cases with lower score than T1. If T2 threshold was applied we observed a different pattern, since 6/35 (17%) cases showed even lower TAMs presence than T2, therefore T1 and T2 classifications cannot be compared ( $P < 0.0001$ , Fisher's test).

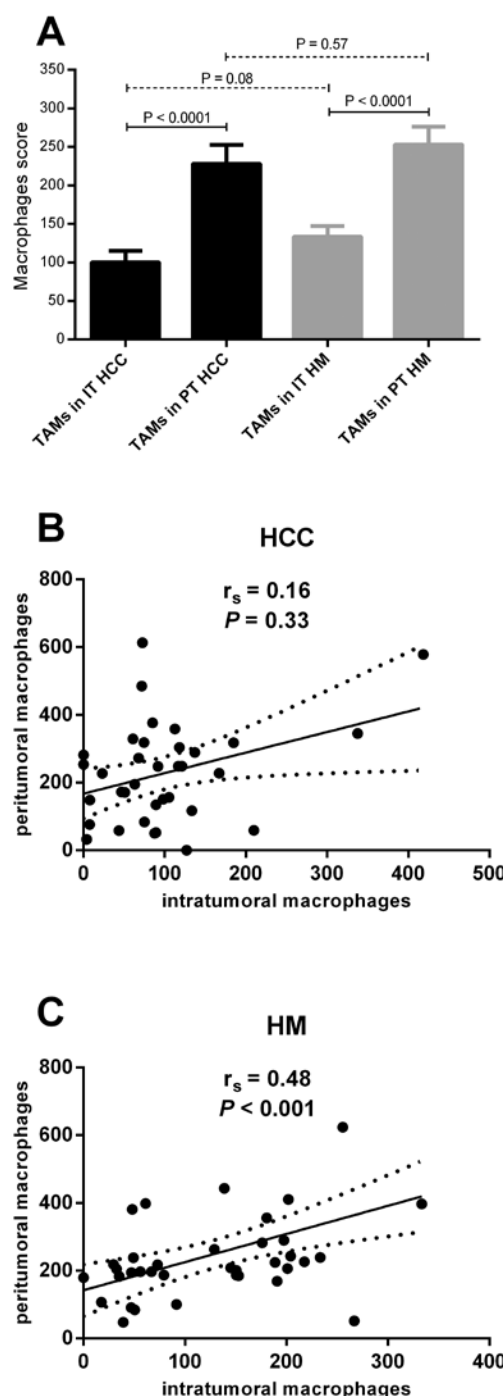
The analysis of IT macrophages in peritumoral territory of HCC revealed that 10/35 (28.5%) and 6/35 (17%) cases showed lower number of TAMs when T1 or T2 were utilized, respectively. In this approach (PT in HCC), both methods of threshold determination can be compared ( $P = 0.39$ ; Fisher's test).

We found no statistical associations between TAMs scores and clinical data of HCC cases (data not shown). We did not find any correlation between number of macrophages in intratumoral and peritumoral tissue (Figure 5B).

#### TAMs in hepatic metastases

Similarly to HCC, twice more TAMs were present in peritumoral than in intratumoral territories of HM patients ( $P < 0.0001$ , Table 4; Figure 5A). T1 and T2 for IT area were calculated as 162.29 and 44.46, respectively. Within IT tissue we observed decreased number of TAMs for 24/39 (61.5%) or 6/39 (15.3%) when T1 or T2 values were applied, respectively. For peritumoral HM cases, with T1 and T2 set for 163 and 84.33, respectively, we found decreased number of TAMs in 6/39 (15.3%) if T1 criterion was used or 3/39 (7.7%) if T2 was applied.

For PT in HM, T1 and T2 can be compared ( $P = 0.48$ ), whereas for IT in HM there was no agreement between T1 and T2 classification methods ( $P = 0.001$ , Fisher's  $2 \times 2$  test).



**Figure 5.** Comparison between macrophages numbers classified by cancer origin and liver territory. **A.** Plot of mean ( $\pm$  SEM) number of TAMs and the results of Mann-Whitney U test. **B** and **C.** correlation plots of TAMs number in matched HCC and HM cases with results of Spearman's test. HCC — hepatocellular carcinoma, HM — hepatic metastasis, IT — intratumoral, PT — peritumoral territory

Although we did not find any statistical associations between TAMs scores in HM and clinical data, we noticed a positive correlation between IT and PT scores ( $r_s = 0.48$ ,  $P < 0.0001$ ; Spearman's test, Figure 5C).

**Table 5.** HCC and HM clinical data in relation to decreased number of macrophages in intratumoral and peritumoral territories

Clinicopathological factor	Intratumoral territory		Peritumoral territory	
	T1 ↓ vs. ↑	T2 ↓ vs. ↑	T1 ↓ vs. ↑	T2 ↓ vs. ↑
<b>HCC</b>				
Age	13 vs. 3	2 vs. 14	3 vs. 13	1 vs. 15
< 63	<i>P</i> = 0.6	<i>P</i> = 0.66	<i>P</i> = 0.28	<i>P</i> = 0.18
≥ 63	17 vs. 2	4 vs. 15	7 vs. 12	5 vs. 14
Tumor stage				
I–II	16 vs. 2	5 vs. 13	5 vs. 13	2 vs. 16
	<i>P</i> = 0.65	<i>P</i> = 0.17	<i>P</i> = 1	<i>P</i> = 0.4
III–IV	14 vs. 3	1 vs. 16	5 vs. 12	4 vs. 13
Histological grade				
G1–G2	25 vs. 3	5 vs. 23	9 vs. 19	5 vs. 23
	<i>P</i> = 0.25	<i>P</i> = 1	<i>P</i> = 0.64	<i>P</i> = 1
G3	5 vs. 2	1 vs. 6	1 vs. 6	1 vs. 6
<b>HM</b>				
Age				
≤ 68	10 vs. 8	3 vs. 15	2 vs. 16	1 vs. 17
	<i>P</i> = 0.2	<i>P</i> = 0.65	<i>P</i> = 0.66	<i>P</i> = 1
> 68	21 vs. 7	3 vs. 25	4 vs. 17	2 vs. 19
Histological grade				
G1–G2	14 vs. 9	3 vs. 20	2 vs. 21	1 vs. 22
	<i>P</i> = 0.2	<i>P</i> = 0.67	<i>P</i> = 0.2	<i>P</i> = 0.55
G3–G4	6 vs. 10	3 vs. 13	4 vs. 12	2 vs. 14
Tumor extension				
One lobe	18 vs. 8	5 vs. 21	4 vs. 22	2 vs. 24
	<i>P</i> = 0.18	<i>P</i> = 0.64	<i>P</i> = 1	<i>P</i> = 1
Many lobes	6 vs. 7	1 vs. 12	2 vs. 11	1 vs. 12

Furthermore, there was no difference between number of macrophages in HM or HCC when IT or PT territories were compared (Figure 5A).

In the explorative analysis of patients with observed lower number of TAMs, either based on T1 or T2 scoring method, we did not observe any association between occurrence of low/high numbers of TAMs and age, TNM, histological G grade in HCC or age, histological G grade and number of involved lobes in HM (Table 5).

### Analysis of prognostic factors

Univariate and multivariate Cox regression analysis was used in order to find the factors which can influence patients' survival or death. The summary analysis of all variables was presented in Table 6. In HCC group we observed that increasing tumor stage is associated with poorer prognosis (*P* = 0.013, HR 2.16), which is related to × 13.6 increase of chance of death for patients with stages III–IV in comparison to HCC cases with diagnosed HCC at stages I–II. In liver metastasis we observed that increasing histological grades of cancer cells (G1–G2 vs. G3–G4) as

well as increasing number of metastatic lobes (> 1) are the factors of poorer prognosis (*P* = 0.004 for both factors). We further included both factors in Cox multivariate analysis and retained their significance (*P* = 0.021 and *P* = 0.045 respectively). For the prognostic significance of TAMs scores, we did not find relations in any of analyzed groups, no matter which threshold was applied.

### Survival analysis

The possible influence of all clinical factors as well as TAMs scores based either on T1 or T2 classifications were assessed separately for IT and PT areas. We found that HCC cases with PT TAMs scores lower than 1/3 mean value (T2) were characterized by a shorter survival rate that cases with higher PT TAMs values (median survival 1 vs. 8 months, 3 years survival 0% vs. 38%; Figure 6A). Other survival relations to TAMs were not significant. However, certain clinical factors had significant influence on HCC and HM survival rates: Stages I–II vs. stages IIIA–IV (median survival 28 vs. 3.5 months, 3 years survival 42% vs. 21%, Figure 6B).



**Table 6.** Univariate and multivariate Cox regression analysis of hepatocellular carcinoma and hepatic metastases cases

Parameters	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	P value	HR (95% CI)
<b>HCC</b>				
Age	0.836	1.004 (0.961–1.049)		
Histological grade G1–G2 vs. G3	0.316	1.382 (0.733–2.603)		
Tumor stage ≤ II vs. ≥ III	<b>0.013</b>	<b>2.614 (1.209–5.410)</b>		
Intratumoral TAMs T1 ↑ vs. ↓ T2 ↑ vs. ↓	0.666 0.968	1.269 (0.429–3.751) 1.022 (0.347–3.009)		
Peritumoral TAMs T1 ↑ vs. ↓ T2 ↑ vs. ↓	0.432 0.062	1.434 (0.582–3.532) 2.457 (0.954–6.326)		
<b>HM</b>				
Age Histological grade G1–G2 vs. G3–G4	0.187 <b>0.004</b>	1.025 (0.987–1.064) <b>1.701 (1.024–2.827)</b>	<b>0.021</b>	<b>0.395 (0.179–0.870)</b>
Tumoral extension one lobe vs. multilobe	<b>0.004</b>	<b>0.498 (0.225–1.101)</b>	<b>0.045</b>	<b>0.438 (0.195–0.984)</b>
Intratumoral TAMs T1 ↑ vs. ↓ T2 ↑ vs. ↓	0.966 0.973	0.983 (0.444–2.175) 1.018		
Peritumoral TAMs T1 ↑ vs. ↓ T2 ↑ vs. ↓	0.161 0.914	2.028 1.082		

Cox analysis of ↑ vs. ↓ or ↑ vs. ↓ means that the HR and P values relate to occurrence of decrease number of TAMs

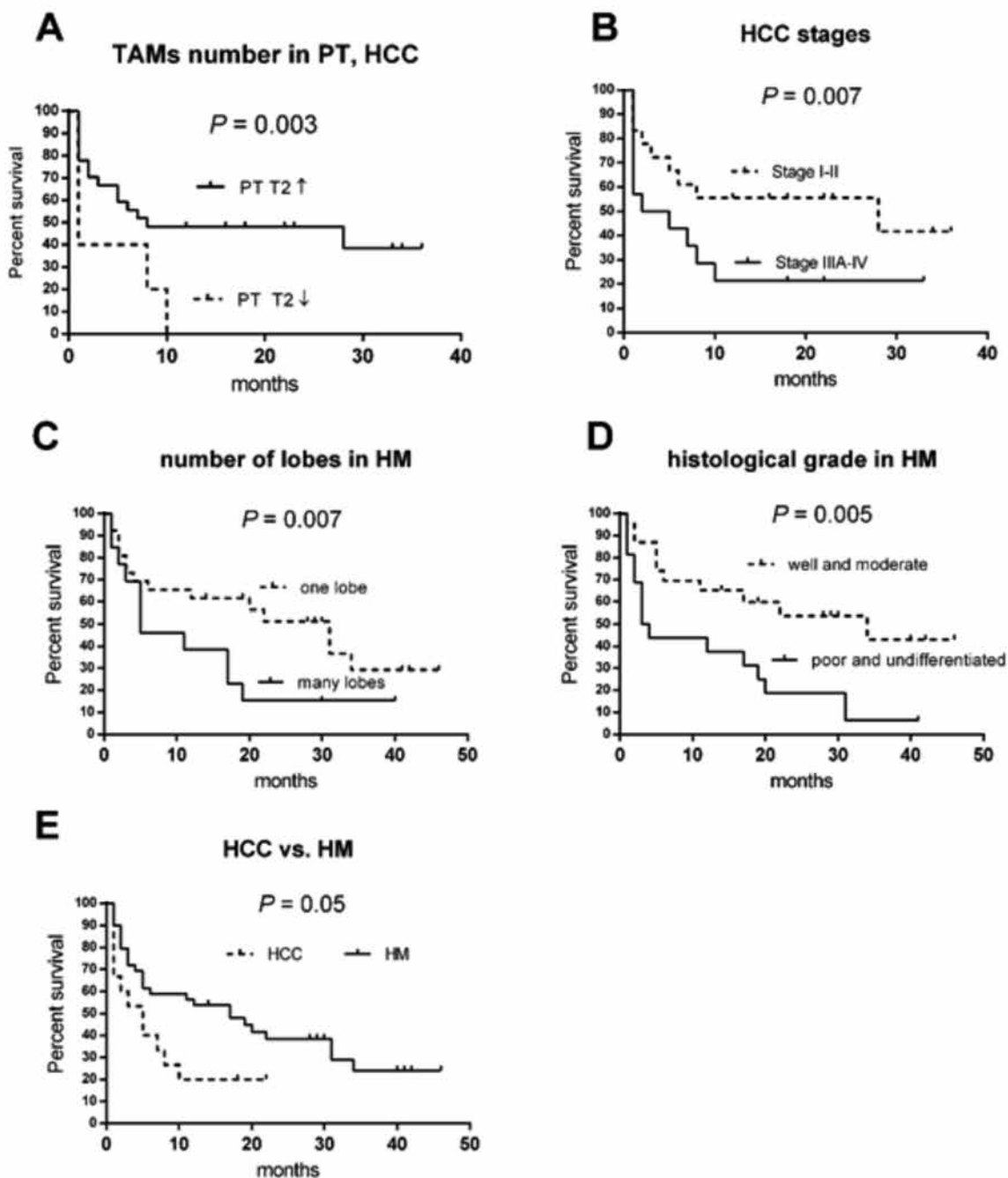
For HM we observed the poorer survival if many lobes were involved: one vs. multilobe involvement (median survival 31 vs. 5 months, 3 years survival 29% vs. 15%, Figure 6C) or if more aggressive tumor cells were found in tumor biopsies: G1–G2 vs. G3–G4 (median survival 34 vs. 3.5 months, 3 years survival 43% vs. 6%, Figure 6D). We also noted that patients with HCC had worse survival outcome than HM cases ( $P = 0.0501$ , Figure 6E).

## Discussion

Recent data presented the modulatory role of hepatic microenvironment both in initiation and progression of the primary carcinogenic process as well as in establishment of optimal conditions for metastatic colonization and development [5, 12, 21, 26]. A necessary condition for HCC initiation and development seems to be the presence of activated inflammatory cells in the liver, through their action on signaling pathways, which results in enhanced cellular turnover and acquisition of critical modifications for the malignant transformation [27, 28].

Studies on inflammatory cells in cancer development focused mainly on the population of tumor-infiltrating lymphocytes (TILs), widely reported in CRC [29–35], however, within liver microenvironment the largest physiological population of immune cells is represented by macrophages, namely Kupfer cells. Our study focused on the parallel analysis of hepatic TAMs in two different tumor pathologies, HCC and HM by evaluating the presence of macrophages in intra- and peritumoral territories, followed by TAMs quantification in respective territories.

Regarding the method of counting TAMs and due to the fact that no accurate correlation between number of macrophages and patients' survival could be found in literature, we established two threshold values. T1 was calculated with the use of widely used Cox method, however, the setting of a second threshold, T2, permitted a convenient mathematical manipulation and had a heuristic motivation (based on the large panel of numerical results). Choice of T2 intended to balance the following two issues: 1) the use of the mean value of the number of macrophages does not ensure a reliable classification of tissues with decreased number



**Figure 6.** Results of Kaplan-Meier survival analyzes of chosen ( $P < 0.05$ ) factors. **A.** HCC cases classified by higher (↑) and lower (↓) number of peritumoral macrophages. **B.** HCC cases divided by pTNM stages according to Benson et al. [43]. **C.** and **D.** Relates to survival analyzes of HM cases divided by tumoral extension and histological grade of differentiation. **E.** Plot relates to survival outcome in relation to tumor disease type.  $P$  values according to Mantel-Cox log-rank test

of TAMs, 2) a proper evaluation of the association of macrophages' number on survival requires a reasonable value above the zero. We however found that our method for T2 assignment could not be applied for TAMs in IT territories (both in HCC and HM), which was probably associated with lack of normal distribution of the macrophages' number in the intratumoral sections.

Our finding of decreased presence of intratumoral TAMs in comparison to peritumoral territories both in HCC and HM are in accordance with the relatively few studies of TAMs in HCC [13, 15, 16] and HM [18, 20, 36], confirming their predominant presence in peritumoral area and in the vicinity of invasion front. Moreover, our results support the

hypothesis of decreased macrophage infiltration or the inhibition of their function which resulted in tumor growth and metastasis [28]. Surprisingly, despite the diversity of the origin of primary and secondary liver tumors, lack of differences in TAMs numbers between primary vs. secondary tumors in respective territories could have been caused by specific characteristics of hepatic microenvironment in recruiting and inducing the phenotype of tumor-associated macrophages either in HCC or HM [28]. Consequently, the macrophage distribution pattern suggests that the anti-tumoral defense mechanism follows a similar algorithm in the liver, without any correlation to the tumor origin or to the primary or secondary tumor status [1, 11].

Our observation of positive correlation of TAMs numbers in IT and PT tumor sections of HM patients could be probably associated with the impact of malignant cells on hepatic macrophages. It was reported in HM that highly aggressive Dukes C and D malignant cells of CRC-origin can trigger apoptotic death of TAMs and monocytes by the secretion of high mobility group box 1 (HMGB1) protein [38]. On the other hand, malignant cells can attract TAMs by secretion of carcinoembryonic antigen (CEA) followed by the release of growth factors by enticed macrophages [21].

The presented study focused on the selection of prognostic factors for HCC and HM patients. Our results confirmed the usefulness of AJCC/UICC pTNM division for the prediction of the survival rate and hazard ratio of HCC patients [39, 40]. The influence of poorly/undifferentiated cells and involvement of many liver lobes in secondary cancer on the poorer outcome of HM patients has been previously reported [41].

Although TAMs presence in tumor microenvironment was observed in many types of cancer [37], the value of estimating number of macrophages in tumor infiltrates as a prognostic factor in HCC is still uncertain since some studies found TAMs number as a negative [13, 14], while other — as a positive prognosis factor [15, 16]. Our results suggest that the low number of PT macrophages ( $P = 0.07$ ) could be assumed as a negative prognostic factor for HCC, which was further demonstrated by Kaplan-Meier analysis, however, we found no association with other clinical factors. Although this preliminary result should be confirmed by larger independent analyses, we wanted to emphasize such interesting observation.

One of the limitations of our study is related to the division of mature macrophages into two subpopulations: M1 and M2. M1 macrophages activated mainly by bacterial lipopolysaccharides and immune stimuli such as interferon- $\gamma$  (IFN- $\gamma$ ) have anti-tumor role due to the elimination of tumor cells, antigen presentation

to T cells, and synthesis of numerous proinflammatory cytokines [6–8]. M2 macrophages differentiate following the contact with Th2 cells or after stimulation in the tumor microenvironment by cytokines (e.g. IL-4, IL-10, IL-13) and growth factors such as TGF- $\beta$ 1 [42]. Since we performed only basic identification for macrophages, future studies should assess the participation of M1 and M2 subpopulation of macrophages in HCC and HM. In this report we were not able to simultaneously detect CD68+, CD80+ and CD163+ cells in histological sections but the use of flow-cytometry to study isolated TAMs may provide valuable data.

In summary, our results lead to the following two conclusions. (i) Low number of intratumoral TAMs in comparison to peritumoral territories both in HCC and HM may be related to negative influence of cancer cells. (ii) Increased TAMs number in peritumoral territory of hepatocellular carcinoma is associated with better prognosis.

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