

## Levels and Values of Circulating Hematopoietic and Endothelial Progenitor Cells in Patients with Hepatocellular Carcinoma

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Received date: August 09, 2014; Accepted date: October 23, 2014; Published date: October 28, 2014

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### Abstract

**Objective:** Development of HCC is associated with the process of angiogenesis and leads to the increment of the number of stem cells in the peripheral blood circulation. This study evaluated whether the level of hematopoietic stem cells and endothelial progenitor cells (HSCs & EPCs) could indicate the unfavorable tumour biology and the progress of disease in HCC.

**Methods:** The study covered 146 HCC patients; 53 selected for liver resection, 49 for liver transplantation, 44 for palliation. Control consisted of 42 patients with liver cirrhosis and 43 healthy individuals. The cells were enumerated with CD45, CD34, CD133, CD309 markers. The cell rates were measured by phenotypic analysis of 2 ml fresh blood in a flow cytometer. The data were evaluated statistically.

**Results:** There were significant differences in the levels of HSCs and EPCs between patients with HCC, with liver cirrhosis and healthy volunteers (Chisq = 45.92,  $p < 0.001$ , Chisq = 16.22,  $p < 0.001$ ), as well as between the groups of patients with HCC selected for liver resection, liver transplantation and palliation (Chisq=40.86,  $p < 0.001$ , Chisq=18.81,  $p < 0.001$ ), respectively. The multivariate analysis of regression indicated the rates of hematopoietic stem cells and the endothelial progenitor cells as the factor predicting poor tumour differentiation ( $W=3.95$ ,  $p < 0.04$  and  $W=7.11$ ,  $p < 0.008$ ).

**Conclusions:** Liver cirrhosis and the development of hepatocellular carcinoma cause significant changes in the levels of circulating hematopoietic and endothelial progenitor cells. The cell levels correlate with the advances of liver pathology and allow anticipating the unfavorable biology of the tumour.

**Keywords:** Hepatocellular carcinoma; Angiogenesis; Hematopoietic stem cells; Endothelial Progenitor cells; HCC histologic grading

evaluation of patients with liver cirrhosis and patients with hepatocellular carcinoma, so far [9,10,17,18].

### Introduction

Extensive studies conducted by a number of centers confirm the importance of angiogenesis and vasculogenesis in tumor development and the role of newly created pathological arterial perfusion in the progress of the malignancy [1-4]. Different subpopulations of stem and progenitor cells were indicated as the source of cancer angiogenesis, the cell fractions coming from hematopoietic, endothelial or even tumor cells [5-7]. Studies of some tumor models seem to show that endothelial progenitor cells are mobilized in peripheral blood and contribute to tumor vascularization [8, 9]. Some studies also indicate that the level of hematopoietic stem cells and endothelial progenitor cells in peripheral blood circulation increases with the stage of tumor development [10-12]. It is suggested that they may serve as a biomarker in the evaluation of tumor progress and prediction of treatment outcome [13-16]. However, only a few studies point to the importance of endothelial progenitor cells in the clinical

There are at least several patient- and tumor-related factors responsible for the ultimate outcome of the patient after surgical treatment. The C hepatitis virus is one of the most important [11,13-15]. The tumor-related factors include tumor number, size, location, and histologic grade of differentiation, presence of macro and microvascular invasion and extrahepatic spread of the disease. The grade of tumor differentiation and the tumor histological type are the universally accepted criteria used to classify HCC, and the microvascular invasion is known to be the strongest predictor of tumor relapse in patients undergoing liver resection and liver transplantation. There is also clear evidence that the less differentiated tumor is, the higher the risk of vascular invasion to the surrounding liver tissue [7,9,14-18]. Unfortunately, there is no standard method of identifying the unfavorable tumor biology likely to entail a high risk for tumor recurrence [5-8]. Histologic information on tumor characteristics is available no sooner than at the time of the pathologic examination of the explanted liver.

The aim of the study was to evaluate levels of Hematopoietic Stem Cells (HSCs) and Endothelial Progenitor Cells (EPCs) in surgical patients with HCC as compared to patients with liver cirrhosis and to healthy individuals. The assumption was that the level of the selected cell fraction in the peripheral blood circulation correlate with the transformation to a neoplasm in a patient with liver cirrhosis and indicates both the unfavorable tumor biology and the progress in tumor development.

## Materials and Methods

The study was carried out on 231 patients of the Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Poland. The study protocol was approved by the Bioethics Committee of Medical University of Warsaw, and an informed consent was obtained from all individuals undergoing the procedure. The assessment of liver function and cancer staging were based on the clinical view and the laboratory tests (blood morphology, levels of albumin, bilirubin, activity of ASAT, ALAT, levels of alkaline phosphatase, coagulation factors as INR, platelet count and serum level of alpha-fetoprotein) in all, and on screening tests (ultrasound and Doppler ultrasound, computed tomography and magnetic resonance imaging) in patients with HCC.

There were 146 patients with HCC (Group 1). They were assigned to an appropriate treatment procedure according to the standard clinic classification and the Milan criteria.

Group 1A consisted of 53 patients (33 males, 20 females, median age 56 years) with a single HCC tumor at the I-II clinical stage (T1/T2,N0,M0), A1-A4 BCLC classification, with proper liver function (healthy liver or cirrhotic liver in the Child-Pugh class A). The patients were selected for liver resection (Group 1A). In 19 patients HCC developed in a healthy liver, in 11 in a cirrhotic liver caused by alcoholic liver disease (ALD) and in 23 in cirrhotic liver caused by viral hepatitis (HBV/HCV). No evidence of gross vascular invasion and no regional nodes or distant metastases were present. The on-explant histopathology analysis indicated well (GI), moderately (GII) and poorly differentiated (GIII) tumor in 6 (11.3%), 32 (60.4%), 15 (28.3%), respectively. The trabecular, acinar and solid architectural growth pattern of the tumor was found in 12 (22.6%), 13 (24.5%) and 28 (52.9%), respectively. Microvascular invasion of the tumor was present in 20 (37.8%) cases. The mean serum alpha-fetoprotein (AFP) for all 53 patients was 3544 ng/mL, SD+/-3607, median 12.3 (896-6192 95% CI), and mean platelets count was 163.35×103/mcL, SD+/-77.0 median 152.1×103/mcL (142.1-184.5×103 95% CI).

Group 1B consisted of 49 patients (F17, M32, median age 58 years) presented with liver cirrhosis and hepatocellular carcinoma within the Milan criteria. The patients were selected for liver transplantation. In 38 (77.5%) patients liver cirrhosis developed due to HCV infection, but 9 of them had the component of alcoholic liver disease (ALD), as well. Eleven patients (22.5%) had liver cirrhosis due to HBV infection. HCC was diagnosed pre-operatively by characteristic appearance on radiologic imaging studies that confirmed a single lesion  $\leq$  5 cm in 27 (55.1%), and up to three separate lesions, not larger than 3 cm in 22 (44.9%). No evidence of gross vascular invasion and no regional nodes or distant metastases were present. None of the patients had been pretreated for HCC but all presented with a history of treatment for liver dysfunction and portal hypertension; 35 (71.4%) patients were clinically evaluated as belonging to Group A, and 14 (28.6%) to Group B, according to the Child-Pugh classification. The on-explant

histopathology analysis indicated well (GI), moderately (GII) and poorly differentiated (GIII) tumor in 7 (14.3%), 37 (75.5%), 5 (10.2%), respectively. The trabecular, acinar and solid architectural growth pattern of the tumor was found in 24 (48.9), 14 (28.6%) and 11 (22.5%), respectively. Microvascular invasion of the tumor was present in 13 (26.5%) cases. The mean serum alpha-fetoprotein (AFP) for all 49 patients was 1233.32 ng/mL, SD+/-3292,1 median 43.7 (287.7-2178.9 95% CI), and mean platelets count was 83×103/mcL, SD +/-42.8 median 71.1×103/mcL (71.1-95.7×103 95% CI).

Group 1C consisted of 44 patients (24 males, 20 females, median age 63 years) presented with advanced HCC. (Group 1C). The tumor developed in a healthy liver in 8 patients, in a cirrhotic liver caused by viral hepatitis (HBV/HCV) in 28, and in a cirrhotic liver caused by alcoholic liver disease (ALD) in 8. In 35% of them, evidence of gross vascular invasion was present, in 15% regional lymph nodes were invaded, in 12% distant metastases were present, as well. None of them had been pretreated for HCC but 62% presented with a history of treatment for liver dysfunction and portal hypertension; 5 patients were clinically evaluated as belonging to Class A, 28 to Class B, and 11 to Class C, according to the Child-Pugh classification. All patients underwent USG guided biopsy of the tumor prior to the treatment. The histopathology analysis indicated well (GI), moderately (GII) and poorly differentiated (GIII) tumor in 2 (4.55%), 33 (75.0%) and 9 (20.5%), respectively. The trabecular, acinar and solid architectural growth pattern of the tumor was found in 19 (43.2%), 12 (27.3%) and 13 (29.5%), respectively. Microvascular invasion of the tumor was present in 25 (43.1%) cases. The mean serum alpha-fetoprotein (AFP) for all 44 patients was 8196.9 ng/mL, SD+/-17048, median 60.5 (2920-13473 95% CI), and mean platelets count was 139.4×103/mcL, SD+/-42.8 median 123.0×103/mcL (114-164×103 95% CI).

There were two control groups. Group 2 consisted of 42 patients with liver cirrhosis and portal hypertension, regardless of the etiology, but without hepatocellular cancer, hospitalized in order to be qualified for liver transplantation (negative control group). Group 3 consisted of 43 healthy volunteers and patients admitted for the surgical treatment of inguinal hernia and family donors of a liver section to their descendants (positive control group).

Samples of 2 ml of fresh peripheral venous blood were collected from each patient at the time of primary clinical setting. The blood sample was collected into a tube containing K3EDTA and processed within 1 hour. Identification of the desired cell fraction was achieved by the standard enumeration of human endothelial progenitor cells by surface markers expression [19-21]. Seven test tubes containing 100µL of fresh blood each + 40 µL EPC Staining Cocktail composed of surface antibodies (10 µL CD34 FITC BD Biosciences, 10 µL CD133 PE Miltenyi Biotec, 10 µL CD45 Per CP BD Biosciences and 10 µL CD309 APC Miltenyi Biotec) constituted the measured sample; 3 test tubes containing 100 µL of fresh blood each + 40 µL EPC Cocktail (10 µL CD34 FITC BD Biosciences, 10 µL CD133 PE Miltenyi Biotec, 10 µL CD45 Per CP BD Biosciences but 10 µL Isotype Control Miltenyi Biotec) constituted the control. After 20 minutes of incubation, red cells were lysed with FACS Lysing Solution (BD Biosciences), incubated again for 10 minutes, rinsed in CellWash (BD Sciences) and pooled. Then the samples were consecutively analyzed with the flow cytometer FACS CANTO II (BD Biosciences). The data were analyzed by using the multiparametric cells gating strategy approved by the International Society of Hematology and Graft Engineering (ISHAGE). The circulating hematopoietic stem cells were defined by the phenotype of CD34+,CD133+,CD45dim and quantified as

percentage within the white blood cells population (%HSCs/WBC). The endothelial progenitor stem cells were defined by the phenotype of CD34+,CD133+,CD45dim,CD309+, and quantified as percentage within the hematopoietic stem cells subpopulation (% EPCs/HSCs) [19-21].

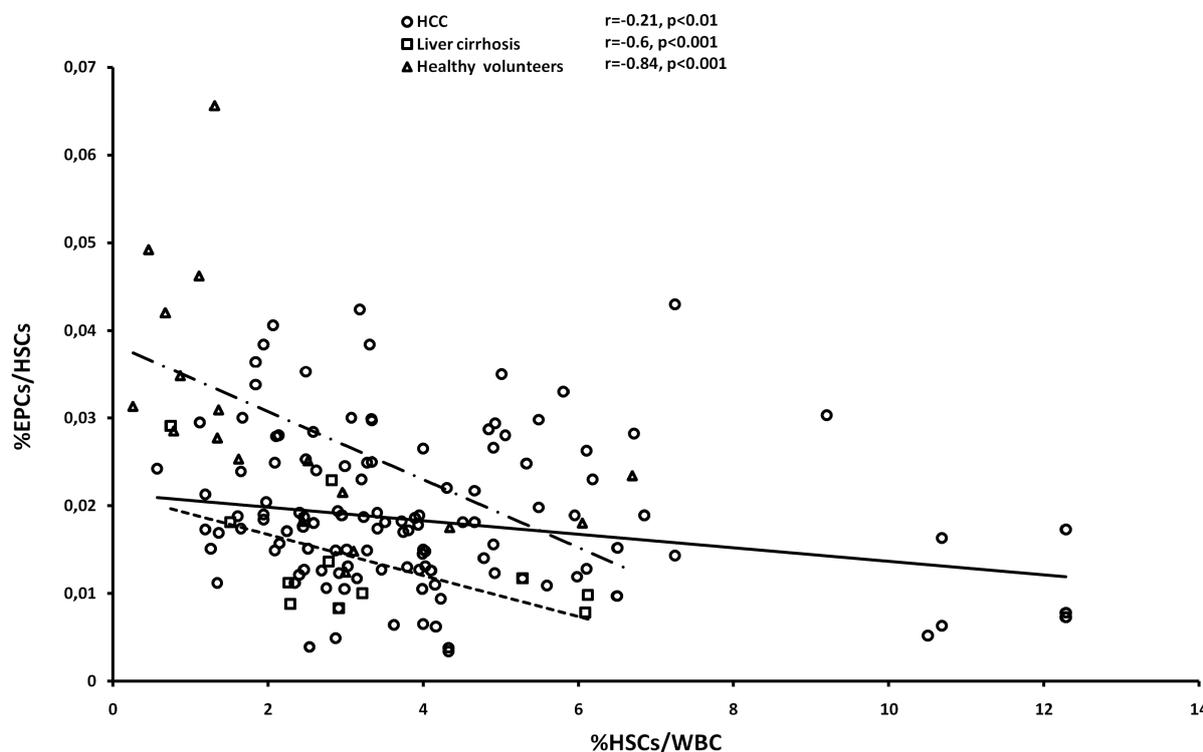
### Statistical Analysis

The descriptive analysis was computed for all variables. The data of pre-operative clinical settings and the on-explant pathologic study, including the type of cirrhosis, number of lesions, tumor grading and architectural growth pattern, and tumor microvascular invasion, were collected. The quantitative data of pre-treatment rates of HSCs and EPCs enumeration were expressed as mean +/- SD and median 95% CI. The rates of circulating hematopoietic stem cells (%HSCs/WBC) and endothelial progenitor cells (%EPCs/HSCs) were compared between the groups categorized by the type of liver pathology and by the advances of the disease in patients with HCC by the use of Kruskal-Wallis one-way analysis of variance. Correlation between continuous variables was also estimated with the Spearman correlation coefficient (r). The multivariate analysis (Program Statistica-10.0) was performed in the group of patients with HCC (Group1) to assess factors associated with the unfavourable tumour biology. The variables tumor stage, histologic-pathologic data relating the liver disease, number of tumors, grade of tumor differentiation and architecture, microvascular invasion, the biochemical data indicating status of the

liver, platelets count and level of AFP, and the rates of circulating hematopoietic stem and endothelial progenitor cells were included. An association between the increased levels of circulating hematopoietic stem cells and endothelial progenitor cells on the one hand to patient's characteristic, clinical-biochemical features of the disease and the parameters of tumor biology (grade of differentiation, tumor architecture, micro vascular invasion) on the other hand were assessed by the logistic analysis of regression - Wald test. In search for the optimal rate of the HSCs and EPCs cutoff value the recursive partitioning method was used and the analysis was perform for the group of HCC patients with the rates lower and with the rates higher than the cutoff point value, respectively. Results with a p-value of less than 0.05 were considered as statistically significant.

### Results

The Spearman test of correlation indicated a statistically significant relationship between the rates of hematopoietic stem cells (%HSC/WBC) and the rates of endothelial progenitor cells (%EPCs/HSCs) in all subjects. The correlation appeared to be negative - the highest level of hematopoietic stem cells was, the lowest level of endothelial progenitor cells. In healthy volunteers the correlation between the cell fraction was strong (Group 3:  $r = -0.84$ ,  $p < 0.001$ ), and became to be weak in patients developing liver pathology, as liver cirrhosis (Group 2:  $r = -0.6$ ,  $p < 0.001$ ) and especially HCC (Group 1:  $r = -0.21$ ,  $p < 0.01$ ) (Figure 1).



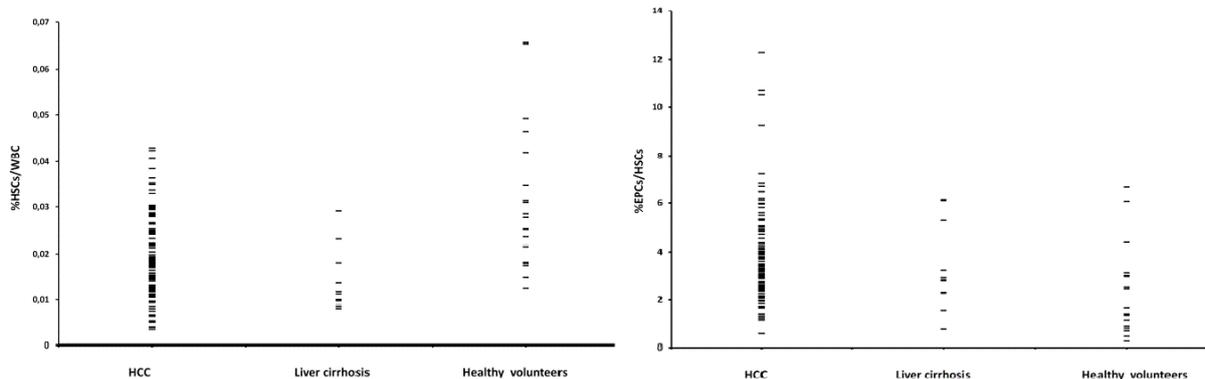
**Figure 1:** Spearman correlation between the rates of endothelial progenitor cells (%EPCs/HSCs) and hematopoietic stem cells (%HSCs/WBC) in HCC patients, patients with liver cirrhosis and a healthy volunteer.

The study indicates significant changes in the rates of circulating hematopoietic stem cells and the endothelial progenitor cells in the patients who developed hepatocellular carcinoma in comparison to the

patients with liver cirrhosis and the volunteers with a healthy liver. The mean rates of the hematopoietic stem cells within the white blood cells population (%HSCs/WBC) were established to be 0.0182, SD

+/-0.008, median 0.0173 (0.0168-0.0197 95% CI) for patients with HCC (Group 1), 0.0139, SD +/-0.0067, median 0,0112 (0.0118-0.0159 95% CI) for patients with non-cancer liver cirrhosis (Group 2), and 0.0293, SD+/-0.0131, median 0.0277 (0.0253-0.0334 95% CI) for volunteers with a healthy liver (Group 3). Consequently, the mean rates of the endothelial progenitor cells fraction within the hematopoietic stem cells (%EPCs/HSCs) were established to be 4.06, SD +/-2.48, median 3.37 (3.65-4.47 95% CI) for HCC (Group 1), 3.21,

SD+/-1.7, median 2.82 (2.68-3.75 95% CI) for liver cirrhosis (Group 2), and 2,36, SD +/-1.93, median 1.35 (1.76-2.95 95% CI) for the volunteers with a healthy liver (Group 3). The differences in the rates of hematopoietic stem cells (%HSCs/WBC) and endothelial progenitor cells (%EPCs/HSCs) between patients with HCC (Group 1), patients with liver cirrhosis (Group 2) and the volunteers with a healthy liver (Group 3) were significant (Chisq=44.89, p<0.001 and Chisq=24.89, p<0.001, respectively) (Figure 2).

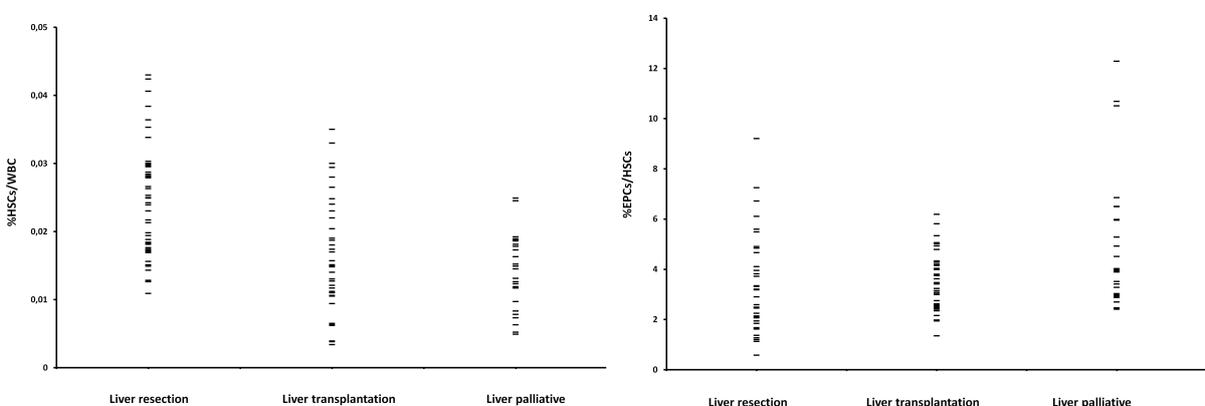


**Figure 2:** The rates of hematopoietic stem cells (%HSCs/WBC) and endothelial progenitor cells (%EPCs/HSCs) in the peripheral blood of HCC patients, patients with liver cirrhosis and a healthy volunteers.

Significant differences were also found in the rates of both cell fractions within the HCC patients (Group 1) that were selected to the appropriate treatment options according to the clinical classification systems. The changes seemed to be depended upon the stage of disease.

The highest rate of hematopoietic stem cells within the white blood cells (%HSCs/WBC) was detected in patients selected for liver resection (Group 1A – mean 0.0245, SD +/-0.008, median 0.0249, 0.0222-0.0269 95% CI), lower rates in patients selected for liver transplantation (Group 1B – mean 0.0151, SD +/-0.0079, median 0.0148, 0.0128-0.0174 95% CI) and in patients selected for palliative treatment (Group 1C – mean 0.0142, SD +/-0.005, median 0.0147,

0.0126-0.0157 95% CI). The differences were statistically (Chisq=40.86, p<0.001). Conversely, the lowest rate of endothelial progenitor cells within hematopoietic stem cells (%EPCs/HSCs) was detected in patients suitable for liver resection (Group 1A – mean 3.3, SD +/-1.9, median 2.58 2.77-3.83 95% CI). Then, the rates of EPCs increased gradually to the level detected in patients suitable for liver transplantation (Group 1B – mean 3.46, SD +/-1.08, median 3.41 3.15-3.77 95% CI) and to the level detected in patients suitable just for palliative treatment (Group 1C – mean 5.64, SD +/-3.38, median 3.96 3.77-6.67 95% CI). The differences were statistically significant (Chisq=18.81, p<0.001) (Figure 3).



**Figure 3:** The rates of hematopoietic stem cells (%HSCs/WBC) and endothelial progenitor cells (%EPCs/HSCs) in the peripheral blood of HCC patients selected for the different treatment options.

The univariate analysis by the Chisq test indicated significant differences in the rates of hematopoietic stem cells (%HSCs/WBC) and endothelial progenitor cells (EPCs/HSCs) in HCC patients who presented with the microvascular invasion of the tumour (Chisq=3.86, p<0.04 and 0.27, p<0.3), with mediate and poor tumour differentiation

(Chisq=5.43, p<0.06 and 14.5, p<0.001) and with acinar growth pattern tumors (Cisq=8.01, p<0.01 and Chisq=1087, p<0.004) in compare to these who did not present with such factors on explanted livers. Details are presented in (Table 1).

A	No. patients	HCC Grading	Cell phenotype	Mean	SD	Median	-95%CI	95%CI
	15	G1	%HSCs/WBC	0.020	0.007	0.019	0.017	0.024
			%EPCs/HSCs	4.766	1.308	4.902	4.041	5.490
	102	G2	%HSCs/WBC	0.017	0.009	0.016	0.016	0.019
			%EPCs/HSCs	4.179	2.743	3.339	3.640	4.718
	29	G3	%HSCs/WBC	0.020	0.008	0.019	0.017	0.023
			%EPCs/HSCs	3.301	1.748	2.948	2.636	3.966
B	No. patients	HCC Arch. Pattern	Cell phenotype	Mean	SD	Median	-95%CI	95%CI
	55	Trabecular	%HSCs/WBC	0.015	0.008	0.015	0.013	0.017
			%EPCs/HSCs	4.837	3.135	4.025	3.990	5.685
	39	Acinar	%HSCs/WBC	0.021	0.010	0.018	0.017	0.024
			%EPCs/HSCs	3.958	1.776	3.407	3.382	4.534
	52	Solid	%HSCs/WBC	0.020	0.008	0.019	0.018	0.022
			%EPCs/HSCs	3.328	1.893	2.967	2.801	3.855
C	No. patients	Microvascular invasion	Cell phenotype	Mean	SD	Median	-95%CI	95%CI
	88	Negative	%HSCs/WBC	0.017	0.009	0.016	0.015	0.019
			%EPCs/HSCs	3.992	2.276	3.564	3.510	4.474
	58	Positive	%HSCs/WBC	0.020	0.009	0.019	0.017	0.022
			%EPCs/HSCs	4.176	2.788	3.274	3.443	4.909

**Table 1:** The rates of hematopoietic stem cells and endothelial progenitor cells in patients with HCC according to histological grading, tumor growth pattern and microvascular invasion found on explants analysis.

The Chisq test indicated also the significant association between the increased rates of both cell fractions and the type of underlying primary liver disease – the lowest rates were detected in 40 patients developing HCC in a healthy liver, intermediate in these with HBV/HCV liver cirrhosis and the highest in patients with the ALD (Chisq=21.23, p<0.001 for %HSCs/WBC and Chisq=9.73, p<0.007 for

%EPCs/HSCs). On the other hand, there were just slight (not significant) differences in the cell rates between patients with normal and elevated level of serum alfa-fetoprotein (AFP) and alkaline phosphatase (ALP) (Chisq=1.99, p<0.15 and Chisq=0.7, p<0.3 for HSCs/WBC, and Chisq=0.94, p<0.2 Chisq=3.3, p<0.06 for EPCs/HSCs, respectively). Details are presented in (Table 2).

A	No. patients	Liver status	Cell phenotype	Mean	SD	Median	-95%CI	95%CI
	40	normal	%HSCs/WBC	0.024	0,008	0,024	0,021	0,027
			%EPCs/HSCs	3.335	2,462	2,467	2,547	4,122
	84	HBV/HCV cirrhosis	%HSCs/WBC	0.016	0,007	0,015	0,014	0,017
			%EPCs/HSCs	3.809	1,598	3,668	3,462	4,156
	22	ALD	%HSCs/WBC	0.018	0,011	0,016	0,013	0,023
			%EPCs/HSCs	6,368	3,798	4,366	4,684	8,052

B	No. patients	Serum AFP level	Cell phenotype	Mean	SD	Median	-95%CI	95%CI
	24	<10	%HSCs/WBC	0,020	0,008	0,019	0,017	0,023
			%EPCs/HSCs	3,252	2,806	2,083	2,067	4,437
	122	>10	%HSCs/WBC	0,018	0,009	0,017	0,016	0,019
			%EPCs/HSCs	4,225	2,396	3,680	3,795	4,654
C	No. patients	Serum ALP level	Cell phenotype	Mean	SD	Median	-95%CI	95%CI
	63	<120	%HSCs/WBC	0,019	0,009	0,018	0,017	0,021
			%EPCs/HSCs	3,550	1,822	3,230	3,092	4,009
	83	>120	%HSCs/WBC	0,018	0,009	0,016	0,016	0,019
			%EPCs/HSCs	4,455	2,837	3,934	3,836	5,075

**Table 2:** The rates of hematopoietic stem cells and endothelial progenitor cells in patients with HCC according to status of the liver and the levels of serum Alfa-Fetoprotein (AFP) and Alkaline Phosphatase (ALP).

No significant associations were also noted in the rates of both cell fractions between patients differed by gender and age, normal or elevated levels of albumin, bilirubin, aminotransferases, platelets count, number and size of the tumour nodules.

According to the recursive partitioning, the optimal cutoff point for the rate of HSCs within WBC (%HSCs/WBC) and EPCs within HSCs (%EPCs/HSCs) in terms of anticipation the unfavorable tumour biology was estimated on 0.018% for the rate of HSCs/WBC and 4.01% for the rate of EPCs/HSCs. The values corresponded to the mean rates of both cell fractions detected in the circulation of 146 patients with

HCC (Group1). The multivariate analysis indicated the rates of hematopoietic stem cells and the endothelial progenitor cells as the independent preoperative factors predicting the disadvantageous tumour propriety. Increasing rate of HSCs/WBC appeared to predict poor tumour differentiation (W=3.95, p<0.04, OR=4.01, (0.82-19.83) 95% CI) and the acinar growth pattern of the tumour (W=5.4, p<0.02, OR=3.5, (1.22-10.07) 95% CI)). On the other hand the increasing rate of endothelial progenitor cells appeared to predict just poor differentiation of the tumour (W=7.11, p<0.008, OR=12.09, (1.93-75.42) 95% CI)) (Table 3).

Variables	Tumor propriety	Odds ratio	Wald	p-value	95% CI for EXP(B)
%HSCs/WBC	Poor differentiation	4.04	3,95	0,046	0,82-19,83
	Acinar growth pattern	3,50	5,41	0,020	1,22-10,07
Variables	Tumor propriety	Odds ratio	Wald	p-value	95% CI for EXP(B)
%EPCs/HSCs	Poor differentiation	12,09	7,12	0,008	1,93-75,42
	Acinar growth pattern	0,45	3,02	0,082	0,18-1,1

**Table 3:** Multivariate analysis of the risk factors associated with the unfavorable tumor biology.

## Discussion

Patients with hepatocellular carcinoma are usually referred to the Department of General Transplant & Liver Surgery, Medical University of Warsaw, as to the Reference Center. Almost 80% of them present with liver cirrhosis. Radical treatment by liver resection or liver transplantation is indicated in a mere 25 – 30 % of patients. The 5-year overall survival rate approximated up to 82%, and the tumour relapse during the 5-year follow-up did not exceed 25%. The experience we have gained so far seems to indicate a need for new selection criteria that would be based, to a larger extent, on tumour biology [22-24].

The present study on hematopoietic stem cells and endothelial progenitor cells in hepatocellular carcinoma and liver cirrhosis highlights the implications of the cell evaluation for clinical settings of

HCC patients. Many studies support the suggestion that the levels of stem/progenitor cell correspond to the stage of HCC development and may help in disease monitoring and outcome predicting. Lyden et al. [30] and Beerepoot et al. [32] showed that the combination of hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs) is essential for tumour growth. In fact, the EPCs are the subpopulation of hematopoietic stem cells that could be recognized by the phenotype of CD34+, 133+, 45dim. The EPCs express additionally the cell surface marker CD309 [6,11,12,30,32]. According to the experience of our study, the relationship between the rates of both cell fractions seems to be mutual. The study shows that the highest rates of hematopoietic stem cells within white blood cells account was, the lowest rate of endothelial progenitor cells within the hematopoietic stem cells population. The high rate of hematopoietic stem cells accompanied by low rate of endothelial progenitor cells seems to be

usual for a healthy liver. Pathologic process within the liver, such as B/C virus hepatitis and cirrhosis and especially cancer change the relations between the cells fraction and results in decreasing rates of hematopoietic cells and increasing rates of endothelial progenitor cells. Basic research data indicate angiogenesis in a healthy liver as a natural process involved in the tissue proliferation and regeneration [25,26]. Liver cirrhosis makes the processes faster, more intensive and warped [5,6,7,25,27]. Hepatocellular carcinoma promotes cells proliferation much more and significantly accelerates the process of angiogenesis [6,7] Yet, the development of a serious liver pathology, such as advanced liver cirrhosis and HCC, results in decreasing rates of hematopoietic stem cell and increasing rates of their endothelial progenitor cell fraction. The patients with liver cirrhosis and patients with HCC could be distinguished from those with a healthy liver by a lower level of hematopoietic stem cells and a higher level of endothelial progenitor cells in the peripheral blood circulation. The divergence between the cell levels seems to be the hallmark of a developing serious liver pathology, i.e. advanced liver cirrhosis and hepatocellular carcinoma [2,7]. The number of changes and the scale of the divergence between the stem/progenitor cells fractions seems to indicate the intensity of angiogenesis. Thus, they could imply the development of a malignancy in a cirrhotic liver, as it is suggested by some other studies [8,12,13,35].

The levels of circulating progenitor cells changed gradually according to the stage of cancer disease. Our findings correspond with some other studies and support the suggestions that the levels of stem/progenitor cell indicate on the stage of HCC development and may help in disease monitoring and outcome predicting [9,12,13,34]. Ho et al. [4] found the circulating EPCs levels significantly elevated in patients with advanced HCC as compared to patients with early resectable HCC, patients with non-cancer liver cirrhosis, and healthy controls, exactly as established in our study. Beerepoot et al. [32] showed that levels of circulating endothelial cells (CECs) are increased in patients with a progressive disease. The clinical data gathered by Yang et al. [7] show that the level of circulating EPCs in HCC patients is significantly higher than in patients with cirrhosis and normal subjects. Patients with an advanced stage of cancer have a higher level of EPCs compared to those with an early stage. Recently, Xi-Tai Sun et al. demonstrated that the role of EPCs in neovascularization becomes more important as HCC growth progresses, and Zhu et al. revealed that EPCs are mobilized and incorporated into tumour vessels throughout the whole process of HCC growth [6,11]. Although the results were obtained on animal models of the tumour, they confirm the findings of many studies conducted since the first study of the role of EPCs was reported by Lyden et al. [30].

The study indicates also some correlations between the levels of stem and progenitor cells and the manifestation of clinical and biochemical symptoms in patients with liver cirrhosis and HCC. The increased rates of both cell fractions were found to be associated significantly with the type of underlying primary liver disease. Such a tendency was reported in some other studies [7,11,36]. Yu et al. suggest that a developing hepatocellular carcinoma coupled with cirrhosis creates a very specific microenvironment that encourages endothelial progenitor cells to settle at the site of the tumour [9,12]. Peri-tumoral cells are responsible for the recruitment of EPCs by angiogenic factors expressed. They also find the cirrhotic liver itself a precancerous change resulting in increased angiogenesis [9,12]. The results of our study seem to confirm the increment of angiogenesis activity in patients with liver cirrhosis and HCC. Interestingly, the changes were more advanced in the HCC patients with liver cirrhosis

that developed due to alcoholic liver disease, not in the patients with cirrhosis due to B and C virus hepatitis, as it is generally thought [10,28,29]. Surprisingly, the levels of hematopoietic stem cells and endothelial progenitor cells were not found to correlate with the high level of serum AFP. Our observations differ from those reported previously by Ho et al. who found a significant positive correlation of circulating EPCs levels with the circulating AFP level [4]. No differences in the levels of both cell fractions were observed also in these of our patients who presented with an elevated serum level of alkaline phosphatase (AFP) indicating cholestasis and a decompensated liver function due to cirrhosis and terminally advanced cancer. In the contrary to our observations, Goon et al. [35] found decreased levels of EPCs in the spread of breast cancer and Sun XT et al. [6] suggest that a decline in levels of serum cytokines and EPCs in advanced HCC may be the result of a disordered environment and systemic failure.

It was essential to evaluate whether the quantification of the circulating hematopoietic stem cells and endothelial progenitor cells may be beneficial for prediction of the unfavorable tumour biology in the preoperative settings. In general, parameters of the tumour biology are not assessed prior the liver resection or liver transplantation because they cannot be reliable predicted by the diagnostic settlements. The systems of classification that are currently used for selection of patients with HCC to the treatment options base mostly on radiologic findings indicating the localization, the size and the number of tumors. Clinical evaluation allows also estimating the grade of liver function, the stage of tumour development, and providing the rationale for treatment option. Unfortunately, the prognosis remains partially unpredictable due to unique biology of the tumour [7,11,33,36-40]. There are four factors that are generally accepted as the significant predictors of poor outcome in patients treated with surgical procedures. Two of them, microvascular invasion of the tumour and the large tumour size are noted as the prerequisite for disease dissemination, thus the tumour recurrence and the treatment failure [36-40]. The two others, multifocal tumour development and poor tumour differentiation are also considered as the factors fostering the failure, however they were found to be not significant predictors in some studies [36-40]. The need for the identification of effective markers that may allow the patients stratification more accurately by tumour features is stressed in many reports [36,37]. The present study seems to indicate a mutual and positive relationship between the cells' activity, the progress of tumour development and the unfavorable tumour biology. Patients with non-malignant liver cirrhosis tend to be distinguished from healthy subjects by a decrease in the level of hematopoietic stem cells and an increase in the level of endothelial progenitor cells. Patients with hepatocellular carcinoma at different stages of disease development tend to differ from one another by a significant elevation of the circulating HSCs and EPCs levels. The multivariate analysis of prognostic influence of various variables including the rates of circulating hematopoietic stem and endothelial progenitor cells and a series of clinical and laboratory parameters identified the increased level of both cell fraction as the predicting factor of the unfavorable tumour biology. The increased levels of hematopoietic stem cells and endothelial progenitor cells seem to be a predictor of poor tumour differentiation (OR=4.01, (0.82-19.83) 95% CI and OR=3.5, (1.22-10.07) 95% CI, respectively). Such objectionable tumour characteristics are considered as one of the most important factor of the tumour recurrence and the failure of radical surgical treatment [36-41]. On the other hand, the increased rates of hematopoietic stem cells indicates also the probability of the acinar

growth pattern of the tumour, that could be of particular clinical interest in patients with HCC tumors developing in a healthy liver (OR=12.09, (1.93-75.42) 95% CI). There were 40 of such patients analyzed in the study. All were presented with high rates of the hematopoietic stem cells (mean 0.024 +/-0.08, median 0.024, 0.021-0.027 95% CI) and the relatively low rates of the endothelial progenitor cells (mean 3.33 +/-2.26, median 2.46, 2.54-4.12 95% CI). The on explant pathologic examination showed the acinar pattern of the tumour in 27 (67.5%) of them. It is generally agreed that the acinar tumors exhibit significant features, such as genetic alterations and the beta-catenin mutation, and they frequently associate with the tendency to cholestasis. According to some reports, the genetic alterations are frequent in Caucasian patients in accordance to the non-cirrhotic liver. The beta catenin mutation associating with the chromosome stability is anticipated as one of the main pathways of hepatocarcinogenesis. It is assumed that some of these cases may developed from a pre-existing adenomas with mutant beta-catenin [42]. From this point of view, quantification of the circulating hematopoietic stem cells in patients with adenoma could be of distinctive value that help to recognize HCC in patients with a healthy liver.

Certainly, tumour recurrence is a major limitation of long-term survival after radical surgical treatment. The ultimate patients outcome and the prognosis are dependent on the different patient/tumour-related factors. Assessment of the tumour angiogenesis activity by the evaluation of the pre-transplant rate of circulating hematopoietic stem cells and endothelial progenitor cells could allow for better the stratification of patients undergoing liver resection and liver transplantation for HCC not only by patient/tumour-related factors, but also by characterizing intrinsic tumour features. The findings of our study consolidate also the premises for the adjuvant therapy with agents inhibiting cell proliferation in these of our patients who express high rates of circulating hematopoietic stem and endothelial progenitor cells [15,17,18,34].

## Study Limits

The major limitation of our study is that it was conducted in a single center. However, our patients were treated and followed uniformly on the basis of consensus algorithms widely used by many centers. Unfortunately, it was not possible to find a more numerous population of the patients in the desirable range of time, especially patients with HCC tumor within Milano criteria that would be suitable for liver transplantation. Thus the study concerned a limited cohort of patients. However, the number of patients evaluated in the study corresponds to the number of cases presented in many other reports concerning biomarkers and factors predicting the angiogenic propriety of HCC. Notably, the levels of hematopoietic stem cells and endothelial progenitor cells were found to correlate significantly with the manifestation of clinical and biochemical symptoms of the disease. Thus, the enumeration of the circulating hematopoietic stem and endothelial progenitor cells should be considered as an additional factor contributing to better evaluation of surgical patients with HCC in pre-treatment settings and a helpful determinant of the optimal management.

Our study should be also considered by the limits of the ISHAGE methodology that was used for the cell enumeration. The debates are ongoing about the definition of 'true' EPC, as well as the availability of a reliable method to assess their quantity and quality, functional status, and therapeutic application. We have adopted the ISHAGE protocol as

a standardized method that allows avoiding the generation of widely divergent data. The ISHAGE protocol was validated as a simple, rapid, and sensitive flow cytometric method to quantitate hematopoietic stem and progenitor cell (HSC) that bear the CD34 antigen in peripheral blood and apheresis products. The standardisation was based on the use of state-of-the-art bright fluorochrome conjugates and the combination of the HSC marker CD34 with CD45 counterstaining, the gating strategy was established to separate the CD34+ HSC from irrelevant cell populations and the enumeration at least 100 CD34+ cells was recommended to ensure a 10% precision. Many reports indicate that EPC are found just in cell fractions staining positive for CD34 and CD34+CD133+CD309+(KDR). It is stressed that human CD34+CD133+CD309+ are just the primitive hematopoietic progenitors. The presence of CD133 positivity indicates both the stemness and the hematopoietic lineage of the cells. Especially the fraction of CD45dim cells harbours the "true" circulating EPCs. Thus, it was justified to apply of the ISHAGE protocol for cells quantification in the study, as recommended method for quantification of hematopoietic stem and progenitor cells in the fresh blood. We added the surface marker AC133 and CD309 to the original ISHAGE protocol and after identification of HSC, immunofluorescence of the cells for CD309 was assessed. The sequential strategy for quantification was strictly followed. However, at least two limitations of the method should be considered: first, the precise antigenic phenotype of EPCs is really unknown, mainly because it overlaps with that of other cell lineages; second, definition of EPCs by flow cytometry implies a conceptual abstraction, because a presumed function is attributed to a relatively simple antigenic phenotype. It should be also noticed that endothelial progenitor cells are very rare in the circulation and the cells positive to CD34, CD133 and KDR produce the lowest counts among them. Identification of the cells with one of the above-mentioned phenotypes by flow cytometry is a "rare event", described by the Poisson distribution probability. According to this distribution, the coefficient of variation ( $CV\% = 100 \times \sqrt{n/n}$ ) depends exclusively on the number of positive events (n). The indirect cell staining may produce an exceeding proportion of false positive identification, as well as, expressing cell counts per unit of volume that could not prevent hemodilution and do not reflect variations in body liquids [19,20]. This is why we increased the total number of acquired events to at least 2 000 000, which is generally not needed for most other applications of flow cytometry. We also expressed the results as percentage of WBC for HSCs and percentage of HSCs for EPCs. Bearing in mind the mentioned above limitations we consider the method of flow cytometry as sensitive enough when count of peripheral blood HSCs and EPCs is conceived as a disease biomarker.

## Conclusions

Liver cirrhosis and the development of hepatocellular carcinoma causes significant changes in the levels of circulating hematopoietic and endothelial progenitor cells. The cell levels correlate with the advances of liver pathology and allow to anticipate the unfavorable biology of the tumour.

## Acknowledgments

The authors wish to thank all cooperators from the Department of General, Transplant & Liver Surgery, the Department of Oncology, Hematology & Internal Medicine, and the Department of Pathology,

Central University Hospital, Medical University of Warsaw for their contribution to this project.

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