UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA
IN
"Scienze Cliniche"
CICLO XXIX

COORDINATORE Prof. Annunziato Francesco

Role of Circulating Endothelial Progenitors cells (EPC) in patients with hepatocellular Carcinoma treated with Sorafenib

Settore Scientifico Disciplinare MED/09

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Dedication

To my father, Hussein Aburas and my mother Fatima Mokhtar for their undying love and support

Acknowledgment

This has been an interesting journey which had its ups and downs. However, if it wasn't for the support of all the wonderful people around me, I would not be in this position right now. And to them all, I present my deepest gratitude.

First, I would like to thank my family for their endless love and support. My sincerest gratitude goes to my father for his constant encouragement. In addition, I would like to thank my sister Dr abir aburas and my uncles Dr Ali Aburas, Prof. Abdurazzag Aburas for their endless support.

I am ever so thankful to my supervisors. I would first like to express my sincere gratitude to Professor Laffi Giacomo, my supervisor, for his patient supervision and for his guidance and his mentorship. His enthusiastic support of my work is vital to the success of this thesis.

Next, I am grateful to my co-supervisor, Professor Marra Fabio, he has introduced me to the field of liver cancer. for his kind guidance, His patience, understanding, personal encouragement, insightful comments, generosity and endless support are all highly appreciated. His invaluable advice and support throughout all these years are vital to the success of this thesis and my academic career, I have the utmost respect for him and am honored to have worked under his wing.

I would also like to acknowledge his lab group members especially Nadia for all their technical help.

I should give the real thanks to Dr Inghilesi Andrea who helped in my research work.

I have also greatly appreciated my colleagues in the clinic, Dr Dragoni Gabriele, Dr Campani Claudia for the generous support and creating a friendly working environment.

I would also like to acknowledge professor Annunziata and his group members: Dr Maria Caterina Rossi for their assistance in the project.

Further, I want to extend my acknowledge to Prof.ssa Balzano for her generous support during my PhD program.

I gratefully acknowledge Cianferoni Chiara and Galli Federica in PhD office for their kind helping since I was admitted into PhD program in 2013.

I would like to thank Ciurcina Giuseppe, Tripodi Emanuele,Lodovico Osnato, Stefano Osai, Bartolini Luciana,Danielo, for being my family and friends here in Italy .

I like to thank Dr Shaya Alshaya and Dr Abdulla Alsaleh and Dr Omar al shaya , from Al Majmaah University of Saudi Arabia for your support and I am grateful for your friendship.

I would also like to thank my friends Dr Rastm Kashur,Dr Mohamed alturban,Dr alrobae esam ,Firas Gadgud ,Waled Ngiga ,Waleed Ahshad ,Ahmed Almahjub for your support.

To all my friends, old and new ones, I thank you all for your support.

Florence, November 2016

Sami Aburas

Abstract

Role of Circulating Endothelial Progenitors cells (EPC) in patients with hepatocellular Carcinoma treated with Sorafenib

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Hepatocellular carcinoma (HCC) is one of the most frequent carcinomas throughout the world, an aggressive malignant tumor with high mortality rate being responsible for more than 1 million deaths annually. This is attributable to a frequent occurrence of recurrence, intrahepatic and distant metastases after resection or transplantation. Owing to its ability of self-renewal and contribution to angiogenesis and neovascularization, hepatocellular carcinoma (HCC) is generally resistant to chemotherapy and conventional radiation therapy. Since 2008 sorafenib is the therapy of choice in the advanced stage of HCC. Sorafenib is an anti-angiogenic drug, which showed statistically significant increase in overall survival in patients with hepatocellular carcinoma in advanced stage. However, only a small portion of patients achieve a real clinical benefit from the treatment, and a very small percentage of them shows a partial or complete radiological response. No single agent or combination therapies have been shown to impact outcomes after sorafenib failure. It is therefore essential to identify the variables that can predict the early course of treatment, in order to optimize costs and above all to avoid a reduction in quality of life related to a therapy that still remains palliative.

Sorafenib is a small-molecule with potent inhibitory action of Raf-1, an enzyme included in the signaling pathway RAF/MEK/ERK, implicated in the control of cell growth, and tyrosine kinase receptors such as PDGFRs, FLT3 and KIT, or vascular endothelial growth factor (VEGFR) which is still considered as one of the most potent angiogenic cytokine and a survival factor for endothelial cells. Vasculogenesis and angiogenesis are the main processes for growth and

development of blood vessels, are essential in normal physiological neovascularization during tissue growth, wound healing, and organ regeneration. Postnatal vasculogenesis also contributes to endogenous neovascularization of developing tumors. In the adult, circulating endothelial progenitor cells (EPC) are believed to be recruited from the bone marrow, migrate to sites that need neovascularization, and participate in the assembly of newly-forming blood vessels in both animal models and humans. These cells derived from the bone marrow include different categories of hematopoietic progenitors. Although the precise role of the EPC is still a matter of debate, recent recent studies showed higher circulating levels of EPCs in patients with advanced unresectable HCC as compared to patients with resectable HCC. Moreover, numerous studies have highlighted the importance of these cells in disease progression and vascularization of metastatic lesions.

In the period of my Doctoral training, I have been involved in the management of patients with HCC at two levels, experimental (focused on aim 1) and clinical (focused on aim 2). These aims are detailed below:

Aim 1: Experimental study in patients with advanced HCC treated with sorafenib. Our objectives were:

i. To investigate the level and frequencies of endothelial progenitor cells (EPC) circulating in the blood of HCC patients before and after sorafenib therapy measured in 3 occasions according to the number of weeks (T0, T2, T8). T0, before the start of therapy, (T2) after 2 weeks, and (T8) after 8 weeks from sorafenib initiation.
ii. To evaluate the possible correlation between the level of endothelial progenitor cells and side effects of sorafenib,
iii. To search for a possible association of the EPC frequency with the basic parameters and the response to treatment, comparing contrast-enhanced CT or MRI images, 8 weeks after initiation of therapy.
iv. To evaluate the role of EPC in disease progression of the and their possible prognostic value in patients with HCC.

Aim 2: Contribution to the Italian national database of liver cancer (Ita.Li.Ca): The HCC cases consecutively observed in this Unit were analyzed and recorded the database. We collected, analyzed and included more than 100 consecutive cases of hepatocellular carcinoma followed in the last four years in our center, together with detailed epidemiological. The Ita.Li.Ca cohort is the largest HCC database in Italy.

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List of Abbreviations

AFP	Alpha fetoprotein
AIH	Autoimmune hepatitis
BCLC	Barcelona-Clinic Liver Cancer
BM	Bone Marrow
BMC	Bone Marrow Cells
CEC	Circulating Endothelial Cells
CEP	Circulating Endothelial Progenitors
CD	Cluster of Differentiation
CXCR	CXCR CXC Chemokine Receptor
cKit	Cytokine Receptor
CI	Confidence interval
CLIP	Cancer of the Liver Italian Program
CR	Complete response (RECIST)
CT	Computed tomography
DFS	Disease-free survival
EC	EC Endothelial Cells
EPC	EPC Endothelial Progenitor Cells
ECOG	European Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
Fig.	Figure
FGF	FGF Fibroblast Growth Factor
HSC	HSC Hematopoietic Stem Cells
PB	PB Peripheral Blood
PMNC	PMNC Peripheral Mononuclear Cells
PIGF	PlGF Platelet Growth Factor
PEI	Percutaneous ethanol injection
PET	Positron emission tomography
PR	Partial response (RECIST)

PSC	Primary sclerosing cholangitis
RCT	Randomized controlled trials
RECIST	Response evaluation criteria in solid tumors
RFA	Radiofrequency ablation
SD	Stable disease (RECIST)
Tab.	Table
TACE	Transarterial chemoembolization
TAE	Transarterial embolization
TNM	Tumor-Node-Metastasis
VEFGA	Vascular endothelial growth factor A
VS	Versus
WHO	World Health Organization

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I. S. Colagrande, A.L. Inghilesi, **S. Aburas**, G.G. Taliani, C. Nardi, F. Marra, Challenges of advanced hepatocellular carcinoma, World J Gastroenterol 22(34) (2016) 7645-59.

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CHAPTER 1

INTRODUCTION TO HEPATOCELLULAR CARCINOMA

1.1. INTRODUCTION:

Hepatocellular carcinoma (HCC) is a primary malignant tumor of the liver arising from the liver cells (hepatocytes). One of the deadliest malignancies, HCC is considered to be the third leading cause of all cancer-related deaths and fifth common cancer worldwide [1]. Worldwide, liver cancer is the sixth most common cancer, the 2nd cause of cancer-related death[2]. The male population is more prone to the liver cancer than the female population [3]. **Figure 1** shows the higher prevalence of liver cancer in men versus women. Hepatocellular Carcinoma (HCC) also called malignant hepatoma, accounts for around 80–90% of all liver cancers. Globally, the occurrence of HCC is increasing by 3–9% annually [4]. Hot spots around the globe are Asia, Africa, Europe and North America where the western regions have seen an increase due to the spread of the hepatitis C virus (HCV), alcoholism, obesity and poor lifestyle choices [3]. Early detection of Hepatocellular carcinoma is difficult and usually results in death within a few months of diagnosis [5] Despite the recognition of cirrhosis as the major risk factor for HCC, more than 50% of patients with HCC present an advanced disease at diagnosis [6].

1.2. Etiology and risk factors:

Nearly all cases of HCC occur in the presence of cirrhosis or advanced fibrosis up to 90% of cases.[7] Thus, any cause of liver disease that can result in cirrhosis should be considered a potential risk factor for HCC. The 5-year cumulative risk of acquiring HCC in the presence of liver cirrhosis vary from 5% to 30%, depending on the cause of cirrhosis, the region, ethnicity, and the stage of cirrhosis.[3, 8] The risk factors differ widely that develop HCC depending on the region. Hence, it is easiest to outline the most important risk factors in terms of global region although it must be noted that some overlap between regions does occur. **Table.1** show the main differences in risk factor distribution.

Common contributors to liver inflammation and eventual cirrhosis include: infection with Hepatitis B virus (HBV) or Hepatitis C virus (HCV), alcoholic liver diseases, non-alcoholic fatty liver diseases (NAFLD) and aflatoxins, also contributors to liver inflammation, that can lead to cell transformation, as showed in **Figure 2.** [9]. In Asia and Africa carriers of HBV can be at high risk of developing HCC while in Europe and North America carriers of HCV and alcoholics can have a potentially high rate of developing the disease[10, 11]. Carriers of HBV or HCV that also abuse alcohol are known to have an even higher risk of developing HCC [8]. The risk due to hepatitis B viral infections are significantly less prevalent in developed nations due to vaccination programs[12]. The management of these factors has demonstrated to decrease the risk of developing HCC. In fact, the rates have declined in some historically high-risk countries due to increased administration of HBV immunizations[5]. Preventative strategies against HCV infections and maintenance of a healthier lifestyle have definitively shown a direct relation to the decline of HCC rates.

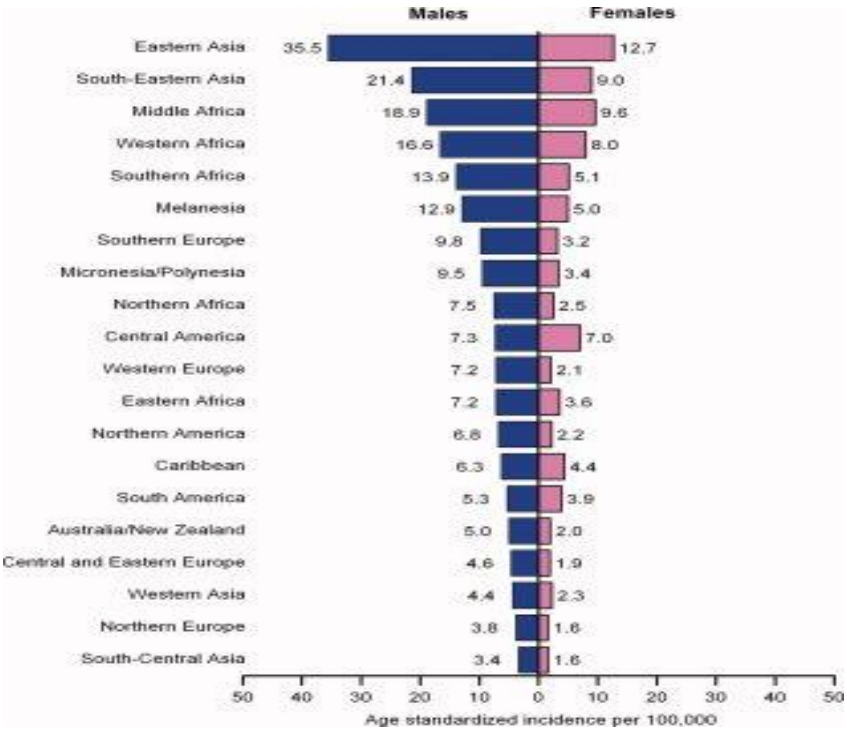


Figure 1: Age-standardized liver cancer incidence rates by sex and world area. Adapted from [5]

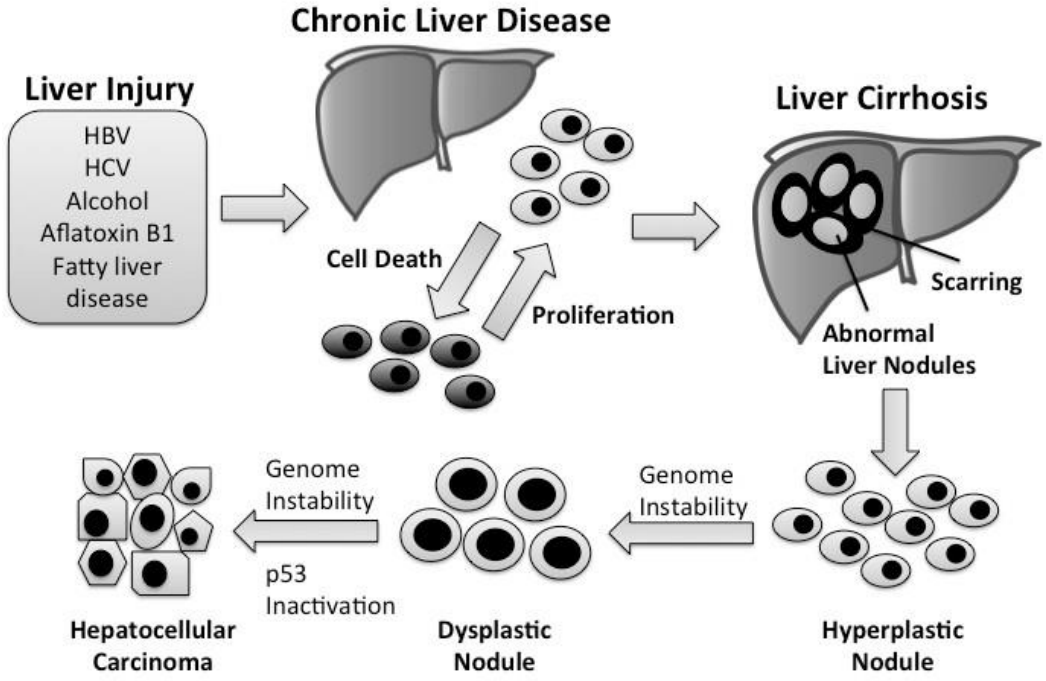
Region	HBV	HCV	Alcohol	Other
Europe	10-15 %	60-70 %	20 %	10 %
North America	20 %	50 %	20 %	< 10 %
Asia and Africa	70 %	20 %	10 %	< 10 %

Table 1. Global causes of HCC[11]

1.2.1 Hepatitis B virus (HBV):

On a worldwide scale, chronic infection with the hepatitis B virus (HBV) is by far the most important risk factor (400 million people are infected with HBV globally), due to its endemic nature in Asian and African regions. In these areas, HBV is transmitted at childbirth from mothers to newborns and thus the peak age in incidence for HCC is much younger than in developed countries, where new infections generally occur in adults. In an effort to prevent exposure to risk factors, immunization programs have been introduced by the World Health Organization (WHO) with the aim of universally vaccinating infants against HBV. As of 2011, this had been achieved in 93% of countries [13].Despite the existence of an effective prevention strategy, Worldwide HBV infection is the primary reason for HCC in half of the patients.[3, 11] it can cause HCC via HBV-induced cirrhotic transformation of the liver, it can develop HCC also in non-cirrhotic livers. HBV can induce the development of HCC through several mechanisms. Firstly, the development of inflammation and increased hepatocyte regeneration in response to HBV infection may provide an environment permissive to cell transformation and tumor growth[12]. In addition to the inflammation caused by chronic viral infection, HBV may accelerate tumor initiation through insertional mutagenesis[14], and also inactivates the tumor suppressor p53 via direct binding[15].

Figure 2. Development of hepatocellular carcinoma. (Adapted from [16]).



1.2.2 Hepatitis C virus (HCV):

In developed areas, such as Europe, North America and Japan, the main risk factors are chronic infection with the hepatitis C virus (HCV) followed by excessive alcohol intake[3, 5].

Unlike HBV, relatively few individuals with acute hepatitis C will clear the infection, and 85-90% develop chronic disease. Of individuals with chronic HCV infection, 50-60% of them will develop liver disease, and up to 20% will develop cirrhosis[17]. 1-6% per year of HCV induced cirrhosis they will develop HCC [18]. Recent developments in antiviral therapies for hepatitis C can reduce virus below detection levels for two years or more[19], so the HCV induced HCC rate may decrease as these therapies become more commonplace. However, there is no currently available vaccine for hepatitis C [20], so worldwide infection rates remain high.

1.2.3 Alcohol:

Prolonged and excessive alcohol intake is also a well-established risk factor and the second most common cause of HCC in developed countries. Excessive alcohol consumption is known to contribute to hepatic steatosis. Excessive intake of alcohol is defined as a daily ingestion of at least 40-60 g of alcohol (one standard drink contains 13.7 g)[9]. In the setting of the cirrhotic liver there is a predisposition for HCC to occur (the risk of developing HCC among alcoholics appears to increase 10-fold in the presence of cirrhosis) [8, 21]. Persons co-infected with HCV are more prone to HCC in combination with the heavy use of alcohol than in HBV coinfection[22].

1.2.4 Aflatoxin:

Aflatoxin B1 (AFB1) is a fungal metabolite that contaminates food products stored in damp, warm conditions, particularly in tropical regions such as Southeast Asia and Sub-Saharan Africa where the climates are most suitable for growth of the fungi that produce it [23]. This toxin is known to induce hepatitis and increase risk of HCC in those who continually ingest it [24]. AFB1 acts as a powerful carcinogen in the liver and causes a characteristic mutation in the p53 tumor suppressor gene [25, 26]. which is thought to facilitate hepatocarcinogenesis[27]. Studies performed in China showed that increased HCC risk ranging from 3-fold [28] to 60-fold [29] for individuals exposed to HBV infection and AFB1.

1.2.5 Nonalcoholic fatty liver disease (NAFLD):

Nonalcoholic fatty liver disease (NAFLD), which is present commonly in up to 90% of all obese persons and up to 70% of persons with type 2 diabetes, has been proposed as a possible risk factor for hepatocellular carcinoma [30]. NAFLD is a group of conditions including non-alcoholic steatohepatitis and mild hepatic steatosis. In these circumstances, fat accumulate in the liver.
The fat causes inflammation and damage, which may lead to cirrhosis. 20-25% of those with NAFLD will progress to NASH, of whom 11% will progress to cirrhosis within 15 years [EL-SERAG, AASLD 2016]. Large epidemiologic studies performed in developed countries have observed a positive significant association between diabetes and HCC[9, 31], with a risk of HCC increasing

approximately 2-fold in men with diabetes, independent of alcoholic liver disease, viral hepatitis or demographic characteristics[32]. The presence of both obesity and diabetes are known to contribute substantially to the development of NAFLD. This in turn can progress to the more severe form of the disease, NASH, which may lead to cirrhotic transformation of the liver and HCC [33]. Generally, all the risk factors contribute to the development of HCC through fibrosis and cirrhosis.

1.3. Staging of Hepatocellular Carcinoma:

Several staging systems have been established in order to determine the severity of the HCC condition (**Table 2**) but the most frequent used system for classifying HCC is the Barcelona Clinic Liver Cancer (BCLC) system (**Figure 3**). BCLC combines features of the tumor(s), such as size, number, and presence of vascular invasion, with the addition of liver function tests. The combination is used to classify tumor stage and patient prognosis[34]. The BCLC establishes four major categories: early, intermediate, advanced and terminal stage. The treatment options that are most commonly used with respect to the staging systems include surgical options (resection and liver transplantation), ablative techniques (ethanol injection, radiofrequency ablation), and transarterial chemoembolization.[34] Early HCCs are characterized by their small size, typically less than 2cm, and their well-differentiated cytology and shows good liver function, excellent performance status. This stage is open to possible curative treatments with high survival rates. These tumors often display fatty change, but otherwise do not display structural atypia[35].The intermediate stage is in those who do not fit the early stage criteria and show moderate compromise in liver function, excellent performance status with multi nodular tumors. Here non-curative methods are chosen, with TACE the treatment of choice. While the advanced stage shows moderate liver function, vascular invasion, extra hepatic spread and symptoms [36]. Before a treatment method can be chosen, the function of the liver is assessed and a stage is assigned depending on the status of the condition, according to the Child Pugh scoring system, which is based on the levels of bilirubin and albumin, prothrombin time, encephalopathy and the presence of ascites. Points are are calculated to give the Child Pugh score of A-C (**Table 3)**. Stage A shows good function allowing curative treatment methods

to chosen, stage B indicates a vulnerable function where TACE is commonly chosen and C shows poor function where transplantation is offered to selected patients [36, 37].

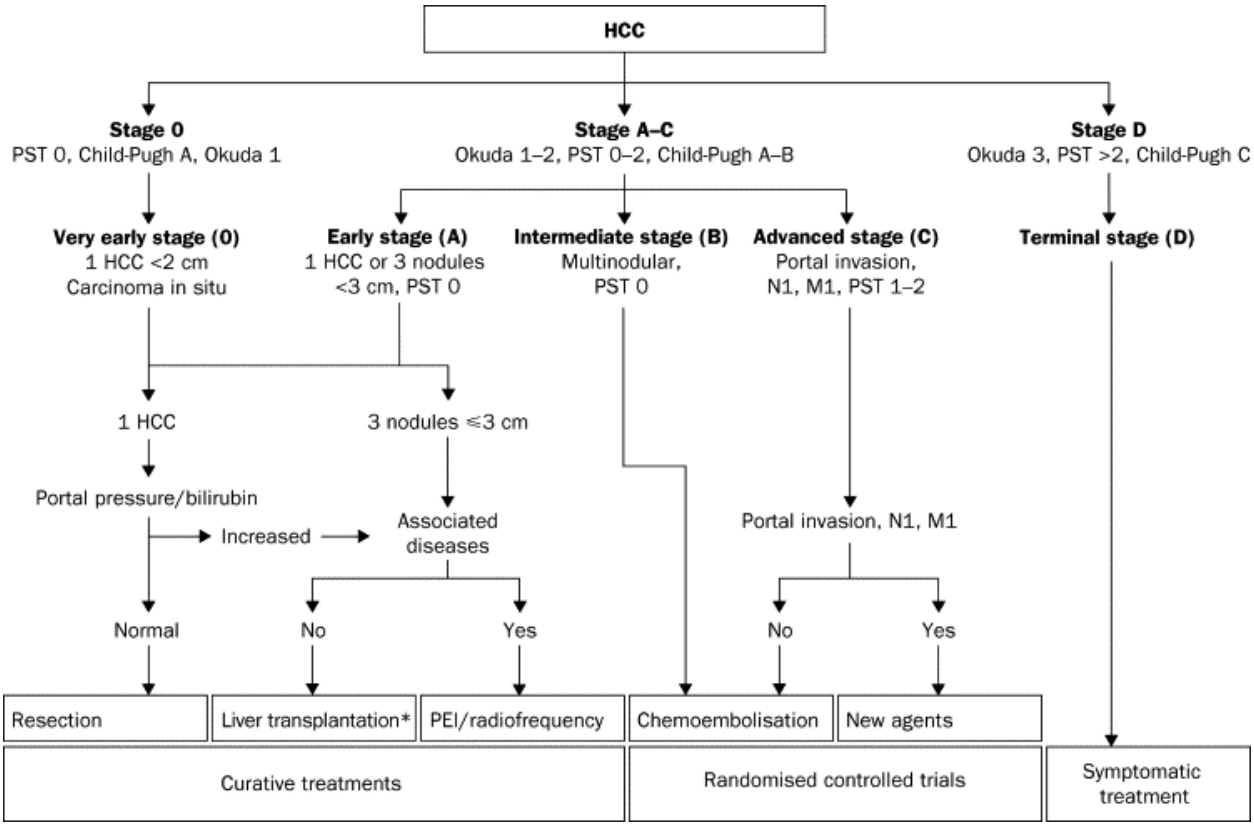


Figure 3. BCLC staging classification and treatment schedule for HCC. N: nodule; M: Metastasis; PS: Performance status; TACE: Transarterial chemoembolization; BSC: Best supportive care. Adapted from[34].

Staging system	Ascites	Tumor burden	Albumin	Bilirubin	INR	HE	AFP	PVT	EHS	PS	ALP
Okuda	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No
CLIP	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
BCLC	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No
GRETCH	No	No	No	Yes	No	No	Yes	Yes	No	Yes	Yes
TNM 7^{th} edition	No	Yes	No	No	No	No	No	Yes	Yes	No	No

Table 2. Variables included in the most widely used hepatocellular carcinoma staging systems. AFP: Alpha fetoprotein; ALP: Alkaline phosphatase; EHS: Extrahepatic spread; HE: Hepatic encephalopathy; INR: International normalized ratio; PS:Performance status; PVT: Portal vein thrombosis.

1.4. Advanced Hepatocellular Carcinoma (HCC):

Advanced stage HCC includes heterogeneous groups of patients with different clinical condition and radiological features and sorafenib is the only approved treatment. Despite the recognition of cirrhosis as the major risk factor for HCC, more than 50% of patients with HCC present an advanced disease at diagnosis [6]. Moreover, increased survival and better care for patients in earlier stages, allow their survival until they reach a more advanced stage.

The concept of “advanced” disease varies considerably analyzing the different staging systems utilized in the past ten years. One peculiarity of HCC is its association with chronic liver disease, especially cirrhosis. This makes prognosis of an individual patient dependent not only on the size, biologic behavior and spread of the tumor, but also on the degree of functional failure of the liver due to the presence of cirrhosis. The role of chronic liver disease in the prognosis of HCC is witnessed by the inclusion of the Child-Pugh score or other aspects linked to liver functions in several staging systems used for HCC (Table 2). In the Barcelona Clinic Liver Cancer (BCLC) staging system[38] , advanced HCC is considered as an unresectable HCC with/without extra-hepatic spread (metastases or lymph nodes involvement) and/or vascular invasion (portal or segmental invasion) and/or systemic symptoms, defined by an Eastern Cooperative Oncology Group performance status 1 or 2, with a liver function defined by a Child Pugh stage not greater than B[39, 40].

Parameter	Points assigned		
	1	2	3
Ascites	Absent	Slight	Moderate
Hepatic encephalopathy	None	Grade 1-2	Grade 3-4
Bilirubin micromol/L (mg/dL)	<34.2 (<2)	34.2-51.3 (2-3)	>51.3 (>3)
Albumin g/L (g/dL)	>35 (>3.5)	28-35 (2.8-3.5)	<28 (<2.8)
Prothrombin time Seconds over control INR	 <4 <1.7	 4-6 1.7-2.3	 >6 >2.3
CPT classification: Child A: score 5-6 (well compensated); Child B: score 7-9 (significant functional compromise); Child C: score 10-15 (decompensated)			

Table .3 – The child Pugh score staging system.

1.4.1. SORAFENIB IN THE TREATMENT OF ADVANCED HCC:

The treatment of patients with advanced HCC has been for a long time disappointing for physicians. Curative options such as surgical resection or liver transplantation did not show any efficacy in prolonging overall survival (OS). Trans-arterial chemoembolization (TACE) in patients with advanced HCC due to portal vein thrombosis has been suggested to improve OS compared to patients receiving supportive care, in retrospective studies [41] and in a recent meta-analysis, but is not currently recommended by practice guidelines [42]. Early systemic therapies with hormone analogues (*e.g.,* tamoxifen) or classic chemotherapeutic agents (*e.g.,* doxorubicin) failed when tested in randomized controlled trials[43]. In 2008 the approval of sorafenib in the until then desolated scenario of advanced HCC therapy radically changed the therapeutic approach, opening the era of molecular targeted therapy. Till now, no additional molecules have been added to our pharmaceutical armamentarium. Sorafenib is a multi-kinase inhibitor that suppresses tumor neo-angiogenesis and proliferation, inhibiting the tyrosine kinase activity of vascular endothelial growth factor receptors 1, 2 and 3 and of the platelet derived growth factor receptor. It also inhibits the serine-threonine kinases Raf-1 and B-Raf [44, 45]. The efficacy of sorafenib has been demonstrated in two large independent randomized controlled trials. In the SHARP and Asia-Pacific studies the Authors reported an improvement in OS of almost 3 mo between the sorafenib and placebo arms (10.7 mo *vs* 7.9 mo and6.5 mo *vs*

4.2 mo, respectively) [46, 47]. These results led to the approval of sorafenib for the treatment of advanced HCC. According to the technical schedule, the drug should be administered orally 400 mg b.i.d. until radiological progression or unacceptable adverse events occur. Therapy is currently recommended in patients with preserved liver function, defined by a Child-Pugh score not greater than A, due to the exclusion of patients with more compromised liver function from randomized controlled trials. This represents a first major problem for sorafenib administration, as only a portion of patients can actually be treated. From the time of sorafenib approval, many field-practice studies have tried to evaluate the efficacy and tolerability of sorafenib in Child B patients, with conflicting results.

The GIDEON study is so far the only prospective study that evaluated the impact of liver function in a large cohort of patients (> 3000), with a robust portion of subjects in Child-Pugh B class (666 patients)[48]. In the final analysis, overall adverse events were similarly observed in both Child A and B patients, but a significant increase in serious adverse events was found in the Child B group. Moreover, Child-Pugh score was confirmed as a strong independent predictor of OS (5.2 mo in Child B vs 13.6 mo in Child A). The Authors concluded that sorafenib at full dosage is safe irrespective of the liver function. However, the use of full-dose sorafenib in a Child B patient is still far to be included in the clinical practice, as many physician's fear that the patients are too fragile in this subgroup. Additional trials specifically addressing this issue are ongoing (Sorafenib in First-line treatment of Advanced B Child Hepatocellular Carcinoma, clinicaltrial.gov).

An approach popular in the Hepatology community and potentially applicable to Child B patients is to start sorafenib at lower dosage (e.g., 400 mg/d), ramping up to 800 mg/d in case of good tolerability. In case of poor tolerability, sorafenib should be continued at lower dosage, since data reported from the SOFIA group in 2011 did not show a reduction in OS in patients receiving half-dose sorafenib, whereas they actually had a significant survival advantage with respect to the group receiving full-dose sorafenib [49]. Another rationale for the implementation of a ramp-up strategy could be the lower tolerability profile of sorafenib that seems to emerge from clinical practice. According to those studies, some of the most common adverse events (fatigue, diarrhea, hand-foot syndrome, bleeding, arterial hypertension,

elevation of aminotransferase and/or bilirubin) are observed more frequently, in terms of incidence and severity, than reported in the registration trials. This leads to take into consideration a primary issue in sorafenib therapy, i.e. that an appropriate quality of life represents an essential goal in a non-curative treatment. Another hot issue that has emerged from the recent literature is linked to the wide variability in survival and time to progression (TTP) observed in clinical practice. It is a general opinion that sorafenib therapy may be truly effective in a subgroup of patients, while it shows no real benefit in others. Identifying early predictors of response represents therefore a crucial research area, that becomes even more important if we consider the economic burden of the therapy [50]. Numerous studies have explored the role of biochemical markers as prognostic factors or predictors of response. The concentrations of alpha-fetoprotein, alkaline phosphatase, angiopoietin 2, Vascular Endothelial Growth Factor have been linked to improved survival, while soluble c-Kit and Hepatocyte Growth Factor have been proposed as predictive markers infield practice studies[51-53] and in the SHARP trial. Observational studies have also linked the early development of adverse events like arterial hypertension, diarrhea or the hand-foot syndrome to a better response[54-56]. Finally, clinical features such as the presence of macrovascular invasion have been associated with a worse prognosis [56]. However, despite the large numbers of studies and the interesting results, no predictors have reached enough strength to be commonly used in clinical practice, due to the small sample size of most studies or to the lack of external validation of the findings. Therefore, although the aim of tailoring sorafenib therapy still appears exciting, tangible progresses will not be obtained without validation of parameters in large studies. Radiologic parameters also may represent an important tool in the management of sorafenib therapy. As the majority of HCC develops in patients with chronic liver disease, treatment of the underlying condition and especially management of its complications, is mandatory. HBV infection accounts for about 60% of the total liver cancer in developing countries and for about 23% in developed countries [57, 58]. The benefits of antiviral nucleot(s)ide analogue therapy in improving recurrence-free survival and OS after curative treatment of HCC [59] may suggest a possible role in improving outcomes also in advanced HCC, but at this time data on this topic are lacking.

BEYOND SORAFENIB, OTHER PHARMACOLOGIC APPROACHES TO THE MANAGEMENT OF ADVANCED HCC:

The discovery of alternative lines of treatment for advanced HCC is an urgent unmet need. Sorafenib therapy is very expensive, and healthcare costs have become one of the main problems confronting governments and patients worldwide [60]. Thus, in countries with limited health resources and a high incidence of HCC, a cost-effectiveness analysis to show the overall advantages of sorafenib is necessary. A Chinese study showed that the total cost was $897 for patients in the best supportive care (BSC) group, while in the sorafenib group, the total cost was $19495[60]. Second, sorafenib is often discontinued for patients in whom the disease is progressed after sorafenib treatment [61]. Many compounds and combinations have been explored in phase Ⅱ or even phase Ⅲ studies. Nevertheless, none of these have proven to be more effective than sorafenib as first-line therapy [62, 63] nor to be superior to placebo in second-line studies.

1.4.2.1 First-line treatments:

The results of the SHARP trials have been a milestone opening the way to systemic therapy in advanced HCC. Nonetheless, the limited results in terms of survival benefit over placebo indicate that more effective first line treatments are needed (**Table 4).**

In the phase Ⅲ SUN trial, **sunitinib**, a multi-kinase inhibitor inhibiting all vascular endothelial growth factor and platelet derived growth factor receptors, was compared to sorafenib (400 mg) in patients with advanced HCC and the median OS was significantly shorter in the sunitinib arm (7.9 mo *vs* 10.2 mo) while TTP was not significantly different (4.1 mo *vs* 3.8 mo with sunitinib and sorafenib, respectively) [64]. Of note, sunitinib was associated with severe adverse events, especially bleeding. The trial was prematurely discontinued for futility and safety reasons [64].

Brivanib is a dual inhibitor of Vascular Endothelial Growth Factor and fibroblast growth factor receptors. A randomized phase Ⅲ clinical trial has been conducted to evaluate the role of this drug as first-line therapy. The BRISK-FL study compared brivanib with sorafenib in patients with

advanced HCC. This trial failed to meet the primary endpoint of improving OS (with 9.5 mo for brivanib and 9.9 mo for sorafenib) or other endpoints, including objective response rate, TTP (4.2 mo *vs* 4.1mo) or disease control rates [65].

Linifanib is another multi-targeted tyrosine kinase inhibitor, which has been evaluated as first-line therapy in comparison to sorafenib. Linifanib inhibits members of the Vascular Endothelial Growth Factor and Platelet derived growth factor receptors families. In the LIGHT phase Ⅲ trial, linifanib was compared to sorafenib for efficacy and tolerability in patients with advance HCC without prior systemic therapy. However, median OS was 9.1 mo on the linifanib arm and 9.8 mo on the sorafenib arm[66], although TTP with linifanib was prolonged as compared with sorafenib (5.4 mo *vs* 4.0 mo, *P* = 0.001). Therefore, this trial failed to meet its primary endpoint and safety results favored sorafenib, as grade 3/4 or serious adverse events leading to discontinuation, dose interruption or reduction were more frequent with linifanib[66].

Erlotinib is an orally active, potent and selective inhibitor of the human epidermal growth factor receptor, and its gene amplification has been reported in HCC [67], although recent large scale results indicate that this occurs in a limited number of cases [68]. This drug was tested in a phase Ⅲ trial, where the efficacy and safety of a first-line treatment with sorafenib and placebo *vs* the combination sorafenib/erlotinib was evaluated in patients with advanced HCC[69].

This trial failed to meet its primary endpoint, *i.e.,* an improvement in OS, the median values of which were 9.5 mo in the sorafenib plus erlotinib arm *vs* 8.5 mo in the sorafenib plus placebo group. Moreover, the median TTP (3.2 mo *vs* 4.0 mo) was not significantly different between the two arms [69]. Withdrawal rates for adverse events were higher in the sorafenib/erlotinib arm. Regarding the drugs combination, a randomized phase Ⅱ trial conducted in Child-Pugh A patients, comparing doxorubicin plus sorafenib or doxorubicin alone, combination therapy led to a longer median TTP (6.4 mo *vs* 2.8 mo, *P* = 0.02), OS (13.7 mo *vs* 6.5 mo, *P*= 0.006) and progression-free survival (6.0 mo *vs* 2.7 mo, *P* = 0.006) were observed[70].

The results of a phase Ⅲ study comparing sorafenib alone *vs* sorafenib plus doxorubicin have been recently presented in abstract form [71]. The addition of doxorubicin to sorafenib resulted in higher toxicity and did not improve OS or progression-free survival.

In another phase Ⅱ study, first-line combination therapy with sorafenib and gemcitabine/oxaliplatin did not result in longer OS or progression-free survival compared to sorafenib alone, although the primary endpoint (4-mo progression-free survival > 50%) was reached [72]. Conventional cytotoxic chemotherapy has been investigated in a first line, phase Ⅲ trial conducted in Asia and comparing the effects of oxaliplatin/fluorouracil with doxorubicin [73]. Significant benefits of FOLFOX were found on progression free survival, while OS resulted significant only in a post-hoc analysis (6.5 mo *vs* 4.9 mo). Sorafenib in combination with other chemotherapeutic regimens, *e.g.* gemcitabine/oxaliplatin or capecitabine/oxaliplatin is currently being investigated in phase Ⅱ studies. Although the combination of cytotoxic chemotherapy and sorafenib is still being evaluated in clinical trials, this combination does not appear particularly promising.

Table 4. Results of studies with molecular targeted therapies as first line in advanced hepatocellular carcinoma.

Treatment	Trial	OS	TTP	Ref.
Sorafenib	Phase Ⅲ *vs* placebo (SHARP)	10.7 mo *vs* 7.9 mo, *P* < HR = 0.69; 95%CI: 0.55-	5.5 mo *vs* 2.8	[47]
Sorafenib	Phase Ⅲ *vs* placebo (Asia-Pacific)	6.5 mo *vs* 4.2 mo, *P* = HR = 0.68; 95%CI: 0.50-	2.8 mo *vs* 1.4 HR = 0.57;	[46]
Sunitinib	Phase Ⅲ *vs* sorafenib (SUN)	7.9 mo *vs* 10.2 mo, *P* = 0.0019; 95%CI: 1.13-1.50	4.1 mo *vs* 3.8 mo, two-sided *P* =	[64]
Brivanib	Phase Ⅲ *vs* sorafenib (BRISK-FL)	9.5 mo *vs* 9.9 mo, *P* = HR = 1.07; 95%CI: 0.94-	4.2 mo *vs* 4.1 HR = 1.01;	[65]
Linifanib	Phase Ⅲ *vs* sorafenib	9.1 mo *vs* 9.8 mo, *P* = NS; HR = 1.05; 95%CI: 0.90-	5.4 mo *vs* 4.0 HR = 0.759;	[66]
Erlotinib	Phase Ⅲ erlotinib plus eorafenib plus placebo (SEARCH)	9.5 mo *vs* 8.5 mo, *P* = HR = 0.929	3.2 mo *vs* 4.0 HR = 1.135; *P* = 0.18	[69]

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; CI: Confidence interval; HR: Hazard ratio; NS: Not significant.

1.4.2.2. Second-line:

Patients who fail first-line systemic therapy are considered to have poor prognosis, and second-line trials are warranted [74] (**Table 5**). Just recently reported, the RESORCE trial (Study of Regorafenib After Sorafenib in Patients With HCC, NCT01774344) demonstrated a significant improvement in median OS for patients treated with regorafenib vs. placebo as a second-line treatment after radiologic progression under sorafenib (10.6 vs. 7.8 months, HR = 0.62, 95% CI 0.50–0.78, p<0.001) [75]. Brivanib was also investigated in the BRISK-PS (brivanib-post sorafenib) trial, where brivanib and placebo were compared in patients who progressed on/after or were intolerant to sorafenib. Although TTP was significantly longer in the brivanib arm than with placebo (4.2 mo *vs* 2.7 mo), the primary end point of the study was not reached, as no differences in OS were observed comparing brivanib and placebo (9.4 and 8.2 mo, respectively) [76]. It is possible that imbalances in patients' recruitment, favoring the placebo arm in terms of some parameters associated with a better prognosis, contributed to the failure of the BRISK-PS trial [77]. The human anti-vascular endothelial growth factor Receptor 2 antibody, ramucirumab, has been recently studied in a second-line, phase Ⅲ in comparison to placebo [78]. Median OS for the ramucirumab group was 9.2 mo *vs* 7.6 mo for the placebo group (p = 0.14), and thus the primary endpoint of the study was not reached. However, a subgroup analysis showed that patients with elevated alpha-fetoprotein could benefit from this treatment. Therefore, a phase 3, placebo-controlled trial testing ramucirumab as a second-line treatment in patients with elevated basal alpha-fetoprotein is currently recruiting patients (NCT02435433, clinicaltrials.gov, accessed April 25, 2016). Similarly, administration of everolimus to patients who failed sorafenib as a first-line treatment did not result in an improved OS over placebo (7.6 mo *vs* 7.3 mo) [79]. Other mammalian target of rapamycin inhibitors have been tested in phase Ⅰ-Ⅱ trials, but conflicting results have been reported[77].

Treatment	Trial	OS	TTP/PFS	Ref.
Brivanib	Brivanib *vs* placebo (BRISK-PS)	9.4 mo *vs* 8.2 mo, $P = 0.3307$; HR = 0.89; 95%CI: 0.69-1.15	4.2 mo *vs* 2.7 mo, $P < 0.001$; HR = 0.56; 95%CI: 0.42-0.76	[76]
Everolimus	Everolimus *vs* placebo (EVOLVE-1)	7.6 mo *vs* 7.3 mo, $P = 0.68$; HR = 1.05; 95%CI: 0.86-1.27	3.0 mo *vs* 2.6 mo, $P = 0.01$; HR = 0.93; 95%CI: 0.75-1.15	[79]
Ramucirumab	Ramucirumab *vs* placebo (REACH)	9.2 mo *vs* 7.6 mo, $P = 0.14$; HR = 0.87; 95%CI: 0.72-1.05	2.8 mo *vs* 2.1 mo, $P < 0.0001$; HR = 0.63; 95%CI: 0.52-0.75	[78]

Table 5. Results of studies with molecular targeted therapies as second line in advanced hepatocellular carcinoma. OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; HR: Hazard ratio.

1.4.2.3. Ongoing studies:

Other compounds are currently under investigation in phase Ⅲ trial, the final results of which have not been yet reported. These include other compounds acting as antiangiogenic agents, including lenvatinib and dovitinib. These are summarized in **Table 6**. For a more complete discussion of ongoing studies and additional targets refer to a recent comprehensive review [77]. A promising approach has been obtained with the phase Ⅱ study investigating tivantinib, an inhibitor of the Met tyrosine kinase, the receptor for hepatocyte growth factor. In this study, patients overexpressing Met, the target of tivantinib, had a significant benefit over placebo [48]. Remarkably, expression of Met in patients receiving placebo was associated with a more aggressive behavior of the tumor, indicating that Met is both a therapeutic target and a prognostic biomarker. A phase Ⅲ trial comparing tivantinib and placebo as a second line therapy is currently underway. Along the same lines, a trial comparing placebo and cabozantinib, a dual Met and Hepatocyte growth factor inhibitor, has been undertaken. One of the most promising areas in the field of HCC is represented by immunotherapy. Expression of PD-1 and CTLA-4 on immune cells is associated with blockade of the anti-tumor immune response, favoring the progression of cancer[80]. In a Phase Ⅰ/Ⅱ study recently presented in abstract form nivolumab, an anti-PD-1 monoclonal antibody, induced tumor size stabilization or reduction in 67% of the patients[81]. In addition, the effects of this treatment were durable, as

previously observed in other types of cancer. A phase Ⅲ study comparing the effects of sorafenib and nivolumab in advanced HCC is currently underway (**Table 6**).

Table 6. Principal ongoing studies in advanced hepatocellular carcinoma with new molecular targeted therapies.

Study	Drug	Status
A multicenter, open-label, phase 3 trial to compare the efficacy and safety of lenvatinib (e7080) *vs* sorafenib in first-line treatment of subjects with unresectable hepatocellular carcinoma	Lenvatinib *vs* sorafenib	Active, not recruiting
A study of dovitinib *vs* sorafenib in adult patients with hepatocellular carcinoma as a first line treatment	Dovitinib *vs* sorafenib	Completed (phase 2)
A study of nivolumab *vs* sorafenib as first-line treatment in patients with advanced hepatocellular carcinoma	Nivolumab *vs* sorafenib	Recruiting

1.4.3. Combination therapy:

Sorafenib combined with classic chemotherapy: HCC is considered a poor responder to chemotherapy, which is not routinely used because of adverse events, particularly in patients with advanced cirrhosis. However, shrinkage of the tumor has been reported, although the magnitude of response is lacking consistency. This has led to the possibility to add sorafenib to a chemotherapeutic agent, as above reported, although the toxicity profile of any chemotherapeutic drug to be added to sorafenib should be kept in mind [82, 83]. Sorafenib and TACE could be promising strategies in advanced HCC treatment. The high rate of HCC recurrence after TACE may be due to its enhancement of angiogenesis and upregulation of Vascular Endothelial Growth Factor and platelet-derived growth factor receptors expression, which increases tumor angiogenesis. Therefore, combination of antiangiogenic agents with TACE, could potentially decrease the recurrence of HCC and improve survival. A phase Ⅲ study has been conducted in Japan and Korea using sorafenib in combination with TACE *vs* TACE

alone. However, combination therapy failed to show any benefit in terms of TTP (sorafenib *vs* placebo 5.4 mo *vs* 3.7 mo) or OS[84]. The results of the SPACE trial comparing sorafenib and placebo in patients undergoing TACE have been recently published. The combination of sorafenib plus TACE with drug-eluting beads was technically feasible, but the combination did not improve TTP in a clinically meaningful manner [85].

1.5. PROGNOSIS IN ADVANCED HCC:

As described before, advanced HCC is a condition where multiple actors can play a determinant role, resulting in large variability of the disease even in the same BCLC stage. Portal vein thrombosis the presence/absence of portal vein thrombosis and its extension, as well as extra-hepatic spread and alterations in liver function, can jeopardize the efficacy of specific treatments. Moreover, the natural history of the disease - even in absence of treatment - is strictly related to these variables. Finally, both natural history and the response to treatment may be influenced by molecular characteristics of the tumor. It is easy to understand how talking of prognosis "in general" for advanced HCC - as well as for all stages of HCC -sounds simplistic. The natural history of the disease is difficult to evaluate through randomized controlled trials for ethical reasons. Nonetheless, some interesting studies have tried to clarify the prognosis of untreated HCC.A meta-analysis published in 2010 evaluated more than 4000 patients included in the placebo or inactive treatment arms of 30 randomized control trials in order to estimate survival in untreated HCC patients and to evaluate factors related to a different survival [86].

The 1-year survival rate in BCLC B + C patients was 34%, with a pooled estimate 1-year survival of 25% in the subgroup of advanced HCC patients. ECOG performance status, albumin levels, prothrombin activity, portal vein thrombosis and Child Pugh score A emerged as predictors of longer survival in all HCC untreated patients. In the BCLC B + C group ECOG performance status, presence of ascites and an Okuda stage Ⅰ were significantly related with a longer survival.

A more recent retrospective cohort study evaluated 320 untreated HCC patients, 39% in advanced stage according to BCLC[87]. The 1-year survival rate for advanced HCC patients was

12%, with a median survival of 6.9 mo. ECOG performance status, INR and alpha-fetoprotein emerged as independent predictors of mortality at multivariate analysis.

Distant metastases A related emerging issue, analyzed in recent studies, has been the attempt to establish a correlation between progression and survival in patients with HCC. In order to do that, attention has been focused not only on classic OS but also on two new parameters, which were not considered in the past studies on HCC during systemic therapy. The first is TTP, defined as the time from the date of starting therapy to disease progression, evaluated by imaging (CT or MRI). The second is the post-progression survival, which is the time from disease progression to death. Along these lines, four different kinds of progression (progression patterns) have been established: intrahepatic or extrahepatic tumor growth (> 20% increase in tumor size of viable target lesion), new intrahepatic lesion and new extrahepatic lesion (including new metastasis and/or vascular invasion). According to data reported by Lee *et al* [61], patients with only metastatic disease have a better post-progression survival than those with vascular invasion or both of them (respectively 7.7, 3.8 and 3 mo), probably because of a higher rate of liver failure in patient with vascular invasion. TTP also seems to be related with survival: in fact, patients with early radiologic progression during sorafenib treatment have a much shorter survival than progressive disease patients at 4 mo (respectively 4.9 and 16.6 mo)[61]. Similar results in term of survival had already been reported by Reig *et al* [88], who showed how the progression pattern may impact on prognosis. In particular, presence of new extrahepatic lesions and/or vascular invasion appear to be correlated to a shorter post progression survival. The purpose of correlating pattern progression with survival is to identify those patients who are eligible for second line treatment, and to appropriately stratify them. In order to do that, the concept of “BCLC upon progression”, which evaluates the progression pattern of PD patients, has been introduced. In advanced HCC patients (BCLC-C) two kinds of progressions have been identified: patients who show an increase in size of an existing lesion or a new intrahepatic lesion (probably candidates for second line treatment) and patients who show extrahepatic lesions or vascular invasion, associated with a poor prognosis [88].

CHAPTER 2

Aims of the Doctoral Thesis

Aims of the doctoral thesis project can be summarized as follow:

Aim 1: Experimental study in patients with advanced HCC treated with sorafenib. Our objectives were:

i. To investigate the level and frequencies of endothelial progenitor cells (EPC) circulating in the blood of HCC patients before and after sorafenib therapy measured in 3 occasions according to the number of weeks (T0, T2, T8). T0, before the start of therapy, (T2) after 2 weeks, and (T8) after 8 weeks from sorafenib initiation.
ii. To evaluate the possible correlation between the level of endothelial progenitor cells and side effects of sorafenib,
iii. To search for a possible association of the EPC frequency with the basic parameters and the response to treatment, comparing contrast-enhanced CT or MRI images, 8 weeks after initiation of therapy.
iv. To evaluate the role of EPC in disease progression of the and their possible prognostic value in patients with HCC.

Aim 2: Contribution to the Italian national database of liver cancer (Ita.Li.Ca): The HCC cases consecutively observed in this Unit were analyzed and recorded the database. We collected, analyzed and included more than 100 consecutive cases of hepatocellular carcinoma followed in the last four years in our center, together with detailed epidemiological. The Ita.Li.Ca cohort is the largest HCC database in Italy.

CHAPTER 3

Role of Circulating Endothelial Progenitors cells (EPC) in Cancer Progression

3.1 Introduction of Angiogenesis

Vasculogenesis, angiogenesis and arteriogenesis are the three main processes for growth and development of blood vessels. See **Figure 4.**

Vasculogenesis, is the formation of vascular structures from circulating or tissue-resident endothelial progenitor cells, which differentiate and proliferate into a de novo endothelial cell tube like structure (blood vessel). During embryonic development, the process of vasculogenesis involves the growth and fusion of multiple blood islands that eventually result in the formation of a capillary network within a previously avascular yolk sac [89], then differentiation into an arteriovenous vascular system after which blood circulation begins [90]. The proliferation of blood vessels formed during neovascularization is necessary for repairing injured tissues or meeting increased metabolic demands [91]. Until a decade ago, it was thought that postnatal neovascularization occurred primarily via angiogenesis and occurring in early embryogenesis, and it does not occur in adult tissues [92, 93].

Asahara's group was the first to isolate endothelial progenitor cells (EPC) from bone marrow (BM) and showed the mobilization and contribution of EPC to neovascularization in an ischemic injury model[94, 95]. This initiated the investigation of the involvement of BM-derived EPC in tumor vascularization via post-natal vasculogenesis. In recent years, many lines of evidence point to EPCs as critical players in vasculogenesis, which is essential for normal physiological neovascularization during tissue growth, wound healing, and organ regeneration [96, 97]. Postnatal vasculogenesis also contributes to endogenous neovascularization of developing tumors, severe hindlimb ischemia, and myocardial ischemia[97]. In the adult, EPCs are believed to be recruited from the bone marrow, migrate to sites that need neovascularization, and participate in the assembly of newly-formed blood vessels [94].

Angiogenesis is defined as the physiological process involving the formation of new blood vessels from pre-existing vessels [98]. Physiological angiogenesis include embryonic angiogenesis, angiogenesis in and the menstrual cycle and wound healing [99]. Angiogenesis associated with pathological conditions includes arthritis, pulmonary diseases, diabetes,

atherosclerosis and tumor growth[100], it is now well established that tumors grow and metastasize to secondary sites in the body by angiogenesis [101]. The basic process involved in angiogenesis is proliferation of existing, activated vascular endothelial cells (VEC) culminating in the formation of tube-like structures for blood flow. Tumors secrete angiogenic factors that activate VEC vascular endothelial cells in the blood vessels in surrounding tissue. The activated VEC proliferate and release proteases and other enzymes, which modify the basement membrane and extracellular matrix (ECM). Integrins on the surface of VEC interact with their respective ligands in the ECM, which leads to their assisted and directional migration towards the tumor-derived chemotactic signal. The developing blood vessels roll up to form tube-like structures and finally the individual conduits connect to form blood vessel loops. These blood vessels are stabilized by smooth muscle cells (SMC) or pericytes, and the blood flow begins [102].

Arteriogenesis : refers to describe a distinct mechanism of blood vessel modification pertaining to the enlargement of pre-existing vessels by an increase in the vessel wall diameter [103]. Arteriogenesis may be triggered in ischemic tissue to compensate for insufficient blood flow, where resident arterioles enlarge and develop collateral arteries in response to increased shear force [104].

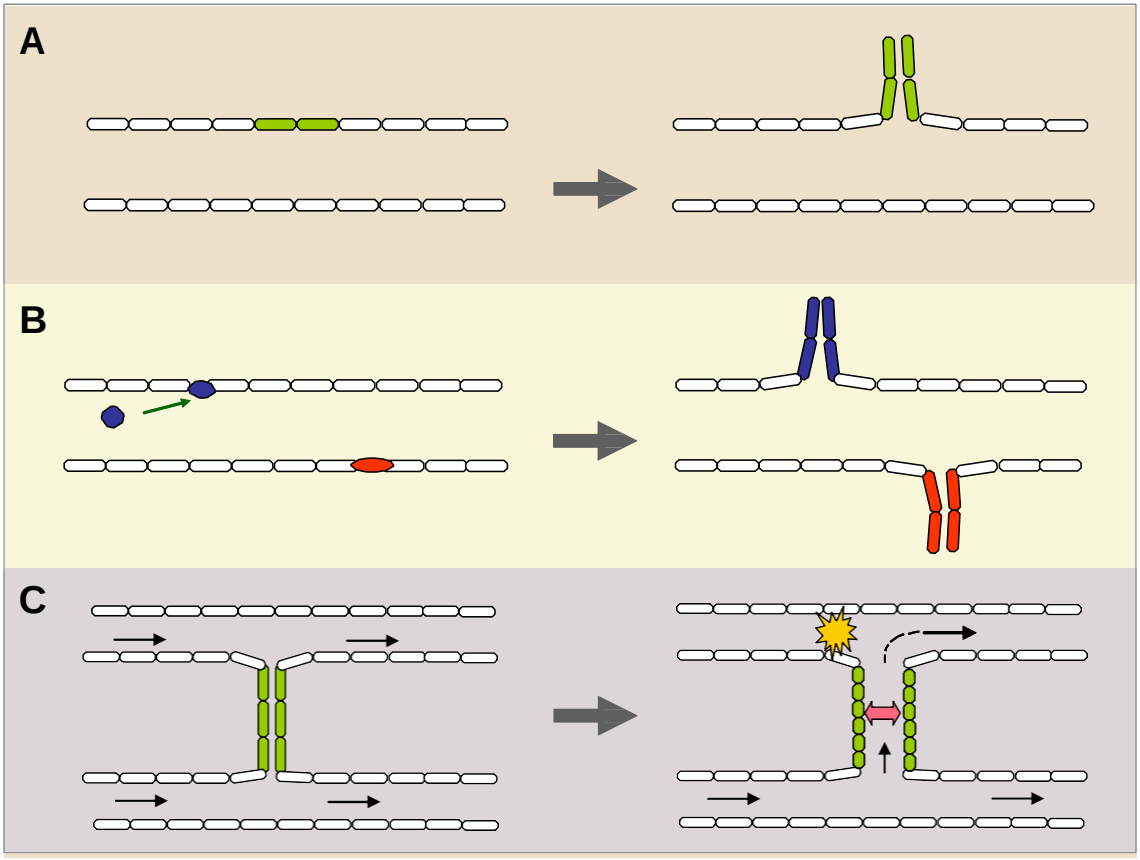


Figure 4. mechanisms of postnatal blood vessel growth.

Three different processes contribute to the formation of new blood vessels. **a)** Angiogenesis; Mature resident ECs proliferate (green) and migrate to form new vessels. **b)** Vasculogenesis; Circulating BM-derived EPCs (blue circle) or vessel- resident EPCs (red circle) proliferate and differentiate into ECs to form new vessels. **c)** Arteriogenesis (collateral growth); Smaller vessels are remodeled to increase their luminal diameter (pink arrow) in response to an increased blood flow demand, such as in the case of an occlusion (yellow). Black arrows are blood flow direction. Figure adapted from [103, 104].

3.2. REGULATION OF TUMOR ANGIOGENESIS

The normal body controls angiogenesis by maintaining ideally a balance between proangiogenic and anti-angiogenic factors ‘‘angiogenic switch’’. In case of tumor angiogenesis this balance is disturbed in favor of proangiogenic factors resulting in the transition from dormant hyperplasia to a growing hypervascularised tumor, angiogenesis remains suppressed by the presence of more anti-angiogenic than pro-angiogenic factors [105].Like normal tissues, tumors require an adequate supply of oxygen, nutrients and an effective way to remove waste products via the vasculature [106]. Tumor demands more oxygen and nutrients than available by the local supply and becomes hypoxic[107].As a response to hypoxia Angiogenesis is initiated by the release of angiogenic growth factors, such as VEGF, PDGF, basic fibroblast growth factor (bFGF), angiopoietins and heparanase from both the tumor cells and the surrounding stromal cell pericytes [108, 109].

VEGF the major stimulating factor of angiogenesis [110], VEGF consists of multiple isoforms that are produced by alternative splicing of a single gene[111], which includes VEGF-A, -B, -C, -D [112] see **Figure 5.** VEGF-A (further referred to as VEGF), the best characterized molecule of the VEGF family, is commonly overexpressed in several solid tumors and interacts primarily with two tyrosine kinase receptors; VEGF receptor-1 (VEGFR-1, Flt-1) and VEGFR-2 (KDR, Flk-2)[113]. VEGFR-2 expression is restricted primarily to the endothelial cells and is the major positive mediator in tumor angiogenesis, VEGF promotes tumor angiogenesis through many processes assisting to changes within the tumor vasculature, including endothelial cell proliferation, migration, invasion, survival, chemotaxis of bone marrow derived progenitor cells, vascular permeability and vasodilatation [107, 114]. And induce the expression of adhesion molecules on endothelial cells, which are critical for leukocyte attraction, rolling and adhesion [115].also depending on the chemoattractant composition of the tumor microenvironment, Bone marrow recruited tumor associated macrophages can act as an antitumor defense or, conversely, can exert immunosuppressive and pro-angiogenic functions [116, 117].

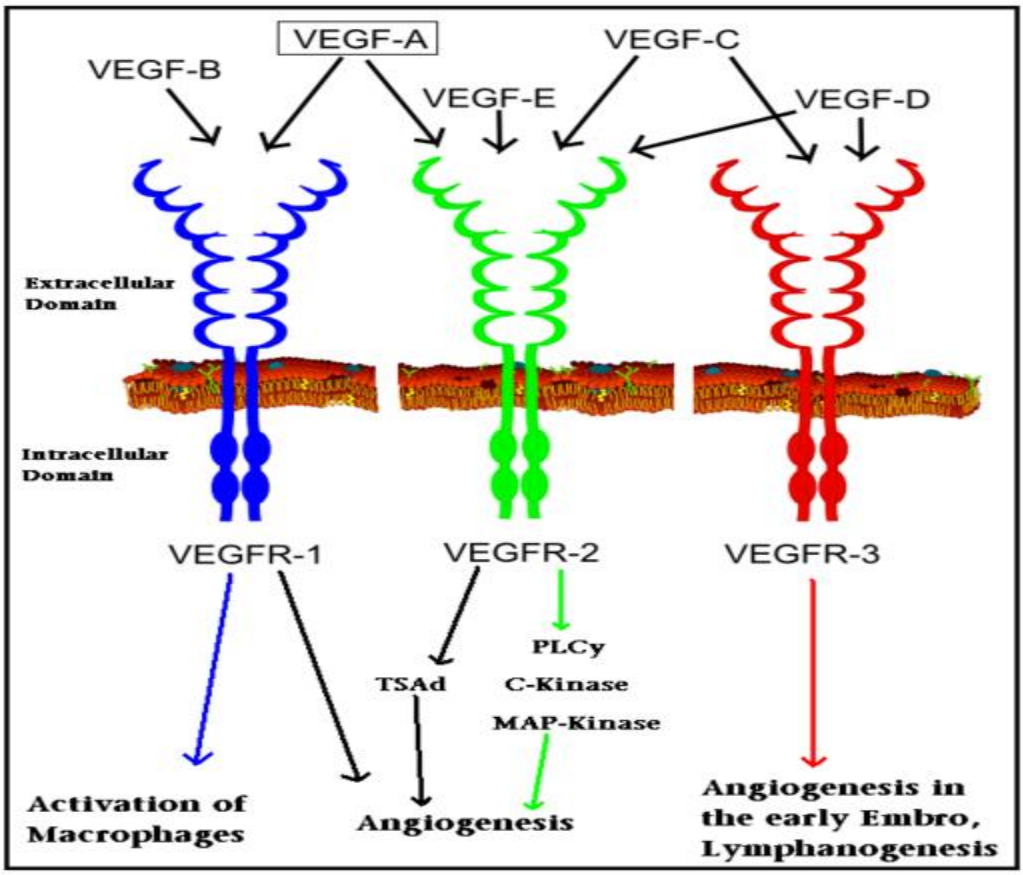


Figure 5. VEGF and its role in angiogenesis. Adapted from [118]

3.3. Mobilisation of Endothelial progenitor cells:

To support vascularization, EPCs must mobilize to the site of neovascularization and differentiate into mature ECs. active arrest and transendothelial extravasation of EPCs into the interstitial space of the growing tumor then EPC incorporation of into neovessels or paracrine support of the nascent microvasculature. Within the bone marrow, EPCs are in a quiescent state. EPCs are activated and migrate into the vascular zone of the bone marrow where proliferation is increased. Various injuries such as ischemia, atherosclerotic lesions, traumatic wound, tumor angiogenesis [119, 120]. Tumor-derived signals instigate the BM compartment to mobilize EPCs and recruit them to the tumor bed [121]. cause the frequency of EPCs in the peripheral blood to increase up to 50-fold [120]. Although the molecular pathways involved in

EPC mobilization are in the early stage of definition, the up-regulation of VEGF is thought to be a significant contributor to this mechanism [122]. VEGF is thought to be a key cytokine that effectively induces the mobilization of EPCs and HSCs into the circulation by interaction with its receptors (VEGFR-2 and VEGFR-1) [123].Hypoxia also mobilizes EPCs [124], malignant tumor growth results in neoplastic tissue hypoxia, which then mobilizes EPCs in a paracrine fashion. EPCs widely express CXCR4, which is the receptor for stromal cell-derived factor-1α (SDF-1α) and a member of the chemokine CXC subfamily . Hypoxia induces SDF-1α secretion by stabilizing hypoxia-inducible factors (HIF) in tumor cells[125] this mobilizes EPCs in the BM . SDF-1 is chemotactic for EPCs and recruits EPCs to sites of neovascularization. additional studies have demonstrated that SDF-1 is essential for the adhesion of BM-derived cells, SDF-1 may significantly help to sequester EPCs at the site of vessel formation [126]. Until now, both SDF-1α/CXCR4 and VEGFA/ VEGFRs pathways are the principal known mediators of EPC BM mobilization during cancer development, and represent potential targets for new anti-vasculogenic therapies.

3.4. Endothelial progenitor Cells:

Endothelial cells (EC) are key players in angiogenesis, forming a thin layer that lines the interior surface of blood vessels, and form a barrier between the blood and the subendothelial extracellular matrix. A major mechanism of angiogenesis is the formation of EC vessel expanding from an existing vessel. Tumor vasculature does not necessarily derive from sprouting angiogenesis, but instead, tumors may well acquire their vasculature by other mechanisms including cooption of existing vessels and by vasculogenesis.

3.4.1 Endothelial Progenitor Cells& markers

There are two types of circulating endothelial (progenitor) cells called: circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs). The term CECs is used with two definitions: First, CECs in general are endothelial cells that circulate in the blood regardless of their function and origin (they may include the endothelial cells with proliferative potential, and may contain progenitors). Second, mature CECs represent endothelial cells in circulation that have been

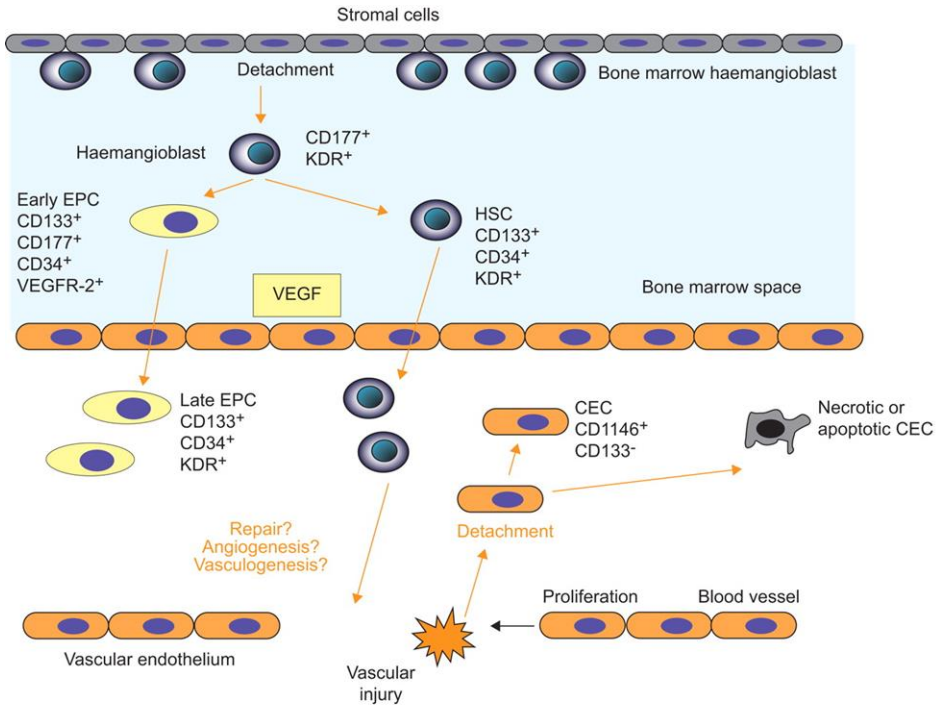
detached from the vessel wall, which have a limited growth capability, and bear a mature phenotype. Endothelial progenitor cells (EPC) are a sub-population of BM-derived cells that have the capacity to proliferate, migrate and differentiate into mature EC and contribute to the process of reendothelialization and neovascularization (**Figure 6**)[94, 95, 127, 128]. It is suggested that CEC can be distinguished from CEP, by the expression CD133 on CEP [129]. Circulating EPCs reside in the bone marrow (BM), in close association with hematopoietic stem cells (HSCs) and the surrounding BM stromal milieu[130, 131], EPCs are derived from hemangioblasts, which are precursor cells that give rise to both EPCs and Hematopoietic Stem Cells (HSCs)[132].EPCs were isolated and identified initially on the basis of their expression of VEGFR-2 and CD34; nevertheless, expression of these markers is also shared by the hemangioblast and by hematopoietic progenitors[94], The EPC and HSC also they share common markers like CD34, CD133, and CD117.EPCs were subsequently shown to also express other markers such as VE-cadherin, CXCR4, CD31, and AC133 (CD133), CD105, CD144, CD106, and CD117 (c-Kit)[130, 133](**Table 7)**.

Table 7. Classification of Human Hematopoietic Stem Cells (HSC), Endothelial Progenitor Cells (EPC), and Circulating Endothelial Cells (CEC) Based on Source of Origin and Protein Markers.

Cell type	Source of origin	Phenotypic marker
HSC	Bone marrow	CD34, CD133, CD117
EPC	Bone marrow, peripheral blood, umbilical cord blood, parenchyma and tissue specific EPC	CD34, CD133, CD117, VEGFR2, VE-Cadherin, FGFR
CEP	Bone marrow	CD31, CD34, CD133, VEGFR2, VE-Cadherin, CXCR4, CD146, vWF
CEC	Mature Endothelium	CD34, VEGFR2, VE-Cadherin, CD146, vWF

EPC markers Studies of hematoendothelial development show that CD34+ embryonic hemangioblasts do not express CD45; they acquire this marker only as they differentiate into hematopoietic progenitor cells (which become CD34+CD45+), but not if they become attached to the endothelial lineage[134, 135].Importantly, the (CD34+CD45−) cell fraction is able to generate the endothelial colony-forming cells (ECFCs) which possess de novo vessel-forming ability while CD34+CD45+ hematopoietic cells are not able to generate it[136, 137].ECFCS is one of the three different cell populations that isolated from total peripheral blood mononuclear cells (PBMCs),in the other hand another separate recent study demonstrate that ECFCs are derived from(CD34+CD45−)mononuclear cell fraction (that co-expresses CD31, CD146 and CD105) or from the (CD146 + CD45−)mononuclear cell fraction [138], So if ECFCs are derived from true EPCs, they should appear in the (CD34+CD45−) cell fraction, but not in the (CD34+CD45+) hematopoietic cell fraction. However, CD45 expression on EPCs remains to be confirmed [139]. Consequently, even the expression of (CD45 —) generally considered to be a specific pan leukocyte marker, does not differentiate between cells of the hematopoietic and endothelial progenitor[140]. Peichev and co-workers [141] showed that immature EPCS express CD34+VEGFR2+CD133+ while they mature lose the expression of CD133;this evidence is compatible with the mature ECs that line blood vessels in the adult, do not express CD133[141], In the other hand Timmermans and co-workers demonstrated that the ECFCs generated by(CD34+CD45−) cell fraction do not express CD133[137]. Many researchers have also been unable to detect the CD133 antigen on (CD34+CD45−) cells [142, 143]. In effect, it was recently shown that CD34+VEGFR2+ CD133+ cells are CD45+ hematopoietic progenitors, rather than true EPCs, and they do not generate ECFCs [136, 137], thus, CD133 should no longer be considered as an EPC-specific marker. These opposing reports make it difficult to interpret previous studies, in which EPCs were classically defined as CD133+ cells.so from all above studies showed that still difficult to identity of EPCs exactly. Recently reported data also showed that high numbers of EPCs circulate in the Peripheral Blood of patients with many types of cancers. Elevated EPC levels have been reported in the PB of patients with hepatocellular [144, 145], lung[146], Colorectal cancers [147], breast cancer [148] and as well as multiple myeloma[149] .

Figure 6. Origin and differentiation of endothelial progenitor cells (EPC). Hematopoietic stem cell (HSC)-derived EPC and circulating endothelial cells (CEC). Adapted from [150]



3.5. Detection of Endothelial Progenitor Cells

In detection of EPC there are some major issues which are number of mature CECs as well as EPCs/CEPs (both < 0.01% of circulating nucleated cells) In healthy individuals[139]. Aging subjects and males have lower levels of CD34+VEGFR2+ EPC than found in young individuals [151], the very low and in females [152], respectively, lack of unique marker proteins for the identification of both populations.

3.5.1. Methods for EPC isolation in vitro :

Currently human EPC are being isolated using 3 general approaches: see **Figure 7**

1) Peripheral mononuclear cells (MNC) cultured on fibronectin-coated tissue culture plates, two subpopulation classes of EPCs with ability to secrete angiogenic factors have been described. They are termed early and late EPCs. They are different in their morphology. Early EPCs are

spindle-shaped cells that have a peak growth in culture at 2–3 weeks and die around week 4 [153]. Late EPCs are cobblestone shaped and usually appear after 2–3 weeks in culture and can be maintained for up to 12 weeks [153, 154]. it is believed that early EPCs which are localized in the bone marrow or found immediately after migration into the circulation are CD133+-CD34+-Flk-1+ cells, whereas later stage circulating EPCs are still positive for CD34+-Flk-1+ but lose CD133 and begin to express other cell surface markers typical to mature ECs [95].

2) CD34+ cells cultured on fibronectin, reportedly giving rise to CD34+VEGFR2+ endothelial progenitor cells, **3)** Peripheral mononuclear cells (MNC) cultured in the colony-forming unit-ECs (CFU-ECs) or colony forming unit-Hill (CFU-Hill) cells; the circulating angiogenic cells (CACs); and the endothelial colony-forming cells (ECFCs). Although the CFU-Hill cells are similar to EC in characteristic properties and protein expression, they neither formed capillaries nor proliferated extensively in vitro. while The ECFC conversely have been shown to express antigens are similar to endothelium and formed capillaries in vitro and in vivo[155-157]. These findings led to the conclusion that ECFCs more recently renamed endothelial outgrowth cells (EOCs), display features that are consistent with the EPC related phenotype [158, 159]. Finally, a disadvantage of the study of in vitro cultured “EPCs” is that these cells during the culture process may have acquired or lost properties, as compared to their non-cultured counterparts (circulating EPCs) from which they originate.

3.5.2. Use of flow cytometry to quantify circulating EPCs:

Flow cytometry is an alternative approach for quantify and study circulating EPCs [160]. With this technique, multichannel Fluorescence-Activated Cell Sorting (FACS), Peripheral mononuclear cells (MNC) can be labeled with endothelial-specific antibodies conjugated with different fluorochromes. [161], This method has the advantage that multiple cell parameters can be measured at the same time, also detect subpopulations such as “bright” vs “dim” or “low” expression and the numbers of cells can be accurately quantified. Most of flow-markers used to identify EPCs are also shared by other circulating hematopoietic derived cells (mostly myeloid cells), as well as by mature ECs. Accumulating evidence supports the concept that strictly interconnects hematopoietic and endothelial cells [140], in particular, a hemogenic

endothelium has been shown to generate hematopoietic cells, at least during development [162]. This discovery of the embryonic process of endothelial to hematopoietic transition which A small subset of cells called hemogenic endothelial cells (HE) undergoes the EHT by becoming pre-hematopoietic stem and progenitor cells (Pre-HSPC) Eventually after losing all their endothelial characteristics they become HSPC. may also occur during postnatal life, this may overlap phenotypically as well as functionally [163].

The lack of EPC-specific markers as stated above has led to the proposal of different flow cytometry protocols makes it necessary to use a combination of markers, but until now there is no universal accepted combination [139, 164] and at this moment based on their morphological and proliferative potential in cell culture.

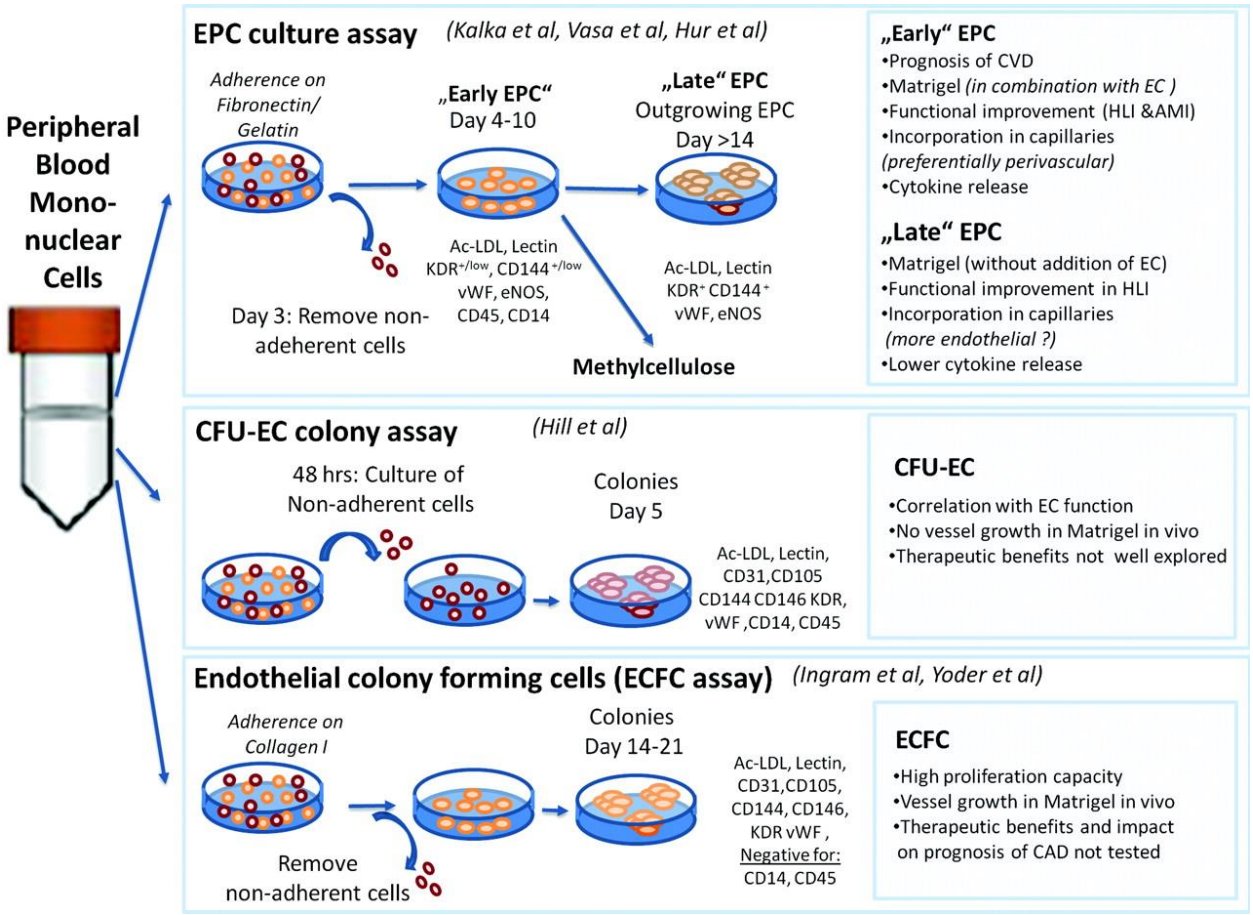


Figure 7. An overview of the most common methods used to isolate EPCs. adapted from [139]

3.6. MATERIALS AND METHODS:

3.6.1. Patients and Eligibility:

This study protocol verified compliance with the standards of Good Clinical Practice of the European Union and was in accordance with the ethical principles expressed in the Declaration of Helsinki. All patients provided written informed consent before study participation. All patients were treated at Florence Hospital AOUC.

To be eligible for the study patients needed to be:

- Greater than 18 years of age
- Histological or radiological diagnosis of HCC.
- Advanced-stage disease (BCLC-C), or presence of extrahepatic metastasis and / or neoplastic portal thrombosis and / or systemic symptoms (ECOG performance status> 0).
- Intermediate stage disease (BCLC-B), or single large HCC or multifocal disease who are asymptomatic and have no vascular invasion or extrahepatic metastasis.
- Unsuitable for surgical treatment or locoregional except TACE.
- Liver function defined as a class of Child-Pugh not exceeding A6.

3.6.2. DURATION OF THE STUDY:

The duration for every patient was about two months, after which the patient continued the treatment provided in international guidelines for the advanced hepatocellular carcinoma.

3.6.3. Treatment and Sample Collection:

Eligible Patients were treated with sorafenib 400 mg once or twice daily depending on their clinical condition, Treatment was continued until progression, unacceptable toxicity. Patients were observed once every 2 weeks for serial laboratory testing and physical examinations, Tumor assessment was performed after 8 weeks at (T8) of beginning of therapy using computed tomography or magnetic resonance imaging according to Modified RECIST criteria.

Blood samples were collected in blood collection tube using EDTA as an anticoagulant before treatment (T0) and at 2 and 8 weeks after the start of therapy (T2, T8). Further processing of blood samples for EPC analysis was conducted within 24 h of collection.

3.6.4. Measurement of EPC Levels:

EPC measurement and enumeration in this study was done through a collaboration with the Immunology Group (Prof. Annunziato). Blood samples were examined and analyzed the subpopulation of cells expressing (TotalCD34+), (CD34+/CD133+), (CD34+/KDR+), (CD34+/133+/KDR+), (CD34+/133-/KDR-) by six-color flow cytometry with a panel of monoclonal antibodies to exclude other hematopoietic cells.

3.6.5. Statistical Analysis:

Statistical analyses were performed with Microsoft excel software. A paired t test was used to examine the change in EPCs levels after treatment at T2, T8. Statistical significance was taken as $p < 0.05$.

3.7. Results:

Clinical data of all patients were collected prospectively in a computer database. Nine patients were enrolled to the study, and their demographic information is listed in (**Table 8**). There were 8 males and 1 female patients with a median age of 69 (33-76) years. Twenty two percent were seropositive for hepatitis B virus surface antigen (HBsAg), 55% of patients had liver cirrhosis, 33% had extrahepatic metastasis and 33% had macrovascular invasion, 77% were classified as BCLC stage C disease.

At the T8 re-evaluation, 44% of patient had demonstrated progression of the disease, 33% patients had stable disease, while the mean of serum Alpha-fetoprotein of the patients at T0,T2,T8 was 429, 1876, 1918 (ng/ml) respectively (**Table 9**). The median progression-free survival (PFS) was 6 months [95% confidence interval (CI) 1.8-10.1].

Baseline and Posttreatment Levels of EPCs:

Mean baseline levels at T0 of EPCs [(total CD34), (CD34+/CD133+), (CD34+/KDR+), (CD34+/133+/KDR+), (CD34+/133-/KDR-)] are indicated in (**table 10**). Further analysis showed that baseline levels were high in all patients and had a decreased level after 4 or 8 weeks of therapy, (**Figure 8**). However, the results were statistically significant only for some phenotypes like in **(total CD34+)**, where the P value was (p = 0.023) at T2 and (0.081) at T8, and in **(CD34+/133-/KDR-)** were it was at T2, T8 (P=0.014, 0.025) respectively. In others phenotypes the decline did not reach statistical significance. In **(CD34+/CD133+)** a significant decline was observed only at T2. (P=0.017).

Next, we classified the patients into two groups, i.e. patients in whom sorafenib induced stability of the disease (responders) and patients with progression of the disease (non-responders) (Table 11,12). In the two groups, we analyzed the EPCs and compared them using t test which were higher at baseline (T0) in patients with stable disease than in patients with progression of the disease. These levels decline after 2 and 4 weeks of treatment, although the decline did not reach statistical significance in all EPCs phenotypes except in **CD34+/133- /KDR-**

the decline was higher in patients with stable disease and was statistically significant when compared to T0 levels.

Baseline EPCs levels were also examined and their correlation with the patient characteristics were investigated as listed in (table 8,9). We found a few significant associations between some phenotypes of EPCs and tumor size (CD34+KDR+, p value= 0,02). Other correlations had a trend toward significance (CD34+CD133+) p value= 0,07, (total CD34+) p value= 0,10, (CD34+CD133+KDR+) p value= 0,19 (CD34+CD133-KDR-) p value= 0,11 .(**Figure 9**)

(CD34+CD133+KDR+) also had significant association with platelet count (p value= 0,03), with absolute lymphocyte count (p value= 0,009), while (total CD 34+) like (CD34+CD133+KDR+) had a significant association with platelet count and absolute lymphocyte count (p value = 0,04, 0,03, respectively). while there is no significant association with other variables.

Table 8. Patient characteristics.

Characteristics	Patients	
	number	%
Total	9	100
Median age (range), years	69 (33-76)	
Females/males	1/8	11/89
Hepatitis virus		
HBsAg positive	2	22
Anti-HCV positive	1	11
Hepatitis C/B coinfection	1	11
Nonalcoholic steatohepatitis(NASH)	3	33
alcoholic steatohepatitis (ASH)	2	22
Liver Cirrhosis	5	55.5
Sites of disease		
Liver	8	89
Lung	1	1
Extrahepatic metastasis or macroscopic vascular invasion		
Extrahepatic metastasis	3	33
Macroscopic vascular invasion	3	33
AFP >400 ng/ml at T0	2	22
AFP >400 ng/ml at T2	2	22
AFP >400 ng/ml at T8	4	44

Characteristics	Patients	
	number	%
ECOG PS AT T0		
0	9	100
1	0	0
2	0	0
ECOG PS at T2		
0	5	55.5
1	4	44.5
2	0	0
ECOG PS at T8		
0	3	33
1	22	22
2	22	22
4	1	11
5	1	11
Child-Pugh score		
A	6	66
B	3	33
BCLC STAGE		
B	2	22.2
C	7	77.7
CLIP score		
0	0	0
1	2	22
2	4	44.5
3	2	22
4	0	0
Treatment response		
Complete response	0	0
Stable disease	3	33
Progressive disease	6	66
HCV = Hepatitis C virus; AFP = a.-fetoprotein; ECOG PS = Eastern Cooperative Oncology Group performance status		

Table 9. Laboratory features of HCC Patients

Parameter	Mean ± SD
Hemoglobin (gr/dl)	12.5 ± 1.47
Platelet (10ˆ3/mmc)	153.5 ± 109
Leucocyte count (g/l)	6.3 ± 4.17
INR	1.13 ± 0.14
Bilirubin (Total)(mg/dl)	1.4 ± 0.98
Creatinine (mg/dl)	0.86 ± 0.11
Albumin (g/dl)	3.5 ± 0.7
ALT (IU/L)	29 ± 14.7
Alkaline phosphatase	153 ±57.7
Gamma GT	183 ± 167.5
Alpha-fetoprotein (ng/ml) T0	429 ± 796
Alpha-fetoprotein (ng/ml) T2	1876 ± 4524
Alpha-fetoprotein (ng/ml) T8	1918 ± 3793

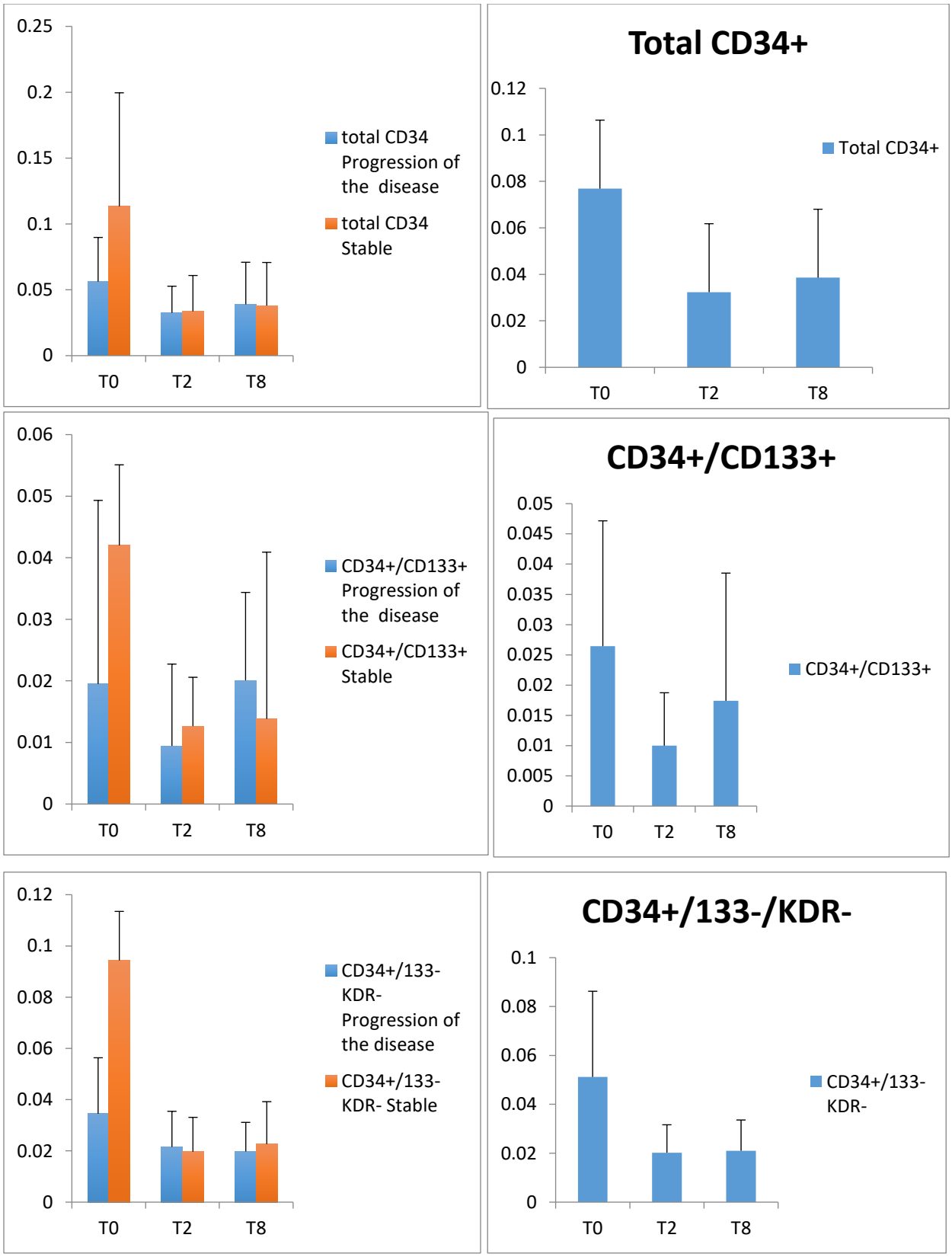


Figure 8. Pretreatment and posttreatment of EPCs levels.

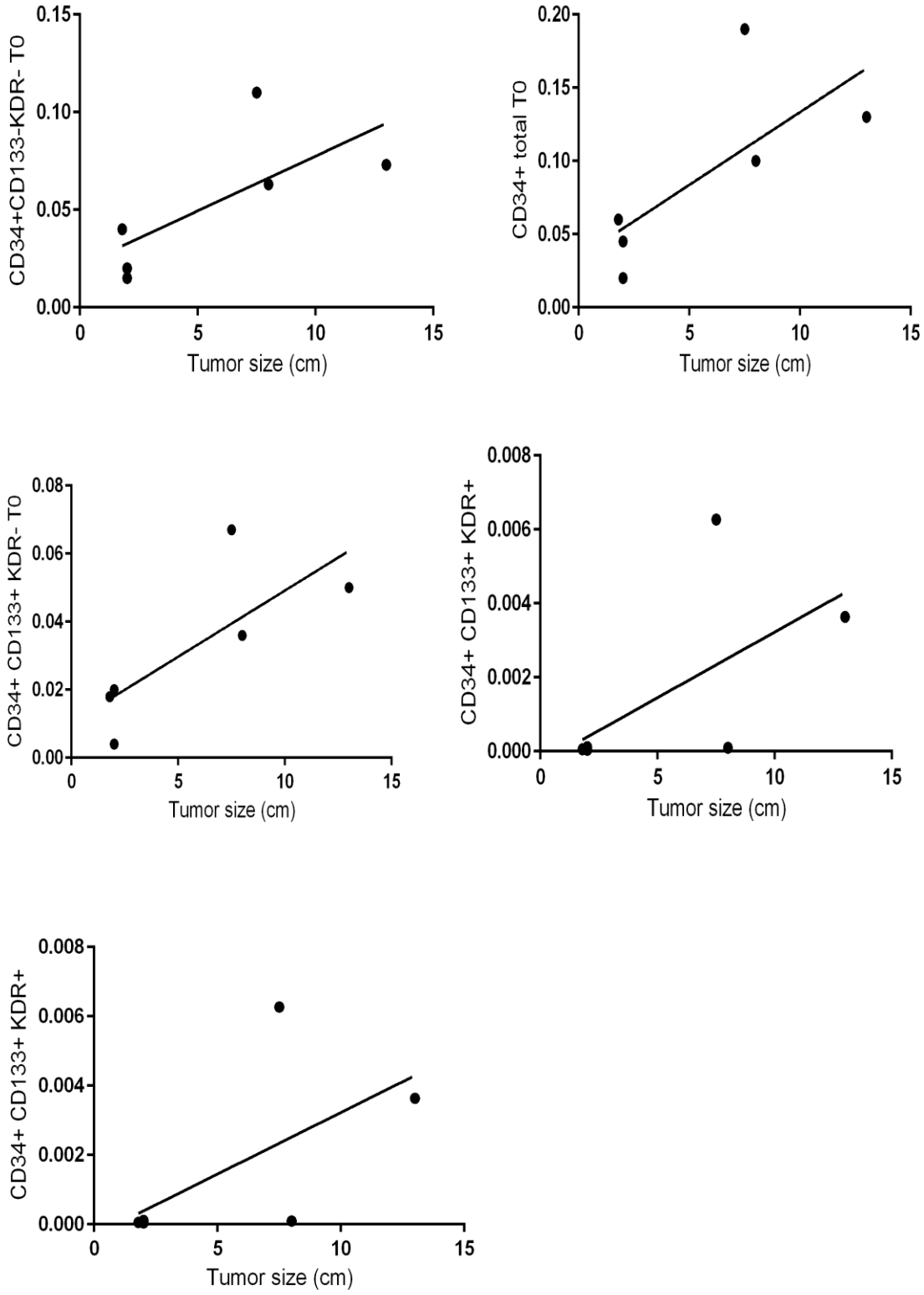


Figure.9. correlation between EPCs baseline level and tumor size using linear regression.

	Patients	T0	T2 (2weeks)		T8 (8 weeks)	
		Mean	Mean	P value	Mean	P value
Total CD34+	Stable	(0.11)	(0.03)	(0.17)	(0.038)	(0.14)
	Progression	(0.05)	(0.03)	(0.22)	(0.039)	(0.45)
	All	(0.07)	(0.03)	(0.023)	(0.0385)	(0.08)
CD34+/ CD133+	Stable	(0.042)	(0.012667)	(0.14)	(0.0138)	(0.12)
	Progression	(0.019)	(0.00945)	(0.20)	(0.02008)	(0.96)
	All	(0.0264)	(0.0099)	(0.017)	(0.01740)	(0.29)
CD34+/KDR+	Stable	(0.00072)	(0.000250)	(0.40)	(0.000215)	(0.35)
	Progression	(0.000562)	(0.000175)	(0.41)	(0.000039)	(0.45)
	All	(0.00140)	(0.000276)	(0.30)	(0.000114)	(0.27)
CD34+/133 +/KDR+	Stable	(0.0033)	(0.00003367)	(0.21)	(0.000038)	(0.20)
	Progression	(0.00008125)	(0.0000325)	(0.16)	(0.000039)	(0.27)
	All	(0.00115)	(0.00003233)	(0.17)	(0.0000386)	(0.18)
CD34+/133 -/KDR-	Stable	(0.094)	(0.0196)	(0.01)	(0.022)	(0.02)
	Progression	(0.034)	(0.0215)	(0.30)	(0.019)	(0.21)
	All	(0.0557)	(0.0202)	(0.01)	(0.021)	(0.02)

Table 10. Pretreatment and posttreatment EPC levels.

(p values by paired t test to compare the post-treatment T2, T8 EPC levels to the baseline levels).

3.8. Discussion:

HCC is a tumor where blood supply is very important. An indirect demonstration is the efficacy of anti-angiogenic drugs such as sorafenib, a multikinase inhibitor which represents the only approved pharmacotherapy for HCC. Considering the fact that only a portion of patients with advanced HCC respond to sorafenib treatment, we evaluated the levels of EPC in a group of patients with advanced HCC, to establish possible correlation with disease characteristics at baseline and with the response to treatment.

Previous studies have investigated the possible role of EPC in HCC. Higher circulating levels of EPCs were found in patients with unresectable HCC as compared to patients with resectable HCC[144], and higher levels were detected in resected patients undergoing recurrence. Sieghart et al. also showed that EPC are increased in patients with HCC [165]. In patients receiving sorafenib, it was found that while EPC did not correlate with baseline characteristics, a high baseline level was a significant independent predictor of poor survival [166]. Our data indicate that baseline levels of some of the different EPC phenotypes, analyzed by flow cytometry, correlated with tumor burden. No other correlations with tumor characteristics were found, while some EPC phenotypes correlated with selected types of blood cells.

Additional data have indicated that mobilized EPCs could participate in tumor vasculogenesis of HCC, contributing to progression of this type of cancer [167]. Similar data were provided in a study in patients undergoing liver transplantation, where enhanced CXCL10/CXCR3 signaling in small-for-size liver grafts directly induced EPC mobilization, which further promotes tumor growth. [168].

We also tested the effects of sorafenib on the levels of circulating EPC. One of the critical points in the therapy of advanced HCC is related to the fact that we do not have any tests which could predict the likelihood of a patient with advanced HCC to respond to sorafenib therapy[169]. In the studies presented herein we have obtained preliminary data separating patients in whom sorafenib induced stabilization or regression of the tumor (responders) from those who did not benefit from treatment with this drug. It should be underscored that only a small number of

patients was analyzed, and the study is still ongoing. Nonetheless, there was a clear trend towards a higher frequency of different types of EPC at T0 (baseline) in patients who responded to sorafenib than in those with progressive disease. This observation, if confirmed in a larger series of cases, may have several implications in the field of HCC. EPC are a proxy of the angiogenic process, and therefore it is biologically conceivable that patients with tumors with the more active angiogenic process are more likely to respond to angiogenesis inhibition with sorafenib. In this respect, further studies will also establish whether elevated EPC in baseline conditions may be validated as a reliable biomarker to predict the likelihood to respond to sorafenib.

Another element which deserves attention is the fact that EPC, in most studies, have been shown to correlate with tumor aggressiveness. Also in this case, there is biological plausibility, as angiogenesis is a feature of the aggressive phenotype of HCC. This implies that even though the patients with higher EPC frequencies may be more likely to respond to sorafenib, their ultimate outcome may be worse than in the other group of patients.

In conclusion, these data, albeit preliminary, provide novel information that may be useful, if confirmed in larger studies, for the management of patients with HCC and specifically those treated with sorafenib.

CHAPTER 4

Italian Liver Cancer (ITA.LI.CA) Group

4.1. What is ITA.LI.CA?

Its abbreviation of Italian liver cancer, it is group of 24 Italian medical institutions, the ITA.LI.CA database contains data from 6595 patients with HCC diagnosed consecutively from 1987 to 2015. Since 2007, the ITA.LI.CA. database has included follow-up clinical and imaging data that were collected prospectively and updated every 2 years.

4.2. AIMS OF ITA.LI.CA:

The first goal of the Group was to create a uniquely powerful resource for liver cancer research comprised of clinical and epidemiological data. With an initial goal of developing a cohort of all HCC patient in all Italian liver cancer centers and to undertake specific analyses in the area of liver cancer.

4.3. Our key Contribution to the Italian national database of liver cancer (ITA.LI.CA):

We collect the cases of HCC in Florence hospital diagnosed from 2011 to 2015. We analyzed and included more than 100 consecutive cases of hepatocellular carcinoma followed in our center and detailed epidemiological and clinical data. Many variables in the database like: age, gender, etiology of the underlying liver disease, presence of cirrhosis, Child–Pugh [C–P] class, modality of HCC diagnosis, surveillance interval, serum alpha-fetoprotein (AFP), Barcelona Clinic Liver Cancer (BCLC) tumor stage and others.

4.4. Data concluded and published from our contribution to Ita.Li.Ca:

The data collected from our center played a role in two major studies published by Ita.li.ca group, providing very important and insights to hepatocellular carcinoma epidemiology, surveillance and treatment approach in Italy.

4.4.1. The evolutionary scenario of hepatocellular carcinoma in Italy: an update[170]

This study was aimed at updating the summary of HCC in Italy, comparing the epidemiological and clinical features collected over the last three quinquiennia (5 years) by about 24 centers in Italy. they included about 5,192 patients diagnosed with HCC from January 1st 2000 to December 31st 2014. Patients were allocated into three groups according to the year of diagnosis: G1=2000–2004 (1147 [22.1%] patients); G2=2005–2009 (1,624 [31.3%]) and G3=2010–2014 (2421 [46.6%]). In this study it is described that many changes have occurred in HCC features and management. The most important ones are:

1. the fast-growing prevalence of tumors related to metabolic disorders and cryptogenic liver disease, emphasizing the need of specific programs of primary prevention:
   HCCs developing in the setting of NAFLD and cryptogenic liver disease are breaking out even in Italy, where about 13% of HCCs currently have these backgrounds. The prevalence of NAFLD/cryptogenic was changed (from 1.1% to 12.6%) and non-viral multietiological tumors (from 0.02% to 4.9%) strikingly increased over time, while The prevalence of HBV infection and viral multi-etiology also decreased in the last 5 years Among non-viral patients, “pure” alcoholic and “other causes” cases significantly decreased in the last period, whereas HCV infection remained the main risk factor in all periods, but its prevalence significantly decreased during the two-first periods, remaining stable thereafter.
2. The favorable stage migration because of the wider and more appropriate surveillance of patients at risk:
   The most commonly used surveillance was semi-annual, and this modality progressively increased over time from 79.8% to 87.7% (*P*<.001), Hepatocellular

carcinoma was diagnosed under surveillance is more than half of the cases, and this proportion significantly increased over the three periods from 55.0% to 61.6%, at the expense of incidental detection (from 32.8% to 25.5%) (**Fig. 10).**

The percentage of HCCs detected during surveillance in our series (around two-thirds) is much higher than in the USA, it remains lower than in Japan, where this practice is managed through a national health program. Significant changes also occurred in cancer size at diagnosis: small tumors (≤2 cm) increased in the first two periods, intermediate-size tumors (2.1–5 cm) progressively decreased, whereas large cancers (>5 cm) increased. the increment of large HCCs may be as a result of two causes: (i) the rising proportion of NAFLD/NASH and cryptogenic cases, which are surveyed more rarely; (ii) the increased prevalence of Child–Pugh B patients, as the liver echo pattern becomes progressively coarser with the advancement of cirrhosis, making it more difficult to recognize small HCCs.

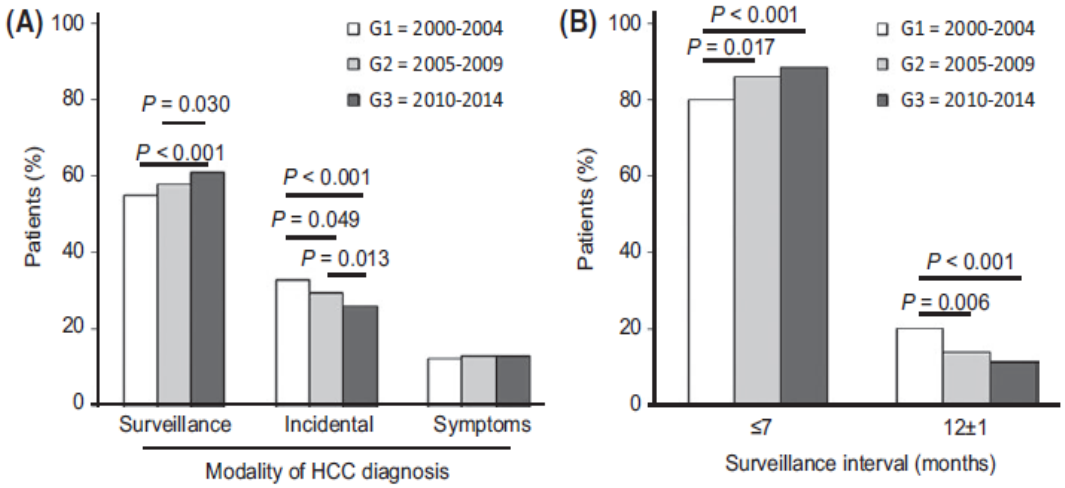


Figure.10. Modality of HCC diagnosis (A) and surveillance interval (B) of patients with HCC across the periods: 2000–2004,2005–2009 and 2010–2014.adapted from [170].

3. The refinement of all therapeutic approaches, likely accounting for the improved survival observed in the last *quinquennium:*

The median lead-time- adjusted overall survival (OS) progressively increased across the three periods assessed in this study; The improvement in survival occurred in both viral and non-viral patients and this improvement became statistically significant in the last 5 years (from 30.7 months [95% CI 27.7–33.7] to 32.2 months [28.9–35.6] to 40.4 months [34.3–46.5] [*P*<.001]). The corresponding 1, 3 and 5-year survival rates were as follows: 72.3%, 73.2%, 73.6%, and 44.2%, 47.0%, 51.2% and 27.7%, 33.0%, 39.2% respectively.

Also, the prognosis of patients undergoing percutaneous ablation and TACE significantly improved over the three periods (*P*=.001 and *P*=.002, respectively). Patient survival significantly increased after 2009. This improvement was attributed to three major factors:(i) the increased prevalence of very early HCCs; (ii) the replacement of PEI by the more effective RF and (iii) the refinement of patient selection for each treatment, ensuring better outcomes. Moreover, survival improved in all treatable BCLC stages except the advanced stage, where this trend did not reach statistical significance despite the advent of Sorafenib.

4.4.2. Hepatocellular carcinoma recurrence in patients with curative resection or ablation: impact of HCV eradication does not depend on the use of interferon [171]:

This study investigates the benefit of SVR on HCC recurrence using the Italian Liver Cancer (ITA.LI.CA.) group cohort, and to compare the observed recurrence rate with that of patients with SVR achieved by IFN based or IFN-free regimens, it demonstrates that in patients with successfully treated, HCV-related early HCC, SVR obtained by IFN-based or IFN-free regimens reduces tumor recurrence significantly without differences related to the anti-viral strategy used. The present study suggests that the positive impact of anti-viral therapy on HCC recurrence is only the effect of virological eradication, these data support the current use of IFN-free regimens in this particular

clinical setting and provide proof of their clinical effectiveness in terms of reducing HCC recurrence.

We evaluated 443 patients with HCV-related cirrhosis and Barcelona Clinic Liver Cancer Stage A/0 HCC who had a complete radiological response after curative resection or ablation. Active HCV infection was present in 328, selected from the Italian Liver Cancer group cohort; 58 patients had SVR achieved by IFN-free regimens after HCC cure, and 57 patients had SVR achieved by IFN-based regimens after HCC cure.

In the present study, it was found that HCC recurrence developed in 142/328 (43.3%) patients with active HCV infection, in 16/58 (27.6%) patients achieving SVR following IFN-free regimens, and in 22/57 (38.6%) patients achieving SVR following IFN-based therapies **(Table 11**). The 6-month recurrence rates were 9.5%, 5.2% and 3.7%, and the 2-year recurrence rates were 40.6%, 26.3% and 15.2% in patients with active HCV infection, with SVR achieved following IFN-free treatment, and with SVR achieved following IFN-based therapies, respectively. This demonstrates that SVR by IFN-based or IFN-free regimens reduced the HCC recurrence rate significantly without differences related to the therapeutic strategy.

Consistent with these data, TTR by Kaplan–Meier curves was significantly shorter in patients with active HCV infection compared with those with SVR achieved following either IFN-free ($P = 0.02$) or IFN-based ($P < 0.001$) treatments. TTR was similar in patients with SVR achieved following IFN-free or IFN-based ($P = 0.49$) strategies. Consistent with these data, recurrence rates at 6 months, 1 year, 2 years and 3 years did not differ significantly between these two groups ($P = 0.66$, $P = 0.19$, $P = 0.34$ and $P = 0.95$ respectively).

We also showed that baseline AFP, bilirubin and creatinine levels as independent predictors of HCC recurrence, bilirubin (HR = 1.43; 95% CI = 1.02–2.00; $P = 0.03$), creatinine (HR = 1.42; 95% CI = 1.11–1.83; $P = 0.006$) and a-FP (HR = 1.001; 95% CI = 1.000–1.003; $P = 0.04$) ,indicating that factors related to tumor aggressiveness (i.e. AFP), advanced liver disease (i.e. bilirubin) and liver disease prognosis (i.e. bilirubin and creatinine) increase tumor recurrence risk.

Table 11 | Follow-up of 443 patients with complete response after HCC treatment, stratified according to HCV infection status.[171]

	Groups		
	Active HCV infection (*N* = 328)	SVR by IFN-free therapies (*N* = 58)	SVR by IFN-based Therapies* (*N* = 57)
Recurrence during follow-up, *n* (%)	142(43.3)	16(27.6)	22 (38.6)
Follow-uplength,median(range)	17 (1–95)	18 (3–90)	34 (0–138)
Recurrence rates			
6-month	9.5%	5.2%	3.7%
1-year	21.0%	12.9%	5.6%
2-year	40.6%	26.3%	15.2%
3-year	54.5%	33.5%	29.3%
4-year	60.7%	39.1%	41.1%
5-year	64.5%	39.1%	41.1%
Median time to recurrence, mo. (95% CI)	31(26–38)	72.0 (40.8–N.A.)	82.3 (39.8–N.A.)

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