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DOCTORAL COURSE IN
BIOMEDICAL SCIENCES AND BIOTECHNOLOGY

CYCLE XXX

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eNOS and *ANGPT2* polymorphisms in advanced hepatocellular carcinoma patients receiving Sorafenib

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1. INTRODUCTION

1.1 Hepatocellular carcinoma (HCC)

1.1.1 Epidemiology

Primary liver cancer, and specifically hepatocellular carcinoma (HCC), represent the sixth most common cancer and the third most frequent cause of cancer-related death worldwide, with 841,080 new cases and 781,631 deaths in 2018 (Figure 1). High rates were in East/Southeast Asia, several areas of Africa and in southern Europe (Figure 2)[1].

HCC represents about 90% of primary liver cancers and constitutes a major global health problem. The incidence of HCC increases progressively with advancing age in all populations and HCC has a strong male preponderance, with a male to female ratio estimated to be 2–2.5:1 (Figure 3)[1].

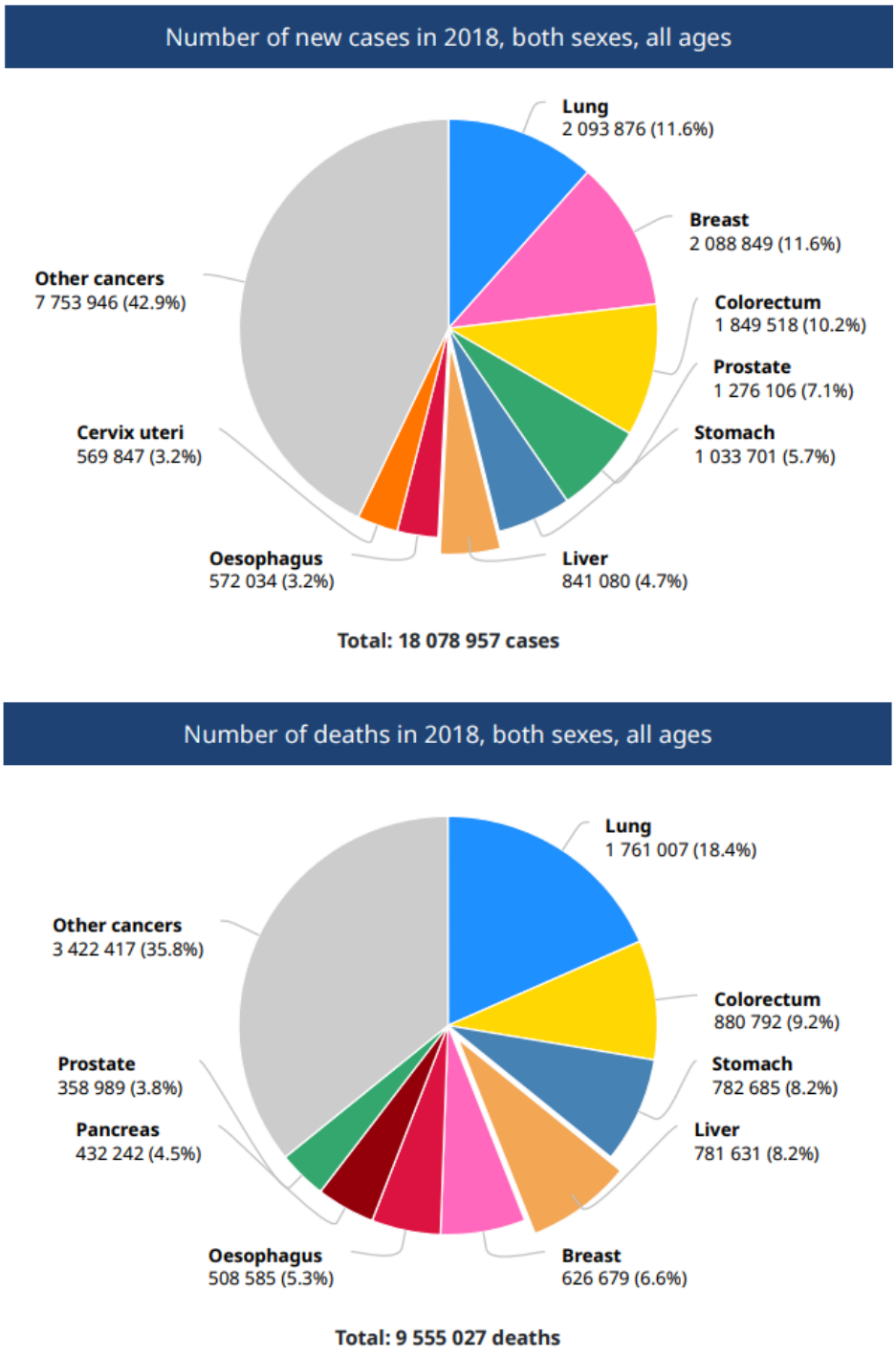


Figure 1. Incidence and mortality of the most common cancers, in both sexes worldwide. (Adapted from "The Global Cancer Observatory", September, 2018[1])

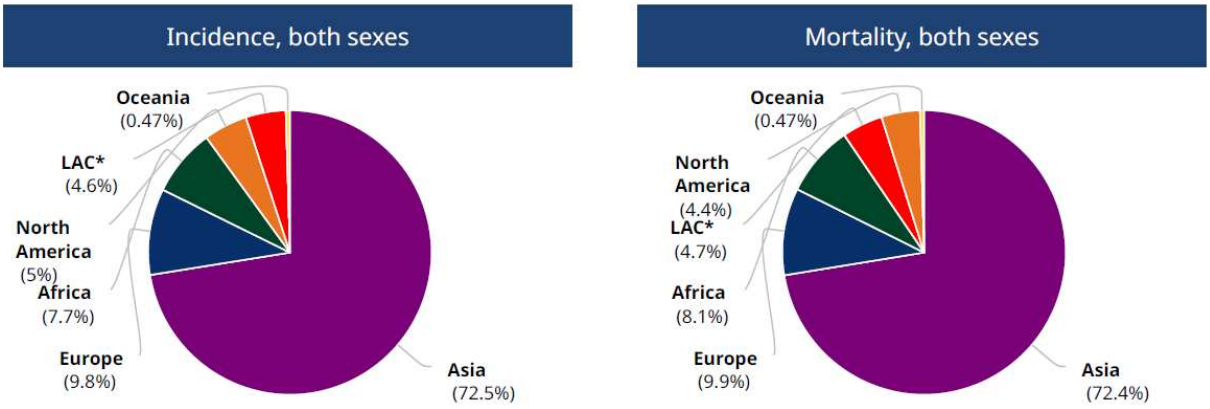


Figure 2. Liver cancer incidence and mortality worldwide. (Adapted from "The Global Cancer Observatory", September, 2018[1])

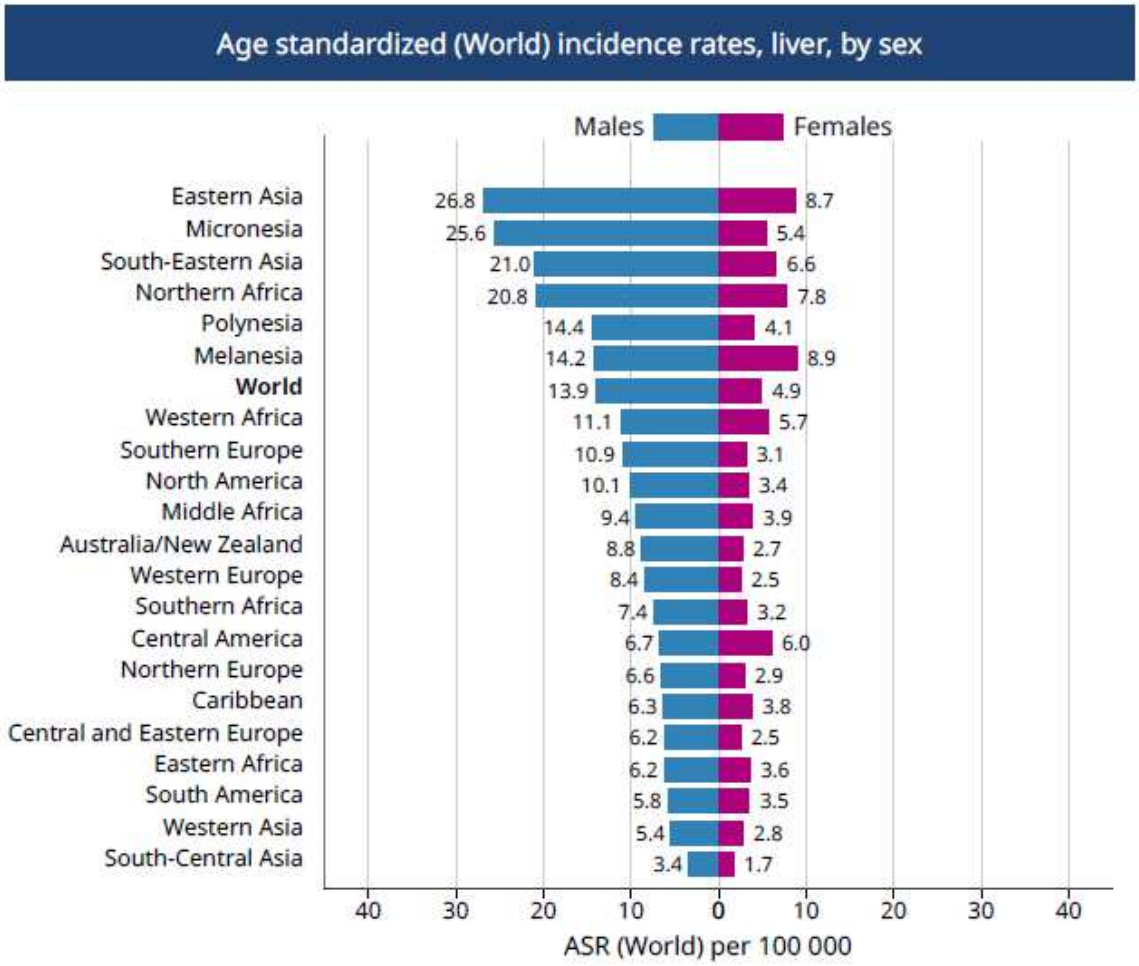


Figure 3. Age standardized incidence rates* (ASR) per 100.000 people. *ASR is the number of new cases or deaths per 100,000 people a year that a population should have if it had a standard age structure. Standardization is needed when comparing many populations that differ in age, because

age has a great influence on cancer risk) (Adapted from "The Global Cancer Observatory", September, 2018[1])

1.1.2 Etiology and risk factors

Approximately 90% of HCCs are associated with a known underlying etiology[2] (Table 1). Chronic infections with hepatitis B (HBV) and C viruses (HCV) are the main risk factors for HCC and they are responsible for about 85% of HCC cases worldwide, with a prevalence of hepatitis B in Asia and Africa and of hepatitis C in Japan and Western world[2].

Other relevant risk factors alcohol intake[3], tobacco[4], some inherited metabolic diseases and the metabolic syndrome, represented by obesity, diabetes, hyperlipemia and hypertension [5,6].

Cirrhosis is an important risk factor for HCC, and may be caused by chronic viral hepatitis, chronic alcohol abuse, acquired and inherited metabolic diseases, such as NAFLD, as well as genetic haemochromatosis, or in some cases alpha-1-antitrypsin deficiency. All etiologic forms of cirrhosis may be complicated by tumor formation, but the risk is higher in patients with chronic viral hepatitis[7].

	Alcohol (%)	HBV (%)	HCV (%)	Others (%)
Europe				
Western	32	13	44	10
Central	46	15	29	10
Eastern	53	15	24	8
North America	37	9	31	23
Andean Latin America	23	45	12	20
Asia				
East Asia	32	41	9	18
Asia-Pacific	18	22	55	6
South-East Asia	31	26	22	21
Africa				
North Africa, Middle East	13	27	44	16
Southern (sub-Saharan)	40	29	20	11
Western (sub-Saharan)	29	45	11	15

Table 1. Geographical distribution of main risk factos for primary liver cancer world-wide. (Adapted from EASL guidelines 2017[7])

1.1.3 Screening and surveillance

HCC is one of the tumor whose causes are better defined and therefore, at least theoretically, preventable. The population at risk are patients with liver cirrhosis and some patients with chronic viral hepatitis. It has been estimated that, in the presence of currently available treatments, periodic surveillance of patients with cirrhosis by liver ultrasonography for early identification of HCC produces a satisfactory cost / benefit ratio when the incidence of disease in the population subject to surveillance exceeds 1.5%[8]. There is only one large, randomized prospective study in Chinese patients with chronic HBV infection reporting data in six-monthly ultrasound surveillance plus alpha-fetoprotein assay, reporting lower HCC mortality in cases under surveillance to those not subjected to this practice[9].

Several observational studies and a recent meta-analysis have provided results in line with the Asian trial also in patients with cirrhosis[10–12].

There is no indication to perform surveillance every 3 months, because it does not reduce the overall mortality and does not increase the percentage of patients diagnosed with a tumor <= 2 cm[13]. The recommended screening interval is 6 months.

The addition of periodic alpha-fetoprotein dosage to ultrasound surveillance does not substantially increase (about 6%) the early-stage HCC recognition and it worsens the cost-effectiveness of surveillance, increasing the number of false-positive results[14]. However, this marker remains important as an indicator of the risk of HCC development and alpha-fetoprotein should be measured when a focal liver lesion on cirrhosis is found to contribute to diagnosis and prognosis.

1.1.4 Diagnosis

The hepatic carcinogenesis occurs in steps in 90% of cases, with a progression from the regenerative micronodule (not visible to the imaging techniques) to the regenerative macronodule (sometimes visible at imaging, with a size > 5mm) in which histological changes occur leading to dysplasia, initially mild and then progressively more serious, until the onset of a micro-outbreak of carcinoma[15].

From the histological point of view, the transformations that occur during carcinogenesis are generally accompanied by the formation of anomalous arterial vessels (tumor neoangiogenesis) and loss of the portal component[16].

This imbalance between the components of the vascular support provides a peculiar behavior of HCC in the different contrast phases of imaging techniques: an increase in the arterial phase signal inside the lesion compared to the surrounding parenchyma (commonly called wash-in), followed by "washout" of contrast in the venous-delayed phases.

Diagnosis of HCC should be based on imaging techniques and/or biopsy. The diagnostic algorithm for suspected HCC is shown in Figure 4. Diagnosis of HCC in cirrhotic patients is often based on contrast-enhanced imaging and/or pathology[7]. In non-cirrhotic patients, diagnosis of HCC should be confirmed by pathology. Biopsy of the lesion is indicated when the imaging-based diagnosis remains inconclusive, especially in lesions smaller than 2 cm in diameter where the diagnostic performance of contrast-enhanced imaging is lower. Pathological diagnosis of HCC should be based on the International Consensus recommendations using the required histological and immunohistological analyses. Because of their higher sensitivity and the analysis of the whole liver, computed tomography (CT) or magnetic resonance imaging (MRI) should be used first[7].

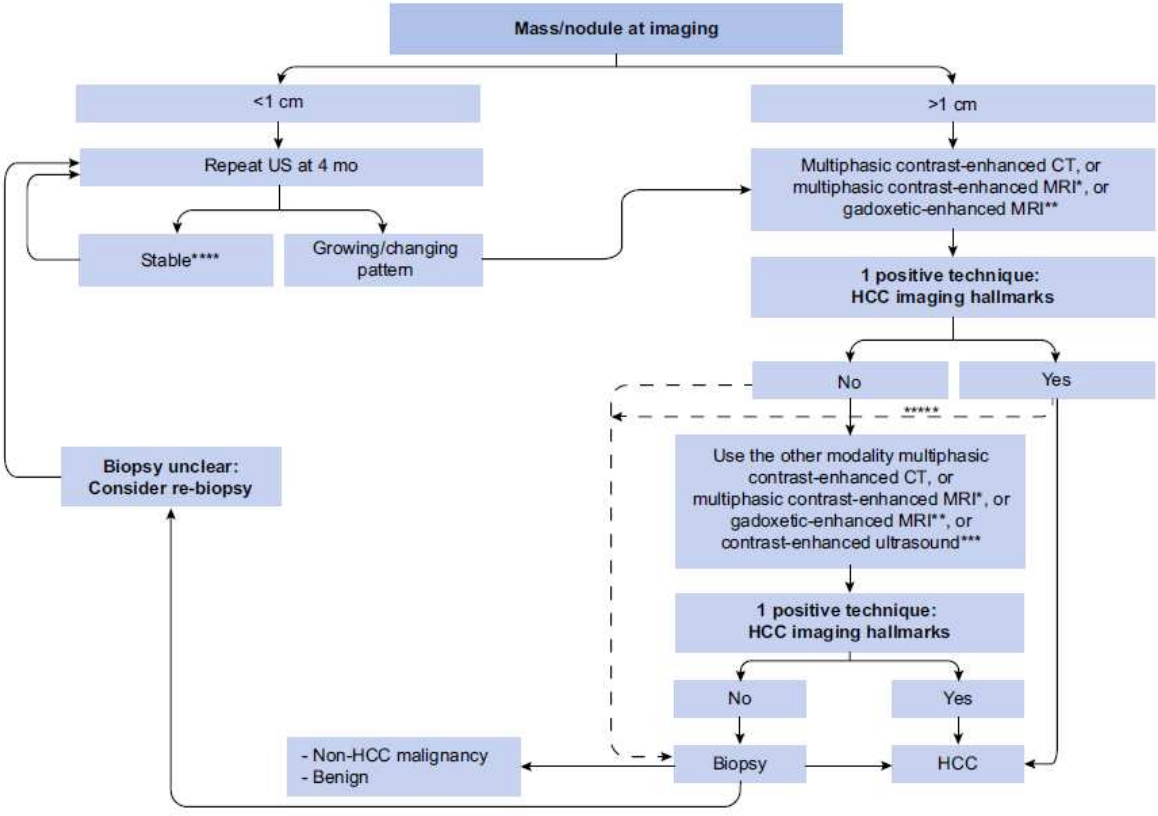


Figure 4:Diagnostic algorithm. CT, computed tomography; MRI, magnetic resonance imaging; US, ultrasound. (Adapted from EASL guidelines 2017[7])

1.1.5 Staging and Treatment of HCC

The Barcelona Clinic Liver Cancer (BCLC) staging system[17,18] has come to be widely accepted in clinical practice and is also being used in several major trials to define the patient population to be recruited and to stratify them into separate prognosis categories. BCLC classification includes prognostic variables related to tumor status (size, number, vascular invasion, N1, M1), liver function (bilirubin, portal hypertension, liver function preservation) and health performance status (ECOG PS) and it is an evolving system that links tumor stage with treatment strategy in a dynamic manner[19].

Liver function has traditionally been assessed through the Child-Pugh classification, that includes some subjective variables (for instance, ascites detected by imaging).

Regarding serum markers, increased alfa-fetoprotein (AFP) is associated with poorer prognosis and seem to predict risk of tumor recurrence, in several studies and clinical setting[20–23], but the heterogeneity of this studies prevents the formulation of a clear recommendation on AFP dosage.

Other markers such as vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang2) have been shown to have independent prognostic value in large cohorts of advanced hepatocellular carcinoma[22].

The BCLC system establishes a prognosis in accordance with the five stages (0, A, B, C and D) that are linked to first-line treatment recommendation (Figure 5).

Given the complexity of the disease, patients diagnosed with HCC should be referred to multidisciplinary teams involving hepatologists, surgeons, radiologists, interventional radiologists, pathologists, and oncologists. The aim of treatment is to increase survival while maintaining the highest quality of life.

Surgical resection, transplantation, ablation, transarterial chemoembolisation[24–26] and the tyrosine-kinase inhibitors sorafenib[27,28], lenvatinib[29] and regorafenib[30] are treatments with proven survival benefit.

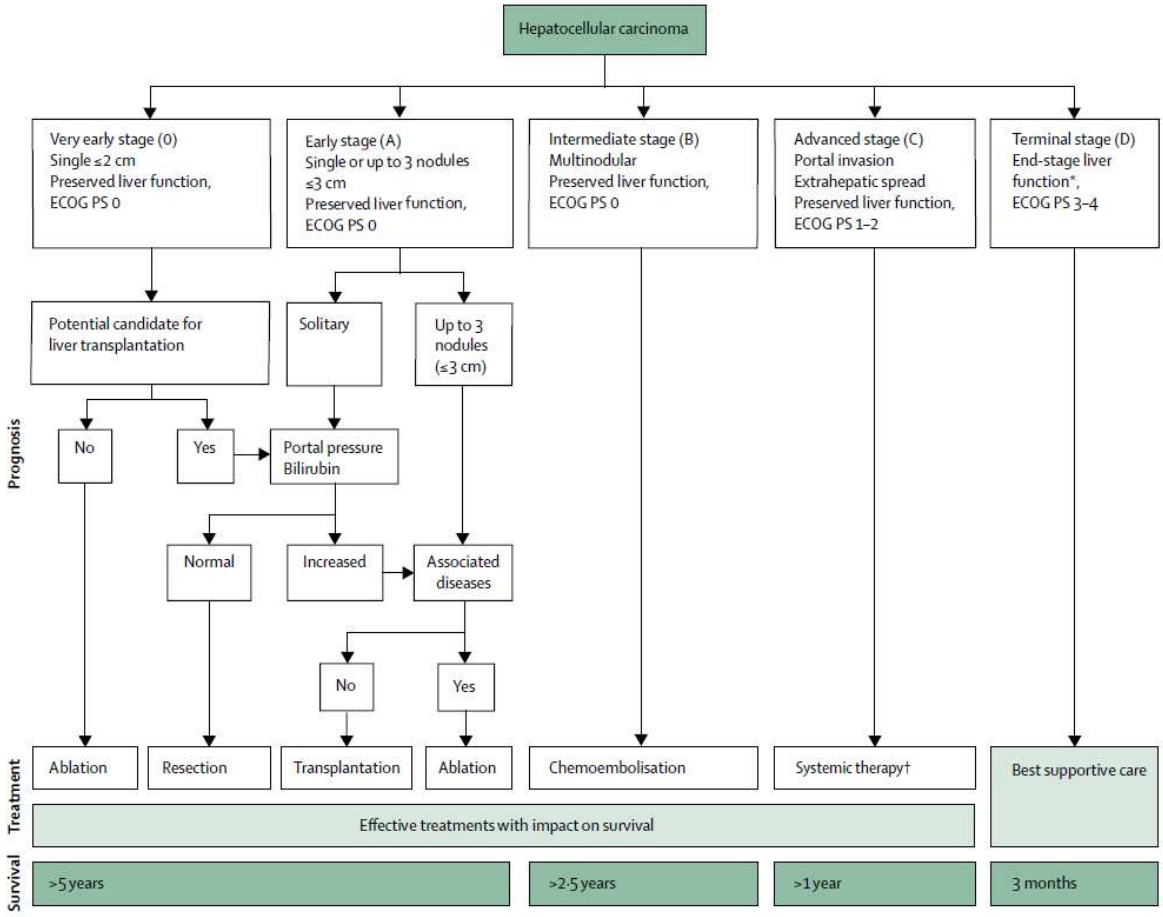


Figure 5. BCLC staging system and treatment strategy .ECOG PS=Eastern Cooperative Oncology Group Performance Status. †Currently, sorafenib followed by regorafenib has been shown to be effective. Lenvatinib has been shown to be non-inferior to sorafenib (Adapted from Forner *et al.*[18]).

1.2 Systemic therapy for advanced HCC

1.2.1 Sorafenib and angiogenesis

Hepatocellular carcinoma is recognized as among the most chemo-resistant tumor types, and until 2007 no systemic drug was recommended for patients with advanced-stage hepatocellular carcinoma or patients who transitioned into it after other therapies failed. Sorafenib was the first systemic therapy approved in hepatocellular carcinoma and it was shown to improve survival in two positive randomized placebo-controlled trials: SHARP[27]

and Asia-Pacific[28] trials. Consequently sorafenib become the standard of care for patients with advanced unresectable HCC[27,28].

Sorafenib works by inhibiting the activity of several tyrosine kinases involved in tumor angiogenesis and progression, including vascular endothelial growth factor receptor (VEGFR-2/3), platelet-derived growth factor receptor (PDGF-R), Flt3 and c-Kit, and also targets Raf kinases involved in the MAPK/ERK pathway[31–33](Figure 6).

The molecular mechanisms by which sorafenib exerts its activity have still not been fully elucidated, and both Raf/MEK/ERK-dependent and -independent mechanisms have been observed[34].

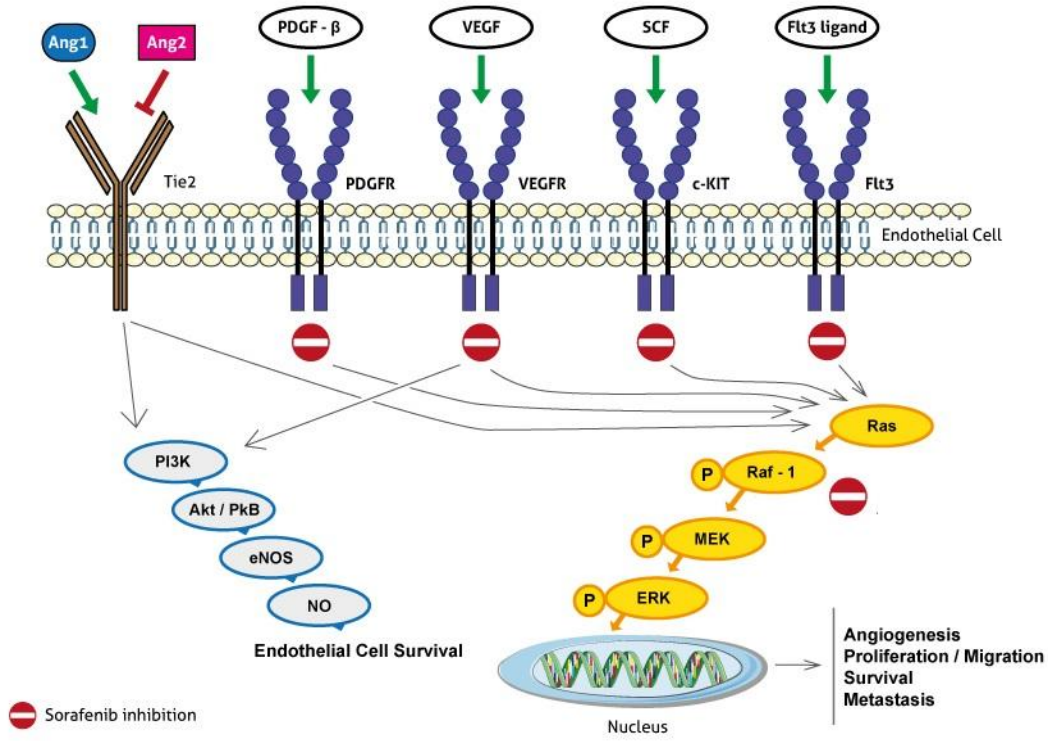


Figure 6. Sorafenib pathway and the main molecular factors. Ang: Angiopoietin; Tie2: Tyrosine-protein kinase receptor; PDGFR: Platelet-derived growth factor receptors; VEGFR: Vascular endothelial growth factor receptor; SCF: Stem cell factor; PI3K: PhosphatidylInositol 3-Kinase; Akt/PKB: Protein-chinasi B; eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; P: Phospho-; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal–regulated kinase (Adapted from Marisi *et al.*[35]).

The inhibition of VEGFR by sorafenib is known to repress phosphoinositide 3-kinase (PI3K) and its downstream serine protein kinase (Akt), decreasing the activity of endothelium-derived nitric oxide synthase (eNOS) and reducing the production of the potent vasodilator nitric oxide (NO)[36–38]. NO, constitutively expressed by vascular endothelial cells, controls a variety of physiologic functions including neovascularization, angiogenesis[36,37,39] and pathological conditions[40,41]. NO appears to play a proangiogenic role in tumor angiogenesis[42].

Numerous studies have reported that specific *eNOS* single nucleotide polymorphisms (SNPs) affect gene transcription, resulting in a variation in eNOS protein levels and activity and consequently influencing NO[43,44].

eNOS gene (*NOS3*) is located on chromosome 7q36.1 and among known polymorphisms, *eNOS*-786 T>C in the promoter region, a 27bp variable number of tandem repeats in intron 4 (*eNOS* VNTR 4a/b) and *eNOS*+894 G>T in exon 7 have received the greatest attention[45,46].

Numerous studies have investigated the role of *eNOS* polymorphisms in the risk of cancer development[47,48] and cardiovascular diseases[49–51], with conflicting results. However, it is still unclear how these polymorphisms affect gene expression and enzyme activity in cells and how they influence response to anti-angiogenic drugs[52].

Angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) are ligands for the tyrosine kinase receptor Tie2 and are widely expressed in many embryonic tissues. Ang2 is a partial agonist and antagonist of Ang1 and is expressed during vascular remodeling, thus preventing vascular stability. Ang1 promotes recruitment of pericytes and smooth muscle cells, stabilizing vascular networks by binding to Tie2[53]. Angiopoietin-2 is a protein that in humans is encoded by the *ANGPT2* gene. This gene is located on chromosome 8p23.1 and genetic variants of this gene may lead to altered activities of *ANGPT2* gene[54,55]. Genetic variants of *ANGPT2* are studied in association with the development of late unexplained intrauterine fetal death (IUFD)[56], the development of retinopathy of prematurity (ROP)[57] and the risk of acute respiratory distress syndrome (ARDS)[58]. However no work studied the impact of *ANGPT2* genetic variants in relation to sorafenib treatment in advanced HCC patients.

1.2.2 Prognostic and predictive factors of sorafenib efficacy

Sorafenib is expensive and associated with adverse events (AEs). Furthermore, a proportion of treated patients showed no response to the drug. It would thus be useful to have predictive markers capable to identify those patients who are more likely to benefit from therapy. The availability of more accurate predictive or prognostic factors would also help to spare potentially resistant patients from unnecessary toxicity[35]. A recent pooled analysis of two phase III randomized trials[59] showed that the neutrophil-to-lymphocyte ratio (NLR), etiology and extra-hepatic spread are predictive factors of response to sorafenib, but did not identify any predictive biological markers (Figure 7).

Numerous studies have focused on the role of markers involved in the angiogenesis process at both expression and genetic levels. The largest biomarker study conducted to date is the SHARP trial[22], which included an adequate number of participants and a placebo-controlled group. Smaller single-arm studies exploring predictive or prognostic markers for sorafenib have also been conducted, but the results of these have yet to be validated (Table 2).

Baseline Ang-2 and VEGF-A plasma levels independently predicted survival in both the entire patient population and the placebo cohort[22]. Genetic alterations, such as SNPs in genes encoding for proteins involved in the angiogenic process, have been studied as potential biomarkers for antiangiogenic therapy[60–62] (Table 2).

The main AEs of sorafenib are skin toxicity, hypertension (HTN) and diarrhea. Several papers evaluated a correlation between AEs and survival in patients treated with sorafenib, in particular patients with skin toxicity reported better OS than patients without this toxicity during the first 60 days of treatment[63]. HTN is frequently associated with the use of angiogenesis inhibitors and some studies showed that early HTN was associated with better clinical outcome[64–66], but this finding has not been confirmed in others[67,68]. Diarrhea was an independent positive prognostic factor in patients with advanced HCC[69,70].

In summary, after 10 years of sorafenib research there are still no validated prognostic or predictive factors of response to the drug in HCC. The fact of having only one drug for the treatment of these patients has certainly not stimulated extensive researches to identify and validate predictors of response and prognosis.

However, given the recent publication of a positive phase III trial[29] and the ongoing CheckMate459 immunotherapy study (NCT02576509), the race is now on seeing who will be the first study capable to identify a prognostic and predictive factor for sorafenib and/or new drugs in this setting.

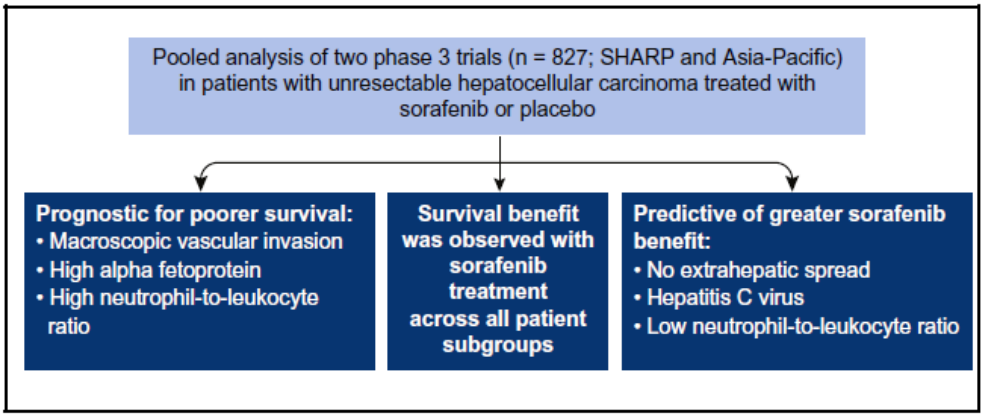


Figure 7. Prognostic and predictive factors of sorafenib benefit (Adapted from Bruix *et al.* [59])

Table 2. Predictive and/or prognostic value of biological markers in HCC

Biological markers	Predictive value	Prognostic value	References
Serum and plasma proteins			
VEGF-A	No	Uncertain	[22,71]
Ang-2	No	Yes	[22]
IGF-1	No	No	[72]
Single nucleotide polymorphisms			
VEGF-A rs2010963	No	Yes	[60]
VEGF-C rs4604006	No	Yes	[60]
HIF-1alpha rs12434438	No	Yes	[62]
Amplifications			
VEGF	No	Uncertain	[61]
FGF3/FGF4	No	Uncertain	[73]
miRNAs			
miR-425-3p	No	Yes	[74]
miR-224	No	Yes	[75]
miR-181a-5p	No	Yes	[76]
miR-339-5p	No	Yes	[76]
miR-423-5p	No	Yes	[77]
miR-10b-3p	No	Yes	[78]
miR-221	No	Uncertain	[79]
Tissue bimarker expression			
phospho-(p-) ERK	Uncertain	Uncertain	[80,81]
PDGFR-b	No	Yes	[82]
c-Met	No	No	[82]
VEGFR	No	No	[82]
p-c-Jun	No	Yes	[83]

1.2.3 Emerging therapies in HCC

Since the introduction of Sorafenib, several first-line clinical trials have been conducted with the aim of developing molecular targeted agents showing better efficacy or safety than sorafenib, both in first and second line of treatment of advanced-stage HCC[84] (Table 3).

In the first line setting, lenvatinib, a multi-targeted tyrosine kinase inhibitor (TKI) (Figure 8) was non-inferior to sorafenib in a recent phase III trial[29], a candidate to be an alternative to sorafenib.

In the second line setting, regorafenib, another multi-targeted TKI, was proven to have efficacy in most recent clinical trials[30], in HCC patients who tolerated sorafenib in the first line of treatment. Phase 3 trials of cabozantinib[85], multi-targeted TKI, and ramucirumab[86], a monoclonal antibody against VEGFR-2, have demonstrated survival benefit in sorafenib-experienced patients.

Immunotherapy has been changing the landscape of oncology in recent years and appears very promising also in HCC. In particular, nivolumab, a PD-1 immune check-point inhibitor, obtained approval for second line HCC[87]. Interesting clinical trials studying immunotherapy in the HCC first line setting are ongoing.

Table 3. Phase III clinical trials of advanced-stage HCC

Target Population		Design	Trial Name	Result	Presentation	Publication
Advanced	First line	1. Sorafenib vs. Sunitinib	SUN1170	Negative	ASCO 2011	*JCO* 2013
		2. Sorafenib +/− Erlotinib	SEARCH	Negative	ESMO 2012	*JCO* 2015
		3. Sorafenib vs. Brivanib	BRISK-FL	Negative	AASLD 2012	*JCO* 2013
		4. Sorafenib vs. Linifanib	LiGHT	Negative	ASCO-GI 2013	*JCO* 2015
		5. Sorafenib +/− Doxorubicin	CALGB 80802	Negative	ASCO-GI 2016	
		6. Sorafenib +/− HAIC	SILIUS	Negative	EASL 2016	*Lancet GH* 2018
		7. Sorafenib +/− Y90	SARAH	Negative	EASL 2017	*Lancet-O* 2017
		8. Sorafenib +/− Y90	SIRveNIB	Negative	ASCO 2017	*JCO* 2018
		9. Sorafenib vs. Lenvatinib	REFLECT	Positive	ASCO 2017	*Lancet* 2018
		10. Sorafenib vs. Nivolumab	CheckMate-459	Ongoing		
		11. Sorafenib vs. Durvalumab + Tremelimumab vs. Durva	HIMALAYA	Ongoing		
		12. Sorafenib vs. Atezolizumab + Bevacizumab	IMbrave150	Ongoing		
		13. Sorafenib vs. Tislelizumab		Ongoing		
	Second line	1. Brivanib vs. Placebo	BRISK-PS	Negative	EASL 2012	*JCO* 2013
		2. Everolimus vs. Placebo	EVOLVE-1	Negative	ASCO-GI 2014	*JAMA* 2014
		3. Ramucirumab vs. Placebo	REACH	Negative	ESMO 2014	*Lancet-O* 2015
		4. S-1 vs. Placebo	S-CUBE	Negative	ASCO 2015	*Lancet GH* 2017
		5. ADI-PEG 20 vs. Placebo	NA	Negative	ASCO 2016	*Ann Oncol* 2018
		6. Regorafenib vs. Placebo	RESORCE	Positive	WCGC 2016	*Lancet* 2017
		7. Tivantinib vs. Placebo	METIV-HCC	Negative	ASCO 2017	*Lancet-O* 2018
		8. Tivantinib vs. Placebo	JET-HCC	Negative	ESMO 2017	
		9. DT vs. Placebo	ReLive	Negative	ILCA 2017	
		10. Cabozantinib vs. Placebo	CELESTIAL	Positive	ASCO-GI 2018	*NEJM* 2018
		11. Ramucirumab vs. Placebo	REACH-2	Positive	ASCO 2018	
		12. Pembrolizumab vs. Placebo	KEYNOTE-240	Ongoing		

Red: positive trials; blue: ongoing trials; black: negative trials. HAIC: hepatic arterial infusion chemotherapy; ADI-PEG 20: arginine deiminase-conjugated with polyethylene glycol; DT: doxorubicin-loaded nanoparticles (modified from Kudo *et al.* [88]).

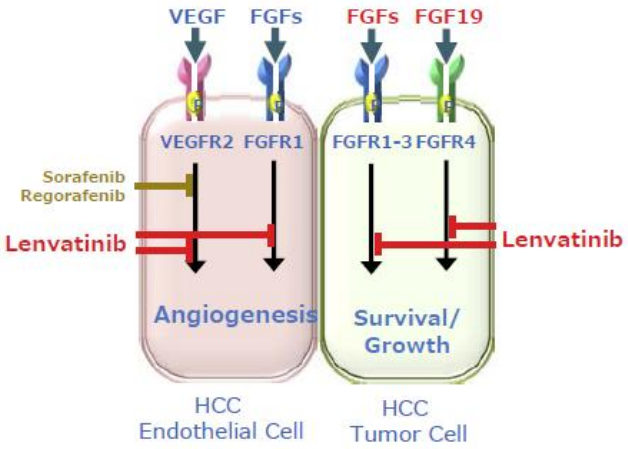


Figure 8. Lenvatinib molecular action. FGF, fibroblast growth factor (adapted from Kudo *et al.*[89]).

2. AIMS

Sorafenib, an oral multi-tyrosine kinase inhibitor, has been considered the standard of care for patients with advanced unresectable hepatocellular carcinoma (HCC), however a large proportion of patients still do not seem to benefit from this treatment approach. Biomarkers of sorafenib efficacy or resistance have yet to be identified. Angiogenesis is one of the most involved pathways in the mechanism of action of sorafenib. The identification of markers could allow a more appropriate administration of the drug, improving the clinical response and reducing the side effects.

The aim of the present study, performed at the Biosciences Laboratory of the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS in Meldola (FC), was to evaluate the prognostic value of different single nucleotide polymorphisms (SNPs) on *eNOS* (endothelial nitric oxide synthase) and *ANGPT2* (angiopoietin 2) genes in patients with advanced HCC receiving sorafenib treatment.

The primary aim was to study the prognostic value of the SNPs in relation to overall survival (OS).

The second aim was to verify whether these polymorphisms are related or not to progression-free survival (PFS), disease control rate (DCR) and toxicities.

3. PATIENTS AND METHODS

3.1 Patient enrollment

This a retrospective multicenter Italian study carried out on 135 HCC patients consecutively treated at Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori from 2012 to 2015.

Patients receiving sorafenib with advanced- or intermediate-stage HCC (either histologically proven or diagnosed according to the AASLD [American Association for the Study of Liver Diseases 2005] guidelines) refractory or no longer amenable to locoregional therapies, were eligible for our study.

Eligibility criteria were the same as those of Llovet's pivotal study on sorafenib in HCC[27]: Eastern Cooperative Oncology Group (ECOG) performance status score ≤2 ; Child-Pugh liver function class A; adequate hematologic function (platelet count, ≥60×109/L; hemoglobin ≥8.5 g/dL; and prothrombin time international normalized ratio ≤2.3 or prothrombin time ≤6 seconds above control, adequate hepatic function (albumin ≥2.8 g/dL; total bilirubin ≤3 mg/dL [51.3 μmol/L]; alanine aminotransferase and aspartate aminotransferase ≤5 times the upper limit of the normal range); and adequate renal function (serum creatinine ≤1.5 times the upper limit of the normal range).

All patients received sorafenib according to the standard schedule (400 mg twice a day continuously), dose reductions were applied when clinically indicated. Follow-up consisted of a CT/MRI scan every 8 weeks or as clinically indicated. Tumor response was evaluated by modified Response Evaluation Criteria in Solid Tumors (mRECIST)[90].

Treatment with sorafenib was continued until disease progression, unacceptable toxicity or death. The study was approved by the Local Ethics Committees of each center and informed consent was obtained from each patient for their biological material to be used for research purposes.

3.2 DNA isolation and genotyping

We performed *eNOS* and *ANGPT2* genotyping using DNA extracted from whole blood samples.

Peripheral blood samples was collected in EDTA tubes and genomic DNA was extracted from 200 μl of whole blood by QIAamp DNA Minikit (Qiagen SPA, Milan, Italy) in

accordance with the manufacturer's instructions. DNA quantity and quality were assessed by Nanodrop 1000 (Celbio, Milan, Italy).

Genotyping was performed for two *eNOS* SNPs (*eNOS* -786,*eNOS*+894), one *eNOS* variable number tandem repeat (VNTR) of 27 nucleotides and eight *ANGPT2* SNPs (rs3739390, rs3739391, rs3739391, rs1961222, rs3020221, rs6559167, rs2916747, rs17063434).

The localizations and refSNP (rs) numbers of these polymorphisms are shown in Figure 9.

eNOS-786 T>C (rs2070744) is located in 5′ promoter region, *eNOS* VNTR 27bp 4a/b in intron 4 and *eNOS*+894G>T (rs1799983) in exon 7. *eNOS* VNTR 27bp 4a/b in intron 4 has 2 common alleles: "4a" with 4 repeats and "4b" with 5 repeats.

For *ANGPT2* polymorphisms, three polymorphisms are located in the promoter region and the others are found in the exons of the gene (Figure 9).

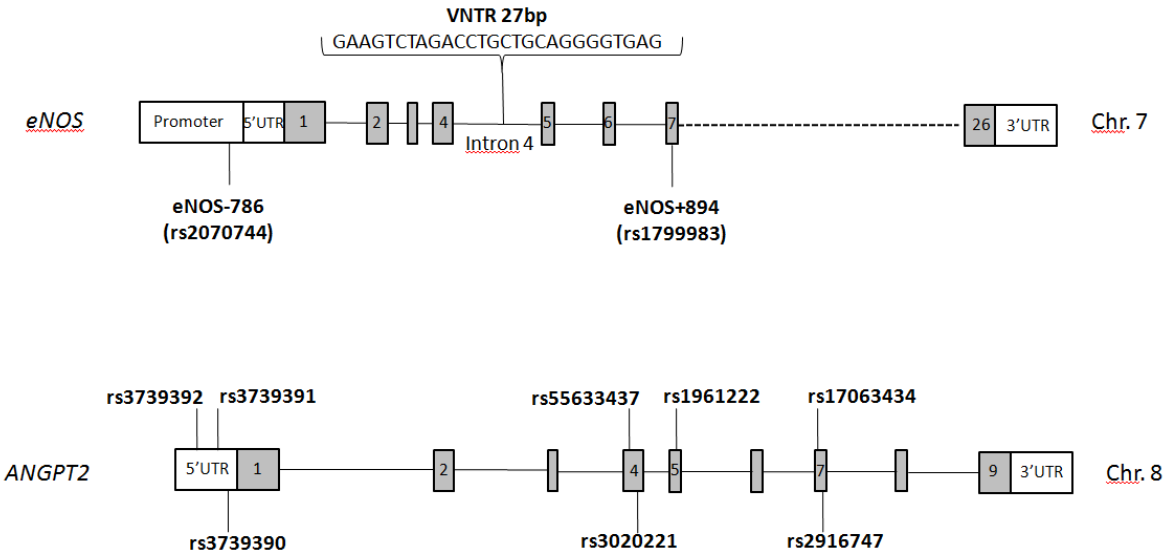


Figure 9: *eNOS* and *ANGPT2* polymorphisms.

We selected these polymorphisms through a review of the Single Nucleotide Polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/SNP) and of medical literature. We selected polymorphisms with some degree of likelihood to alter the structure or the expression of the gene in a biologically relevant manner (i.e., affecting ESE sequences, 3'UTR or promoter region) and with the minor allele frequency (MAF) above 5% (with the only exception of rs rs17063434).

Genotyping analyses of *eNOS*-786 and *eNOS*+894 were performed by TaqMan technology using SNP genotyping assays (Assay ID C_15903863_10 and C_3219460_20, respectively, Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) was performed and genotypes were analyzed on the 7500 Real-Time PCR System (Applied Biosystems) using a 7500 Software version 2.3. PCRs were performed starting from 20 ng of genomic DNA and following this standard protocol: Hold 10 min at 95°C, 40 cycles of denature (15 sec at 92°C) and anneal/extend (1 min at 60°C).

eNOS VNTR and all *ANGPT2* polymorphisms were determined by standard PCR and direct sequencing analysis on an ABI 3130 Genetic Analyzer (Applied Biosystems). Primer sequences and PCR conditions are reported in Table 4. PCRs were performed starting from 50 ng of genomic DNA.

All samples were analyzed at the same institution (Biosciences Laboratory, IRST IRCCS, Meldola, Italy) and laboratory personnel was blinded to patient status performed genotyping.

Table 4 : Primer sequences for *eNOS* and *ANGPT2* SNPs and PCR programs

SNPs	Primer Sequences (5'-3')	PCR programs
eNOS		
VNTR 27bp	F: AAA-CTG-TGG-GGG-AGA-TCC-TT R: GGG-CAG-CTT-GCT-TCT-CTT-AG	Step 1: 39 cycles of 94ºC for 60s, 62ºC for 60s and 72ºC for 60s; Step2: 72ºC for 5min
ANGPT2		
rs3739390 rs3739391 rs3739392	F: CCTGGAGAGAACACAGCAGT R: CGGCCAAGACAAGATCACAG	Step 1: 39 cycles of 94ºC for 60s, 62ºC for 60s and 72ºC for 60s; Step2: 72ºC for 5min
rs3020221 rs55633437	F: GCTACAGGTGTTAGTATCCAAGC R: TGAGAAATAGCGCCTTTTCTGA	Step 1: 39 cycles of 94ºC for 60s, 58ºC for 60s and 72ºC for 60s; Step2: 72ºC for 5min
rs1961222	F: AGGACCCCACTGTTGCTAAA R: GTGAGGCTGGGGAAGATCTT	Step 1: 39 cycles of 94ºC for 60s, 62ºC for 60s and 72ºC for 60s; Step2: 72ºC for 5min
rs17063434 rs2916747	F: ACTTGCATTACAGGGATTTGGT R: GCCCGGCCACAAATCTTTTA	Step 1: 39 cycles of 94ºC for 30s, 60ºC for 30s and 72ºC for 30s; Step2: 72ºC for 5min

Abbreviation: F, forward primer; R, reverse primer.

3.3 Statistical analysis

Data were summarized as median, minimum and maximum values for continuous variables and as absolute frequencies and percentages for categorical variables. The association between SNPs and patients or clinical categorical variables was assessed by means of Chi-square test or the Fisher Exact test, when appropriate, and among patients and clinical continuous variables by means of Wilcoxon-Mann-Whitney test.

The two main time-to-event end-points considered were progression-free survival (PFS) defined as the time since the beginning of the treatment with sorafenib until disease

progression or death for any cause (whichever occurred first) and overall survival (OS) defined as the time since start of treatment with sorafenib until death for any cause. Patients not experiencing the event of interest were censored at last follow-up available.

Disease Control Rate (DCR) was defined as the proportion of patients with a complete, partial response or with stable disease. Pearson chi-square test or the Fisher Exact test were used to evaluate the association between SNPs and DCR or toxicity, when appropriate.

Kaplan-Meier (K-M) method and log-rank test were used to compare PFS and OS between groups of patients. Median follow-up was computed on censored observations only. Median PFS and OS values and corresponding 95% confidence intervals (CIs) in square brackets were reported.

SNPs were prescreened prior to statistical analyses to determine the correct genetic model by analyzing the Kaplan-Meier curves, following the approach by Savas *et al.*[91].

When the number of patients with the minor allele homozygous genotype ($n \leq 10$) was not sufficient, dominant genetic model was considered.

Hazard ratios (HRs) were estimated by means of the Cox proportional hazards regression model.

Hardy-Weinberg equilibrium, linkage disequilibrium and haplotype analyses were performed using the Haploview v. 4.2 software package[92]. This software provides Lewontin's disequilibrium coefficient (D') as the measure of the nonrandom association of alleles at different loci. The D' coefficient is equal to 1 only if 2 SNPs have not been separated by recombination (or recurrent mutation) during the history of the sample (complete degree of linkage disequilibrium [LD]).

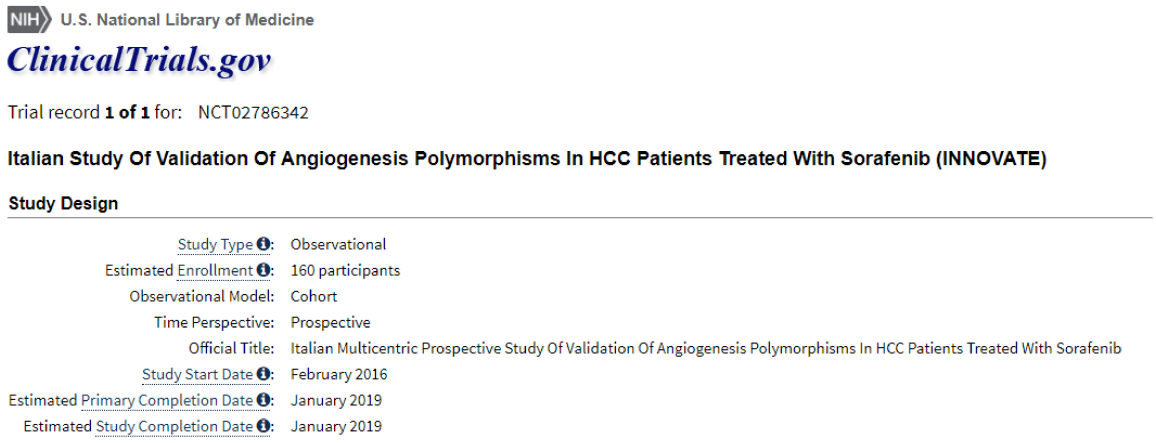
Haplotypes blocks were found using Haploview v. 4.2 software package using the algorithm by Gabriel *et al.*[93]. The association between haplotypes and PFS or OS was performed by means of the weighted haplotype combination method proposed by French *et al.*[94] using a dominant model due to low frequencies

To select the variables to include in the final Cox models, one for PFS and one OS respectively, we proceeded as follows: we considered those variables significantly associated at 10% level at univariate analysis as well as SNPs found to be significantly associated at level of 10% at univariate analysis or after adjustment for clinical covariates. Moreover, correlation among variables, especially among SNPs, was taken into consideration for variables selection. SNPs correlation was measured by estimating tetrachoric correlation coefficients.

All statistical analyses were performed using STATA 15.0 statistical software (StataCorp, College Station, TX, USA) and R version 3.4.1.

3.4 Description of the prospective INNOVATE study

The INNOVATE study (NCT02786342) is an Italian multicenter prospective study of validation of angiogenesis-related gene polymorphisms in HCC patients treated with sorafenib (Figure 10).


NIH U.S. National Library of Medicine

ClinicalTrials.gov

Trial record **1 of 1** for: NCT02786342

Italian Study Of Validation Of Angiogenesis Polymorphisms In HCC Patients Treated With Sorafenib (INNOVATE)

Study Design

Study Type:	Observational
Estimated Enrollment:	160 participants
Observational Model:	Cohort
Time Perspective:	Prospective
Official Title:	Italian Multicentric Prospective Study Of Validation Of Angiogenesis Polymorphisms In HCC Patients Treated With Sorafenib
Study Start Date:	February 2016
Estimated Primary Completion Date:	January 2019
Estimated Study Completion Date:	January 2019

Figure 10. Overview of study details (https://clinicaltrials.gov/)

The primary aim of the study is to validate the prognostic role of *eNOS*, *ANGPT2* and other angiogenesis-related gene polymorphisms in relation to the clinical outcome (OS and PFS) of HCC patients treated with sorafenib.

The study involves 160 HCC patients treated with sorafenib. The study population consisted of patients with advanced-stage HCC and patients with intermediate HCC not eligible to loco-regional therapies or liver transplantation. None of the patients have received previous systemic therapy.

Blood samples (serum, plasma, corpuscles part and PAXgene) from each patients are collected at the baseline, 14 days after sorafenib, 28 days after sorafenib, 60 days after sorafenib and at the time of disease progression (PD). The sample collection and storage are performed at the Biosciences Laboratory of the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS in Meldola (FC) and at the participant centers laboratories with the identical procedures.

The molecular analysis are performed at the Biosciences Laboratory of IRST IRCCS.

4. RESULTS

4.1 Patient characteristics and clinical variables

The main clinical pathological characteristics of patients are shown in Table 5. Median follow-up for PFS was 2.96 months (95% CI:1.87-3.91) whereas for OS was 8.9 months (1.71-48.92). Median PFS was 5.75 months (95% CI: 5.06-6.60) and median OS was 14.39 months (95% CI: 11.83-15.74).

Univariate analysis regarding PFS and OS data in relation to baseline patient characteristics are shown in Table 6. In particular we found that patients with HBV etiology showed worse OS than patients with HCV etiology (8.57 *vs* 14.3 months; HR 1.95 95% CI:1.17-3.26; *P*=0.011) and patients without extra hepatic spread showed better outcomes in terms of PFS (6.27 *vs* 2.83 months; HR 0.50 95% CI:0.34-0.73; *P* <0.001) and OS (15.6 *vs* 10.84 months; HR 0.65 95% CI:0.43-0.99; *P*=0.043) than patients with metastatic disease. No significant correlation was found between the other clinical characteristics and clinical outcomes.

Table 5. Patient characteristics

Clinical and pathologic indexes	**No. (%)**
Median age at start of treatment (min-max)	70 (25-88)
Gender	
Male	109 (80.7)
Female	26 (19.3)
Etiology	
Metabolic syndrome	18 (13.3)
Alcoholic	10 (7.4)
Viral- HBV	22 (16.3)
Viral - HCV	78 (57.8)
Biliary cirrhosis/cryptogenic	7 (5.2)
BCLC stage	
B	37 (27.4)
C	98 (72.6)

Child-Pugh	
A	125 (92.6)
B	10 (7.4)
ECOG Performance Status	
0	83 (61.5)
1-2	52 (38.5)
Resection	
No	87 (64.4)
Yes	48 (35.6)
Sorafenib dose reduction	
No	59 (61.5)
Yes	37 (38.5)
missing	39
Extra hepatic spread	
No	81 (64.3)
Yes	45 (35.7)
Serum α-FP level	
≤ 400	54 (40)
> 400	35 (25.9)
missing	46

Abbreviations: BCLC, Barcelona clinic liver cancer; ECOG, Eastern Cooperative Oncology Group; α-FP, alpha-fetoprotein.

Table 6. PFS and OS in relation to baseline patient characteristics

	Median$_{PFS}$ [95% CI]	**HR$_{PFS}$ [95% CI]**	**P_{PFS}**	**Median$_{OS}$ [95% CI]**	**HR$_{OS}$ [95% CI]**	**P_{OS}**
Gender						
Female	6.11 [3.22-8.18]	1		12.35 [5.72-20.89]	1	
Male	5.62 [5.03-6.27]	0.96 [0.62-1.50]	0.873	14.85 [11.83-15.80]	0.94 [0.59-1.52]	0.814
Median age at start of treatment (min-max)*		0.92 [0.85-0.995]	**0.038**		0.94 [0.87-1.02]	0.118
Etiology						
Viral - HCV	6.11 [5.06-6.90]	1		14.29 [11.14-17.77]	1	
Biliary cirrhosis/cryptogenic	3.98 [3.19-21.91]	0.96 [0.44-2.11]	0.926	14.39 [1.41-17.15]	0.70 [0.29-1.76]	0.453
Alcoholic	5.26 [1.41-8.71]	0.98 [0.50-1.92]	0.956	15.64 [6.80-21.68]	1.23 [0.59-2.59]	0.580
Metabolic syndrome	6.01 [3.25-8.51]	1.21 [0.71-2.06]	0.478	8.57 [4.66-15.24]	1.11 [0.63-1.96]	0.710
Viral - HBV	5.06 [2.33-6.90]	1.45 [0.90-2.34]	0.131	8.57 [4.66-15.24]	1.95 [1.17-3.26]	**0.011**
Resection						
No	6.11 [5.26-6.90]	1		15.08 [11.24-17.77]	1	
Yes	5.06 [3.75-6.14]	1.26 [0.88-1.81]	0.206	12.81 [8.57-15.74]	1.23 [0.84-1.81]	0.294
Child-Pugh						
A	5.75 [5.06-6.60]	1		14.59 [11.83-15.74]	1	
B	6.11 [0.69-11.37]	0.76 [0.37-1.57]	0.459	13.53 [1.48-26.41]	1.16 [0.56-2.40]	0.683
BCLC						
B	6.60 [5.06-8.77]	1		14.36 [11.24-16.43]	1	
C	5.32 [4.11-6.14]	1.27 [0.86-1.88]	0.225	14.59 [9.99-16.43]	1.05 [0.68-1.62]	0.819
ECOG Performance Status						
0	5.75 [5.03-6.60]	1		14.39 [11.7-15.74]	1	
1-2	6.01 [2.69-7.42]	1.01 [0.71-1.45]	0.949	15.01 [8.18-18.92]	1.23 [0.84-1.80]	0.294
Extrahepatic spread						
Yes	2.83 [1.94-5.22]	1		10.84 [6.96-15.08]	1	

No	6.27 [5.72-7.65]	0.50 [0.34-0.73]	**<0.001**	15.60 [12.81-18.00]	0.65 [0.43-0.99]	**0.043**
Serum α-FP level						
≤ 400	5.75 [3.75-6.64]	1		13.57 [10.35-16.66]	1	
> 400	5.72 [2.92-7.23]	0.87 [0.56-1.34]	0.526	13.86 [8.15-15.80]	1.26 [0.80-2.01]	0.320

*5-year increment

Abbreviations: BCLC, Barcelona Clinic Liver Cancer; ECOG, Eastern Cooperative Oncology Group; α-FP, alpha-fetoprotein; PFS, progression-free survival; OS, overall survival; HR, hazard ratio

4.2 *eNOS* and *ANGPT2* genotypes

Genotype frequencies of *eNOS* and *ANGPT2* polymorphisms are shown in Table 7 and all genotype frequencies followed the Hardy-Weinberg equilibrium. Polymorphisms missing data are due to the lack of input DNA.

Table 7. Genotype frequencies of *eNOS* and *ANGPT2* polymorphisms

	No.	**(%)**
ANGPT2		
rs3739392		
CC	3	(2.33)
TC	34	(26.36)
TT	92	(71.32)
missing	6	
rs3739391		
CC	81	(62.79)
CT	45	(34.88)
TT	3	(2.33)
missing	6	
rs3739390		
CC	1	(0.78)
GC	22	(17.05)
GG	106	(82.17)
missing	6	
rs55633437		
GG	111	(87.40)
GT	14	(11.02)
TT	2	(1.57)
missing	8	
rs3020221		
AA	24	(18.90)
GA	58	(45.67)
GG	45	(35.43)
missing	8	
rs1961222		
AA	21	(16.28)
GA	53	(41.09)

GG	55	(42.64)
missing	6	
rs17063434		
CC	1	(0.78)
TC	18	(14.06)
TT	109	(85.16)
missing	7	
rs2916747		
TC	14	(10.94)
TT	114	(89.06)
missing	7	
eNOS		
***eNOS*+894 (rs1799983)**		
GG	58	(46.40)
GT	56	(44.80)
TT	11	(8.80)
missing	10	
VTNR 4a4b		
4aa	3	(2.48)
4ab	33	(27.27)
4bb	85	(70.25)
missing	14	
***eNOS*-786 (rs2070744)**		
CC	20	(16.00)
TC	54	(43.20)
TT	51	(40.80)
missing	10	

Abbreviations: VNTR, variable number of tandem repeat

Some *eNOS* and *ANGPT2* SNPs were highly correlated (Table 8).

Table 8: Correlation coefficient between polymorphisms

	rs1799983 (dom)	vntr4a4b (dom)	rs2070744 (dom)	rs3739392 (dom)	rs3739391 (dom)	rs3739390 (dom)	rs55633437 (dom)	rs3020221 (rec)	rs1961222 (dom)	rs17063434 (dom)	rs291674701
rs1799983 (dom)	1.00										
vntr4a4b (dom)	-0.26	1.00									
rs2070744 (dom)	0.59*	0.77*	1.00								
rs3739392 (dom)	-0.23	0.06	0.02	1.00							
rs3739391 (dom)	-0.20	-0.02	0.06	1.00*	1.00						
rs3739390 (dom)	-0.26	0.09	-0.14	1.00*	1.00*	1.00					
rs55633437 (dom)	0.02	-0.20	0.17	0.17	0.30	-0.09	1.00				
rs3020221 (rec)	0.09	-0.08	-0.06	-0.06	-0.13	-0.02	-1.00	1.00			
rs1961222 (dom)	-0.01	-0.13	0.03	0.11	0.08	0.06	-0.59*	0.72*	1.00		
rs17063434 (dom)	0.07	0.12	0.01	0.14	0.16	0.31	-0.06	-1.00*	-0.35	1.00	
rs291674701	0.46*	0.10	0.18	0.24	0.11	0.01	0.19	-0.27	0.39	-0.02	1.00

*Values with an asterisk means that they differ statistically significantly from 0 (no correlation).

4.3 *eNOS* and *ANGPT2* genotypes and clinical outcomes

In univariate analysis we found that two *eNOS* SNPs and three *ANGPT2* SNPs were associated with outcome. In particular patients with at least one copy of the minor allele C for *eNOS*-786T>C polymorphisms had a significantly higher median PFS (7.03 *vs.* 3.5 months, HR 0.43 95% CI 0.30-0.63; $P < 0.001$) and OS (15.6 *vs.* 9.1 months, HR 0.65 95% CI 0.44-0.97; $P=0.036$) than patients homozygous for T allele (Table 9 and Figure 11A-B). Moreover, for *eNOS* VNTR4a/b patients with at least one copy of the minor allele "a" showed a significantly higher median PFS (7.65 *vs.* 5.06 months, HR 0.54 95% CI 0.36-0.80; $P = 0.002$) than patients homozygous for "b" allele (Table 9 and Figure 11C).

No statistically significant differences were observed for the other *eNOS* polymorphisms.

Regarding *ANGPT2* SNPs rs55633437 was associated with both PFS and OS. For this polymorphism we chose the dominant genetic model. Patients with at least one copy of the minor allele T had a significantly lower median PFS (1.58 *vs.* 6.27 months, HR 4.79 95% CI 2.73-8.35; $P<0.001$) and OS (4.66 *vs.* 15.51 months, HR 4.86 95% CI 2.73-8.67; $P<0.001$) than those homozygous for G allele (Table 9 and Figure 12A-B).

ANGPT2 rs3020221 and rs1961222 were associated only with OS. For rs3020221 we chose the recessive genetic model and patients homozygous for A allele showed a significantly better median OS than those with other genotypes (18.99 *vs* 12.81 months, HR 0.53 95% CI 0.31-0.92; $P=0.024$). (Table 9 and Figure 13A). For rs1961222 we chose the dominant genetic model and patients carrying at least one copy of the minor allele A showed a significantly better median OS (16.43 *vs.* 11.24 months, HR 0.67 95% CI 0.46-0.99; $P=0.044$) than those homozygous for G allele (Table 9 and Figure 13B).

No statistically significant differences were observed for other *ANGPT2* polymorphisms and PFS and OS.

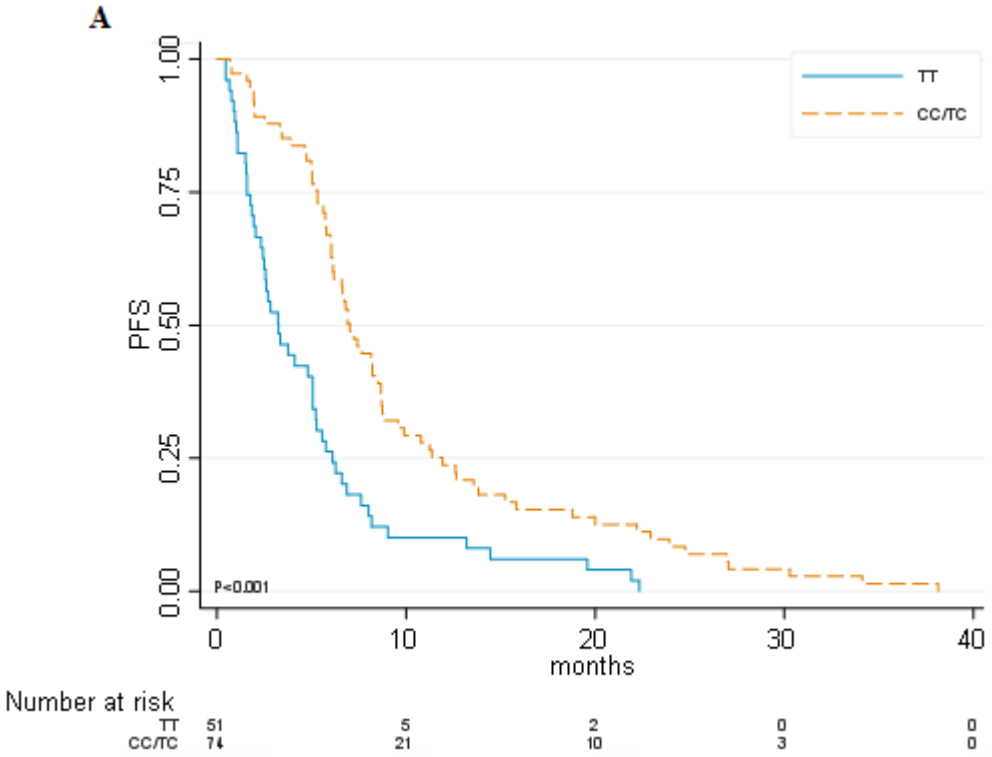
No significant correlation was found between the main clinical-pathologic characteristics of patients and *eNOS* polymorphisms. For *ANGPT2* SNPs, rs55633437 was associated with the extra hepatic spread, in particular 64% of patients with at least on copy of T allele showed a metastatic disease. Conversely, 32.7% of patients homozygous for G allele showed extra hepatic spread.

Table 9. Univariate analysis of PFS and OS in relation to *eNOS* and *ANGPT2* polymorphisms

		PFS			OS		
	Genetic model	**Median$_{PFS}$ [95% CI]**	**HR$_{PFS}$ [95% CI]**	**P_{PFS}**	**Median$_{OS}$ [95% CI]**	**HR$_{OS}$ [95% CI]**	**P_{OS}**
eNOS							
***eNOS*+894 (rs1799983)**	DOM						
GG		5.22 [2.83-6.11]	1		15.74 [9.23-18.50]	1	
GT/TT		6.60 [5.32-8.18]	0.81 [0.57-1.16]	0.251	14.29 [11.14-15.51]	1.14 [0.77-1.68]	0.527
VNTR4a4b	DOM						
4bb		5.06 [3.75-6.11]	1		11.99 [9.10-14.85]	1	
4aa/4ab		7.65 [6.08-12.61]	0.54 [0.36-0.80]	**0.002**	17.15 [14.59-20.89]	0.68 [0.44-1.05]	0.080
***eNOS*-786 (rs2070744)**	DOM						
TT		3.25 [2.33-5.06]	1		9.10 [6.80-14.29]	1	
CC/TC		7.03 [6.08-8.67]	0.43 [0.30-0.63]	**<0.001**	15.60 [13.86-19.51]	0.65 [0.44-0.97]	**0.036**
ANGPT2							
rs3739392	DOM						
TT		5.62 [5.03-6.73]	1		14.39 [11.24-16.43]	1	
CC /TC		6.14 [3.91-8.54]	0.94 [0.64-1.39]	0.765	13.57 [8.15-18.00]	0.92 [0.60-1.39]	0.679
rs3739391	DOM						
CC		5.75 [5.06-6.90]	1		15.11 [12.65-18.50]	1	
TT/CT		6.04 [3.91-6.80]	1.13 [0.79-1.63]	0.506	11.14 [8.54-15.64]	1.17 [0.79-1.73]	0.445
rs3739390	DOM						
GG		5.75 [5.03-6.60]	1		14.36 [11.24-15.74]	1	
CC/GC		6.27 [2.60-12.61]	0.83 [0.52-1.31]	0.416	12.81 [8.15-20.89]	0.88 [0.54-1.44]	0.621
rs55633437	DOM						
GG		6.27 [5.75-7.23]	1		15.51 [13.57-18.40]	1	

TT/GT		1.58 [0.76-3.32]	4.79 [2.73-8.35]	**<0.001**	4.66 [2.69-8.57]	4.86 [2.73-8.67]	**<0.001**
rs3020221	REC						
GG/GA		5.78 [5.06-6.60]	1		12.81 [10.35-15.24]	1	
AA		8.77 [4.01-10.78]	0.72 [0.45-1.14]	0.163	18.99 [13.57-36.47]	0.53 [0.31-0.92]	**0.024**
rs1961222	DOM						
GG		5.32 [3.19-6.60]	1		11.24 [8.54-15.01]	1	
AA /GA		6.04 [5.09-8.02]	0.93 [0.65-1.34]	0.712	16.43 [13.57-18.99]	0.67 [0.46-0.99]	**0.044**
rs17063434	DOM						
TT		5.62 [5.03-6.14]	1		0.84 [11.99-17.77]	1	
CC /TC		6.80 [6.14-8.54]	0.93 [0.55-1.56]	0.779	11.24 [8.15-15.64]	1.59 [0.94-2.69]	0.084
rs2916747							
TT		5.78 [5.06-6.60]	1		14.39 [11.99-16.43]	1	
TC		6.27 [1.97-13.83]	0.61 [0.34-1.10]	0.102	11.70 [4.20-27.79]	1.09 [0.61-1.94]	0.784

Abbreviations: DOM, dominant; REC; recessive; VNTR, variable number of tandem repeat; PFS, progression-free survival; OS, overall survival; HR, hazard ratio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PFS
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0.75
0.50
0.25
0.00
TT
CC/TC
P<0.001
0
10
20
30
40
months
Number at risk
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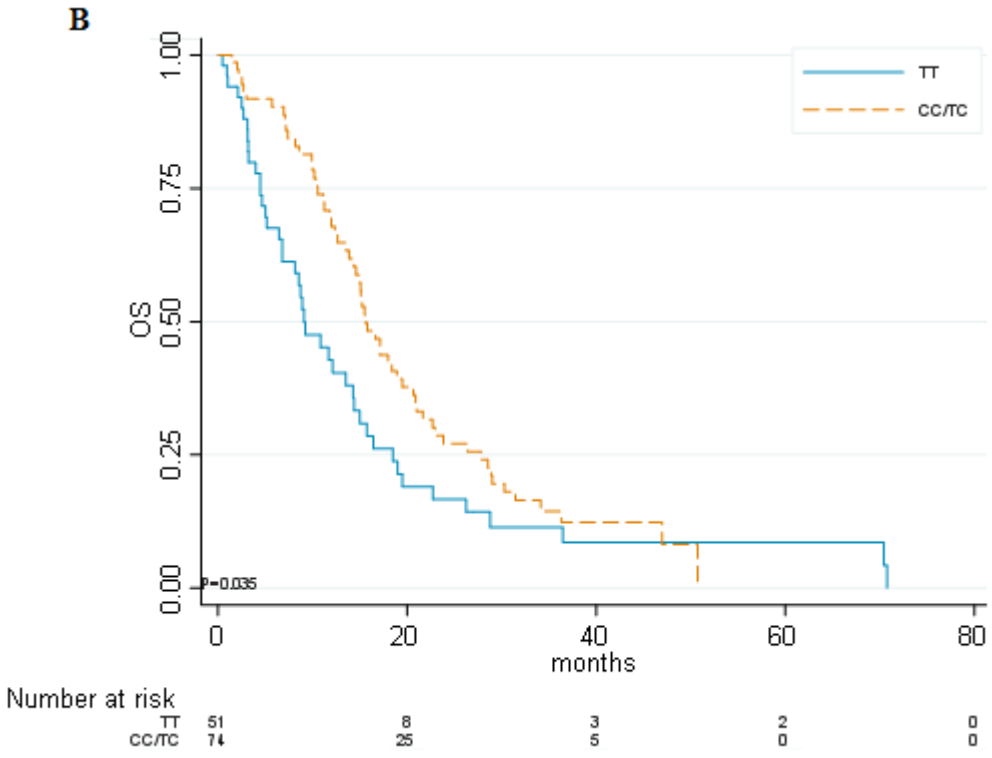
CC/TC 74 21 10 3 0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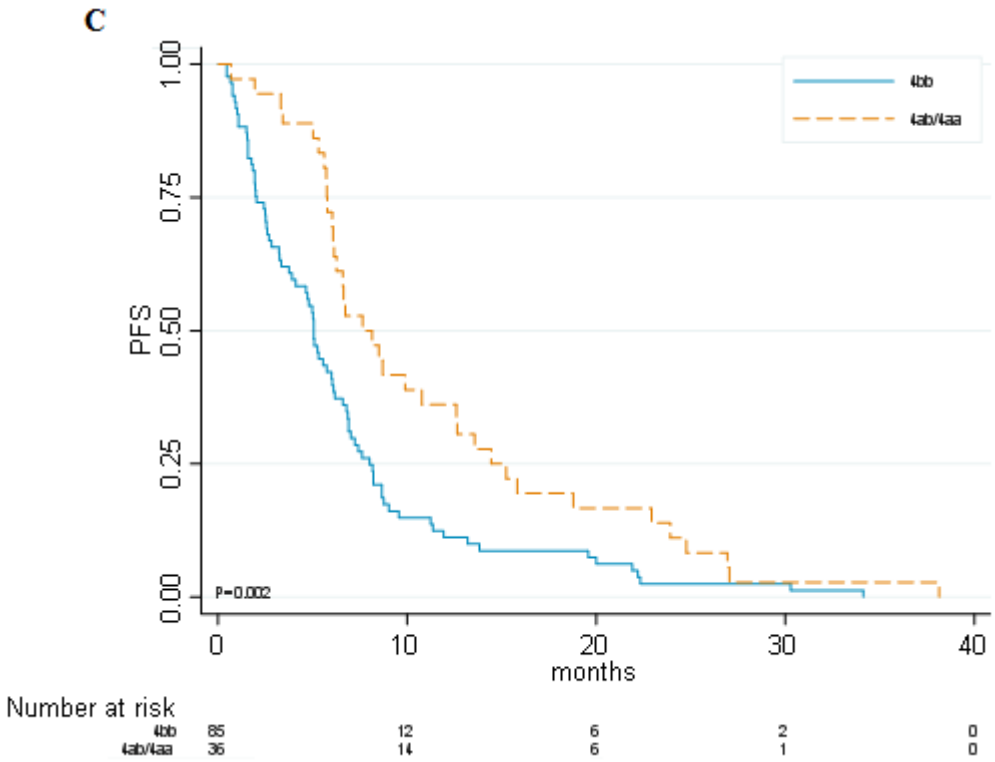


Figure 11. Kaplan Meier curves for *eNOS* SNPs. (A) Progression-free survival (PFS) and **(B)** overall survival (OS) in relation to *eNOS*-786T>C genotypes **(C)** OS in relation to *eNOS* VNTR4a/b genotypes .

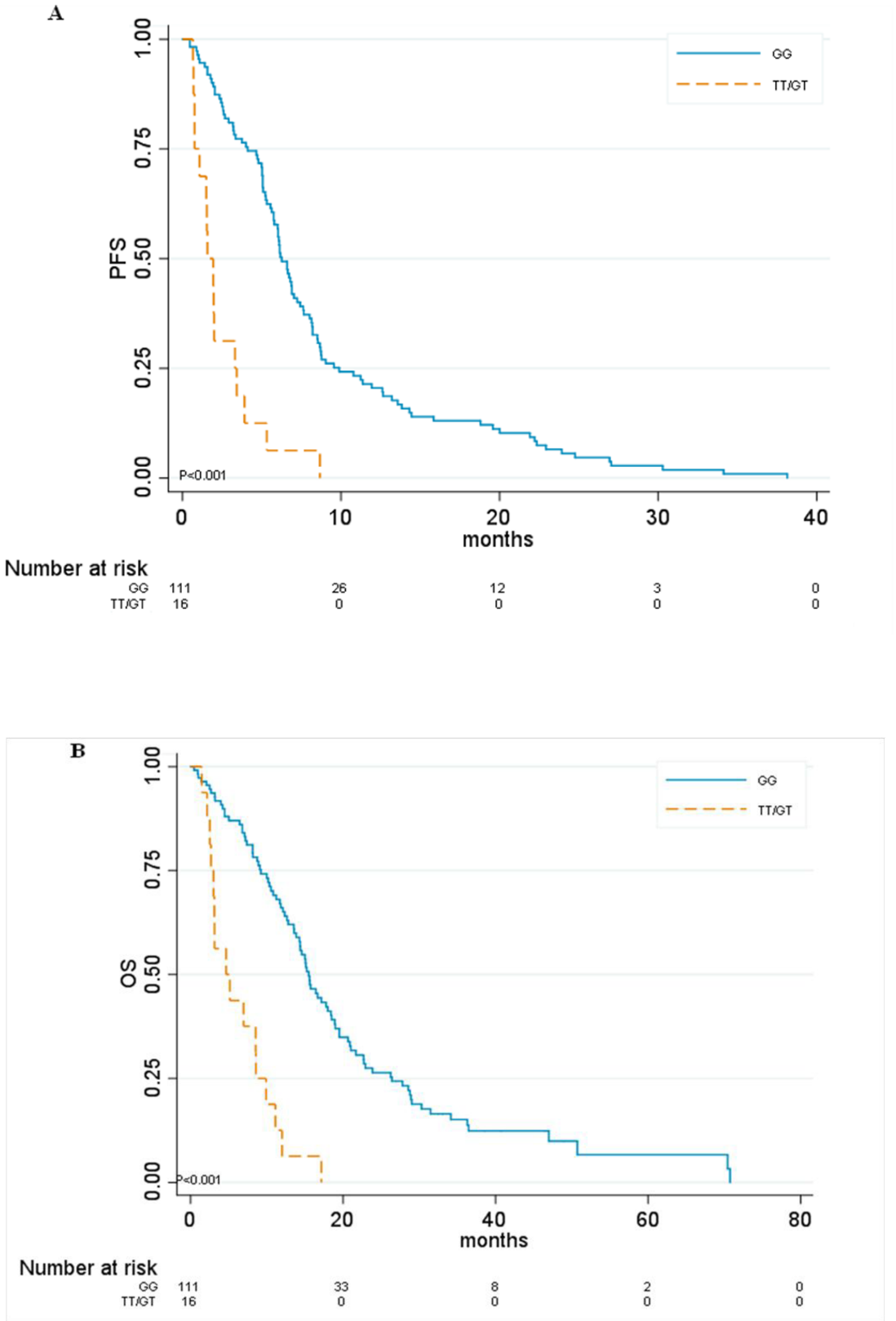


Figure 12. Kaplan Meier curves for *ANGPT2* rs55633437. (A) Progression-free survival (PFS) and (**B)** overall survival (OS) in relation to rs55633437 genotypes .

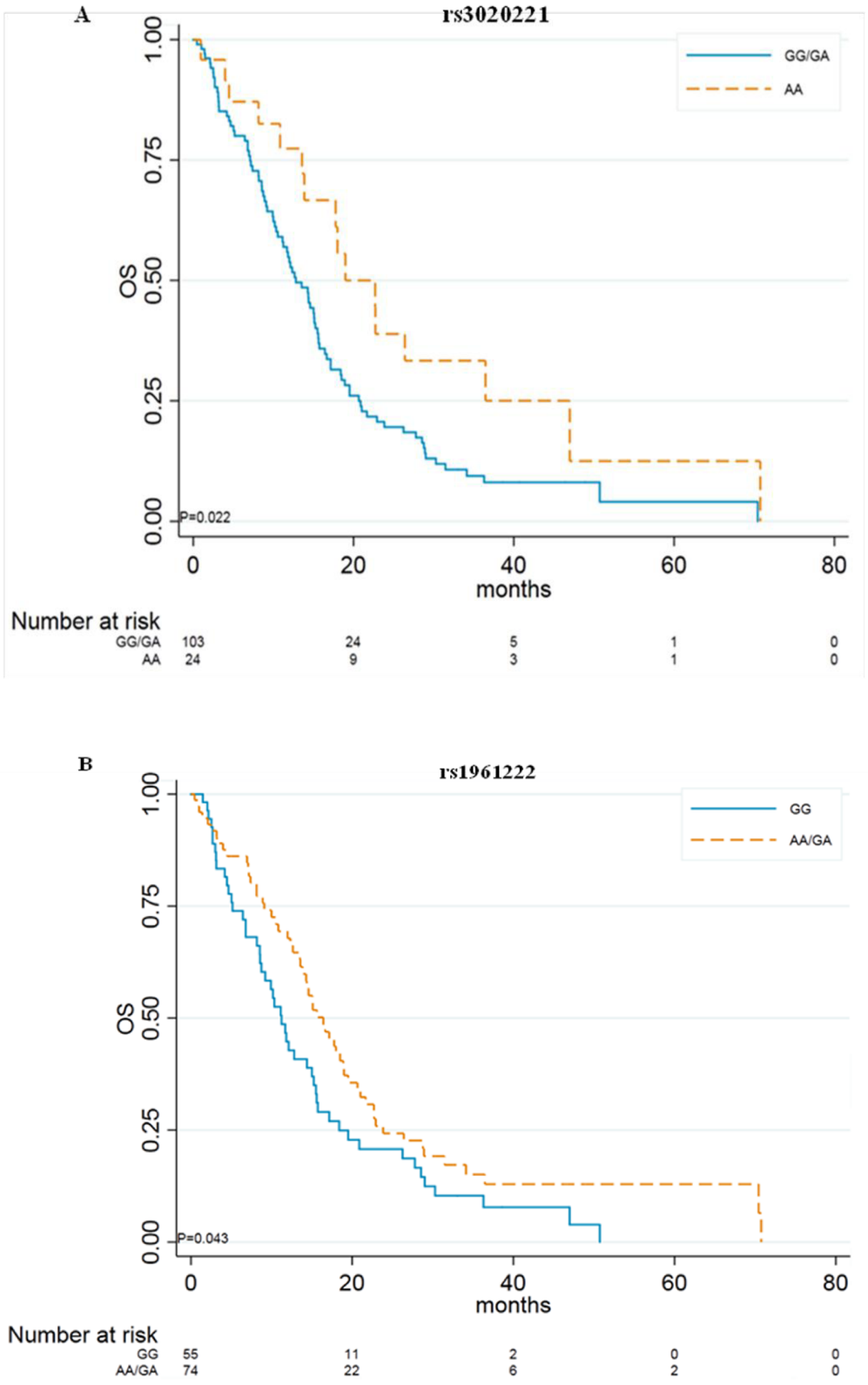


Figure 13. Kaplan Meier curves for *ANGPT2* rs3020221 and rs1961222. OS in relation to rs3020221 genotypes **(A)** and rs1961222 genotypes **(B).**

Following adjustment for clinical covariates (age, etiology and extra hepatic spread), the final model of multivariate analysis confirmed *eNOS*-786 and *ANGPT2* rs55633437 as the independent prognostic factors predicting PFS (HR 0.24, 95% CI 0.15-0.38, $P < 0.001$; HR 6.32, 95% CI 3.32-12.04, $P<0.001$, respectively) and OS (HR 0.67, 95% CI 0.47-0.96, $P = 0.03$; HR 5.48, 95% CI 2.85-10.54, $P<0.001$, respectively) (Table 10). Regarding clinical parameters extra hepatic spread and HBV etiology remained the independent prognostic factors predicting OS (Table 10).

Table 10. Multivariate analysis of OS

	HR_{OS} [95% CI]	**P_{OS}**
Extrahepatic spread		
Yes	1	
No	0.54 [0.35-0.84]	**0.007**
Etiology		
Viral-HCV	1	
Biliary cirrhosis/cryptogenic	0.33 [0.08-1.39]	0.131
Alcoholic	1.69 [0.75-3.84	0.207
Metabolic syndrome	1.50 [0.80-2.83]	0.209
Viral-HBV	2.42 [1.38-4.26]	**0.002**
***eNOS*-786 (rs2070744)**		
TT	1	
CC/TC	0.67 [0.47-0.96]	**0.030**
***ANGPT2* rs55633437**		
GG	1	
TT/GT	5.48 [2.85-10.54]	**<0.001**

4.4 *eNOS* and *ANGPT2* genotypes and disease control rate (DCR)

Four (3.96%) patients showed a complete response (CR) , 28 (27.72%) a partial response (PR), 34 (33.66%) a stable disease (SD) and 35 (34.65) patients showed disease progression (PD). Forty-four patients did not have this information, due to the retrospective nature of the study design.

eNOS and *ANGPT2* polymorphisms were also investigated in relation to the DCR.

Patients carrying at least one copy of the minor allele C for *eNOS*-786 showed a higher percentage of DCR at the first CT re-evaluation than those carrying the TT genotype (81.1% *vs* 48.8% respectively).

For *ANGPT2* polymorphisms, patients carrying at least one copy of the minor allele T for rs55633437 showed a lower percentage of DCR at the first CT re-evaluation than those carrying the GG genotype (13.3% *vs* 75.3% respectively). Patients carrying at least one copy of the minor allele A for rs1961222 showed a higher percentage of DCR at the first CT re-evaluation than those carrying the GG genotype (75.4% *vs* 48.8%, respectively) (Table 11).

No substantial differences were seen between other SNPs and response.

Table 11. *ANGPT2* SNPs and DCR

***ANGPT2* SNPs**	**Patients No.**	**CR/PR/SD No. (%)**	**PD No. (%)**	***P***
rs55633437				
GG	81	61 (75.3)	20 (24.7)	
TT/TG	15	2 (13.3)	13 (86.7)	**<0.001**
rs1961222				
GG	41	20 (48.8)	21 (51.2)	
AA/GA	57	43 (75.4)	14 (25.6)	**0.036**

4.5 *eNOS* and *ANGPT2* genotypes and toxicities

We also investigated the relationship between these polymorphisms and the main toxicities (skin toxicity, asthenia and diarrhea). We divided these toxicities into early, within a month of sorafenib treatment, and late, after a month of treatment.

We found that *eNOS*+894 (rs1799983) was associated with late skin toxicity (*P*=0.021) and with a higher grade (CTCAE 4.0) of this toxicity (*P*=0.003).

Moreover, we found that *ANGPT2* rs1961222 and rs17063434 were associated with late skin toxicity with grade >=2 (CTCAE 4.0) (*P*=0.030 and *P*=0.003, respectively).Moreover, *ANGPT2* rs2916747 was associated with any late toxicities (*P*=0.031), in particular with diarrhea and skin adverse events.

No significant associations were observed between other *ANGPT2* polymorphisms and skin toxicity, asthenia and diarrhea (data not shown).

4.6 Haplotypes analysis

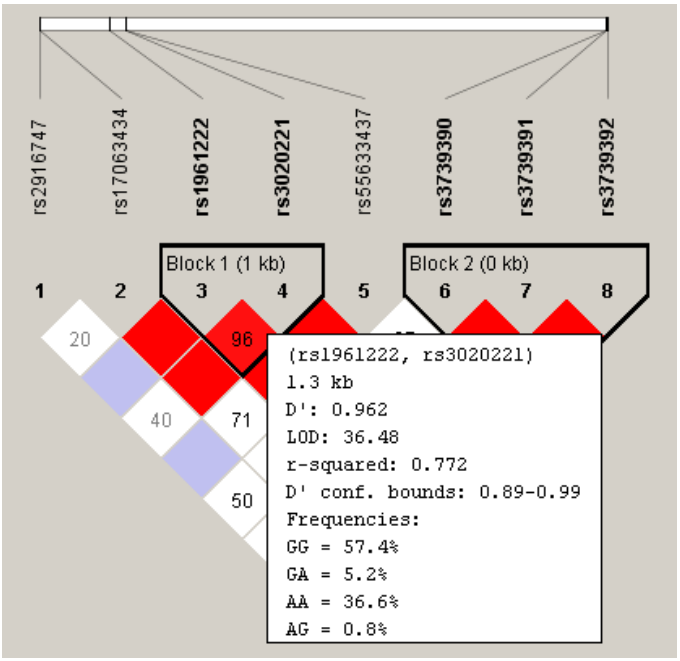
We did not observe linkage disequilibrium between *eNOS* polymorphisms and we did not identified any haplotypes.

We instead observed linkage disequilibrium between *ANGPT2* polymorphisms. Lewontin's disequilibrium coefficient (D') and correlation coefficient (r^2) are reported in Figure 14.

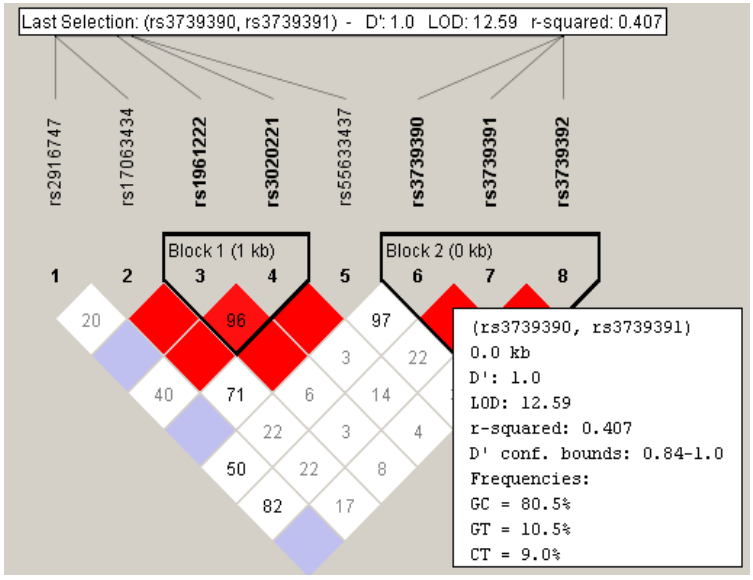
We identified two blocks of SNPs by Haploview software version 4.2 and for both blocks we identified a total of four haplotypes. For Block 1, including rs1961222 and rs3020221, the most frequent haplotype was HT1 (G-G at rs302022 /rs1961222) (57.1%), followed by HT4 (A-A) (38%), HT3 (A-G) (5.2%) and HT2 (G-A) (1%) (Figure 15A).

For Block 2, including rs3739392, rs3739391 and rs3739390, the most frequent haplotype was HT1 (T-C-G at rs3739392/rs3739391/rs3739390) (80.2%), followed by HT4 (C-T-C) (9.1%), HT3 (C-T-G) (6.3%) and HT2 (T-T-G) (4.4%) (Figure 15B).

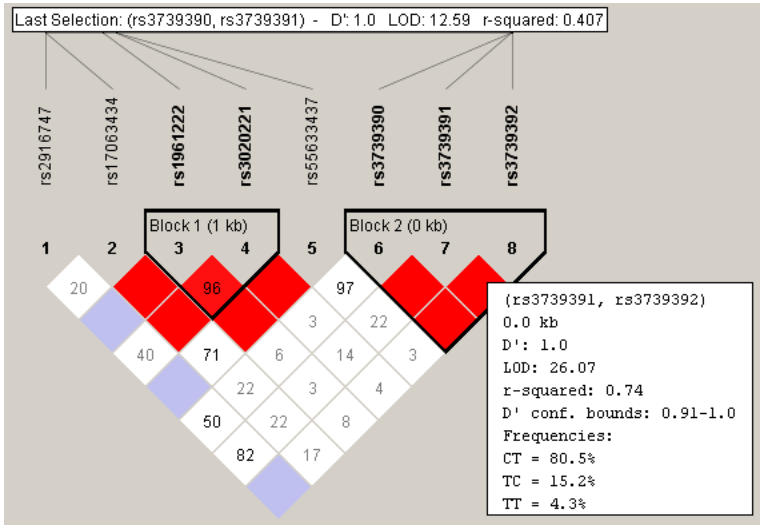
A



B


Last Selection: (rs3739390, rs3739391) - D': 1.0 LOD: 12.59 r-squared: 0.407
rs2916747
rs17063434
rs1961222
rs3020221
rs55633437
rs3739390
rs3739391
rs3739392
Block 1 (1 kb)
Block 2 (0 kb)
1 2 3 4 5 6 7 8
20
96
97
3 22
40 71 6 14
22 3 4
50 22 8
82 17
(rs3739390, rs3739391)
0.0 kb
D': 1.0
LOD: 12.59
r-squared: 0.407
D' conf. bounds: 0.84-1.0
Frequencies:
GC = 80.5%
GT = 10.5%
CT = 9.0%

C


Last Selection: (rs3739390, rs3739391) - D': 1.0 LOD: 12.59 r-squared: 0.407
rs2916747
rs17063434
rs1961222
rs3020221
rs55633437
rs3739390
rs3739391
rs3739392
Block 1 (1 kb)
Block 2 (0 kb)
1 2 3 4 5 6 7 8
20
96
97
3 22
40 71 6 14 3
22 3 4
50 22 8
82 17
(rs3739391, rs3739392)
0.0 kb
D': 1.0
LOD: 26.07
r-squared: 0.74
D' conf. bounds: 0.91-1.0
Frequencies:
CT = 80.5%
TC = 15.2%
TT = 4.3%

D

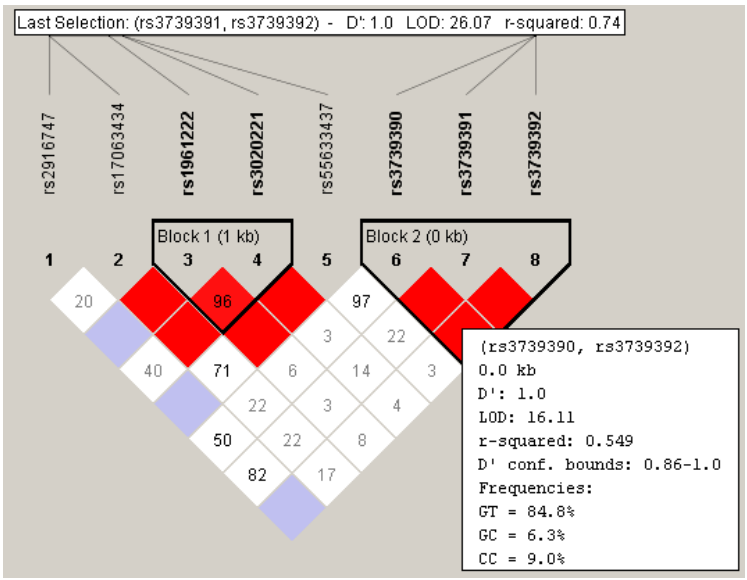

Last Selection: (rs3739391, rs3739392) - D': 1.0 LOD: 26.07 r-squared: 0.74
rs2916747
rs17063434
rs1961222
rs3020221
rs55633437
rs3739390
rs3739391
rs3739392
Block 1 (1 kb)
Block 2 (0 kb)
1 2 3 4 5 6 7 8
20
96
97
3 22
40 71 6 14 3
22 3 4
50 22 8
82 17
(rs3739390, rs3739392)
0.0 kb
D': 1.0
LOD: 16.11
r-squared: 0.549
D' conf. bounds: 0.86-1.0
Frequencies:
GT = 84.8%
GC = 6.3%
CC = 9.0%

Figure 14: Haploview linkage disequilibrium plot and identification of haplotype block in *ANGPT2* gene. Lewontin's disequilibrium coefficient (D') and correlation coefficient (r^2) between the two SNPs of Block 1 **(A)**; rs3739390 and rs3739391of Block 2 (**B);** rs3739391 and rs3739392of Block 2 (**C);** rs3739390 and rs3739392of Block 2 **(D).** Pairwise linkage disequilibrium (LD) coefficients D′×100, indicating extent of LD between SNPs, are shown in each square (D′ values of 1.0 are not shown). Higher color intensity of the squares indicates higher LD between SNPs. The inverted black triangle represents a single haplotype block.

A

	rs3020221	rs1961222	hap.freq
1	1 (G)	1 (G)	0.57143
2	1 (G)	2 (A)	0.00794
3	2 (A)	1 (G)	0.05159
4	2 (A)	2 (A)	0.36905

B

	rs3739392	rs3739391	rs3739390	hap.freq
1	1 (T)	1 (C)	1 (G)	0.80159
2	1 (T)	2 (T)	1 (G)	0.04365
3	2 (C)	2 (T)	1 (G)	0.06349
4	2 (C)	2 (T)	2 (C)	0.09127

Figure 15: *ANGPT2* haplotypes frequencies. (A) Block 1, including rs1961222 and rs3020221 **(B)** Block 2, including rs3739392, rs3739391 and rs3739390. "1"represented the major allele, "2" represented the minor allele.

4.7 *ANGPT2* haplotypes and clinical outcomes

Haplotypes analysis was performed considering only those subjects for whom there were no missing data on the five *ANGPT2* SNPs involved in the two haplotypes blocks (n=126). Regarding the block 1, at univariate analysis patients carrying at least one copy of HT1 had a lower median OS than those without any copies of HT1 (12.8 *vs* 21.7 months; HR 1.75 95%CI 1.04-2.95; *P*=0.037) (Table 12). No statistically significant differences were observed for other *ANGPT2* haplotypes of block 1 in relation to PFS and OS (Table 12).

Table 12: Univariate analysis of PFS and OS in relation to Block 1 *ANGPT2* haplotypes

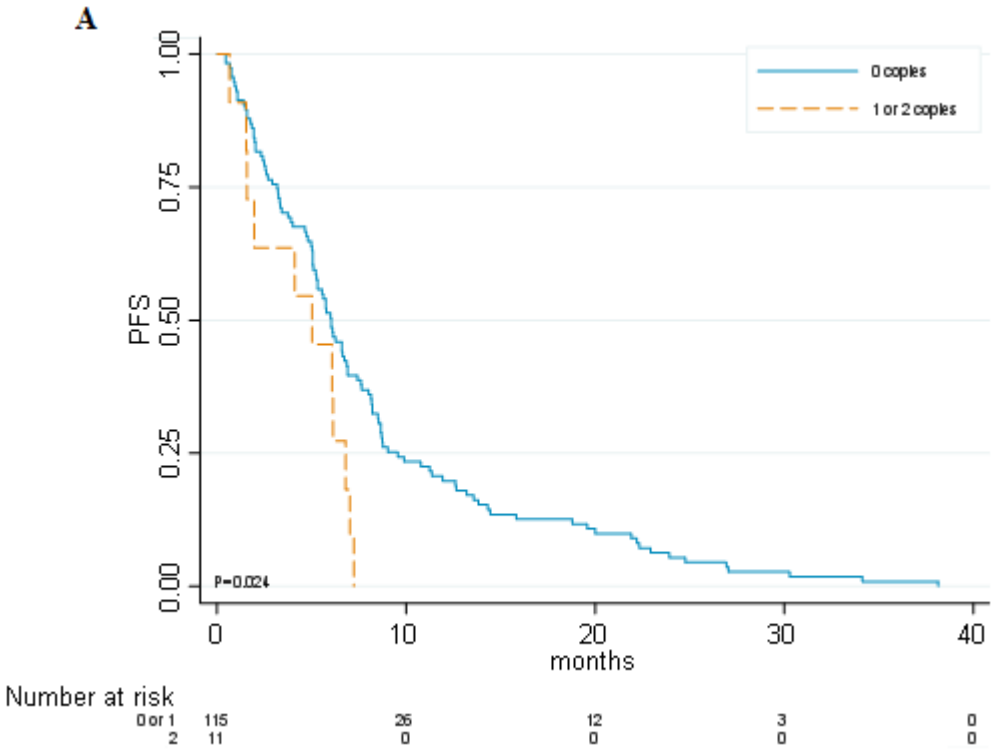
		PFS			OS		
	No. (%)	**Median$_{PFS}$ [95% CI]**	**HR$_{PFS}$ [95% CI]**	**P$_{PFS}$**	**Median$_{OS}$ [95% CI]**	**HR$_{OS}$ [95% CI]**	**P$_{OS}$**
HT1 (G-G)							
0 copies	26 (20.6)	8.21[5.03-11.37]	1		21.7 [13.9-NA]	1	
1 or 2 copies	100 (79.4)	5.78 [5.06-6.64]	1.33 [0.85-2.09]	0.213	12.8[10.6-15.5]	1.75 [1.04-2.95]	**0.037**
HT2 (G-A)							
0 copies	124 (98.7)	6.01 [5.22-6.8]	1		14.4[11.9-16.7]	1	
1 or 2 copies	2 (1.3)	5.27 [2.33-NA]	1.41 [0.35-5.74]	0.631	12.4 [3.19-NA]	1.40 [0.35-5.72]	0.64
HT3 (A-G)							
0 copies	114 (90.5)	6.01 [5.09-6.8]	1		14.3 [12.0-16.7]	1	
1 or 2 copies	12 (9.5)	6.07 [2.69-NA]	0.65 [0.35-1.23]	0.185	15.0 [10.8-NA]	0.76 [0.39-1.48]	0.42
HT4 (A-A)							
0 copies	52 (41.3)	5.32 [3.75-6.87]	1		11.2 [8.74-15.5]	1	
1 or 2 copies	74 (58.7)	6.04 [5.22-8.15]	0.97 [0.67-1.4]	0.862	16.4 [13.86-19.5]	0.68 [0.46-1.00]	0.05

After adjustment for age, etiology, extrahepatic spread, and specific SNPs significant from the previous analysis, none of the haplotypes was associated with PFS and OS (data not shown).

Interestingly, regarding the block 2, at univariate analysis, patients carrying at least one copy of HT2 had a lower median PFS (5.03 *vs* 6.04 months; HR 2.05 95%CI 1.08-3.89; *P*=0.027) and OS (9.9 *vs* 15.1 months; HR 2.71 95%CI 1.37-5.38; *P*=0.004) than those without any copies of HT2 (Table 13 and Figure 16).

Table 13: Univariate analysis of PFS and OS in relation to Block 2 *ANGPT2* haplotypes

		PFS			OS		
	No. (%)	**Median$_{PFS}$ [95% CI]**	**HR$_{PFS}$ [95% CI]**	***P*$_{PFS}$**	**Median$_{OS}$ [95% CI]**	**HR$_{OS}$ [95% CI]**	***P*$_{OS}$**
HT1 (T-C-G)							
0 copies	3 (2.4)	4.73 [1.02-NA]	1		7.39 [1.02-NA]	1	
1 or 2 copies	123(97.6)	6.04 [5.22-6.87]	1.06 [0.34-3.36]	0.921	14.39 [11.9-17.1]	1.20 [0.36-4.05]	0.763
HT2 (T-T-G)							
0 copies	115(91.3)	6.04 [5.26-6.9]	1		15.08 [12.81-18.4]	1	
1 or 2 copies	11(8.7)	5.03 [1.97-NA]	2.05 [1.08-3.89]	**0.027**	9.99 [5.16-NA]	2.71 [1.37-5.38]	**0.004**
HT3 (C-T-G)							
0 copies	111(88.1)	6.04 [5.22-6.90]	1		14.6 [11.9-17.1]	1	
1 or 2 copies	15 (11.9)	6.01 [2.50-9.89]	1.33 [0.77-2.3]	0.303	13.6 [7.39-NA]	1.13 [0.62-2.07]	0.689
HT4 (C-T-C)							
0 copies	104(82.5)	5.75 [5.06-6.8]	1		14.4 [11.83-16.7]	1	
1 or 2 copies	22 (17.5)	6.45 [3.22-13.8]	0.82 [0.52-1.31]	0.407	14.2 [8.74-28.9]	0.86 [0.52-1.40]	0.536



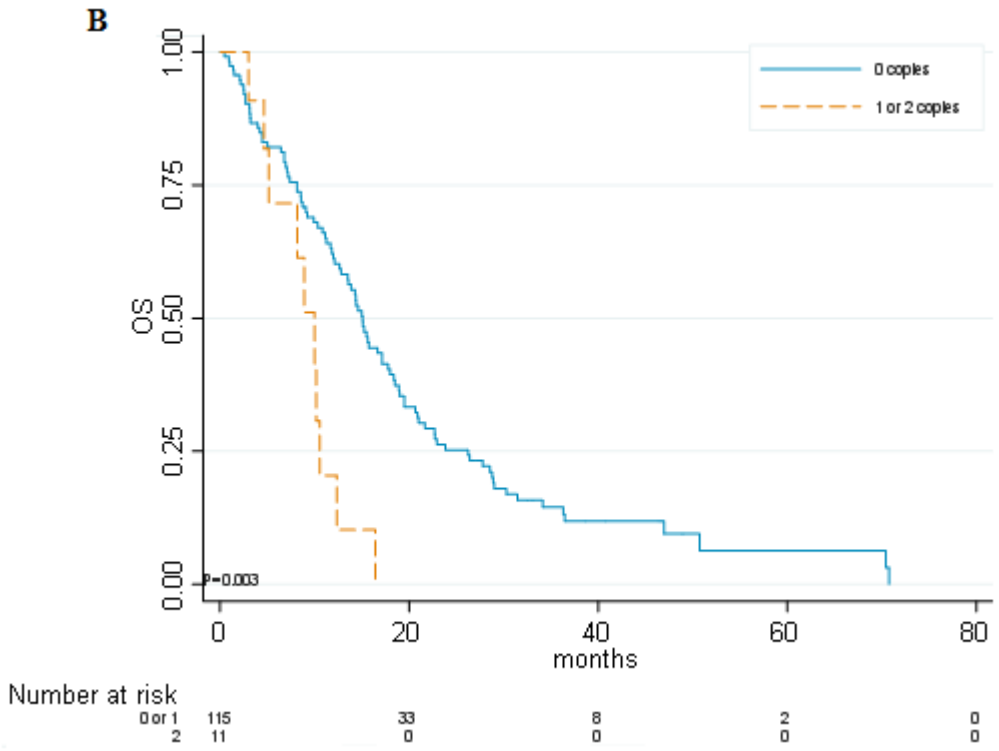


Figure 16. Kaplan Meier curves for *ANGPT2* haplotype 2 (Block 2). (A) Progression-free survival (PFS) and **(B)** overall survival (OS).

The final model of multivariate analysis, including age, etiology and extra hepatic spread, confirmed previous *eNOS* and *ANGPT2* polymorphism and haplotype 2 (HT2) of block 2 as the independent prognostic factors predicting PFS (HR 0.24, 95% CI 0.15-0.38, $P <$ 0.001; HR 6.03, 95% CI 3.1-11.6, P<0.001; HR 2.48, 95% CI 1.2-5.2, P =0.015, respectively) and OS (HR 0.46, 95% CI 0.29-0.73, P = 0.001; HR 4.88, 95% CI 2.99-11.5, P<0.001; HR 2.30, 95% CI 1.02-5.2, P =0.044, respectively). Regarding clinical parameters extra hepatic spread and HBV etiology remained the independent prognostic factors predicting OS (Table 14).

Table 14. Multivariate analysis of OS, considering *eNOS*, *ANGPT2* SNPs and haplotypes

	HR_{OS} [95% CI]	**P_{OS}**
Extrahepatic spread		
Yes	1	
No	0.56 [0.36-0.89]	**0.015**
Etiology		
Viral-HCV	1	
Biliary cirrhosis/cryptogenic	0.28 [0.07-1.2]	0.088
Alcoholic	2.04 [0.89-4.68]	0.092
Metabolic syndrome	1.58 [0.84-2.99]	0.158
Viral-HBV	2.57 [1.43-4.59]	**0.002**
***ANGPT2* rs55633437**		
GG	1	
TT/GT	4.88 [2.99-11.53]	**<0.001**
***eNOS*-786 (rs2070744)**		
TT	1	
CC/TC	0.46 [0.29-0.73]	**0.001**
***ANGPT2* HT2 (T-T-G)**		
0 copies	1	
1 or 2 copies	2.30 [1.02-5.19]	**0.044**

4.8 Preliminary data of INNOVATE study

This is a prospective Italian multicenter study, that includes 160 HCC patients receiving sorafenib. For this interim analysis we analyzed *eNOS*-786 (rs2070744) polymorphism on 119 patients. *eNOS*-786 was analyzed by Real Time PCR in relation to the primary end point (OS).

119 HCC patients (102 males and 17 females), prospectively treated with sorafenib from May 2015 to September 2018 were included. Median age was 69 years (range 28-88 years). 95 patients had Child-Pugh A and 23 had Child-Pugh B7. 42 had BCLC-B and 77 patients had BCLC-C.

At univariate analysis, we confirmed that *eNOS*-786 TT genotype were significantly associated with a lower median OS than the other genotypes (13.3 *vs* 18.7 months

respectively *P*= 0.021, HR 1.96, 95% CI 1.08-2.94 *P*=0.023) (Figure 17). Moreover, patients carrying a TT genotype for *eNOS*-786 showed a lover percentage of Disease Control Rate at the first CT re-evaluation than those carrying other genotypes (50.5% *vs*. 25%).

These preliminary data confirm the prognostic role of *eNOS*-786 in HCC patients treated with sorafenib and this *eNOS* polymorphisms may identify patients who more likely may benefit from sorafenib treatment.

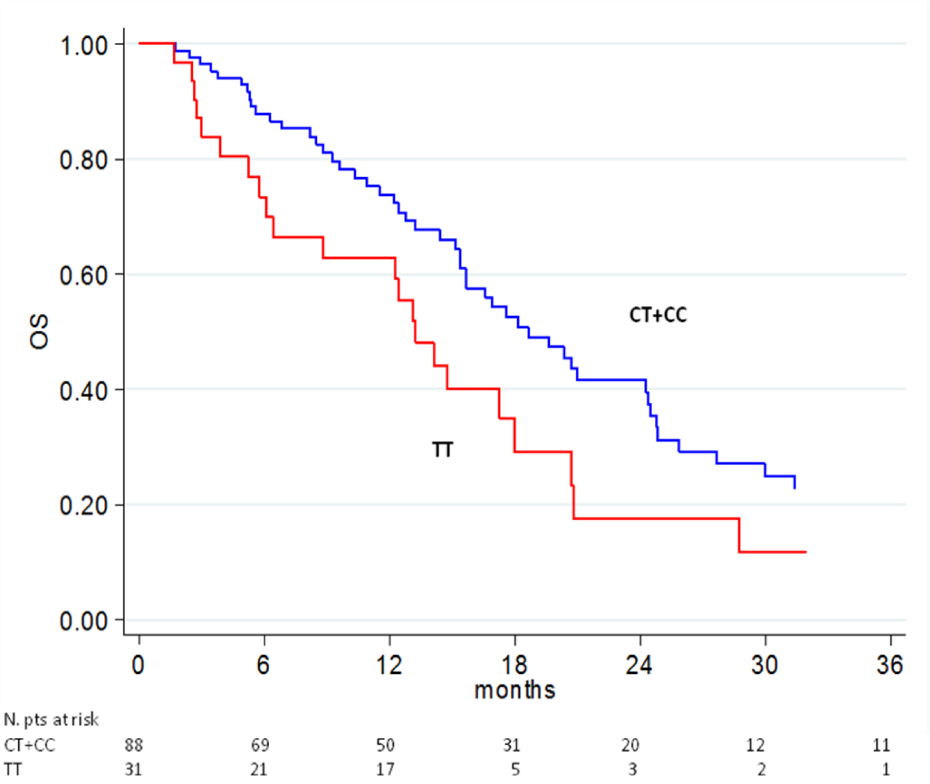


Figure 17. OS in relation to *eNOS*-786 polymorphisms in the INNOVATE study

5. DISCUSSION

The present study analyzed *eNOS* and *ANGPT2* polymorphisms related to clinical outcome in patients with advanced hepatocellular carcinoma receiving sorafenib. In particular we found that patients with *eNOS*-786 TT and *ANGPT2* rs55633437 TT/GT genotypes had a significantly lower median OS and PFS than patients with other genotypes. We identified also a specific *ANGPT2* haplotype (characterized by *ANGPT2* rs3739392, rs3739391 and rs3739390) that was significantly associated with worse OS and PFS.

Moreover, we found that patients with HCV etiology and without extra hepatic spread showed better outcomes in terms of OS, in agreement with Bruix et al's pooled analysis[59]. They demonstrated that the benefit of sorafenib is significantly higher in patients with the disease confined into the liver (without extra hepatic spread), with HCV, or low NLR (neutrophil lymphocyte ratio).

Our results showed also that patients with a more advanced age at start of sorafenib treatment show better clinical outcome. A possible explanation for this phenomenon can be that patients with HBV chronic infections develop the disease at an earlier age and show a worse prognosis. Conversely, metabolic syndrome causes the disease in subjects with a more advanced age and these patients show a better prognosis.

We found that patients with *eNOS*-786 TT genotypes and patients carrying at least one copy of the minor allele T for rs55633437 showed a lower percentage of DCR at the first CT re-evaluation Moreover, patients with other genotypes associated with a better PFS and OS showed higher response rates.

Currently, the measurement of specific predictive biomarkers for cancer therapy is mandatory in patients with different cancer types[95], but for hepatocellular carcinoma biomarkers of sorafenib efficacy or resistance have yet to be identified[35].

In the literature, only a few studies have identified possible markers of response to sorafenib in HCC patients. In the SHARP trial, Llovet and co-workers found that low VEGF-A and Ang-2 plasma baseline concentrations predicted survival in patients with advanced HCC in both the entire patient population and the placebo cohort[22]. Conversely, none of the tested biomarkers significantly predicted response to sorafenib[22].

However, other authors did not find any association between VEGF-A and prognosis in patients treated with sorafenib[71].

In the presence of VEGF, Ang-2 destabilizes blood vessels, promotes vascular sprouting, and it is

associated with an invasive and metastatic cancer phenotype[96].

Llovet and co-workers demonstrated also that high baseline plasma Ang-2 levels were related with a more aggressive disease[22]. Moreover, Ang-2 protein levels increased during treatment in the placebo group, suggestive of poor outcome related to disease progression in this cohort, whereas they remained constant during treatment with sorafenib, reflecting the generally more favorable outcome of patients in the sorefenib-treated group[22]. Overall increased Ang-2 expression levels were associated with poorer outcome in both groups, suggesting that this marker could be useful in monitoring treatment response. In agreement with Llovet's study, Miyahara *et al.* reported that high baseline Ang-2 serum levels were associated with poor outcome in advanced HCC patients receiving sorafenib[71].

Single nucleotide polymorphism (SNP) analysis seems to have more advantages than protein or gene expression analysis. The latter is performed on biological material collected at a specific time point in the natural history of the disease and it can be also under the influence of a number of laboratory biases. Conversely, SNPs analysis can be performed at any time during the course of the disease, is not substantially influenced by laboratory biases and furthermore is also less expensive.

In this regard, Scartozzi *at al.* in the ALICE-1 study[60,97] and Faloppi *et al.* in the ALICE-2 study[62] showed that specific SNPs in *VEGF-A, VEGF-C* and *HIF-1*α genes were independent factors influencing PFS and OS in HCC patients receiving sorafenib.

Our study demonstrate the role of *eNOS* and *ANGPT2* polymorphisms in relation to clinical outcome in advanced HCC patients receiving sorafenib.

eNOS is a constitutively expressed gene in endothelium involved in the production of nitric oxide (NO) and it plays a central role in maintaining endothelial cell functional integrity, regulating hemodynamics and establishing collateral circulation[98]. An adequate NO production is essential for preventing thrombotic and atherogenic processes[99].

Previous studies suggested that DNA variants of the *eNOS* gene can quantitatively control *eNOS* expression[51,100,101].

The point variation at nucleotide-786bp has been associated with a significant reduction in *eNOS* gene promoter activity, resulting in lower levels of *eNOS* mRNA, eNOS protein and enzyme activity[51,101].

Intron 4 *eNOS* VNTR polymorphism plays a role in regulating eNOS expression though the coding for a 27-nt small RNA (sirRNAs) which appears to inhibit eNOS expression at the transcriptional level[102].

Endothelial cells containing 5 repeats show higher quantities of sirRNA and lower levels of eNOS mRNA when compared with cells containing 4 repeats[103].

However, the association between *eNOS* VNTR in intron 4 and eNOS expression is still a much debated issue[104–106].

The rare allele 4-repeat homozygote shows the highest eNOS mRNA levels, which are, however, associated with lower eNOS protein levels and enzyme activity[51,101].

eNOS+894G>T variation in exon 7 of the *eNOS* gene, leading to an amino acid change from Glu to Asp (Glu298Asp), was associated with reduced eNOS protein levels, enzyme activity and basal NO production[107].

Our results showed that patients homozygous for T allele for the *eNOS*-786 variant had lower PFS and OS. Moreover patients homozygous for the five repetitions (4bb) of *eNOS* VNTR had a lower PFS. In agreement with previous studies, these types of variants seem to be associated with higher eNOS protein levels and enzyme activities, and consequently with increased basal NO production.

We therefore hypothesized an association between high levels of eNOS protein/ activity and sorafenib resistance.

ANGPT2 gene is an highly polymorphic gene[54] and single nucleotide polymorphisms may alter gene expression[55]. Some SNPs have been studied in association with obstetric diseases, premature retinopathy and acute respiratory distress syndrome[56–58].

Some authors investigated the role of *ANGPT2* variants in colorectal cancer patients with liver metastases[108]or in breast cancer patients[109] in relation to bevacizumab-based treatmetent, another anti-angiogenic drug, but no work studied the impact of Ang2 genetic variants in relation to treatment in HCC patients.

The functional role of our *ANGPT2* polymorphisms are not well documented in literature, but SNP function prediction tools reveal that these SNPs could be located inside transcription factor binding sites (TFBS) or exonic splicing enhancers/silencer (ESE or ESS).

In particular the three SNPs of Block 2 (rs3739390, rs3739391 and rs3739392), located in the 5' UTR region, could be found in a transcription factor binding site and have an effect on protein synthesis. Thus, it will be interesting to evaluate a correlation between the presence of a specific allele on a polymorphic site and the expression of the respective protein.

eNOS and ANGPT2 are not the direct target of sorafenib, other factors may be involved in the relation between eNOS activity, Ang-2 and sorafenib efficacy. In particular, it is possible that these genetic variants are linked with other functional variants in the regulatory regions of the *eNOS* or *ANGPT2* gene and these variants may create altered funcionality.

With regard to toxicity, we found that *eNOS* rs1799983, *ANGPT2* rs1961222 and rs17063434 were associated with late skin toxicity.

The development of Dermatology Adverse Events (DAEs) early (within the first 60 days of treatment) after treatment initiation is associated to a delayed tumor progression and improved survival[110]. It has been recently demonstrated that the angiotensinogen (AGT) M235T SNPs can predicts early DAEs in HCC patients treated with sorafenib[111].The identification of predictive biomarkers for early DAEs would be important to define a population with a major survival treatment impact.

It has been shown that patients with hypertension during sorafenib treatment showed better PFS and OS[112,113].

In particular, in an our previous work we found that the early onset of hypertension was associated with improved clinical outcome in HCC patients treated with sorafenib[64].

Increase in blood pressure seems to be closely related to eNOS. The activation of VEGFR-2 also stimulates the production of NO and inhibits endothelin-1 (ET- 1), a potent vasoconstrictor[114]. In patients treated with sorafenib, inhibition of VEGFR-2 induces a decrease in eNOS expression and thus in NO production[115] resulting in vasoconstriction and hypertension, one of the most common toxicities of VEGFR inhibitors[116].

Previous studies have demonstrated an association between specific *eNOS* polymorphisms and hypertension[52,117].

Unfortunately, we did not have hypertension data available, due to the retrospective nature of the study, but given the possible correlation between *eNOS* polymorphisms and hypertension, it will be interesting to evaluate this in our ongoing prospective study.

Sorafenib is the first oral molecular targeted agent for unresectable advanced HCC, while transarterial chemoembolization (TACE) is the first-line treatment option for intermediate-stage HCC. Several clinical trials investigated the efficacy of TACE combined with sorafenib[118–121], however, these studies have not reported major treatment outcomes to date[122]. It is important to consider the reasons for the negative results and to carefully plan future clinical trials of combination therapy with TACE, maybe selecting patients on the basis of molecular markers.

The results obtained from our analysis of *eNOS* and *ANGPT2* polymorphisms suggest that they could identify potential candidates for treatment with combined therapies including TACE-sorafenib and could help to evaluate the efficacy of sorafenib in patients without good liver function (Child-Pugh B).

The study has some limitations, *e.g.* its retrospective nature (cases were, however, consecutively selected, thus reducing potential bias). Thus, we were not able to collect

detailed data on toxicities, in particular on hypertension, and on neutrophil-to-lymphocyte ratio (NLR).

As our study was carried out on Caucasian individuals only, our findings cannot be automatically extrapolated to patients of other ethnicities. Another limitation of our study is the absence of a control arm not receiving sorafenib. Thus, a clear distinction cannot be made between the prognostic and predictive role of *eNOS* and *ANGPT2* polymorphisms in relation to survival.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, our results suggest that *eNOS*-786, *ANGPT2* rs55633437 polymorphisms and the presence of a specific *ANGPT2* haplotype may be capable of identifying a subset of HCC patients who are more resistant to sorafenib in terms of OS, PFS and DCR.

These data now require further validation in the ongoing multicenter prospective INNOVATE study (NCT02786342). If confirmed, these biomarkers could represent valid criterions for selecting candidates for treatment with sorafenib.

Moreover, a correlation between polymorphisms and the protein expression levels will be interesting to evaluate in this prospective study.

We will measure plasma eNOS and Ang-2 levels at baseline and during treatment (day14, day28,day60 and at disease progression) to investigate whether changes in their levels might be correlated with therapeutic efficacy and disease outcome.

Given the prominent arrive of immunotherapy in the first line of HCC treatment it will be very important to identify prognostic and predictive factors for sorafenib and/or new drugs in this setting.

In this context, the use of metabolic profiling and whole genome analysis to examine the association between patient outcome and response to sorafenib could become a new approach to search new biomarkers in HCC.

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