Association of NOS3 and ANGPT2 Gene Polymorphisms with Survival in Patients with Hepatocellular Carcinoma Receiving Sorafenib: Results of the Multicenter Prospective INNOVATE Study

Association of *NOS3* and *ANGPT2* gene polymorphisms with survival in patients with hepatocellular carcinoma receiving sorafenib: results of the multicenter prospective INNOVATE study

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Running title: Polymorphisms in HCC patients treated with sorafenib

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were reported.

Abstract

Purpose: After 10 years of clinical practice and research studies, there are still no validated prognostic or predictive factors of response to sorafenibfor hepatocellular carcinoma (HCC).On the basis of the results of our two retrospective studies, we designed the multicenter INNOVATE study with the aim to validate the role of *NOS3* and *ANGPT2* polymorphisms in HCC patients treated with sorafenib [NCT02786342].

Experimental Design: This prospective multicenter study was conducted at 10 centres in Italy. All eligible patients received a continuous oral treatment with 400 mg of sorafenib twice daily. Genotyping analysis was performed for *NOS3* (rs2070744) and *ANGPT2* SNPs (rs55633437).The primary outcome was progression free survival (PFS), while secondary outcomes included overall survival (OS) and disease-control rate (DCR).

Results:165 patients were enrolled between March2016 and June 2018. *NOS3* rs2070744 CC/CT genotypes were significantly associated with a higher median PFS(5.9 *vs* 2.4 months, HR 0.43, p=0.0007) and OS(15.7 *vs* 8.6 months, HR 0.38, p<0.0001) compared to TT genotype.There was no statistically significant association between*ANGPT2* rs55633437 TT/GT genotypes and PFS(2.4 *vs* 5.7 months, HR 1.93, p=0.0833) and OS(15.1 *vs* 13.0 months, HR 2.68, p=0.55) when compared to the other genotype. Following adjustment for clinical covariates,multivariate analysis confirmed *NOS3* as an independent prognostic factor for PFS (HR 0.50, p=0.0128)and OS (HR 0.29, p=0.0041).

Conclusions: The INNOVATE study met the primary endpoint, confirming that advanced HCC patients with *NOS3* rs2070744 CC/CT genotypes had a better prognosis with respect to TT genotype patients.

Keywords: Prognostic factor, Advanced hepatocellular carcinoma, biomarkers, regorafenib, second line, eNOS

Translational relevance

A significant proportion of HCC patients treated with sorafenib are experiencing difficult-to-treat and quality of life impairing side-effects, without any clinical benefit. In this scenario, clinical or biological predictive factors, able to identify up-front responding and refractory patients, would represent an important clinical asset for both research and patient stratification in clinical practice.

Unfortunately, after more than 10 years of clinical and translational research,still there are no validated biomarkers helping physicians in the decision-making process useful in maximizing results, sparing unnecessary toxicities and possibly guiding treatment selection.

This study confirms that *NOS3* rs2070744 CC/CT genotypes are capable to identifysorafenib-treated patients with longer survival. *NOS3* genotyping analysis is a simple analysis that allows us to better stratify patients in future randomized studies.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer andit hasbecome the 5th most common malignancy worldwide and it is the 3rdleading cause of cancer-related death. The estimated incidence of new cases is about 500,000-1000,000 globally, with 600,000 deaths per year.(1)

Following the positive results of two phase III trials, the SHARP study in 2007(2) and the Asia Pacific study in 2008,(3) sorafenib has been approved by regulatory authorities as the standard of care for the first-line treatmentof HCC patients with advanced stage of disease, and for patients with intermediate stages of disease that are refractory to loco-regional therapy, according to Barcelona Clinic Liver Cancer (BCLC).

However, a significant proportion of HCC patients treated with sorafenib are experiencing difficult-to treat and quality of lifeimpairing side-effects, without receiving any clinical benefit. In this scenario, clinical or biological predictive factors able to identify up-front responding and refractory patients might represent an important clinical asset for both research and daily clinical practice. In addition, new therapeutic options are already or will be soon available in this setting, making the selection of the appropriate treatment for the appropriate patient more crucial than ever.

Unfortunately, after more than 10 years of clinical and translational research, there are still no validated biomarkers helping physicians in the decision-making process with the aim of maximizing results, sparing unnecessary toxicities and possibly guiding treatment selection.(4)

To date,the only relevant and available findings in this area come from subset and pooled analysis of the 2maintrials,(2, 3) whereas validation prospective trials are lacking.

Only a few studies identified possible prognostic or predictive markers for 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.(5)

Moreover, a recent study of Harding and co-workers demonstrated that advanced HCC patients harboring PI3K-mTOR pathway alterations had significantly worse outcomes under sorafenib treatment.(6)

In our ePHAS retrospective study, a training cohort of 41 HCC patients and a validation cohort of 87 patients receiving sorafenib were analysedto evaluate the prognostic role of the endothelial nitric oxide synthase (eNOS), also known as nitric oxide synthase 3 (*NOS3*)polymorphisms(7). At univariate and multivariate analyses, patients with *NOS3*rs2070744CC/CT genotypes showed a highermedian overall survival (OS) comparedto patients with *NOS3*rs2070744 TT genotype.(7)In our second retrospective study patients with *ANGPT2* rs55633437 GG genotype showed a significantly higher median OS and progression free survival (PFS) than patients carrying *ANGPT2* rs55633437 TT/GT genotypes (8)

On the basis of these preliminary results,INNOVATE study has been designed with the aim of validatingthrougha multicentre prospective studythe potential role of *NOS3* and *ANGPT2* polymorphisms in patients with HCC treated with sorafenib[NCT02786342].(9)

Patients and Methods

Patients

The study population consists of 165 patients with advanced-stage (BCLC C) HCC and patients with intermediate (BCLC B) HCC not eligible for loco-regional treatments (surgical resection, percutaneous ablation, TACE) or liver transplantation(9). The eligibility criteria also included an Eastern Cooperative Oncology Group (ECOG) performance status score of 2 or less, Child–Pugh liver function class A or B7 with bilirubin <2, a life expectancy of 12 weeks or more, adequate hematologic function (platelet count, ≥60×109 per liter; haemoglobin ≥8.5 gr/dl, adequate hepatic function (albumin, ≥2.8 g/dl; total bilirubin, ≤2 mg per deciliter; and 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).

Patients were required to have at least one untreated target lesion that could be measured in one dimension, according to the modified Response Evaluation Criteria in Solid Tumours (mRECIST)assessed at each centre. Patients were excluded if theypreviously received molecular targeted therapies or any other systemic treatment. All patients provided written informed consent before the enrolment in the study. The study was approved by ethics committee at each centre and complied with the provisions of the Good Clinical Practice guidelines and the Declaration of Helsinki and local laws.

Study Design

This prospective multicenter phase IV study was conducted at 10Italian centers. All eligible patients receiveda continuous oral treatment with 400 mg of sorafenib (consisting of two 200-mg tablets) twice daily. For each patient, a blood sample wascollected at baseline (the same day of sorafenib initiation).

Treatment interruptions and up to two dose reductions (first to 400 mg once daily and then to 400 mg every 2 days) were permitted for drug-related adverse effects (physician's choice). Adverse events were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.03 and seriousness of adverse events was recorded.

Treatment continued until the occurrence of radiologic progression, as defined by mRECIST, symptomatic progression, unacceptable adverse events or death.

The schedule of study procedures was reported in Supplementary Table S1.

Trial registration: Clinical trial NCT02786342, version 1.1.(9)

DNA isolation and genotyping analysis

Peripheral blood samples (3mL)collected in EDTA-tubes were usedfor polymorphism analysis.Genomic DNA was extracted from 200ul of whole blood using QIAamp DNA

Minikit(Qiagen SPA, Milan, Italy) following the manufacturer's protocol. DNA quantity and quality were assessed by Nanodrop 1000 (Celbio, Milan, Italy).

Genotyping analysis was performed for *NOS3* (rs2070744) and *ANGPT2* SNPs (rs55633437).

NOS3 rs2070744 was analysed by Real-Time Polymerase chain reaction (PCR) using a TaqMan SNP Genotyping assay (Assay ID C_15903863_10, Applied Biosystems, Foster City, CA, USA). Genotypes were assessed by a 7500 Real-Time PCR System (Applied Biosystems) using a 7500 Software version 2.3 (Applied Biosystems). PCRs were performed starting from 20 ng of genomic DNA.

ANGPT2 rs55633437 was instead determined by a standard PCR and direct sequencing analysis on an ABI 3130 Genetic Analyzer (Applied Biosystems). PCRs were performed starting from 50 ng of genomic DNA. The primer sequences and PCR conditions are reported in our previous study.(8)

All samples were analysed at the same institution (Biosciences Laboratory at IRST, Meldola, Italy) and all genotyping analysis were performed at the end of patient recruitment.Physicians treating were blinded to polymorphisms results and laboratory personnel were blinded to patient clinical outcome.

Outcomes and Assessments

The primary outcome of the study was PFS. PFS was defined as the time from entry into the study until the first observation of documented disease progression or death due to any cause, whichever occurred first. Patients without tumour progression at the time of analysis were censored at the time of their last follow-up.

Secondary outcomes included OS and disease-control rate (DCR).OS was defined as the observed length of life from study entry to death for any cause or to last contact date of patients lost to follow up. DCR was defined as the percentage of patients who had a best-response rating of complete

response (CR), partial response (PR), or stable disease (SD) (according to mRECIST(10)). Safety was assessed in all patients receiving at least one dose of study drug.

Tumour evaluations were performed at screening, every 8 weeks during treatment (within 10 days before the end of each cycle) and at the end of treatment by abdominal and thorax computed tomography.

Statistical analysis

The primary outcome was assessed according to the intention-to-treat population. On the basis of the results from our previous studies, we assumed a 0.50 prevalence of the polymorphisms to be validated. Through a sample size of 80 patients for each group is 80 (160 total sample size), an exponential maximum likelihood test of equality of survival curves with a 0.05 two-sided significance level reaches 90% power to detect the difference between a group 1 exponential parameter, λ_1 of 0.1155 (median PFS of 6 months) and a group 2 exponential parameter, λ_2 of 0.198 (median PFS of 3.5 months, a constant hazard ratio of 0.58). Patient enrolment periodwas about 24 months. We considered a follow-up period of at least 12 months for each patient.(9)
OS and 95% Confidence Interval (95 % CI) were estimated with Kaplan-Meier product-limit method. Further analyses of PFS/OS were performed using a multivariable Cox model, and we inserted all positive parameters in univariate analysis after Bonferroni correction.
All tests were two-sided at a significance level of 0.05;no interim analyses wereplanned.
MedCalc package (MedCalc® version 16.8.4) was used for statistical analysis.

Results

As shown in Fig.1, 182 patients had been screened between March, 2nd2016 and June, 27th2018. Seventeen patients did not satisfy the inclusion criteria and were considered as screening failure.165 patients were enrolled in the study (“intention-to-treat" population) and were treated with sorafenib. The main characteristics of the 165 patients enrolled in the study are summarized in Table1.Child-Pugh class-A was the most represented (88.5%), 65.5% had a BCLC-C stage disease, and 20.0% of

patients had α-fetoprotein (AFP) level ≥400 ng/ml. The most common underlying aetiology was hepatitis infections from C virus (37.6%).In terms of main characteristics, no differences were found between *NOS3* rs2070744 CC/CT and *NOS3* rs2070744TT (Supplementary Table S2).

At the time of database lock in February 2019, after a median follow-up time of 25.9 months,127 patients had progressed and 114 had died.

Median OS was 13.1 months (95% CI 10.4-15.7) and median PFS was 5.2 months (95% CI 4.5-6.5) (Supplementary Fig.S1).

Best response to sorafenib treatment wereCR in 1% of patients, PR in 8.6% of patients, SD in 25.8% of patients and PDin 64.6% of patients, while the DCR was 35.4%. Twenty-five percent of patients discontinued sorafenib treatment for intolerance and 46.1% of patients received other treatments after sorafenib progression/intolerance.

Genotype frequencies of *NOS3*and *ANGPT2* polymorphisms are shown in Supplementary TableS3 and all genotype frequencies followed the Hardy-Weinberg equilibrium.

At univariate analysis, *NOS3* rs2070744CC/CT genotypes were significantly associated with a higher median PFS (5.9 *vs* 2.4 months, HR 0.43, 95% CI 0.26-0.70, p=0.0007) and OS (15.7 *vs* 8.6 months, HR 0.38, 95% CI 0.24-0.60, p<0.0001) compared to the other genotype(Fig. 2).After Bonferroni correction, these results remained statistically significant.

At univariate analysis, there was no statistically significant association of *ANGPT2* rs55633437 GG genotype with PFS (5.7 *vs* 2.4 months, HR 0.52, 95% CI 0.25-1.10, p=0.0833) and OS (13.0 *vs* 15.1 months, HR 1.20, 95% CI 0.65-2.20, p=0.55)when compared to other genotypes (Fig.2).

Regarding baseline patient characteristics,univariate analysisidentified Portal Vein Thrombosis Yes (*vs* No), BCLC C (*vs* B), child pugh B (*vs* A), LDH > normal value (*vs*<normal value), albumin < 35 g/L (*vs*>35), AST > normal value (*vs*< normal value) and a decrease of sodium and platelets for OS and PLR for PFS(Table 2; Supplementary Fig.S2).

No significant association was found between the main clinical-pathologic characteristics of patients and *NOS3*polymorphisms.

Following adjustment for clinical covariates withBonferroni correction (PLRand *NOS3*for PFS; Portal Vein Thrombosis, BCLC and *NOS3* for OS), multivariate analysis confirmed *NOS3* as an independent prognostic factor for PFS (HR 0.50, 95% CI 0.29–0.86, p=0.0123) and OS (HR 0.44, 95% CI 0.29–0.68, p=0.0002; Supplementary Table S4).

*NOS3*and *ANGPT2*genotypes and objective response

Regarding *NOS3* polymorphisms, no differences were found between *NOS3* rs2070744TT and *NOS3* rs2070744 CT/CC genotypes in terms of DCR (34.6% vs. 35.8%; p=1.00).

Regarding *ANGPT2*, no differences were found between *ANGPT2* rs55633437 GG and *ANGPT2* rs55633437 TT/GT genotypes in terms of DCR (35.0% vs. 38.4%; p=1.00).

NOS3 and *ANGPT2*genotypes and toxicities

Adverse Events (AEs) of any grade were reported in 83.0% of patients (Grade 1: 9.6%; Grade 2: 38.2%; Grade 3: 35.1%) (Supplementary Table S5).

A trend for betterPFS and OS was found in patients that experienced any toxicity compared to patients without toxicity (OS: 15.4 months *vs* 8.1 months, HR 0.66, 95% CI 0.41-1.06, p=0.0869; PFS: 4.5 months vs. 3.7 months, HR 0.56, 95% CI 0.30-1.05, p=0.0733).

Maximum toxicity in grade 0-1 versus > 1 highlighted a trend for OS improvement (15.4 months vs. 10.3 months, HR 0.68, 95% CI 0.44-1.05, p=0.0874) and a better PFS in patients with toxicityof grade >1 (6.7 months vs. 3.7 months, HR 0.40, 95% CI 0.22-0.72, p=0.0027).

AST toxicity grade 0-1 versus >1 was correlated with better OS (13.3 months *vs* 9.2 months, HR 0.38, 95% CI 0.16-0.93, p=0.0343) and diarrhoea grade >1 for better PFS (8.2 months vs. 3.1 months, HR 0.66, 95% CI 0.45-0.96, p=0.0335). No other correlations were found (Supplementary Table S6).

No significant associations were observed between *NOS3*polymorphism and toxicity (any toxicity *p*=1.00; elevated ALT *p*=0.72;elevated AST *p*=0.29; elevated bilirubin *p*=1.00; diarrhoea *p*=1.00; hand-foot skin toxicity *p*=0.83; asthenia *p*=0.30; hypertension *p*=0.64).

Evaluation of blood pressure changes between baseline and different timepoints in patients with *NOS3*rs2070744CC/CT genotype (14°, 28°, 60° days) highlighted a more significant increase of diastolic pressure at 28 days respect to patients with *NOS3*rs2070744 TT genotype (Supplementary Fig.S3).

ANGPT2 rs55633437 GT/TT genotypes were associated with AST toxicity (100% *vs*25.4%, *p*=0.0007), whereas no significant associations were observed for other toxicities (All toxicity *p*=1.00; ALT *p*=0.31; Bilirubin *p*=0.67; Diarrhoea *p*=0.54; hand-foot skin toxicity *p*=0.76; asthenia *p*=0.13; hypertension *p*=1.00).

Second line treatment

Median OS after progression or intolerance to sorafenib was 5.3 months (95% CI 4.3-8.0).

Second line treatment data were available for 89 patients:46.1% of them received other treatments after sorafenib progression/intolerance (22 patients received metronomic capecitabine, 11 patients regorafenib and 7 patients other treatments).

NOS3 rs2070744 CC/TC genotypes were significantly associated with a higher median OS considering all second line population (6.7 vs 3.5 months, HR 0.56, 95% CI 0.32-0.95, *p*=0.0343,Fig 3A) and a trend was observed in the cohort of patients treated with regorafenib (3.4 *vs* 9.9 months, HR 0.13, 95% CI 0.01-1.06, *p*= 0.057) (Fig 3B). No differences were found in patients treated with capecitabine (*p*=0.90), other treatments (*p*=0.10) and in patient without any treatment (*p*=0.07). Interaction test on *NOS3* polymorphisms and treatment efficacy in regorafenib and no regorafenib treated patients was not statistically significant for OS (*p*=0.9666).

Discussion

The INNOVATE met its primary endpoint, confirming that advanced HCC patients with *NOS3* rs2070744 CC/CT genotypes have a better prognosis than those with TT genotype. To the best of our knowledge, this is the first time that a prospective cohort study successfully validates a biological prognostic factor in this patient setting. At the time the study was closed, patients with *NOS3* rs2070744 CC/CT genotypes had a median PFS of 5.9 months and median OS of 15.7 months compared to 2.4 months and 8.6 months for those with *NOS3* rs2070744 TT genotype. This difference remained significant after adjustment for baseline factors that were found to influence survival, thus supporting the primary analysis. However, INNOVATE study not confirmed the data on *ANGPT2* rs55633437 TT/GT genotypes. The functional role of this *ANGPT2* polymorphism is not well documented in literature and other factors and SNPs may cooperate with this variant, directly affecting transcription efficiency.

The results from the IMbrave 150 trial (Phase 3 trial of sorafenib versus atezolizumab plus bevacizumab) were recently presented by Cheng et al. (11).It is not possible to compare our study with IMbrave150 study, but an indirect comparison is possible in order to speculate future studies basingon the results of our study.In particular, if we indirectly would analyze the OS of IMbrave 150 trial and OS from our study, we could speculate that, if we select the patients treated with sorafenib with the best prognosis, they may have a prognosis similar to that obtained in the atezolizumab plus bevacizumab arm of IMbrave 150 trial. In fact, 6-month OS was 80% in our study and 85% in the atezolizumab plus bevacizumab arm of the IMbrave 150 trial. It is clear that this observation currently has no clinical value but could be suggestive for generatinghypotheses for future studies.

Another unmet clinical need for HCC is the management of patients with intermediate stages of disease, candidate to transarterial chemoembolization (12-14). Several clinical trials have been performed on the combined use of Sorafenib and transarterial

chemoembolization or transarterial chemoembolization alone with negative results. In our opinion, these studies did not reach the primary endpoint because patients were not selected on the basis of prognostic molecular markers. We think that in the future it could be interesting to design studies in this setting by selecting patients on basis of *eNOS* polymorphisms.

In recent years, several drugs demonstrated activity in first- and second-line treatment of patients with advanced HCC(15-18), enhancing therapeutic options and further underlining the importance of patient selection for treatment. In addition, the recently reported negative results for nivolumab in the first-line setting (19)and for pembrolizumab in the second-line setting (20)reinforce the need to better characterize patients undergoing first-line treatments such as sorafenib or lenvatinib, to identify the confounding factors that may explain the poor efficacy of immunotherapy in HCC.To the best of our knowledge, very few studies to date have identified potential markers of response to sorafenib in HCC patients, and none have been validated prospectively (4). Low baseline plasma concentrations of vascular endothelial growth factor A (VEGF-A) and Ang-2 have been associated with better OS in some cases (5, 21), but not in others.

Polymorphism analysis has several advantages over gene or protein expression assays. Most importantly, it can be performed on a peripheral blood sample collected at any time and is not influenced by patient conditions. Secondly, it is a more robust and reproducible analysis because it is directly performed on DNA. It is also less expensive than expression analysis, making it a more welcome option for use in clinical practice.

Previous studies have suggested that DNA variants at the *NOS3*gene quantitatively control eNOS expression. The point variation T>C at nucleotide-786bp of *NOS3* gene has been associated with a significant reduction in *NOS3*gene promoter activity, resulting in lower levels of eNOS mRNA, eNOS protein and a lower enzyme activity, with consequent decreased nitric oxide (NO) production(22-24). We therefore hypothesized an association between low levels of eNOS protein/activity and sorafenib efficacy. With regard to the correlation between sorafenib toxicity and *NOS3* polymorphism, we also found that patients carrying the TT genotype for *NOS3*

rs2070744 showed a significant increase in diastolic blood pressure between baseline and the 28th day of therapy. This findingwould seem to be related to eNOS expression. The activation of VEGFR-2 has also been shown to stimulate the production of NO and inhibit endothelin-1 (ET- 1), a potent vasoconstrictor(25). In patients treated with sorafenib, VEGFR-2 inhibition may reduce NO, resulting in vasoconstriction and hypertension.

Another interesting data highlighted in this study is the prognostic value of *NOS3*in the second line setting. Obviously, this is a post-hoc analysis with few evaluable patients, but still our study points out the prognostic role of *NOS3*in the overallpopulation and a trend in favour of regorafenib treatment.In light of this finding, future studies should evaluate the sorafenib and regorafenib sequence in patients with *NOS3* rs2070744 CC/CT genotypes.

The main strength of our multicenter study is that the analyses were prospectively performed in order to validate the prognostic role of polymorphisms. Patients were treated by different specialists (oncologist, gastroenterologist and hepatologist) with different expertise in this field and this reinforces the data of ourstudy. The study also has a number of limitations, *e.g.* as our study was carried out on Caucasian patients, it is not possible to know if *NOS3* rs2070744 genotypes are prognostic in an Asian population where hepatitis B is the prevailing aetiology. Another important limitation of our study is the absence of a control arm not receiving sorafenib, making not possible to evaluate the predictive role of *NOS3*polymorphismsand this limits the completeness of results that the study can give. Furthermore, a low number of patients had been enrolled. These limitations reduce the value of the study and we think that a prospective study enrolling larger case series with two arms will be fundamental to definitively understand the role of polymorphism in this patient setting.

Furthermore, we selected PFS as primary endpoint because this endpoint would have not been influenced by subsequent treatment lines in a period of very intensive clinical research with many ongoing clinical trials at many participating Institutions investigating second-line therapy.

The power for OS is 95%, therefore the results obtained for OS are reliable.

In summary, this study shows that *NOS3* rs2070744 CC/CT genotypes are capable to identify patients receiving sorafenib with longer survival. *NOS3* genotyping analysis is a simple analysis that allows us to better stratify patients in future randomized studies.

Before this biomarker can be used in daily clinical practice as a surrogate marker of efficacy or response to sorafenib, further investigations by other authors are needed to confirm our data.

Authors' Contribution

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Table 1. Patient characteristics

Clinical and pathologic variables	**No (%)**
Median age years(range)	69 (24-87)
Gender	
Male	139 (88.7)
Female	26 (11.3)
Etiology	
HCV	62 (37.6)
HBV	24 (14.5)
Alcohol	13 (7.9)
NASH	24 (14.5)
Criptogenic	12 (7.3)
hemochromatosis	2 (1.2)
Multifactorial	28 (17.0)
Previous Surgery	
Yes	36 (21.8)
No	129 (78.2)
Previous	
Radiofrequency	
Yes	37 (22.4)
No	128 (77.6)
Previous TACE	
Yes	51 (30.9)
No	114 (69.1)
BCLC stage	
B	57 (34.5)
C	108 (65.5)
Child Pugh	
A	146 (88.5)
B	19 (11.5)
ECOG	
0	104 (63.0)
1	61 (37.0)

Macrovascular invasion	
Yes	61 (37.0)
No	104 (63.0)
Portal hypertension	
Yes	50 (30.3)
No	105 (63.6)
Unknow	10 (6.1)
Extrahepatic disease	
Yes	62 (37.5)
No	103 (62.5)
Portal Vein Thrombosis	
Yes	59 (35.7)
No	106 (64.3)
MELD (range)	8 (6-20)
MELD-Na (range)	9 (6-19.9)
AFP (range)	40.7 (0.6->50.000)
<400	104 (63.0)
≥400	33 (20.0)
Unknow	28 (17.0)
ALBI grade	
1	117 (70.9)
2	18 (29.1)
3	0 (0)

Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; BCLC, Barcelona clinic liver cancer; ECOG, Eastern Cooperative Oncology Group; AFP, alpha-fetoprotein; ALBI, albumin-Bilirubin.

Table 2. Univariate analysis of PFS and OS

	PFS		OS	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
eNOS-786 (CC/CT vs TT)	0.43 (0.26-0,70)	0.0007	0.38 (0.24-0.60)	**<0.0001**
ANGPT2 (GG vs GT/TT)	0.52 (0.25-1.10)	0.0833	1.20 (0.65-2.20)	0.55
Child (B vs C)	1.56 (0.72-3.35)	0.2552	2.37 (1.00-5.62)	**0.0491**
BCLC (C vs B)	1.05 (0.72-1.53)	0.81	1.93 (1.29-2.88)	**0.0012**
Extra Hepatic Spread (Yes vs No)	1.31 (0.90-1.89)	0.1579	1.35 (0.90-2.00)	0.1420
ECOG (0 vs >0)	0.78 (0.44-1.39)	0.4023	0.81 (0.44-1.48)	0.4918
Age at start of therapy (>70 vs <70)	1.02 (0.71-1.47)	0.9017	1.08 (0.74-1.57)	0.6967
Etiology				
HCV	1		1	
HBV	1.06 (0.61-1.84)		1.65 (0.87-3.15)	
Alcohol	1.09 (0.55-2.17)		0.97 (0.50-1.88)	
Multifactorial	1.50 (0.87-2.58)		1.74 (0.95-3.21)	
Others	1.34 (0.82-2.19)	0.5064	1.63 (0.96-2.79)	0.1227
Portal hypertension (Ref Yes)	1.04 (0.62-1.73)	0.8777	1.76 (0.96-3.20)	0.0656
Portal vein thrombosis (Yes vs No)	0.91 (0.62-1.35)	0.6531	2.04 (1.30-3.18)	**0.0017**
Macrovascular invasion (Yes vs No)	1.35 (0.79-2.16)	0.3276	1.76 (0.94-2.16)	0.0945
LDH (>NV vs <NV)	1.89 (0.93-3.85)	0.0771	2.21 (1.02-4.80)	**0.0429**
AFP (continuous variable)	1.00 (1.00-1.00)	0.2415	1.00 (1.00-1.00	0.1758
AFP (≥400 vs <400)	1.03 (0.66-1.63)	0.8738	1.21 (0.72-2.03)	0.4563
ALBI (2 vs 0-1)	1.08 (0.53-2.20)	0.8198	1.49 (0.67-3.33)	0.3254
Albumin (NV vs <NV)	0.68 (0.43-1.09)	0.1085	0.52 (0.31-0.86)	**0.0111**
ALT U/L(>NV vs NV)	1.02 (0.62-1.68)	0.9393	1.14 (0.65-1.98)	0.6493
AST U/L(>NV vs NV)	1.54 (0.89-2.65)	0.1175	2.00 (1.05-3.82)	**0.0341**
Bilirubin mg/dl (continuous variable)	1.04 (0.73-1.48)	0.8154	1.05 (0.74-1.51)	0.7624
Bilirubinmg/dl (> NV vs NV)	1.14 (0.76-1.70)	0.5205	1.06 (0.69-1.64)	0.7737
Creatinine mg/dl (>NV vs NV)	1.30 (0.77-2.19)	0.3279	1.43 (0.82-2.48)	0.1979
Creatinine mg/dl (continuous variable)	1.43 (0.70-2.91)	0.3272	1.52 (0.77-3.00)	0.2242
Hemoglobin g/dl (continuous variable)	0.90 (0.80-1.02)	0.0980	0.93 (0.80-1.08)	0.3561
Alkaline phosphatase (>NV vs NV)	1.10 (0.64-1.87)	0.7356	1.57 (0.86-2.84)	0.1378
Sodium	0.96 (0.88-1.04)	0.3184	0.90 (0.82-0.98)	**0.0291**
Phosphorus (NV vs <NCV)	1.07 (0.51-2.21)	0.8634	0.40 (0.16-1.06)	0.0671
INR (>NV vs NV)	1.01 (0.68-1.50)	0.9628	1.40 (0.89-2.20)	0.1470
Lymphocyte 10^9/L(continuous variable)	0.80 (0.59-1.07)	0.1342	0.88 (0.67-1.16	0.3805
Neutrophil 10^9/L(continuous variable)	1.00 (0.99-1.00)	0.5270	1.00 (0.99-1.03)	0.9287
NLR (>3 vs <3)	1.18 (0.72-1.93)	0.5111	1.09 (0.63-1.90)	0.7444
PLR (>15 vs <15)	2.10 (1.19-3.71)	**0.00105**	1.28 (0.71-2.30)	0.4036
SII (>360 vs <360)	1.26 (0.77-2.06)	0.3466	1.16 (0.67-2.01)	0.5826
Platelet 10^9/L(continuous variable)	1.00 (0.99-1.00)	0.2264	1.00 (1.00-1.00)	**0.0150**
Platelet 10^9/L (>100 vs <100)	1.37 (0.94-2.00)	0.1045	1.26 (0.82-1.93)	0.2910
Calcium (>NV vs NV)	0.50 (0.11-2.25)	0.3671	0.20 (0.02-1.93)	0.1651
BMI(continuous variable)	0.98 (0.94-1.03)	0.5260	1.00 (0.94-1.06)	0.8925
TSH				0.9991

<NV	1.96 (0.69-5.56)		0.98 (0.35-2.75)
NV	1		1
>NV	1.06 (0.45-2.48)	0.2316	0.98 (0.30-3.17)

Abbreviations: Ref, reference; NV normal value; BCLC, Barcelona clinic liver cancer; ECOG, Eastern Cooperative Oncology Group; AFP, alpha-fetoprotein; LDH, lactate dehydrogenase; ALBI, albumin-Bilirubin; INR, International normalized ratio; NLR, neutrophil lymphocyte ratio; PLR, platelet to lymphocyte ratio; SII, systemic immune-inflammation index; BMI, body mass index; TSH, thyroid-stimulating hormone

Figure Legends:

Figure 1. Flow chart of the study population.

Figure 2: Kaplan-Meier curves. (A) Progression-free survival (PFS) and (B) overall survival (OS) in relation to *NOS3*rs2070744polymorphism. (C) PFS and (D) OS in relation to *ANGPT2*rs55633437polymorphism.

Figure 3: Kaplan-Meier curves for *NOS3*polymorphism in second line treatment. (A) All population. (B) Patients treated with regorafenib.

FIGURE 1

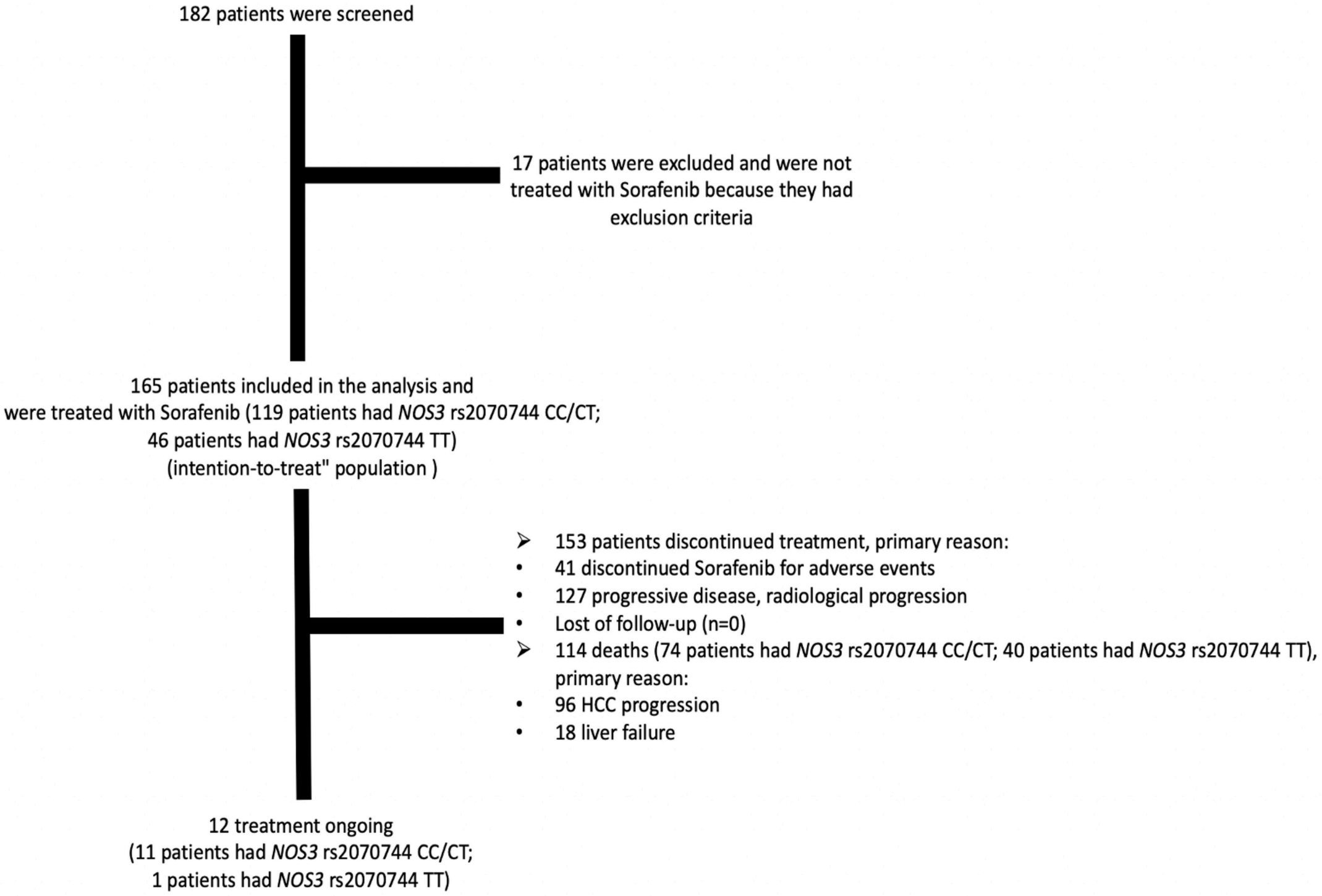

182 patients were screened
17 patients were excluded and were not treated with Sorafenib because they had exclusion criteria
165 patients included in the analysis and were treated with Sorafenib (119 patients had NOS3 rs2070744 CC/CT; 46 patients had NOS3 rs2070744 TT) (intention-to-treat" population)
➢ 153 patients discontinued treatment, primary reason:
• 41 discontinued Sorafenib for adverse events
• 127 progressive disease, radiological progression
• Lost of follow-up (n=0)
➢ 114 deaths (74 patients had NOS3 rs2070744 CC/CT; 40 patients had NOS3 rs2070744 TT), primary reason:
• 96 HCC progression
• 18 liver failure
12 treatment ongoing (11 patients had NOS3 rs2070744 CC/CT; 1 patients had NOS3 rs2070744 TT)

FIGURE 2

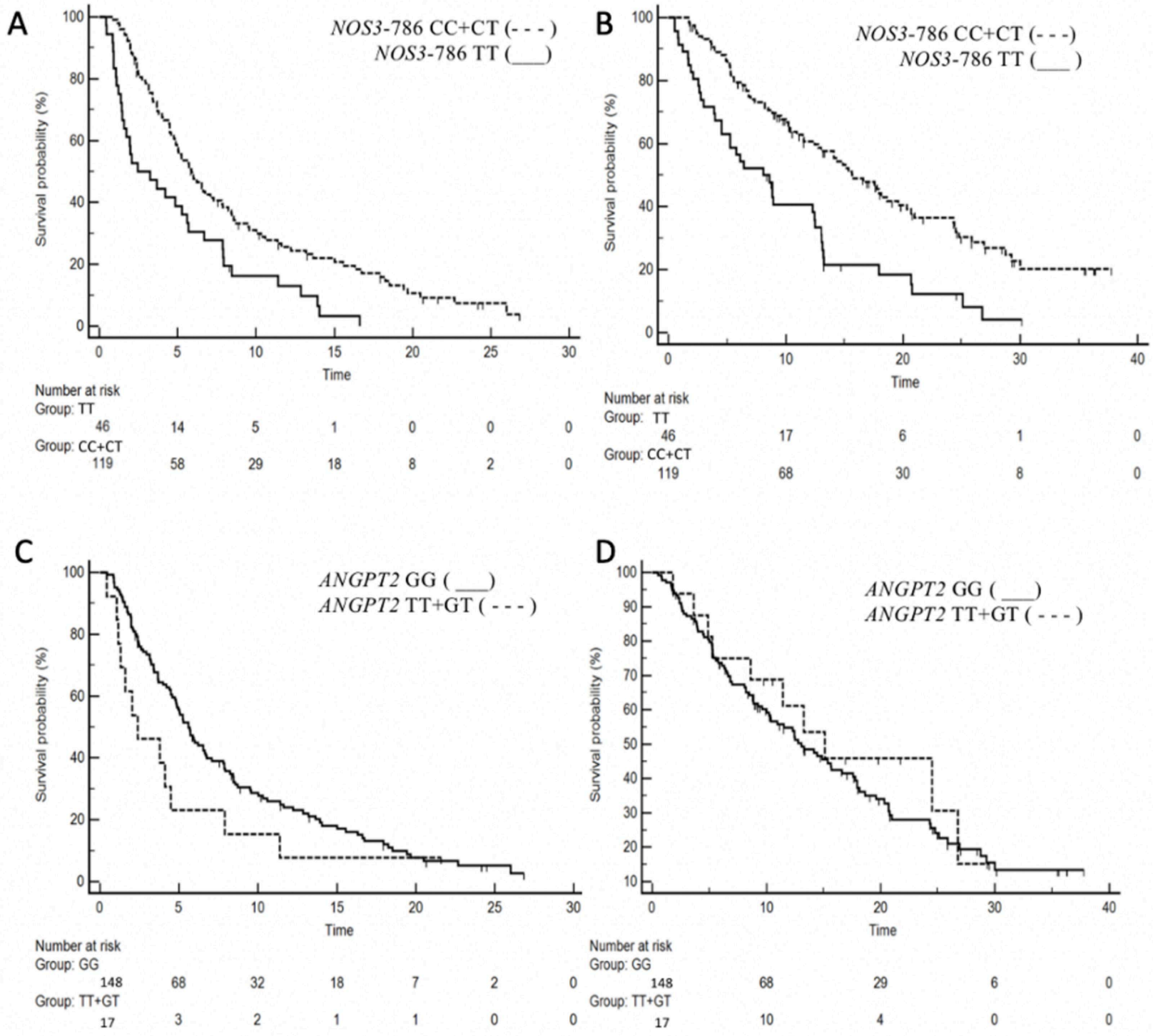
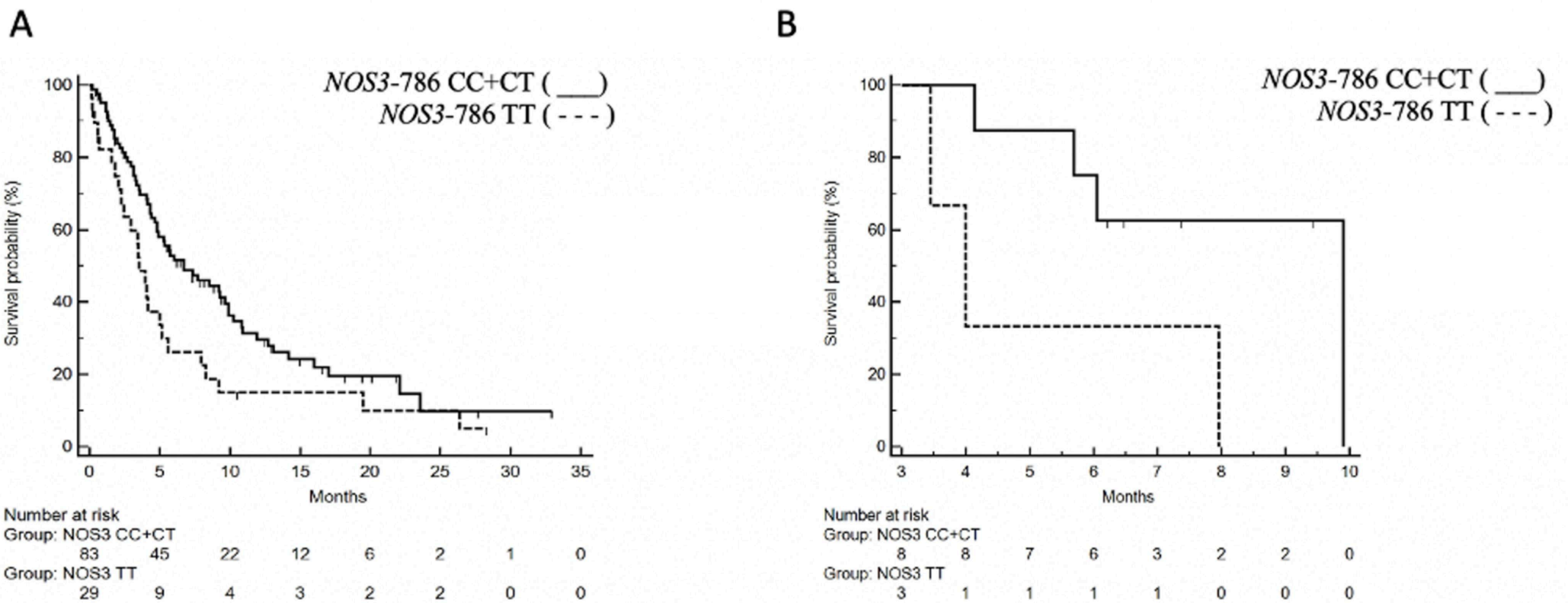

A
NOS3-786 CC+CT (- - -)
NOS3-786 TT (___)
Survival probability (%)
Time
Number at risk
Group: TT
46 14 5 1 0 0 0
Group: CC+CT
119 58 29 18 8 2 0
B
NOS3-786 CC+CT (- - -)
NOS3-786 TT (___)
Survival probability (%)
Time
Number at risk
Group: TT
46 17 6 1 0
Group: CC+CT
119 68 30 8 0
C
ANGPT2 GG (___)
ANGPT2 TT+GT (- - -)
Survival probability (%)
Time
Number at risk
Group: GG
148 68 32 18 7 2 0
Group: TT+GT
17 3 2 1 1 0 0
D
ANGPT2 GG (___)
ANGPT2 TT+GT (- - -)
Survival probability (%)
Time
Number at risk
Group: GG
148 68 29 6 0
Group: TT+GT
17 10 4 0 0

FIGURE 3


A
NOS3-786 CC+CT (___)
NOS3-786 TT (- - -)
Survival probability (%)
100
80
60
40
20
0
0 5 10 15 20 25 30 35
Months
Number at risk
Group: NOS3 CC+CT
83 45 22 12 6 2 1 0
Group: NOS3 TT
29 9 4 3 2 2 0 0
B
NOS3-786 CC+CT (___)
NOS3-786 TT (- - -)
Survival probability (%)
100
80
60
40
20
0
3 4 5 6 7 8 9 10
Months
Number at risk
Group: NOS3 CC+CT
8 8 7 6 3 2 2 0
Group: NOS3 TT
3 1 1 1 1 0 0 0