Università degli Studi di Cagliari

PHD DEGREE

Molecular and Translational Medicine

Cycle XXXII

TITLE OF THE PHD THESIS

Selection with a molec**U**lar **P**an**E**l fo**R** **P**anitumumab **E**ffic**A**cy in **K**-RAS and N-RAS wild type metastatic colorectal cancer (SUPER-PEAK**)**

Scientific Disciplinary Sector(s): MED-06

PhD Student : *Dott. Marco Puzzoni*

Supervisor : *Prof Mario Scartozzi*

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Study Glossary

Abbreviation or Term	Definition/Explanation
5-FU	5-fluorouracil
AE	adverse events
ANC	absolute neutrophil count
ALT	alanine aminotransferase
AST	aspartate aminotransferase
Baseline	unless otherwise indicated, defined as any procedure or measurement taken closest to, but prior to the first dose of protocol-specified therapy; for example, if a hematology sample is collected and analyzed twice during screening, then the sample collected closest to enrollment would be considered the screening value used to assess eligibility
BID	twice a day
BP	blood pressure
BRAF	v-raf murine sarcoma viral oncogene homolog B
BUN	blood urea nitrogen
CBC	complete blood count
CEA	carcinoembryonic antigen
CI	confidence interval
CR	complete response
CRF	electronic case report form
CT	computerized tomography
CTCAE	Common Terminology Criteria for AEs
Disease control	incidence of objective response (CR or PR), or stable disease during treatment
DNA	deoxyribonucleic acid
DoR	Depth of response
ECG	Electrocardiogram
ECOG	Eastern Cooperative Oncology Group
eg	for example
EGF	epidermal growth factor
EGFr	epidermal growth factor receptor
EMEA	European Medicines Agency
End of Study for Subject	the date the subject withdraws full consent from the study, completes the 30-day safety follow-up visit or the final long-term follow-up visit, whichever is later, or death.
mFOLFOX6	Modified FOLFOX6: Oxaliplatin 85 mg/m^2 IV over 2 hours day 1; leucovorin 400 mg/m^{2}racemate (or 200 mg/m^2 l-LV) IV over 2 hours day 1; 5-FU 400 mg/m^2 IV bolus day 1, then 2400 mg/m^2 IV over 46 to 48 hours days 1 and 2
End of Study	when all subjects have completed or have had the opportunity to complete the 30-day safety follow-up visit or 3 years after the last subject is randomized, whichever is later
End of Treatment	defined as the point when all protocol-specified therapy is stopped; the day of the last dose of protocol-specified therapy or the day the decision is made to permanently stop treatment, whichever occurs last

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ETS	Early tumor shrinkage
FDA	Food and Drug Administration
FOLFIRI	irinotecan with infusional 5-fluorouracil and leucovorin
FOLFOX	a combination chemotherapy regimen consisting of infusional 5-FU,leucovorin, and oxaliplatin
GDP	guanosine 5'-diphosphate
GTP	guanosine-5'-triphosphate
HIV	human immunodeficiency virus
ICH/GCP	International Conference on Harmonisation/Good Clinical Practice
ID	Identification
ie	specifically, to be exact, that is
IEC/IRB	Independent ethics committee/institutional review board
IFL	a combination chemotherapy regimen consisting of infusional irinotecan and bolus 5-FU/leucovorin
INR	international normalized ratio
ITT	intent to treat
IV	Intravenous
IVRS	interactive voice response system; the telephone system that is used to register subject activity (eg, screen, screen-fail, enroll/randomization, end of treatment)
KM	Kaplan-Meier
KRAS	Kirsten rat sarcoma virus oncogene homolog
LDH	lactate dehydrogenase
Long Term Follow Up	long-term follow-up; after the safety follow-up visit, long-term follow-up will occur every 3 months (± 28 day window) until approximately 36 months after the last subject is randomized
M1	Distant metastasis according to TNM classification
mCRC	metastatic colorectal cancer
MRI	magnetic resonance imaging
NCI	National Cancer Institute
OR	objective response
ORR	objective response rate
OS	overall survival
PABA	para-aminobenzoic acid
PD	progressive disease
PFS	progression-free survival
PIK3CA	phosphoinositide 3-kinase
PR	partial response
Protocol-specified therapy	panitumumab plus mFOLFOX6
PTT/aPTT	partial thromboplastin time/activated partial thromboplastin time
Q2W	every 2 weeks
RBC	red blood cell
RECIST	Response Evaluation Criteria in Solid Tumors
RNA	ribonucleic acid
RP	resting pulse

RR	response rate
SAE	serious adverse events
Safety Follow-up Visit	occurs 30 days (+ 3 day window) after the subject's last dose of protocol-specified therapy
Screening period	defined as the period of time between when the subject signs the informed consent to randomization via the IVRS
SD	stable disease
SPF	sun protection factor
Study Day 1	the first day protocol-specified therapy is administered to the subject
TGF-α	transforming growth factor alpha
UE	un-evaluable
ULN	upper limit of normal
UPC ratio	urine protein creatinine ratio
USA	United States of America
UV	Ultraviolet
vs	Versus
washout or window	when calculating the protocol windows or washout, keep in mind that today is day 0, tomorrow is day 1
XELOX	a combination chemotherapy regimen consisting of xeloda and oxaliplatin

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Contents

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BACKGROUND

Clinical reports with the use of monoclonal antibodies directed against the ligand-binding site of the epidermal growth factor receptor (EGFR) have shown practice-changing results in the treatment of colorectal cancer and will hopefully further improve, in the next future, available therapeutic options for patients diagnosed with this highly deadly disease.

After several years of intense translational research and clinical absence of predictive factors, the introduction of RAS mutational status seemed to possess the necessary potential for a full translation into clinical practice of the concept of targeted therapy in this setting.

However if on the one hand we are now able to exclude from anti-EGFR treatment more patients with putative refractory colorectal tumours (i.e. those harboring a RAS mutant status), on the other hand we are still incapable to accurately select responding patients among those without RAS mutations. In first-line, randomised trials objective response rate approached in fact 50-60% with the use of chemotherapy in combination with anti-EGFR treatment (cetuximab or panitumumab), thus suggesting that a non negligible proportion of patients, ranging from 50% to 40%, did not fully benefit from the use of anti-EGFR targeted antibodies although in the absence of a mutation of the RAS genes. Most of the biological factors analysed in the attempt to improve patients selection in this setting focused either on the EGFR-downstream signaling pathway or on the receptor itself. The presence of mutated BRAF is one of the most powerful prognostic factors for advanced and recurrent colorectal cancer and BRAF mutational status determination is incorporated in clinical practice. Moreover published research data suggested that EGFR gene copy number, PIK3CA mutations, PTEN mutations or copy number variations, may

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all represent predictive determinants for anti-EGFR therapy, however these factors were not incorporated into clinical practice particularly because prospective validation is lacking.

Biologically enriched, prospective clinical trials are clearly needed in order to further identify the proportion of patients more likely to benefit from the use of first-line anti-EGFR antibodies.

STUDY DESIGN

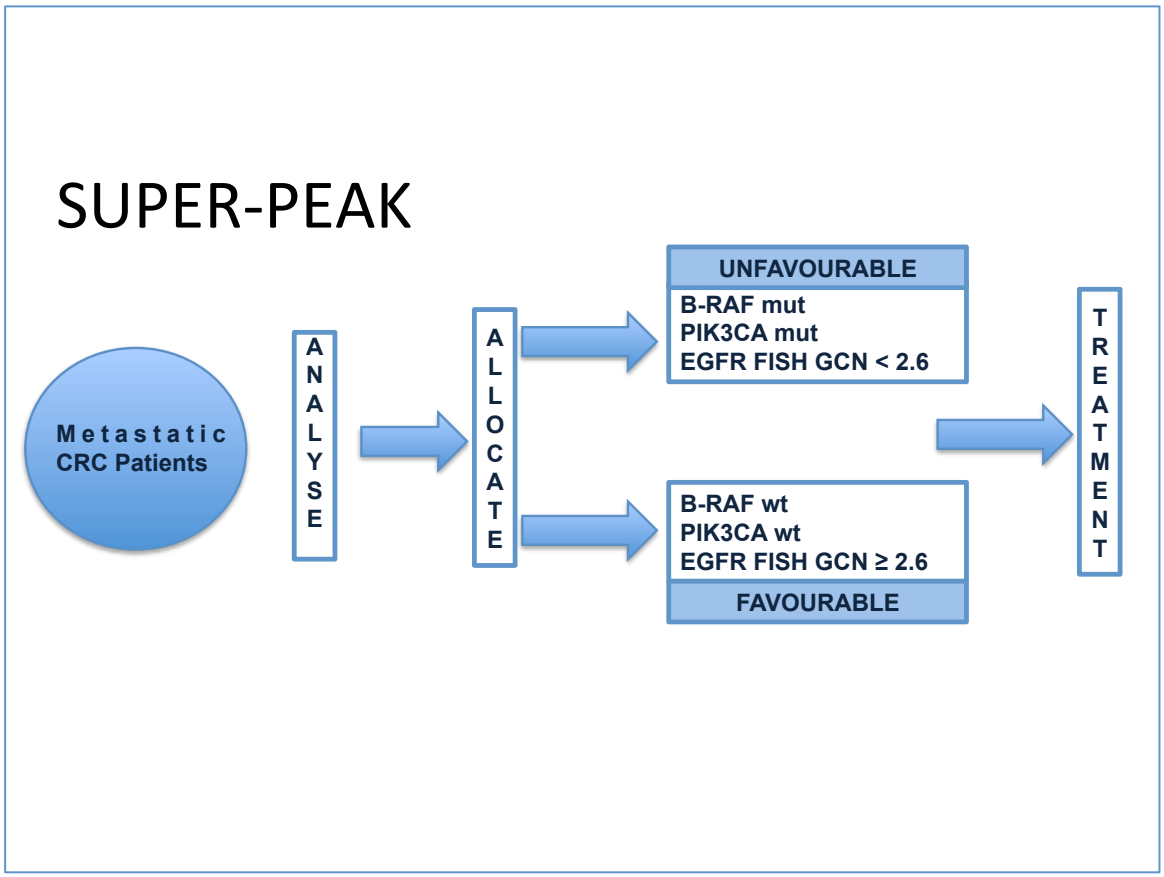

Figure 1: SUPER-PEAK- Study Design.

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The SUPER-PEAK is a multicentre, biologically enriched, prospectively stratified study.

Patients will be divided into 2 prognostic groups (Fig.1) on the basis of their biological and clinical profile: favourable and unfavourable (respectively high and low probability for improved RR). Patients subsequently receive mFOLFOX +panitumumab as per indication.

In more details, patients with RAS wild type metastatic colorectal cancer are tested for BRAF mutational status according to clinical practice. Moreover a supplementary evaluation of EGFR gene copy number and a pre-specified gene panel of markers (using the Ion Torrent technology on DNA samples from formalin-fixed paraffin-embedded -FFPE- tissues) is planned.

The following gene-panel will be used (Table 1: Hotspot regions in Ion Ampliseq Cancer Hotspot panel: ~2,800 mutations of 50 oncogenes and tumor suppressor genes): EGFR gene copy number (GCN) variations are evaluated by FISH analysis.

On this basis patients will be stratified stratification to either the favourable group (PIK3CA and BRAF wild type and EGFR GCN ≥ 2.6) or the unfavourable group (PIK3CA mutation or BRAF mutation or EGFR GCN < 2.6).

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ABL1	*EGFR*	*GNAS*	*KRAS*	*PTPN11*
AKT1	*ERBB2*	*GNAQ*	*MET*	*RB1*
ALK	*ERBB4*	*HNF1A*	*MLH1*	*RET*
APC	*EZH2*	*HRAS*	*MPL*	*SMAD4*
ATM	*FBXW7*	*IDH1*	*NOTCH1*	*SMARCB1*
BRAF	*FGFR1*	*JAK2*	*NPM1*	*SMO*
CDH1	*FGFR2*	*JAK3*	*NRAS*	*SRC*
CDKN2A	*FGFR3*	*IDH2*	*PDGFRA*	*STK11*
CSF1R	*FLT3*	*KDR*	*PIK3CA*	*TP53*
CTNNB1	*GNA11*	*KIT*	*PTEN*	*VHL*

Table 1: Gene panel (Hotspot regions in Ion Ampliseq Cancer Hotspot panel: ~2,800 mutations of 50 oncogenes and tumor suppressor genes).

STUDY OBJECTIVES

Primary objective

To prospectively define a molecular panel able to identify patients more likely to benefit from the use of first-line panitumumab in combination with mFOLFOX in terms of overall response rate (ORR).

Secondary objectives

The secondary objectives are the following:

- To prospectively define a molecular panel able to identify patients more likely to benefit from the use of first-line panitumumab in combination with mFOLFOX in terms of median progression free-survival (mPFS).

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- To prospectively define a molecular panel able to identify patients more likely to benefit from the use of first-line panitumumab in combination with mFOLFOX in terms of median overall survival (mOS).
- As ancillary part of the study, a gene panel of markers (using the Ion Torrent technology on DNA samples from formalin-fixed paraffin-embedded -FFPE- tissues) will be evaluated to correlate the gene profile with clinical outcome in terms of RR, PFS, OS.

SUBJECT ELIGIBILITY

Before any study-specific procedure, the appropriate written informed has to be obtained.

Inclusion Criteria

-Informed written consent

-Histologically or cytologically-confirmed adenocarcinoma of the colon or rectum in subjects with unresectable metastatic disease

-At least 1 uni-dimensionally measurable lesion of at least 20 mm per modified RECIST 1.1 guidelines using conventional techniques (CT scan or MRI).

-Wild-type KRAS-NRAS tumor status of archival tumor tissue confirmed according to regulatory guidelines using a validated test method

-Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1

-No previous treatment for metastatic disease

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- Adjuvant treatment allowed if disease progression is documented at least 6 months after chemotherapy completion

- Previous fluoropyrimidines treatment allowed if administered solely as radiosensitization

Demographic

-Man or woman 18 years of age or older at the time the informed consent is obtained

Laboratory

To be performed ≤ 10 days prior to enrollment, unless otherwise specified:

Hematologic function within the following limits:

-Absolute neutrophil count (ANC)≥1.5x10 ^{9}/L

-Platelet count≥ 100 x10^{9}/L

-Hemoglobin(Hgb)≥ 9.0g/ dL

Renal function within the following limits:

-Serum creatinine≤ 1,5 mg/dl

-Creatinine clearance (GFR) ≥ 50 mL/min calculated by the Cockcroft-Gault method as follows:

Male creatinine clearance = (140 – age in years) x (weight in Kg) / (serum creatinine in mg/dL x 72)

Female creatinine clearance = (140 – age) x (weight in Kg) x 0.85 / (serum creatinine in mg/dL x 72)

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Hepatic function within the following limits:

-Total bilirubin≤1.5xULN

-Alkaline phosphatase≤2. 5xULN (if liver metastases ≤ 5 x ULN)

--Aspartateaminotransferasi (AST) ≤2.5x ULN (if liver metastases,≤5 x ULN)

- Alanine aminotransferase (ALT)≤2.5x ULN (if liver metastases,≤5 x ULN)

Metabolic function within the following limits:

-Magnesium ≥ lower limit of normal

Exclusion Criteria

Disease Related

-History of other malignancy, except:

- Adequately treated melanoma in situ
- Adequately treated cervical carcinoma in situ without evidence of disease

Cancer Therapy

-Prior chemotherapy or other systemic anticancer therapy for the treatment of metastatic colorectal carcinoma

-Prior adjuvant chemotherapy for the treatment < 6 months prior of enrollment with the following exceptions:

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- Subjects may have received prior fluoropyrimidine therapy if administered solely for the purpose of radiosensitization for the adjuvant or neoadjuvant treatment of rectal cancer

- Adjuvant treatment allowed if disease progression is documented at least 6 months after chemotherapy completion

-Radiotherapy≤ 28 days prior to enrollment. Subjects must have recovered from all radiotherapy-related toxicities.

-Unresolved toxicities from prior anti-cancer therapy that, in the opinion of the investigator, excludes subject from participation

Other Medications

-Infection requiring a course of systemic anti-infectives that was completed ≤ 14 days before enrollment (exception can be made at the judgment of the investigator for oral treatment of an uncomplicated urinary tract infection [UTI])

General

-Uncontrolled infections

-Significant cardiovascular risk:

-Myocardial infarction, grade 2 or greater peripheral vascular disease**,** arterial thrombotic event, visceral arterial ischemia, cerebrovascular ischemia, transient ischemic attack, percutaneous transluminal coronary angioplasty/stent or unstable≤ angina 24 weeks prior to enrollment

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-Symptomatic and/or serious uncontrolled cardiac arrhythmia

-Symptomatic congestive heart failure (New York Heart Association Class III or IV)

-Uncontrolled blood pressure defined as > 150 mm Hg systole or > 90 mm Hg diastole. Anti-hypertensive medications are allowed if hypertension is stably controlled at the time of randomization.

-Pulmonary embolism, deep vein thrombosis, or other significant venous event ≤ 8 weeks before enrollment

-History of interstitial lung disease (eg, pneumonitis or pulmonary fibrosis) or evidence of interstitial lung disease on baseline CT scan

-Active inflammatory bowel disease or other bowel disease causing chronic diarrhea (defined as ≥ CTC grade 2, [CTCAE version 4.0])

-Peripheral sensory≥ CTC grade 2 neuropathy2 [CTCAE version 4.0]

-Woman of child-bearing potential is evidently pregnant (eg, positive HCG test) or is breast feeding

-Men and women of childbearing potential who do not consent to use adequate contraception during the course of the study and 6 months after the last dose of protocol specified therapy.

-Subjects allergic to any components that are part of the treatment regimen

-History of any medical or psychiatric condition or addictive disorder, or laboratory abnormality that, in the opinion of the investigator, may increase the risks associated with study participation or study drug administration or may interfere with the conduct of the study or interpretation of study results

-Known drug or alcohol abuse

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-Subject has previously been randomized into this study

-Subject unwilling or unable to comply with study requirements (eg, will not be available for follow-up assessment)

-Subject has any kind of disorder that compromises the ability of the subject to give written informed consent and/or to comply with study procedures (except for subjects with a legally acceptable representative).

TREATMENT PROCEDURES

The term protocol-specified therapy refers to panitumumab plus mFOLFOX6 (table 2). Panitumumab is administered on day 1 of each cycle just prior to the administration of mFOLFOX6 chemotherapy. One treatment cycle is defined as the 14 day (± 3 days) period following the start of treatment with panitumumab plus chemotherapy plus additional time, as needed, for the resolution of mFOLFOX6-related toxicities.

TREATMENT SCHEDULE

Drug	Dose	Administration	Dosing Interval
Panitumumab	6 mg/kg[1]	IV diluted in 100 mL of 0.9% NaCl solution infused over 60 ± 15 minutes[2,3]	Day 1 every 14 (± 3) days (prior to chemotherapy)
Oxaliplatin	85 mg/m^2 [7]	Oxaliplatin infusion in 250-500 mL D5W and leucovorin IV infusion in D5W both given over 120 minutes (±15 minutes)[4] at the same time[5] in separate bags using a Y-line[6]	Day 1 every 14 (± 3) days
Leucovorin	400 mg/m^2 racemate (or 200 mg/m^2 l-LV) [7]		
5-FU	400 mg/m^2 [7]	IV bolus over approximately 2 to 4 minutes	Day 1 every 14 (± 3) days
5-FU	2400 mg/m^2 [7]	IV continuous infusion over 46 to 48 hours	Days 1 to 3 every 14 (± 3) days

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Table 2: Panitumumab plus mFOLFOX6 treatment schedule.

1 The starting panitumumab dose is 6 mg/kg. The total dose may be rounded up or down by no greater than 10 mg. The panitumumab dose will be calculated based on the subject's actual body weight at baseline and re-calculated at subsequent doses per institutional guidelines. At a minimum, the dose will be re-calculated if the actual body weight changes by at least ±10% from the baseline weight. Investigators may recalculate the dose of panitumumab more frequently according to institutional guidelines or clinical practice.

2 The panitumumab dose will be diluted to a total volume of 100mL with pyrogen-free 0.9% sodium chloride solution USP (normal saline solution, supplied by the site). Doses higher than 1000 mg should be diluted to 150mL with 0.9% sodium chloride solution USP. The maximum concentration of the diluted solution to be infused should not exceed 10 mg/mL. The diluted solution should not be shaken excessively and should be mixed by gentle inversion.

3 Panitumumab will be administered IV by an infusion pump through a peripheral line or indwelling catheter using a non-pyrogenic, low protein binding 0.2- or 0.22-micron pore size in-line filter (obtained by each center) infusion set-up over 60 minutes ± 15 minutes by a trained healthcare professional. If the first infusion is well tolerated (ie, without any serious infusion-related reactions) then all subsequent infusions may be administered over 30 minutes ± 10 minutes. The infusion time should be extended to 90 minutes

- 15 minutes for doses higher than 1000 mg

4 Prolongation of infusion time for oxaliplatin from 2 hours to 6 hours to mitigate acute toxicities is allowed at investigator's discretion. The infusion times for 5-FU and leucovorin do not need to be changed.

5 At the investigator's discretion leucovorin and oxaliplatin can be administered sequentially according to standard of care and all applicable guidelines in the region.

.6 Oxaliplatin is not compatible with normal saline solution or 5-FU. The infusion line must be thoroughly flushed with D5W before and after the administration of 5-FU.

.7 The total dose of the mFOLFOX6 components may be rounded up or down per institutional guidelines clinical practice however should be within ± 10% of expected dose.

PANITUMUMAB DOSAGE, ADMINISTRATION, AND SCHEDULE

Panitumumab is administered by IV infusion on day 1 of each cycle just prior to the administration of chemotherapy. In the event a cycle is delayed beyond 14 days due to chemotherapy-related toxicity, administration of panitumumab should also be delayed. However, if panitumumab is delayed, administration of chemotherapy should continue as scheduled. Subjects who have not progressed and become intolerant to chemotherapy may continue on panitumumab monotherapy every 14 days (±3 days) until disease progression, intolerability, withdrawal of consent or death. In the event any component(s) of the protocol-specified regimen is discontinued for intolerability in the absence of disease progression subjects may continue with the remaining component(s)

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every 14 days (±3 days) until disease progression, intolerability, withdrawal of consent, or death.

Dose Adjustement and supportive care are permitted according to institutional guidelines.

MFOLFOX6 DOSAGE, ADMINISTRATION, AND SCHEDULE

mFOLFOX6 regimen is a combination therapy of oxaliplatin (85 mg/m^2) administered as a 2-hour infusion (± 15 minutes) on day 1; leucovorin (400-200 mg/m^2) administered as a 2-hour infusion (± 15 minutes) on day 1; followed by a loading dose of 5-FU (400 mg/m^2) IV bolus administered over approximately 2 to 4 minutes on day 1, then 5-FU (2400 mg/m^2) via ambulatory pump administered for a period of 46 to 48 hours. The total dose of the mFOLFOX6 components may be rounded up or down per institutional guidelines or clinical practice however should be within ± 10% of expected dose.

Prolongation of infusion time for oxaliplatin from 2 hours to 6 hours to mitigate acute toxicities is allowed at investigator's discretion. The infusion times for 5-FU and leucovorin do not need to be changed. Dose adjustement and supportive care are permitted according to institutional guidelines.

To prevent the occurrence of accumulative oxaliplatin-induced neurotoxicity subjects may discontinue oxaliplatin after a minimum of 8 cycles at the investigator discretion but continue to receive other first-line protocol-specified therapy , until disease progression, unacceptable toxicities, death, or withdrawal

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of consent. Reintroducing oxaliplatin after permanent discontinuation will not be allowed as part of the first line treatment aspects of this trial.

Subjects who discontinue oxaliplatin due to associated neurotoxicity should continue all other first-line treatment components of the regimen (5-FU/leucovorin and panitumumab). This modified first-line therapy will continue until 1 of the following occurs: disease progression, unacceptable toxicities, withdrawal of consent by the subjects or death.

MOLECULAR BIOLOGY METHODS

Patients will be tested for a pre-specified gene panel of markers (using the Ion Torrent technology on DNA samples from formalin-fixed paraffin-embedded -FFPE- tissues) including BRAF and PIK3CA mutational status. EGFR gene copy number (GCN) variations will be evaluated by FISH analysis.

The gene-panel previously reported in table 1 will be used (Hotspot regions in Ion Ampliseq Cancer Hotspot panel: ~2,800 mutations of 50 oncogenes and tumor suppressor genes). A >100 reads of coverage and ≥5% mutated alleles filter will be applied.

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TUMOR RESPONSE EVALUATION AND SAFETY

Tumor response evaluation is performed according to RECIST 1.1 every 8 weeks (± 7 days) and treatment continued until disease progression, unacceptable toxicities, death, withdrawal of consent or investigator decision. Patient will be observed for safety at 30 days after the last study drug administration. Adverse event were (AEs) were graded using CTCAE version 4.0.

STUDY ENDPOINTS

Primary endpoint

RR will be evaluated after 8 weeks (± 7 days) of treatment and defined according to the Response Evaluation Criteria in Solid Tumours (RECIST), v. 1.1

Secondary endpoints

- PFS will be defined as the interval between the start of first-line therapy to clinical progression or death or last follow up visit if not progressed;
- OS will be defined as the interval between the start of first-line therapy to death or last follow up visit.

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STATISTICAL METHODS

Statistical methods (for analysis of primary and secondary endpoints or interim analysis): patients are tested for a pre-specified panel of markers and they will be prospectively allocated to either the favourable group (PIK3CA and BRAF wild type and EGFR GCN ≥ 2.6) or the unfavourable group (PIK3CA mutation or BRAF mutation or EGFR GCN < 2.6). Patients subsequently receive mFOLFOX6 +panitumumab as per indication. Differences in RR, will be evalutated using the chi square test. Survival probability over time will be estimated by the Kaplan–Meier method. Significant differences in the probability of survival between the strata will be evaluated by log-rank test. Logistic regression analysis will be used to assess the independent role of variables resulted significant at univariate analysis.

Sample size and justification

To detect a difference in terms of RR among patients with an unfavourable profile (estimated around 40%) and patients with a favourable profile (estimated around 80%), assuming a probability alpha of 0.05 and beta of 0.10, required sample size is 85 patients.

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RESULTS

This report represents an interim analysis since the SUPERPEAK trial is currently ongoing as well as its enrollment. Globally from October 2014 to February 2020 21 patients were enrolled.

Main demographic and clinical patients' characteristics in the global population

The main demographic and clinical characteristics of the study population are summarized in table 3. The global population included 15 males (71.4%) and 6 (28.6%) females. The median age at diagnosis was 65 years (95% CI for the median 56,6 to 68,4). All patients had ECOG PS 0 (57.1%) or ECOG PS:1 (42.9%). As for primary tumour sidedness, 1 (4.8.%) patient had right sided colorectal tumour, whereas 20 patients (95.2.%) had left sided colorectal tumour. All patients received FOLFOX plus panitumumab as for protocol-specified therapy with a median of 11 cycles. Four (4/21) patients received 5-FU plus panitumumab as maintenance therapy after at least 8 cycles of FOLFOX plus panitumumab. As for safety, most common grade 2-3 adverse events reported were neurotoxicity G2-3 (12%) and skin toxicity G2-G3 (22%), whereas no G4 adverse events occurred. As for outcome in the global population, the median overall survival was 27 months (95% CI 22.0 to 38.0 months), the median PFS was 12 months (95% CI 10.0 to 14.0 months), whereas the RR were 81%.

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	Total (%)
SEX Male Female	 15 (71.4%) 6 (28.6%)
Age Median (Range)	65 (95% CI for the median 56,6 to 68,4)
ECOG PS at treatment start 0 1	 58 (40) 86 (60)
Primary tumour sidedness: Left sided CRC Right sided CRC	 20 (95.2%) 1 (4.8%)
Median OS	27 months (95% CI 22.0 to 38.0 months),
Median PFS	12 months (95% CI 10.0 to 14.0 months)
Overall response rate	81%

Table 3: Main demographic and clinical patients' characteristics in the global population.

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Molecular characteristics in the global population (Ion Torrent technology on DNA samples from formalin-fixed paraffin-embedded -FFPE- tissues)

As for the molecular characteristics, they are summarized in Figure 2 and Figure 3.

The most frequent mutations include: P53 (95.2%), APC (61.9%), KIT (42.9%), KDR (38.1%), PIK3CA (28.6%), SMAD 4 (23.8%), GNAQ (19.0%), FBXW7 (14.3), RB 1 (14.3%), KRAS (9.5%), MET(9.5%), PTEN (4.8%) SMARCB1 (4.8%), VHL (4.8%), ERBB4 (4.8%), ALK (4.8%), ATM (4.8%), FGFR1 (4.8%), FGFR2 (4.8%), FGFR 3 (4.8%), MLH1 (4.8%). The molecular data regarding EGFR GCN are not yet available thus preventing us from evaluating differences in the probability of survival between the preplanned strata (i.e. favourable vs unfavourable profile). All patients had BRAF wild type status on tumour tissue.

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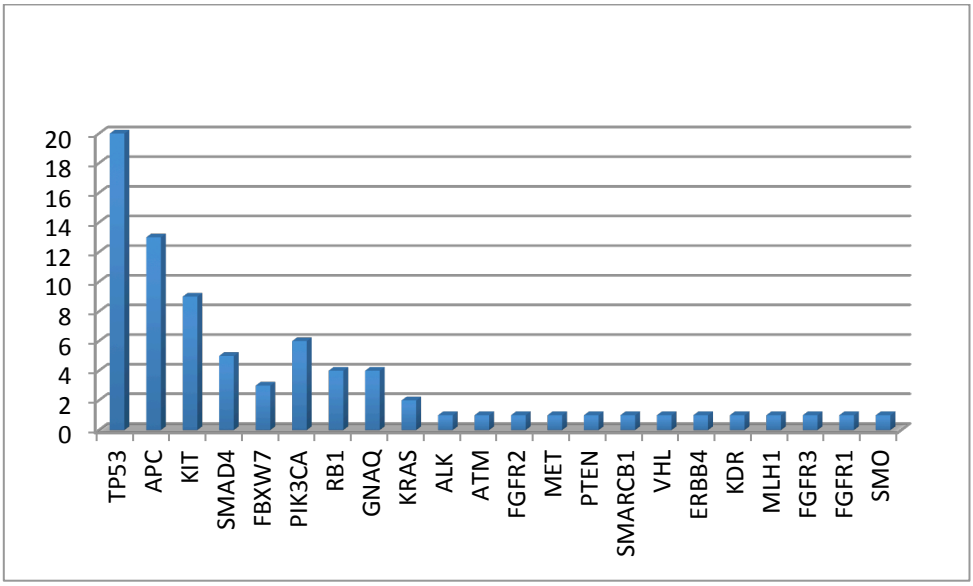

Figure 2: Distribution of gene variants in the global population (Ion Torrent technology on DNA samples from formalin-fixed paraffin-embedded -FFPE- tissues).

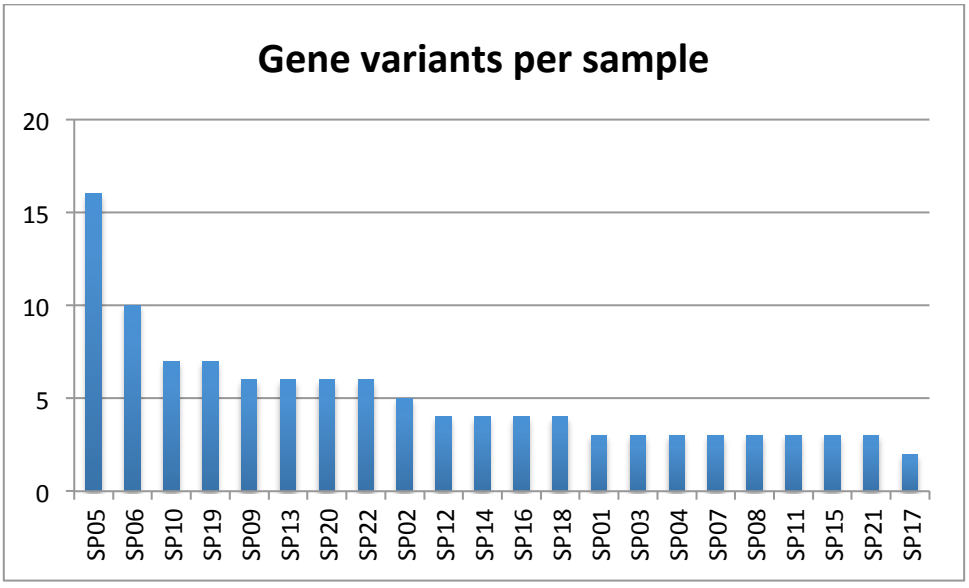

Figure 3: Gene variants per sample.

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Univariate analysis

Several clinical covariates (i.e. ECOG PS, primary tumour sidedness, response to first line, age>70 years) and genetic covariates, have been evaluated as putative prognostic/predictive factors.

As for **clinical covariates**, the sidedeness resulted to be correlated to response rate (0% for right sided colorectal cancer vs 85% for left sided colorectal cancer p:0.03). The response to first line (Fig.4) resulted to be correlated to PFS (14.0 months for responders vs 6.0 months for non responders HR 0.24 p:0.003), whereas it resulted to be correlated to a not statistically significative trend (Fig.5) in terms of improved OS (38 months for responders vs 22.0 months for non responders HR: 0.58 p:0.42).

The ECOG PS 0-1 and the age ≥70 years resulted to be not correlated to outcome.

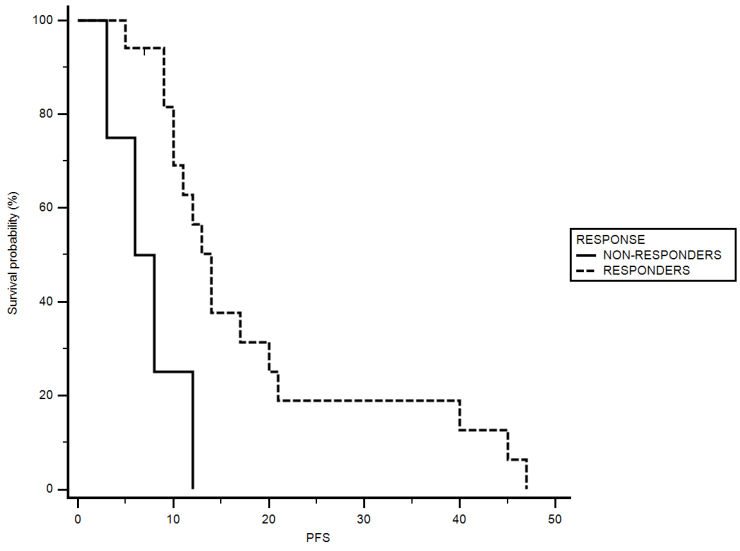

Figure 4: The response to first line FOLFOX PANITUMUMAB resulted to be correlated to PFS (14.0 months for responders vs 6.0 months for nonresponders HR 0.24 p:0.003).

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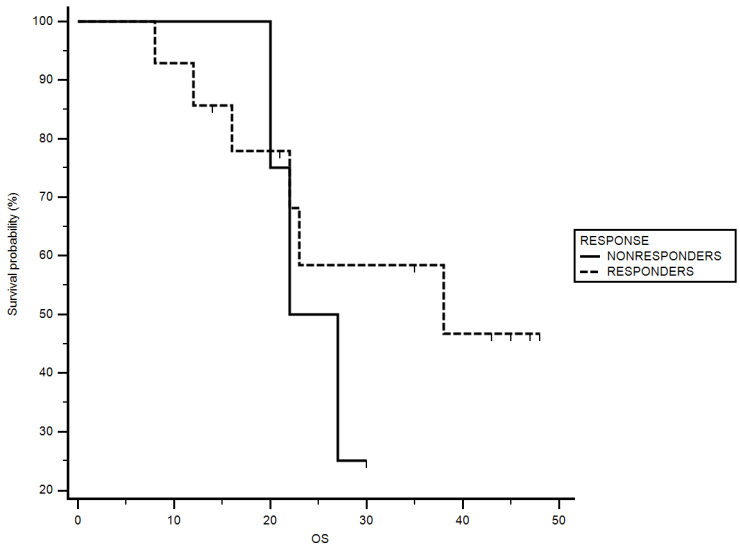

Figure 5: The response to first line FOLFOX PANITUMUMAB resulted to be correlated to a not statistically significative trend in terms of improved OS (38 months for responders vs 22.0 months for non responders HR: 0.58 p:0.42).

The results of univariate analysis for different genetic covariates on patients' outcome in terms of RR, PFS, OS are reported in Table 4 and Figure 6.

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	%	RR	*p*	PFS (months)	HR (95%CI) *p*	OS (months)	HR (95%CI) *p*
P53							
MUT	95.2%	80%	0.62	12.0	2,78 (0.74 to 10.33)	22.0	2.20 (0.52 to 9.26)
WT	4.8%	100%		45.0	0.25	45.0	0.40
APC							
MUT	61.9%	76.9%	0.55	12.0	0.99 (0.40 to 2.43)	27.0	0,54 (0.20 to 1.47)
WT	38.1%	87.5%		12.0	0.99	20.0	0.15
KIT							
MUT	42.9%	77.8%	0.75	12.0	0.94 (0.38 to 2.28)	21.0	1.65 (0.65 to 4.19)
WT	57.1%	83.3%		12.0	0.88	23.0	0.23
KDR							
MUT	38.1%	87.5%	0.55	9.0	1.35 (0.53 to 3.44)	17.0	1.29 (0.51 to 3.25)
WT	61.9%	76.9%		12.0	0.48	22.0	0.55
PIK3CA							
MUT	28.6%	83.3%	0.86	13.0	0.78 (0.31 to 1.95)	17.0	1.43 (0.58 to 3.54)
WT	71.4%	80%		10	0.59	22.0	0.41
SMAD 4							
MUT	23.8%	100%	0.22	11.0	1.22 (0.43 to 3.45)	38.0	0.34 (0.14 to 0.83)
WT	76.2%	75%		12.0	0.70	20.0	0.02
GNAQ							
MUT	19%	75%	0.74	12.0	0.70 (0.23 to 2.10)	45.0	0.34 (0.13 to 0.86)
WT	81%	82.4%		12.0	0.55	21.0	0.04
FBXW7							
MUT	14.3%	100%	0.37	17.0	0.47 (0.15 to 1.43)	17.0	1.23 (0.32 to 4.75)
WT	85.7%	77.8%		12.0	0.27	22.0	0.76
RB1							
MUT	14.3%	100%	0.37	11.0	1.75 (0.53 to 5.74)	45.0	0.28 (0.10 to 0.74)
WT	85.7%	77.8%		12.0		21.0	0.03
MET							
MUT	9.5%	100%	0.48	14.0	0.49 (0.15 to 1.54)	38.0	0.51 (0.16 to 1.60)
WT	90.5%	78.9%		12.0	0.31	21.0	0.33
PTEN				12.0	1.36 (0.13 to 13.88)	30	0.87 (0.13 to 5.87)
MUT	4.8%	0%	0.03	12.0	0.75	22	0.89
WT	95.2%	85%					

SMARCB1 MUT WT	 4.8% 95.2%	 0% 85%	 0.03	12.0 12.0	1.36 (0.13 to 13.88) 0.75	30 22	0.87 (0.13 to 5.87) 0.89
VHL MUT WT	 4.8% 95.2%	 0% 85%	 0.03	12.0 12.0	1.36 (0.13 to13.88) 0.75	30 22	0.87 (0.13 to 5.87) 0.89
ERBB4 MUT WT	 4.8% 95.2%	 100% 80%	 0.62	 13.0 12.0	 0.84 (0.09 to 7.40) 0.86	 17.0 22.0	 3.02 (0.10 to 88.8) 0.24
ALK MUT WT	 4.8% 95.2%	 100% 80%	 0.62	Not reached 12.0	/	Not reached 22.0	/
ATM MUT WT	 4.8% 95.2%	 100% 80%	 0.62	10.0 12.0	2.15 (0.12 to 38.09) 0.42	22.0 22.0	1.35 (0.13 to 13.5) 0.75
FGFR1 MUT WT	 4.08 95.2	 100% 80%	 0.62	Not reached 12.0	/ 0.69	Not reached 22.0	/
FGFR2 MUT WT	 4.8% 95.2%	 0% 85%	 0.03	 12.0 12.0	 1.36 (0.13 to 13.8) 0.75	 30.0 22.0	 0.87 (0.13 to 5.87) 0.89
FGFR3 MUT WT	 4.8% 95.2%	 0% 85%	 0.03	 12.0 12.0	 1.36 (0.12 to 13.8) 0.75	 30.0 22.0	 0.87 (0.13 to 5.87) 0.89
MLH1 MUT WT	 4.8% 95.2%	 0% 85%	 0.03	 12.0 12.0	 1.36 (0.12 to 13.8) 0.75	 30.0 22.0	 0.87 (0.13 to 5.87) 0.89

Table 4: results of univariate analysis for different genetic covariates on patients' outcome in terms of RR, PFS, OS. RR= response rate, PFS = progression-free survival, HR = Hazard ratio, CI = confidence interval, OS = overall survival.

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At univariate analysis, the genetic covariates for which the mutated allele resulted to be correlated to outcome in terms of RR, are PTEN, SMARCB1, VHL, FGFR2, FGFR3 and MLH 1. In particular the mutated allele of these covariates correlates to worse RR as reported in table 4.

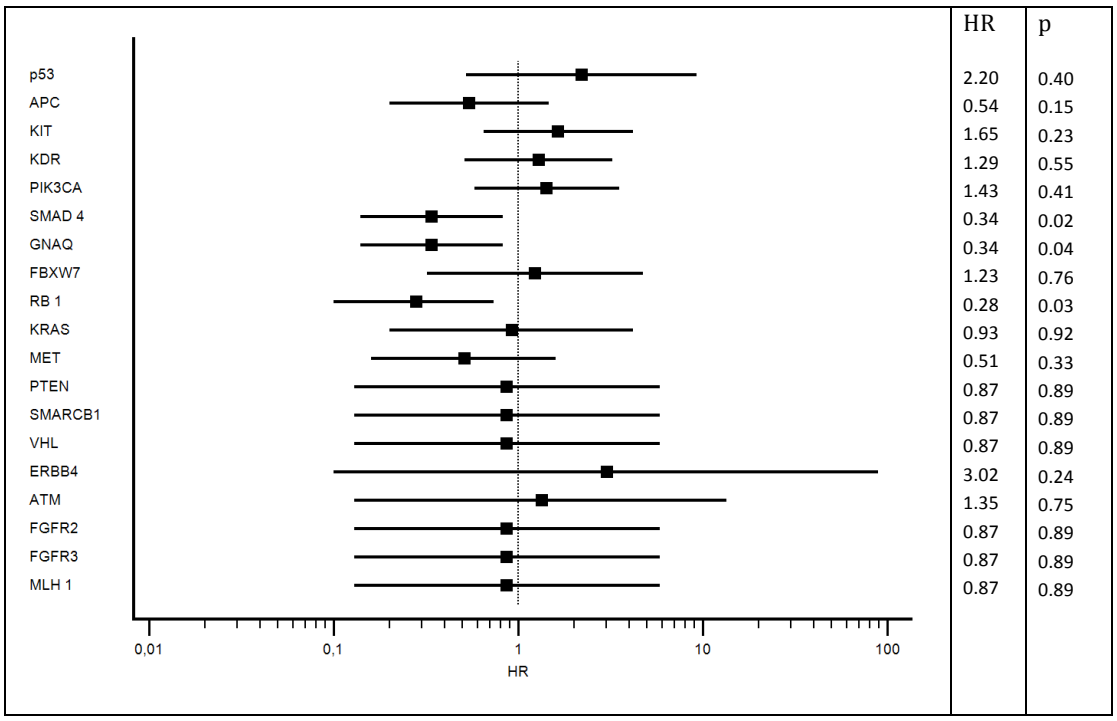

Figure 6: Hazard ratios and 95% CIs for overall survival in prespecified subgroups.

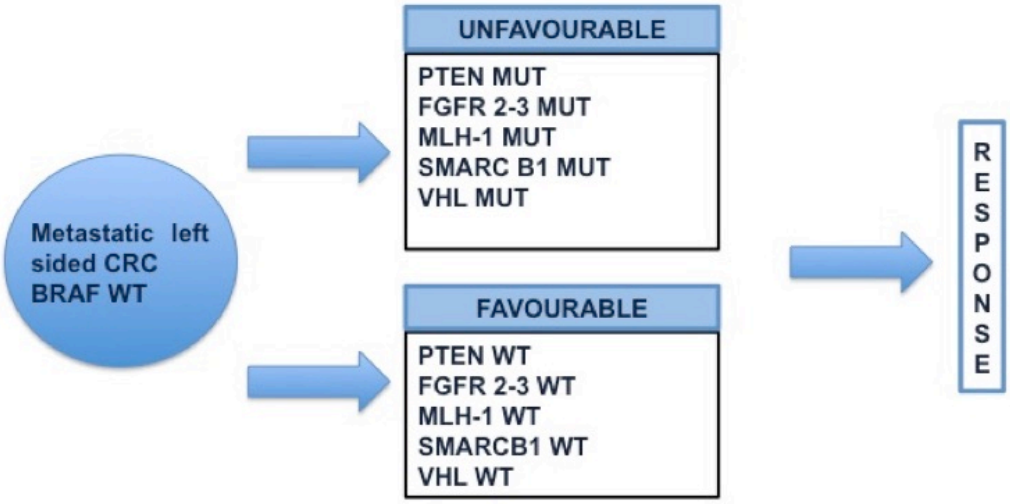

Figure 7: biologic model including unfavourable profile vs favourable profile for prediction of response.

On the basis of the results of univariate analysis for different genetic covariates on patients' outcome in terms of RR, a biologic model for prediction of response to treatment has been evaluated in the left sided subgroup (Fig. 7). Patients were allocated in the unfavourable group or in the favourable group on the basis of the molecular status of

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PTEN, FGFR 2-3, MLH-1, SMARCB1, VHL, as explained in figure 7. The unfavourable (5.0%) profile correlated to worse overall response rate vs the favourable (95%) profile (0% for the unfavourable profile vs 89.5% for the favourable profile respectively p: 0.0173). These profiles, conversely, didn't correlate to PFS (12.0 months for the unfavourable profile vs 13.0 months for the favourable profile; HR: 1.47 p:0.67) and OS (21 months for the unfavourable profile vs 30 months for the favourable profile HR 1.09 p:0.92).

As for overall survival, the mutational status of SMAD 4, GNAQ and Rb1 correlates to improved outcome (the correlation of all genetic covariates to overall survival is reported in Table 4 and Figure 6)

The mutated allele of SMAD 4 correlated with longer mOS (Fig.7): 38.0 month (95% CI 35.0 to 48.0 months) vs 20 months (17.0 to 23.0 months) ; HR: 0.34 (95% CI 0.14 to 0.83); p: 0.02.

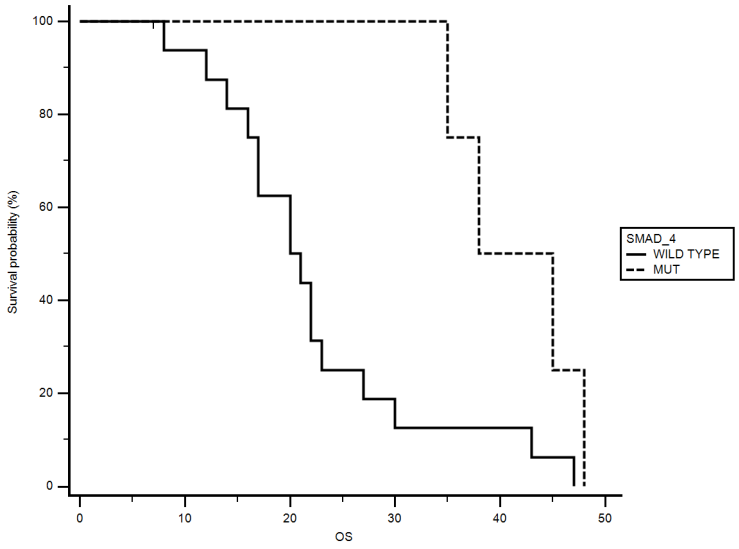

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Figure.7 The mutated allele of SMAD 4 correlated with longer mOS: 38.0 month (95% CI 35.0 to 48.0 months) vs 20 months (17.0 to 23.0 months) ; HR: 0.34 (95% CI 0.14 to 0.83); p: 0.02.

The mutated allele of GNAQ correlated with longer mOS (Figure. 8): 45.0 months (95% CI 30.0 to 48.0 months) to 21.0 months (95% CI 17.0 to 27.0 months); HR: 0.34 (95% CI 0.13 to 0.86); p: 0.04.

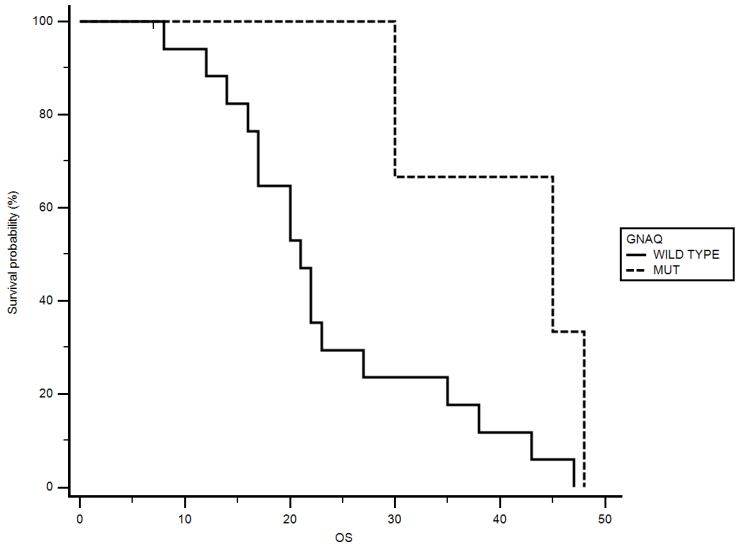

Figure 8. The mutated allele of GNAQ correlated with longer mOS (Figure. 8): 45.0 months (95% CI 30.0 to 48.0 months) vs 21.0 months (95% CI 17.0 to 27.0 months); HR: 0.34 (95% CI 0.13 to 0.86); p: 0.04.

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The mutated allele of Rb1 correlated with longer mOS (Fig. 9): 45.0 months (95% CI 45.0 to 48.0 months) vs 21.0 months (95% CI 17.0 to 27.0 months); HR: 0.28 (95% CI 0.10 to 0.74); p: 0.03.

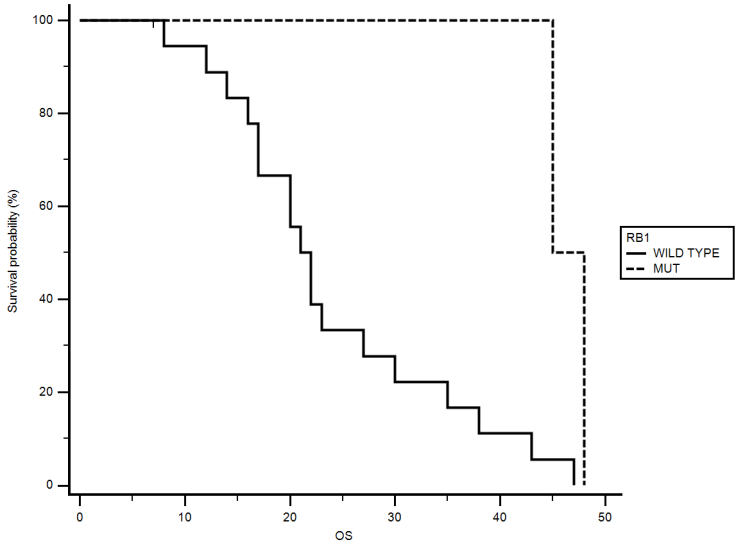

Fig.9 The mutated allele of Rb1 correlated with longer mOS: 45.0 months (95% CI 45.0 to 48.0 months) vs 21.0 months (95% CI 17.0 to 27.0 months); HR: 0.28 (95% CI 0.10 to 0.74); p: 0.03.

Multivariate analysis

Multivariate analysis for OS was performed by including only those variables that resulted to be correlated with a signicantly different OS at univariate analysis, namely SMAD 4, GNAQ, Rb1 and response to first line FOLFOX PANITUMUMAB. None of this variables maintained their independent role for OS (SMAD 4 p:0.33;Exp(b) 0.47. GNAQ p: 0.65; Exp(b) 0.61. Rb1 p: 0.61; Exp(b): 0.42. Response to first line FOLFOX PANITUMUMAB p:0.96; Exp(b) 0.96. Similarly, multivariate analysis for RR was

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performed by including only those variables that resulted to be correlated with a signicantly different rr at univariate analysis, namely PTEN, SMARCB1, VHL, FGFR2, FGFR3, MLH 1 and sidedness. None of these variables maintained their independent role for RR.

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DISCUSSION

The recent scientific evidence regarding the mechanism of primary resistance to anti EGFR therapy in RAS-BRAF wild type colorectal cancer, includes HER 2 amplification, MET amplification and the putative role of the PIK3CA-AKT-mTOR pathway. Secondly many others factors, mostly involved in MAPK or PIK3CA-AKT-mTOR pathways, have often been called into question, those including p53 and ERBB3 alterations. As for BRAF V600 E mutational status, this is currently one of the most utilized predictive and prognostic factor in clinical practice. As for mechanism of secondary resistance to anti EGFR, the EGFR mutations and the KRAS mutations are instead predominant in addition to the factors involved in primary resistance. Moreover the primary tumour sidedness became a fully part of clinical practice as prognostic and predictive factor for first line treatment choice.

The present analysis suffers from a study design temporally non-aligned to the recent insight regarding the role of primary tumour sidedeness and BRAF mutational status role in the treatment choice. The analysis of the patients' clinical characteristics, in fact, shows an imbalanced proportion in terms of primary tumour sidedness and BRAF mutational status. In fact, only one patient (1/21; 4.8%) had a right sided primary tumour, whereas none of the patients had a BRAF mutation. This, on the one hand is reflected in a response rate in the global population (i.e. 81%) which is higher than expected (i.e. 63.6 % in all RAS subgroup of panitumumab+FOLFOX harm in the PEAK trial), on the other hand in a greater difficulty to highlight molecular factor implied in primary resistance to anti EGFR therapy.

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As for the primary endpoint (i.e. RR), the presence of mutations in the factors PTEN, SMARCB1, VHL, FGFR2, FGFR3, MLH1 and sidedness were associated with a reduced ORR in the univariate analysis in the intention to treat population. Conversely, the mutations of p53, PIK3CA, MET and HER3, were not statistically related to RR. None of the patients showed a HER alteration.

The tumour sidedness is currently considered a valuable prognostic and predictive factor for first line treatment choice and the results of the present study are consistent with available literature data.

As for molecular factors resulted significant at univariate analysis, the PTEN mutational status has a strong biological rationale to support the putative role in the primary resistance to anti EGFR influencing the PIK3CA-AKT-mTOR pathway.

The PTEN mutation also would seem to be more frequent in MSI-H tumours . Even in this case the results of the present study are consistent with our present knowledge in this respect.

As for the putative role of the mismatch repair proteins mutations and the microsatellite instability, this is difficult to be discussing in this context. The molecular profile consistent with high microsatellite instability (MSI-H), linked to the mismatch repair proteins mutation related to high mutational burden, thus making the MSI-H profile a predictive factor to immunotherapy. The MSI-H profile has also been associated with better prognosis and reduced response to chemotherapy in the early stages, whereas the insight in stage IV are not conclusive .

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As for the mutations in the factors SMARCB1, VHL, FGFR2 and FGFR3 which resulted to be related to RR at univariate analysis, these may be considered like an epiphenomenon of the mismatch repair proteins mutation related to high mutational burden in our case series. Here, indeed, these mutations, together with PTEN mutation, have been observed aggregated to MLH-1 mutation.

As for secondary endpoints (OS, PFS), the mutations of SMAD 4, GNAQ and Rb1 have been linked to an improved OS. Nevertheless, we are lacking a clear biological rationale in support of this statistical result.

Given the results observed in the intention to treat population and the imbalanced proportion in terms of primary tumour sidedness, we conducted a not preplanned analysis in a selected subgroup totally homogeneous for LSCRC as previously exposed. The molecular stratification for prediction of response to treatment evaluated in the LSCRC subgroup (i.e. favourable profile vs infavourable profile), with the limits peculiar to a not preplanned analysis, showed interesting results which are worthy of further development in the final analysis. As for safety, the treatment was confirmed to be safely and well tolerated with a predictable toxicity profile.

In the present study, from a statistical point of view, on the one hand the internal validity of this analysis is positively influenced by the prospective design, on the other hand it suffers from the imbalanced proportion in terms of primary tumour sidedness and BRAF mutational status. The results of the not preplanned analysis don’t allow us to derive definitive conclusion in the LSCRC subgroup (which however doesn’t represent the objective of an interim analysis).

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The internal consistence of the study is reduced, taking into account that the favourable profile which correlate to response, doesn't statistically correlate to improved prognosis. On the contrary, more in general, the response to treatment linked to a prognostic improvement, thus confirming the effectiveness of the therapy under study.

The study has a good external consistence with regard to the putative predictive factors called into question, which has been previously individually analized with a retrospective method. The prospective nature of the present study represents the strenght of our results compared to the already available data.

Given the above, the present interim analysis doesn't allow us to derive definitive conclusions. The molecular stratification proposed for LSCRC could have a relevant role in identifying different risk categories among LSCRC. Further analysis of the present study would hopefully clarify the role of these potential biomarkers in the near future.

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