Clinical Study Statistical Plan

A prospective, multicentre, single-arm, phase II clinical trial to evaluate biomarkers in advanced and/or metastatic colorectal cancer patients with wild-type KRAS/NRAS status treated with chemotherapy plus bi-weekly cetuximab as first-line therapy.

Protocol Number: GEMCAD-1002

EudraCT Number: 2010-019236-12

Clinicaltrials.gov number: NCT01276379

Acronym: POSIBA

27/JUN/2011

CONFIDENTIAL

Signature pages for clinical study statistical plan

I have read this report and confirm that to the best of my knowledge it accurately describes the conduct and results of the study.

Signed: Date: / / Print name: Dr. [redacted]

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Affiliation: Trial Statistician and Coordinating Investigator

TITLE PAGE

Study title: A prospective, multicentre, single-arm, phase II clinical trial to evaluate biomarkers in advanced and/or metastatic colorectal cancer patients with wild-type KRAS/NRAS status treated with chemotherapy plus bi-weekly cetuximab as first-line therapy.

Name of tested drug: Cetuximab

Indication studied: Advanced and/or metastatic colorectal cancer

Study description: A single-arm, prospective, multicentre phase II clinical trial with central review of RAS status and the identification of predictive biomarkers. Patients were treated either with commercially available FOLFOX6 (m) or FOLFIRI (m) as single-arm treatment combined with cetuximab 500 mg/m^2 bi-weekly as first-line treatment. FOLFIRI (m) or FOLFOX6 (m) was administered once every 2 weeks until the completion of 6 months of therapy, or the presence of progressive disease (PD), or unacceptable toxicity. Cetuximab was administered every 2 weeks until PD.
Tumour sample quality from primary or metastatic tissues was checked, and blood samples were collected at baseline and every 3 months along with abdominopelvic CT scan imaging. The feasibility of bi-weekly cetuximab in combination with chemotherapy (FOLFOX or FOLFIRI) was evaluated by means of progression free survival (PFS), overall survival (OS), objective response rate (ORR), and reported toxicities. The trial hypothesis was that the proposed biomarkers would allow identifying those patients who would benefit the most from bi-weekly cetuximab therapy. KRAS and NRAS mutations were evaluated by pyrosequencing, BRAF and PI3K mutations by RT-qPCR, and PTEN expression by immunohistochemistry. The PFS (time from the signature of the ICF date to PD or death) and OS (time from the signature of the ICF date to death) were analysed and compared between patients with: wild-type (wt) BRAF (wt-BRAF) vs mutant BRAF, and in patients with increased PI3K pathway activation (PI3K mutant or loss of PTEN expression) vs low PI3K pathway activation (wtPI3K and preserved PTEN expression).

Sponsor: Grupo Español Multidisciplinar en Cáncer Digestivo (GEMCAD) Spanish Multidisciplinary Group in Digestive Cancer

Protocol number: GEMCAD 1002 - POSIBA

Clinical Phase: II

Study dates: Study initiation date: first patient enrolled on 27/JUN/2011
Study completion date (last patient completed): 20/JUN/2017

This study, including the archiving of essential documents, was performed in compliance with ICH Good Clinical Practice (GCP)

1. SYNOPSIS

NAME OF SPONSOR: GEMCAD **NAME OF FINISHED PRODUCT** N/A **NAME OF ACTIVE INGREDIENT(S):** CETUXIMAB	
Title of Study	A prospective, multicentre, single-arm, phase II clinical trial to evaluate biomarkers in advanced and/or metastatic colorectal cancer patients with wild-type KRAS/NRAS status treated with chemotherapy plus bi-weekly cetuximab as first-line therapy.
Investigator(s)	[redacted]

Study centre(s)	[redacted]

Objectives	*Primary Objective* To evaluate BRAF, IGF-1RP/MMP-7 (Double-positive Phenotype/DP), and PI3K-PTEN as well as other relevant biomarkers which may arise during study implementation, to predict PFS in advanced and/or metastatic colorectal cancer patients with wt-RAS tumours treated with standard chemotherapy plus bi-weekly cetuximab as first-line therapy. *Secondary Objectives* ● To analyse secondary biomarkers (MMP-7, IGF-1, IGFBP-3, amphiregulin, and epiregulin, as well as other relevant biomarkers, which may arise during the study) in the serum and tumour tissues to predict acquired chemoresistance. ● OS based on the proposed classification ● ORR according to RECIST 1.1 (46) ● Safety of treatment with bi-weekly cetuximab at 500 mg/m^2

Methodology	We conducted a biomarker-evaluation phase II trial. A total of 181 wt KRAS exon 2 patients were evaluated for whole KRAS and NRAS mutations by pyrosequencing, BRAF and PI3K mutations by RT-qPCR, and PTEN expression by immunohistochemistry. Patients were followed every 3 months with abdominopelvic CT-scan imaging and clinical examination. PFS (time from the signature of the ICF date to progression or death) and OS (time from the signature of the ICF date to death) in patients with wt-BRAF vs mutant BRAF and in patients with increased PI3K pathway activation (PI3K mutant or loss of PTEN expression) vs low PI3K pathway activation (wt-PI3K and preserved PTEN expression) were evaluated. Univariate and multivariable analyses of BRAF and PI3K-PTEN together with well-known clinical variables were performed. Safety was evaluated based on type, frequency, and intensity of adverse events related to the combination treatment.
Number of patients	Target events for primary endpoint: Planned: 170 patients Enrolled: Analysed for safety: Analysed for efficacy:

Diagnosis and main criteria for inclusion	*Main inclusion criteria:* Patients, men or women 18 years or older, with advanced and/or metastatic wt-KRAS and NRAS colorectal cancer, histologically or cytologically confirmed and radiologically measurable, with ECOG performance status 0 to 2 and adequate hepatic, renal, and haematological function, all after provision of the patients' signed informed consent. Patients were included only if their tumour samples were of the required quality to perform the proposed biomarker tests. The centralised review laboratory was responsible for evaluating the quality of the tumour blocks. *Main exclusion criteria:* Patients who have received a previous systemic treatment for their metastatic colorectal cancer were excluded from the trial. Those who have previously undergone metastatic surgery (liver, lung, or other), presenting with central nervous system metastasis or significant cardiovascular disease will also be excluded.
Test product, dose and mode of administration	Cetuximab (Erbitux® 5 mg/ml - 500 mg/m^2 every 2 weeks) was administered intravenously with an infusion pump, gravity drip, or a syringe pump. The duration of the cetuximab infusion was 120 minutes for the first cycle, 90 minutes for the second, and 60 minutes for the third and subsequent cycles. Prior to all cetuximab infusions, patients received adequate premedication as prophylaxis against an acute hypersensitivity reaction with an antihistamine and a corticosteroid, according to local practice.

Duration of treatment	Cetuximab (Erbitux®) 500 mg/m^2 bi-weekly until underlying disease progression.
Criteria for evaluation	*Primary:* PFS comparing 2 cohorts of patients, which will be defined according to the classification based on the predictive capacity of each biomarker. *Secondary:* ● ORR: incidence of a complete or partial response according to the revised RECIST 1.1 criteria. ● Duration of response (DoR); calculated only for the subjects who present an objective response and considering the time from the first objective response to radiological disease progression, according to the revised RECIST 1.1 criteria. For responding subjects without disease progression, DoR are censored at the last date of assessable disease evaluation.

	● OS: time from the signature of the ICF date date to date of death. For subjects who have not died, or are lost to follow-up at the closing date for data analysis, OS will be censored at the date of last contact. ● Safety: type, frequency, and intensity of adverse events related to the combination treatment.

Statistical methods	**Sample size considerations** Initially, the number of patients required to be recruited in 24 months was 155 and the total study duration (recruitment + follow-up) was considered to be 28 months for 115 events to occur. After recruiting 157 patients, it was determined that about 70 of these patients had insufficient samples for biomarker analysis preventing their inclusion in the analysis of the primary objective. Therefore, in order to reach the 115 events necessary to evaluate the primary endpoint, the total number of patients to be included in the trial was increased up to 240. Finally, 221 patients were considered sufficient to observe the required number of events. After central reviewing and monitoring, 181 patients with wt-RAS were finally included for efficacy analysis. For sample size calculation, it was assumed that patients could be classified into 2 groups based on their clinical characteristics and their biomarker profile. The log-rank method was planned to be used for the comparison of PFS during follow-up between the two groups. A minimum difference in PFS of 20% (50% vs 30%) was expected between the groups at 12 months, given the following assumptions: ● Alpha error (two-tailed): 5% ● Beta error: 20% **Statistical considerations** The primary and the secondary efficacy objectives were analysed using per-protocol analysis. Patients fulfilling all eligibility criteria and with documented wt-RAS status (KRAS and NRAS) were included in the efficacy analysis. Efficacy analysis was based on radiological imaging performed throughout the study and assessed by site investigators. Any finding considered as non-evaluable by the investigator was excluded from the analysis (it was considered as non-existent). For continuous variables, mean, standard error (for efficacy variables), standard deviation (for other measurements), median, 25th percentile, 75th percentile, minimum and maximum, were considered. Categorical variables are presented using frequencies and percentages. The 95% two-sided confidence intervals (CI 95%) of Kaplan–Meier

	quartiles were calculated according to Brookmeyer and Crowley methods (55,56). Safety data were analysed throughout the entire study. In the first step to develop a prediction model for PFS, univariate Cox regression analysis including all clinical variables collected in the study was performed in order to identify which should be considered for the model selection thereafter in the second step. All factors with a ≤0.15 p value (Appendix 15.2, item 4) were selected for the multivariable Cox analysis. The cut-off of ≤0.15 p-value was used in order to include the presence of resectable liver metastases as that is a well-known prognostic factor in mCRC. In the second step multivariable Cox regression analysis was performed including those variables identified in the of univariate analysis. Importantly, the variable treatment (FOLFIRI vs FOLFOX) was not included for the development of the prediction score because it was not a characteristic intrinsic to the patients.

12-MONTHS PFS RISK MODEL DEFINITION

Clinical variables and biomarkers were used to build a score to predict 12-months PFS, by using a multivariable Cox regression model built with those clinical variables with a p≤0.15 in the multivariable analysis (performance status, chemotherapy regimen, sidedness, resectable only liver metastases). Once the first risk score was built adding up the coefficients of the multivariable model (PS>0= 0.6, left sided=-0.5, resectable liver only metastases=-0.4 and folfiri+cetuximab therapy=Not evaluable), ROC curves were computed for PFS at 12 months using a logistic regression model. All 4 scores (*A.* Clinical variables including PS, sidedness and resectable only liver metastases, *B.* Clinical variables + BRAF mutation; *C.* Clinical variables + PI3K mutation or PTEN ≤3; *D.* Clinical variables + DP) had comparable low areas under the curve, ranging between 0.5 and 0.7, overall: The AUC of the score containing the clinical variables was 0.67 (95% CI) (0.60-0.75).

The AUC of the score with clinical variables and BRAF mutational status was 0.68 (0.61-0.75, p-value = 0.37). The AUC of the score with clinical variables and PI3KCA mutation/PTEN status was 0.69 (0.61-0.76, p-value = 0.32). The AUC of the score with clinical variables and DP phenotype was 0.66 (0.58-0.73, p-value = 0.09).

SAFETY RESULTS

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DATE OF THE REPORT:

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LIST OF ABBREVIATIONS & DEFINITION OF TERMS

Abbreviation/ Acronym Definition

5-FU 5-fluorouracil
AE Adverse event
AEMPS Agencia Española de Medicamentos y Productos Sanitarios ALT
Alanine aminotransferase
AST Aspartate aminotransferase
CEA Carcinoembryonic antigen
CI Confidence interval
CNS Central nervous system
CR Complete Response
CRF Case report form
CrCl Creatinine clearance
CT Computerised tomography
CTCAE Common terminology criteria for adverseevents DP Double-positive
phenotype (Co-expression of p-IGFR-1 and MMP-7) DoR Duration of Response
ECG Electrocardiogram
ECOG PS Eastern Cooperative Oncology Group performancestatus
EGF Epidermal growth factor
EGFr Epidermal growth factor receptor
EoT End of Treatment
FOLFIRI (m) Chemotherapy regimen with irinotecanand 5-FU/leucovorin infusion
FOLFOX-6 (m) Chemotherapy regimen with oxaliplatin and 5-FU/leucovorin
infusion GCP Good Clinical Practice
G-CSF Granulocyte colony stimulatingfactor
HIV Human immunodeficiency virus
ICH International Conference on Harmonisation
ICF Informed Consent Form
IGF Insulin-like growth factor
IGFBP-3 Insulin-like growth factor-binding protein-3
IGFr Insulin-like growth factor receptor
i.v. Intravenous
LDH Lactate dehydrogenase
LLN Lower limit of normal
ULN Upper limit of normal
LV Leucovorin
mAb Monoclonal antibody
mCRC Metastatic colorectal cancer
MMP-7 Matrix metalloproteinase- 7
MRI Magnetic resonance imaging
ORR Objective response rate
OS Overall survival
PFS Progression free survival
PD Progressive disease
PR Partial Response
PTEN Phosphatase & Tensin homolog
RBC Red blood countRECISTResponse Evaluation Criteria In SolidTumours ROC
Receiver operating characteristic
SAE Serious adverse event
SD Stable Disease
SEOM Sociedad Española de Oncología Médica (Spanish Society of MedicalOncology) SIV

Site initiation visit
StD Standard Deviation
SUSAR Suspected unexpected serious adversereaction
WBC White blood count

3. INVESTIGATORS AND STUDY ADMINISTRATIVE STRUCTURE

Table II shows the principal study personnel involved.

Table II: Principal study personnel

Title	Name and affiliation
Coordinating Investigator	[redacted]
Coordinating Investigator	[redacted]
Sponsor	[redacted]
Project Managers	[redacted]
Clinical Research Associate(s)	[redacted]
Medical Advisory	[redacted]
Data Management	[redacted]
Trial Statistician	[redacted]

	[redacted]

Table I: Study sites

Centre name	**1.** **2.** **3.** **4.** **5.** **6.** **7.** **8.** **9.** **10.** **11.** **12.** **13.** **14.** **15.** **16.** **17.** **18.** **19.** **20.** **21.** **22.** **23.** **24.** **25.** **26.** **27.** **28.** **29.**

4. INTRODUCTION

4.1. COLORECTAL CANCER (CRC)

CRC is the third most common cancer in men (746,000 cases, 10.0% of the total) and the second most common in women (614,000 cases, 9.2% of the total) worldwide. Almost 55% of the cases occur in more developed regions. There is a wide geographical variation in cancer incidence across the world, and the geographical patterns are very similar in men and women; incidence rates vary 10-fold in both sexes worldwide, the highest estimated rates being in Australia/New Zealand (ASR 44.8 and 32.2 per 100,000 in men and women, respectively), and the lowest in Western Africa (4.5 and 3.8 per 100,000).

Mortality is higher (694,000 deaths, 8.5% of the total) with more deaths (52%) in the less developed regions of the world, reflecting a poorer survival in these regions. There is less variability in mortality rates worldwide (6-fold in men and 4-fold in women), with the highest estimated mortality rates in both sexes in Central and Eastern Europe (20.3 per 100,000 for men, 11.7 per 100,000 for women), and the lowest in Western Africa (3.5 per 100,000 for men and 3.0 per 100,000 for women). http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx?cancer=colorectal

It is expected that CRC will cause about 50,630 deaths during 2018 in the US. Death rate (the number of deaths per 100,000 people per year) associated with CRC has been dropping in both men and women in the past several decades. One reason for this is that the early diagnosis of colorectal polyps is possible, and these polyps are removed before they can develop into cancers and hinder cancer treatment. In addition, treatment for CRC has improved over the last few decades. As a result, there are now more than 1 million survivors of CRC in the US (1).

According to the Globocan estimation of 2012, 447,136 new cases of CRC were diagnosed in Europe (second most common cancer, accounting for 13.0% of all cancers apart from non-melanoma skin cancers) and 214,866 deaths from CRC in Europe (12.2% of the total number of cancer deaths, second most common cause of cancer-related deaths) (2). The most recent data from REDECAN shows colorectal cancer as the most frequently diagnosed in 2015, followed by prostate (33,370 cases), lung (28,347 cases), breast (27,747 cases), and bladder (21,093 cases) (3)

Data from the SEOM state that 41.441 new cases of CRC were diagnosed in Spain in 2015 (16.3% of all tumour incidences), making CRC the most diagnosed cancer. Moreover, CRC is the second most common cause of cancer death in our country, after lung cancer, with over 15,449 deaths in 2014 (4).

Out of the newly diagnosed cases, 15–20% correspond to patients with metastatic disease at the time of diagnosis (5) and up to 50% of all patients eventually present with metastatic disease (5,6). Generally, CRC is curable if diagnosed at an early stage, and it is confined to the intestinal mucosa, with a 5-year survival rate of 93% (7). However, the 5-year survival rate decreases to 67% if the tumour spreads to adjacent organs and lymph nodes, and it is only 8% in patients with disseminated metastatic disease (5,7).

4.1.1. CRC treatment

Historically, the median survival rate of patients with metastatic disease has been 11–13 months after treatment with a combination of the fluoropyrimidine, 5-fluorouracil (5-FU), and leucovorin (LV) (8). The introduction of oxaliplatin and irinotecan in advanced colorectal chemotherapy has improved the frequency and extent of clinical response compared with that achieved with 5-FU/LV alone, and it has enhanced progression free survival (PFS) and overall survival (OS). Current research is focused on optimising this systemic chemotherapeutic regimen.

Irinotecan, a specific DNA topoisomerase I inhibitor, has shown significant activity as a single agent in the treatment of patients with CRC refractory to 5-FU (9,10). Furthermore, the addition of irinotecan to the combination therapy with 5-FU/LV (FOLFIRI regimen) significantly improves OS, response rate, and time until progression in the therapy of previously untreated mCRC compared to 5-FU/LV alone (11).

Oxaliplatin, a platinum analogue, causes the inter- and intra-strand cross-linking of DNA, thereby preventing DNA replication. It is effective and well tolerated when administered with LV as a bolus and an infusion (FOLFOX regimen); however, neutropenia and sensory neuropathies are more frequent with this combination than with treatment with 5-FU and LV alone (12,13,14,15,16). In recent years there has been significant progress in the treatment of mCRC, which has resulted in the improvement of patient life expectancy.

Therapies targeted against vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFr) have been recently established in mCRC treatment.

It has been proved that targeted therapies, specifically designed to inhibit carcinogenic biochemical processes, are effective in the treatment of mCRC. Cetuximab (Erbitux®), a chimeric monoclonal antibody targeted against EGFr, has been approved by the FDA for use in combination with irinotecan for the treatment of EGFr-expressing mCRC in patients who are refractory to irinotecan-based chemotherapy. It has also been approved as a single agent for the treatment of EGFr-expressing recurrent mCRC in patients who are intolerant to irinotecan-based chemotherapy (17,18,19,20). Within the European Union (Erbitux® SmPC), cetuximab is indicated for the treatment of EGFr-expressing wt-RAS mCRC:

- in combination with irinotecan-based chemotherapy,
- as first-line therapy in combination with FOLFOX,
- as a single agent in patients refractory to oxaliplatin and irinotecan-based therapy and who are intolerant to irinotecan.

Bevacizumab (Avastin®) is another selective product that binds to and neutralises the biologic activity of human vascular endothelial growth factor-A, a protein with a crucial role in tumour angiogenesis. Bevacizumab, in combination with fluoropyrimidine-based chemotherapy, is indicated for the treatment of mCRC (Avastin® SmPC).

There are 2 monoclonal antibodies targeted against EGFR: panitumumab and cetuximab.

Panitumumab is presently indicated for the treatment of wt-RAS mCRC in adult

patients: ● as first-line therapy in combination with FOLFOX or FOLFIRI, ● as second-line therapy in combination with FOLFIRI for patients who have received first-line fluoropyrimidine-based chemotherapy (excludingirinotecan), ● as monotherapy after the failure of fluoropyrimidine-, oxaliplatin-, and irinotecan containing chemotherapy regimens for the treatment of EGFr-expressing mCRC in patients who are refractory to fluoropyrimidine-, oxaliplatin-, and irinotecan-based chemotherapy (Vectibix® SmPC).

The monoclonal antibody, cetuximab, was approved by the EMEA in July 2008 as first line treatment of mCRC in patients who fulfil the criteria detailed below.

Cetuximab is currently indicated for the treatment of patients with EGFr-expressing wt RAS mCRC:
● in combination with irinotecan-based chemotherapy,
● as first-line therapy in combination with FOLFOX,
● as a single agent in patients who have failed oxaliplatin- and irinotecan-based therapy and who are intolerant to irinotecan (Erbitux® Datasheet).

Therefore, the current indication for cetuximab as first-line treatment in patients with EGFr-expressing wt-RAS mCRC, reflected in the SPC, was established after the CRYSTAL and OPUS clinical trials, and it was verified that cetuximab could be used in routine clinical practice in combination with irinotecan and oxaliplatin chemotherapy regimens as first-line treatment.
The PRIME study results, published in NEJM (Douillard and cols, Sep 2013), showed that patients with mutations of KRAS exon 3 and exon 4 or of NRAS exon 2, exon 3, and exon 4 do not benefit from treatment with FOLFOX and panitumumab compared with FOLFOX alone. The FIRE-3 study, presented at ESMO 2013, confirms the same findings with FOLFIRI+cetuximab vs. FOLFIRI+bevacizumab. Therefore, patients enrolled in this current trial were evaluated for mutational status of the mentioned genes at the indicated exons.

4.1.2. CRYSTAL study

The CRYSTAL study was designed to evaluate the efficacy of adding cetuximab to FOLFIRI as first-line treatment of mCRC. It is a phase III trial comparing FOLFIRI vs FOLFIRI+cetuximab. Sample size was 1,198 patients, randomised 1:1 according to geographical site location and Eastern Cooperative Oncology Group performance status (ECOG PS). The CRYSTAL study design contemplated PFS as the primary objective and OS, response, and safety as secondary objectives. Efficacy analysis of KRAS status was retrospectively performed.

Updated survival data on the wt-KRAS status of CRYSTAL study patients treated with the combination of FOLFIRI+cetuximab were presented at the 2009 ECCO/ESMO conference in Berlin (21) and in ASCO GI 2010 (22) after a longer follow-up period and a higher number of KRAS determinations than in the original publication: 45% to 89% (21,22). Significant improvements in outcomes features were observed for the first time including ORR (57.3% vs 39.7%, p<0.0001; OR: 2.07, CI95%: 1.52-2.83), PFS (9.9 vs 8.4 months, p=0.012; HR: 0.70, CI95%: 0.67-0.95), and OS (23.5 vs 20 months,

p=0.0094; HR: 0.8; CI 95%: 0.67–0.95) (21,22).

These data, along with data on their BRAF mutational status (determined in 83% of the patients) (22) confirms the superiority of the combination of standard chemotherapy with cetuximab in wt-KRAS patients, in terms of efficacy. These results proved superior in wt KRAS and wt-BRAF patients, and the presence of mutated BRAF worsened the results, regardless of the treatment used. Therefore, the authors concluded that BRAF mutation is a negative prognostic factor in mCRC.

4.1.3. OPUS 20 study

The OPUS 20 study is a randomised phase II trial, comparing FOLFOX vs FOLFOX+cetuximab as first-line treatment of mCRC. The sample size was 292 patients, randomised 1:1 according to ECOG PS. The OPUS 20 study design contemplated the superiority of response rate in the experimental arm as the primary objective. Efficacy analysis of KRAS status was retrospective, as was in the CRYSTAL study.

Updated data on the efficacy of the FOLFOX-4+cetuximab combination in wt-KRAS patients, following an increment in the number of patients who underwent mutational status determination were presented at the ECCO/ESMO 2009 conference (23) and ASCO GI 2010 (24). A higher number of patients with KRAS status determination 93.5% vs 69.1% in the original publication) was reported then. The OPUS 20 study re-confirmed that the addition of cetuximab to FOLFOX in wt-KRAS is beneficial in terms of PFS (FOLFOX+cetuximab arm: 8.3 vs. 7.2 months; HR 0.57; CI 95% 0.38–0.86, p=0.0064) indicating a 43% reduction in the risk of PD, and ORR (FOLFOX+cetuximab: 57.3% vs 34%, p=0.027; OR: 2.55; CI 95%: 1.38–4.72). Benefit on OS was not statistically demonstrated with median OS of 22.8 vs. 18.5 months (p=0.38) (23,24).

Results of a meta-analysis on treatment efficacy after adding cetuximab as first-line mCRC chemotherapy in the CRYSTAL and OPUS 20 trials were presented at these conferences (25). This study involved independent data on 845 wt-KRAS mCRC patients (666 patients from the CRYSTAL trial and 179 patients from the OPUS 20 trial). The analysis showed that cetuximab addition offers a clear benefit in the ORR (with an odds
ratio above 2) and an increase in PFS for wt-KRAS patients (the risk of PD was reduced by 34%; HR 0.66; p<0.001). Moreover, the study also demonstrated a significant increment in the OS of wt-KRAS patients who received cetuximab (HR 0.81; p=0.0062) (Table 6) (26).

	HR/odds ratio	95% CI	p-value	Heterogenicity p-value
OS	0.81	0.69–0.94	0.0062	0.6696
PFS	0.66	0.55–0.80	<0.0001	0.3332
ORR	2.16	1.64–2.86	<0.0001	0.5568

4.2. RATIONALE FOR THE STUDY

Advanced and/or mCRC is a heterogeneous condition, and the classification of advanced and/or mCRC patients is currently poor. Approximately 20% of the patients

present with advanced and/or mCRC at early stages (<5 nodes in the liver and size <5 cm), and are suitable for local treatment (surgery or local ablative therapy). Additionally, 10–15% of the patients show a poor functional status (ECOG PS>2) or exhibit disability secondary to geriatric syndromes and/or comorbidity with conditions that oppose any strategy other than supportive care. The rest of the patients (patients unsuitable for radical surgery) represent the patient population treated with palliative therapy. Despite this, not all patients display the same prognosis. Patients with an ECOG PS of 0–1 and lactate dehydrogenase (LDH) levels < upper limit of normal (ULN; intermediate risk patients) have shown better PFS and OS independent of the therapy in all previous randomised clinical trials (11,13,27).

The CRYSTAL study shows that FOLFIRI+cetuximab in wt-KRAS patients improves PFS when compared with FOLFIRI treatment alone. Presently, the selection of patients to undergo cetuximab treatment was based on KRAS mutational status, which enabled us to identify those patients who would not respond to therapy. Other activity biomarkers remained to be evaluated.

PTEN

The cytoplasmic expression of PTEN is inversely associated with PFS in CRC. There are 2 primary retrospective studies that evaluated PTEN expression in patients treated with cetuximab. However, this biomarker had not yet been prospectively evaluated.

Bi-weekly cetuximab regimen

The cetuximab standard administration regimen implies a weekly infusion of this drug. Bi weekly administration (i.e. every fortnight) presents 2 advantages: it is more convenient for the patients and allows health systems to save resources.

However, this benefit would be even more significant in the case of treatment with cetuximab based chemotherapy regimens, as most chemotherapy regimens combined with cetuximab are administered every fortnight.

This possibility has been studied via clinical trials, and it has been previously demonstrated that the pharmacokinetics and pharmacodynamics of weekly and bi-weekly regimens are equivalent. Phase II clinical trials of cetuximab in combination with chemotherapy provide data confirming the hypothesis that the toxicity and efficacy of the bi-weekly regimen are similar to those of the weekly regimen.

The decision for using the bi-weekly cetuximab regimen in this trial was based on the findings described below:

Results of a multicentre, prospective, uncontrolled clinical trial, which evaluated the safety profiles of 70 patients treated with irinotecan+cetuximab at 500 mg/m^2 every fortnight, were presented at the ASCO GI 2008 conference. Grade 3 and grade 4 toxicities included neutropenia (6%), diarrhoea (6%), asthenia (7%), and acne-like skin rash (11.4%). A total of 29 patients were eligible for the treatment, and the PFS at 12 months was 52%. The authors concluded that bi-weekly cetuximab is as effective and safe as the standard weekly therapy (28).

An exploratory clinical trial of bi-weekly therapy with irinotecan and cetuximab was

conducted in mCRC patients, who had received at least one previous chemotherapy cycle. In this trial, a total of 40 patients were treated with irinotecan at 180 mg/m^2 and cetuximab at 500 mg/m^2 every 2 weeks in 21-day cycles until unacceptable toxicity or PD. OR was 22.5% (2 complete and 7 partial responses). Disease control rate was 60%. Time to progression was 3.4 months and OS was 8 months. The bi-weekly regimen demonstrated good tolerability. Grade 3 and grade 4 adverse events (AEs) were observed in 12 patients. These study findings show that bi-weekly regimen is similar to weekly administration schedule in terms of toxicity and efficacy (29).

A single-centre clinical trial was conducted on 74 patients with refractory mCRC treated with irinotecan and cetuximab at 500 mg/m^2 every 2 weeks. Median treatment duration, ORR, median time to progression, and median OS were 4.3 months, 25%, 5.4 months, and 8.9 months, respectively. Treatment was well tolerated (30).

A study was conducted on mCRC patients, which retrospectively compared the efficacy and safety of cetuximab combined with irinotecan, administered either weekly or bi weekly. In this study, patients in the first group (n=32) received an initial dose of cetuximab at 400 mg/m^2, followed by a weekly infusion of cetuximab at 250 mg/m^2. Patients in the second group (n=18), received cetuximab at 500 mg/m^2 bi-weekly. ORR, PFS, OS, and toxicity were compared in both groups. All patients had received irinotecan and 5-FU, and most patients had previously received oxaliplatin. Median follow-up duration for all patients was 34.2 months. PFS and OS at 7 months were similar in both groups. Results considering the proportion of patients with the variable "disease control (complete response plus partial response plus those with stable disease)" were not statistically significant (56.3% weekly cetuximab patients vs 77.8% bi-weekly cetuximab patients, p=0.21), and when treatment toxicity was evaluated, no patient experienced an allergic reaction to cetuximab, nor were any treatment-related deaths reported; the most significant AE was dermatological toxicity, and only 1 patient in each group presented with a grade 3 AE. These study findings indicated that the efficacy of weekly and bi weekly cetuximab regimens is similar without increasing toxicity in association with irinotecan (31).

A phase I clinical trial reported that cetuximab at 500 mg/m^2 administered bi-weekly shows the same pharmacokinetic profile as the approved weekly regimen, and that cetuximab at 500 mg/m^2 can be administered safely bi-weekly with a similar pharmacodynamic profile, concluding that the bi-weekly could be considered as an alternative to the weekly regimen (32).

These reports support that the bi-weekly and weekly cetuximab administration regimens are equivalent in terms of efficacy and toxicity.

At the time of trial initiation, there was no published information on the usage of the bi weekly cetuximab regimen in combination with oxaliplatin (FOLFOX-6); however, there were several ongoing clinical trials to evaluate this treatment regimen. Experience gathered from daily clinical practice at some hospitals suggested a benefit of the bi weekly regimen over the weekly administration schedule, indicating that it is more convenient for patients and allows health systems to save resources. Therefore, the sponsor in this study ultimately left the choice of FOLFOX or FOLFIRI treatment to the principal investigators (PIs) at each centre, according to their own experience in the

treatment of the condition. There are prior data on the efficacy of the combination of oxaliplatin with cetuximab-based regimens, and no difference among oxaliplatin regimens has been reported. Moreover, the added convenience of combining the FOLFOX-6 regimen with the bi-weekly administration schedule justifies its use in this study.

After demonstrating differences in clinical benefits obtained with bi-weekly cetuximab treatment among mCRC patients, considering KRAS mutational status, it was justified to conduct a clinical trial with the secondary objective of evaluating the cetuximab toxicity profile when administered as a bi-weekly regimen.

Justification of the investigational biomarkers

Biomarker evaluation constitutes one of the fundamental development fields at GEMCAD. Biomarkers currently play an important role in the detection and treatment of patients with CRC. Biomarkers also direct diagnostic and treatment modalities by facilitating the selection of therapeutic drugs across a broad spectrum of patients. There are attempts to personalise chemotherapy based on the presence or absence of specific biomarkers. Therefore, the design of the trial, from the very beginning, included the primary endpoint to identify the potential role of relevant biomarkers in patient characterisation and treatment selection.

Role of insulin growth factor receptor (IGFr) pathway in mCRC

IGFr is overexpressed in CRC. Neither IGFr amplification nor mutation have been found in CRC; however, it is probable that mechanisms underlying IGFr transcriptional activation are involved in oncogenesis. IGFr activation results in EGFr phosphorylation by an autocrine/paracrine pathway through the cleavage of ligands similar to EGF, such as heparin-binding EGF, amphiregulin, tumour necrosis factor alpha, or metalloproteinases (33,34).

Matrix metalloproteinase (MMP)-7 and its relation to the IGF pathway

MMP-7 is a metalloproteinase secreted within the tumour microenvironment by neoplastic cells, generally facilitating tumour invasion, tumour progression, and metastasis. It can degrade IGFr pathway proteins, such as the proapoptotic insulin-like growth factor binding protein (IGFBP)-3 (35), and activate EGFr (36). In addition, activated EGFr can stimulate MMP-7 expression. Several *in vitro* studies correlate IGFr pathway activation with resistance to EGFr inhibitors (37,38).
In vitro observation has shown that MMP-7 increases after 48 hours of oxaliplatin treatment in the native cell line HT-29, and that baseline MMP-7 levels are 4-fold higher in oxaliplatin-resistant HT-29 cells than in native HT-29 cells (39). We hypothesised that chemotherapy or hypoxia may increase MMP-7. In fact, under hypoxic conditions, MMP 7 expression was found to be upregulated (40).

It has been observed that PD in untreated CRC patients increases MMP-3, which is associated with a reduction in IGFBP-3 levels (41). IGFBP-2 level in oxaliplatin-resistant HT-29 cells was higher than that in native HT-29 cells. This increment in IGFBP-2 level

may induce apoptosis by downregulating IGF-1RP and pAKT; however, this may not be possible in double-positive resistant HT-29 cells (IGF-1RP+ and MMP-7+). Recently, an increase in IGFr-1 phosphorylation was reported by Dallas et al. (2009) in this resistant phenotype. We consider that after treatment with oxaliplatin, IGFBP induces apoptosis through IGFr-1 phosphorylation inhibition in HT-29 cells, unlike in resistant HT-29 cells due to the constitutive activation of MMP-7.

In the clinical setting, our group retrospectively investigated the role of IGF-1RP and MMP-7 in patients with advanced primary or mCRC treated with cetuximab or panitumumab as second- or third-line therapy (42). A total of 168 tissue samples were available for the analysis of RAS and BRAF mutational status, and MMP-7 and IGF-1RP expression. There were no significant differences in the ORR (18.8% vs 15.0%), PFS (3.3 vs 3.0 months), and OS (7.8 vs 7.0 months) among the total and selected cohorts. MMP 7 and IGF-1RP expression was observed in 49% and 52% of patients, respectively. The co-expression of MMP-7 and IGF-1RP ("double-positivity" group) was noted in 27/104 wt RAS patients (26%) and in 15/71 wt-BRAF patients (24%).

There was no correlation between RAS and BRAF mutational status and double-positivity (p=0.52). In the subgroup of patients with wt-RAS and BRAF, the double-positivity group showed a lower ORR than the group without double-positivity (p=0.002); they also showed a declining median PFS of 91 days vs 121 days (p=0.09) and a poor median OS of 196 days (CI 95%: 175–215) vs 294 days (CI 95%: 182–344)(p=0.002).

BRAF

Both prognostic and predictive roles of BRAF mutation in mCRC have been established. BRAF mutation is present in 5–8% of mCRC cases. A retrospective analysis of the prognostic and predictive potential of this mutation was conducted in patients included in the OPUS 20 study. The proportion of patients with mutated BRAF was 7.9%, and this mutation was associated with OS (HR: 1.82; CI 95%: 1.36–2.43) but not with PFS (HR: 1.14; CI 95%: 0.86–1.52). In studies conducted by Di Nicolantonio et al. (43) and Laurent– Puig et al. (44) to evaluate the prognostic and predictive roles of BRAF mutation, the results indicated that BRAF mutation was associated with lower OS and PFS. Our own data confirm these findings. Our interest in investigating this biomarker, other than to identify it as a new prognostic biomarker, lies in determining its significance among other prognostic biomarkers (establishing a scoring system) as proposed in this protocol.

PTEN

PTEN, like BRAF, is another component of the MAPK signalling pathway; therefore, its mutational status may affect cetuximab efficacy. In the study by Laurent–Puig et al. (44), this biomarker was also evaluated. PTEN expression was absent in 19.9% of wt-KRAS patients, and was associated with both PFS and OS. Furthermore, another study (45) evaluated the predictive role of PTEN expression loss in mCRC patients treated with irinotecan+cetuximab. This study demonstrated that the loss of PTEN expression was associated with lower PFS (p=0.005).

Consequently, we consider that PTEN expression, along with BRAF, is another biomarker that is worth investigating for the characterisation of the MAPK signalling pathway in this protocol.

Other biomarkers

The list of investigated biomarkers was left open to avoid proposals being outdated; however, based on GEMCAD preclinical studies and recent publications, no other biomarker of interest was identified at the time of database closure.

5. STUDY OBJECTIVES

5.1. PRIMARY OBJECTIVE

• To evaluate BRAF, IGF-1RP/MMP-7 (DP) and PI3K-PTEN biomarkers to predict PFS in advanced and/or wt-KRAS/NRAS mCRC patients treated with standard chemotherapy along with bi-weekly cetuximab as first-line therapy.

• To identify new biomarkers, which may predict PFS in wt-KRAS/NRAS patients treated with chemotherapy and bi-weekly cetuximab as first-line therapy. • The need to identify additional biomarkers of cetuximab efficacy originates from the low predictive value of the current biomarker classification, based on KRAS mutational status, regarding the efficacy of bi-weekly cetuximab administration in patients with the investigational condition.

- The proposed biomarkers were:
  - BRAF mutation
  - IGF-1RP/MMP-7 (DP)
  - PI3K-PTEN

• The identification of additional biomarkers, which determine the efficacy of bi weekly cetuximab, will provide the scientific community with additional stratification factors for the design of clinical trials and facilitate the selection of patients for bi-weekly cetuximab therapy.

5.2. SECONDARY OBJECTIVES

● To analyse secondary biomarkers (MMP-7, IGF-1, IGFBP-3, amphiregulin, and epiregulin as well as other relevant biomarkers, which may arise during trial implementation) in the serum and tumour tissue, to predict acquired chemoresistance. ● OS based on the proposed classification.

● Objective response rate (ORR) according to RECIST 1.1 *(46)*. ● To describe the safety profile, including AE incidence and significant changes in laboratory parameters after the bi-weekly administration of cetuximab at 500 mg/m^2 q2w as first-line therapy.

● To evaluate the ORR, duration of response (DoR), and OS in wt-KRAS/NRAS mCRC patients treated with bi-weekly cetuximab in combination with FOLFOX6 (m) or FOLFIRI (m) as a first-line chemotherapy regimen, according to the expression of the proposed biomarkers.

6. INVESTIGATIONAL PLAN

Table 2: Schedule of examinations and procedures

Study procedures	**Selection**		**Study treatment**	**Safety follow-up**[c]	**Long-term follow-up**[m]
	≤28 days from cycle 1	≤7 days from cycle 1	Every 2 weeks [a, b]	(30±3 days)	
Informed consent	X				
Eligibility criteria review	X				
Clinical and medical history	X				
Physical examination	X		X	X	
ECG	X				
Vital signs	X		X	X	
Weight	X		X	X	
Height	X				
Body surface area calculation		X	X		
ECOG PS[j]		X	X	X	
AE evaluation[d]		X			
Concomitant medication[d]		X			
Survival assessment				X	
Laboratory tests					
Pregnancy test[e]		X			
Clinical safety blood tests[f]	X	X	X	X	X
Serum CEA[k]	X		Every 3 months until PD		
Serum biomarkers	X		Every 3 months until PD		
Paraffin embedded tumour block[l]	X				
Imaging evaluation of response					
CT scan (MRI optional)[g]	X		Every 3 months until PD		
Response evaluation according to the revised RECIST 1.1 criteria[i]			Every 3 months until PD		
Bi-weekly cetuximab			Until PD		

Evaluations schedule – Footnotes

a. Bi-weekly cetuximab administered through an i.v. infusion at a 500 mg/m^2 dose, every 2 weeks according to the indications established in this protocol. Cetuximab was given at least 1 hour prior to chemotherapy administration. A treatment cycle was defined as the 14-day period subsequent to the treatment start with cetuximab+FOLFIRI (m)/FOLFOX-6 (m).
b. Treatment cycles may be delayed longer than the normal 2 weeks due to bi-weekly cetuximab or FOLFIRI (m)/FOLFOX-6 (m) related toxicity.
c. Safety follow-up visit performed 30±3 days after the last administration of the investigational product. d. Adverse events and concomitant medication are recorded continually, until the safety follow-up visit. SAEs and significant AEs were followed up until resolution or stabilisation.
e. Women of reproductive age underwent a serum or urine pregnancy test ≤72 hours prior to investigational treatment initiation.
f. Haematology tests: complete haemogram, including WBC differential count and platelet count. Biochemistry tests: sodium, potassium, magnesium, chloride, bicarbonate, creatinine, albumin, glucose, calcium, phosphorus, AST, ALT, ALP, LDH, total protein, total bilirubin, uric acid, and blood-urea-nitrogen. g. Tumour imaging evaluations through CT scan or MRI were performed at selection, and then every 12 weeks until PD.
h. If thoracic CT scan at selection did not show any chest abnormality, chest X-ray imaging were performed in subsequent evaluations. A CT scan was performed to confirm any abnormality identified upon chest X-ray imaging performed after selection, and all subsequent evaluations were thoracic CT imaging.
i. Response evaluation was conducted according to the revised RECIST 1.1 criteria, every 12±1 weeks until PD. Subject follow-up was performed regardless of discontinuation or a delay in treatment. j. ECOG PS evaluation (on day 1 of each treatment cycle).
k. Tumour evaluation and serum CEA test was performed every 12 weeks and at the follow-up visit only in subjects who have withdrawn from the trial for reasons different from PD, and in those who have not had a serum CEA test in the previous 12 weeks.
l. Paraffin embedded tumour block was sent to the central laboratory (Fundación Jiménez Díaz) at selection. The centralised evaluation laboratory perform a tumour block quality assessment. It was compulsory that KRAS and NRAS mutational status (wtKRAS and NRAS) was confirmed by a validated laboratory before the inclusion of any subject.
m. Follow-up of all subjects who permanently discontinued the treatment before disease progression (e.g. due to unacceptable toxicities) was conducted to evaluate PFS (i.e., tumour imaging) every 12± 1 week until PD or until the end of study (unless the reason for study discontinuation was total consent withdrawal). After PD, all subjects underwent a follow-up assessment of disease status, subsequent cancer therapy, and survival assessment every 12 ± 1weeks until the end of study (maximum of 24 months).
n. Blood samples for biomarker analysis were withdrawn before the study treatment initiation and every 12 weeks ± 1 weeks until PD.

Table 3: SDV schedule

Form	Variables
Eligibility	Pathology report, staging, prior treatment.
Demographics	Date of birth, ECOG PS, ICF (signature, date, and completion)
Primary tumour	Diagnosis date, location, staging and surgery
Adjuvant or neoadjuvant treatment	Yes/No
KRAS/NRAS	Central reviewing
Baseline biochemistry and SAE biochemistry	LDH, creatinine, bilirubin, and CEA
Baseline haematology and SAE haematology	Haemoglobin, platelets, and leukocytes

Metastasis	Nº affected organs and hepatic M1 (number and size)
CT scans or MRI	All variables (date, lesions measure, location, new lesions) and RECIST criteria
AEs	Grade and need of SAE communication
Treatment	Start date, number of cycles, end date and reason of EOT
Progression	Date of progression and reason
Death	Date of death and reason

If all the aforementioned variables were reviewed by the CRA for all patients included in the site, then dose modifications and minor toxicities were also reviewed.

6.5 STATISTICAL METHODS PLANNED IN THE PROTOCOL & DETERMINATION OF SAMPLE SIZE

6.5.1 STATISTICAL AND ANALYTICAL PLANS

6.5.1.1. Primary endpoint

To quantify if the biomarkers BRAF, IGF-1RP/MMP-7 (DP), DP and PIK3CA-PTEN improve the prediction of 12-months progression-free survival (PFS) over the use of just clinical variables in the study population we compared the area under the curve (AUC) of a ROC curve using scores composed by clinical variables with the AUC of scores with the clinical variables plus each of the biomarkers.

PFS was defined as the time from the signature of the ICF date to the radiological progression date or death (whatever occurs first). Subjects who had not shown PD and had not died at database closing were censored on the last date of radiological assessment. Subjects who had concluded study treatment before 48 weeks, due to any cause, underwent follow-up every 12 weeks as described in the protocol. Subjects who had undergone metastatic surgery were not censored on the surgery date but were followed up every 12 weeks until progression was documented. Subjects who received other treatment schemas maintaining the same strategy (chemotherapy + monoclonal antibody) without documented PD, were not censored at the time of the change of treatment. Progression to first line for these patients was considered when applicable according RECIST, independently of treatment received. If by any cause, the baseline CT scan was the only imaging evaluation performed (e.g., subject refusal to stay in the study or lost to follow-up), the case was censored on the inclusion date +1 day.

Two groups with a higher and lower likelihood for tumor progression until 12 months were determined based on the designated classification of clinical variables and following proposed biomarker categories :

1. wt-BRAF vs mutant BRAF

2. wtPI3K and PTEN >3 vs mutant PI3K or PTEN ≤3
3. Non-DP vs DP

6.5.1.2. Secondary endpoints

- Serum and tumor tissue secondary biomarkers evaluated to predict acquired chemo-resistance including BRAF and PI3K.
- ORR was defined as the incidence of a complete or partial response according to the revised RECIST 1.1 criteria.
- DoR (calculated only for the subjects who present an Overall Response): Time from the first assessment of Overall Response to radiologically-confirmed PD according to the revised RECIST 1.1 criteria. For subjects who showed response and did not show progression, DoR was censored on the last date of assessable disease evaluation reported in the eCRF of the study.
- OS: Time from the signature of the ICF date to date of death. For subjects who did not die or were lost to follow-up on the closing date for data analysis, OS was censored on the date of last contact.
- Safety: Type, frequency, and intensity of AEs related to the combination treatment.

6.5.1.3. Analysis population

Efficacy variables were analysed in all subjects who provided their informed consents, were included in the trial and did not present significant protocol deviations throughout the study period.

An intention-to-treat analysis in all patient enrolled into the study was not performed because the primary study objective was to evaluate the predictive value of the three biomarker BRAF, IGF-1RP/MMP-7 (Double-positive Phenotype/DP) and PI3K-PTEN on PFS in wt-RAS CRC patients receiving standard chemotherapy plus bi-weekly cetuximab as first-line therapy, but patients with significant protocol deviations (e.g. RAS mutations, missing biomarker data and those not receiving cetuximab treatment) could not contribute to the investigation of the primary objective.

All patients who received study medication were included in the safety analysis. .

6.5.1.4. Covariate analysis

The following baseline characteristics were studied by biomarkers in orther to properly categorise the sample:

- Gender
- Age
- Primary Tumour location and sideness (ascending colon, transverse colon, descending colon, sigma and rectum)
- Stage of the disease
- Surgery of the primary tumor
- ECOG Performance Status
- Number of metastatic organs
- Specific locations of metastasis including number, size and loctation node, lung, peritoneal and liver (considering also those patients with only resectable liver metastases for comparison) ● Mean values of haemathological tests including leucocytes, haemoglobin, platelets,. ● Baseline LDH levels >450

● Mean ALP and Mean CEA
● Chemotherapy (FOLFOX+Cetuximab /FOLFIRI+Cetuximab)

These groups have been used for univariate and multivariable analysis in order to evaluate efficacy variables such as ORR and PFS. Disease response was clinically assessed by the investigator based on the radiological imaging data collected throughout the study. Any finding considered as non-assessable by the investigator was excluded from the analysis (it was considered as non-existent).

For continuous variables, mean, standard error (for efficacy variables), standard deviation (for other measurements), median, 25th percentile, 75th percentile, minimum and maximum, were evaluated. Categorical variables were presented using frequencies and percentages.

The 95% two-sided confidence intervals (95%CIs) of Kaplan–Meier quartiles for time to event variables were calculated according to Brookmeyer and Crowley methods (Brookmeyer, 1982). The 95% two-sided CIs for the Kaplan–Meier estimates at various time points were calculated according to the methods described by Collett (Collett, 1992).

Safety data were analysed throughout the entire study and presented by descriptive analysis.

6.5.1.5. Analysis of main study variables

The primary efficacy analysis of this trial was performed based on all subjects who provided their informed consents, were included in the trial, and did not present significant protocol deviations throughout the study period. .
Continuous and ordinal variables between independent groups were compared using the Wilcoxon rank-sum test and proportions were compared with Fisher's exact test. Clinical endpoints were ORR, DoR, PFS, and OS. Tumour assessments by CT scans were performed every 3 months until PD. Patients without a second CT scan evaluation were not assessable for ORR.
PFS was defined as the time of the signature of the ICF date until PD or death from any cause. Patients who did not experience PD or death were censored on the date of last examination. For more information regarding censoring see in Section 7.5.1.1. OS was defined as the time from the signature of the ICF date to death from any cause. Patients lost to follow-up or still in the study at database closure have been censored at the date last known to be alive.

Survival analyses including PFS and OS were performed using Kaplan–Meier methods. Cox proportional hazards regression with the Efron method for ties was used to identify prognostic factors for PFS and OS.

Multivariable analysis was performed using subject-matter knowledge and deciding *a priori* the variables to adjust by age (>65 years), sex, tumour site (right vs left), surgery of primary tumour, liver only metastases (<3 nodules and <5 cm vs >3 nodules or >5 cm), ECOG PS, LDH (>450 U/L) and CEA, BRAF mutational status, and type of therapy. This analysis was later adjusted by variables with p-values <0.10 in the

univariate analysis via the automated stepwise selection of variables (p-value for variable entry into the model was 0.2 and p-value to keep the variable in the model was 0.1).. All p-values were two sided.

The proposed sample size ensures establishing a consistent multivariable analysis. Clinical variables and biomarkers were used to build scores intended to predict 12- months PFS as following:

1. A multivariable Cox model was built with those clinical variables that in the univariate analysis had a p-value≤0.15 (PS, chemo regimen, sidedness, resectable liver metastases).
2. A score was established for each patient by adding up the regression coefficients of the multivariable Cox model present in these patients. If for example PS>0 was a negative predictor for PFS (i.e. regression coefficient is negative; e.g. -0.5) and left side tumour was a positive prognostic factor (regression coefficient was positive, e.g. 0.6) then for a patient with PS>0 and left sided tumour the two scores were added (-0.5 + 0.6=0.1). If a patients has PS=0 then the score to be added is 0, as it is the case for right sided tumour in the example.
3. Using a logistic regression model for the outcome 12-months PFS, a ROC curve was computed to choose the score cut point that classifies most patients correctly 4. Patients were dichotomized according to that cut point.
5. Steps 2 to 4 were repeated, adding each of the three biomarkers.
6. Definition of the discriminating power of the 4 scores for prognosis of or 12-months PFS according to these results were compared via ROC curves.
7. Determination of the most suitable score for 12-months PFS according to these results.

The four scores included in the first multivariate model using clinical factors where PS>0, chemotherapy with FOLFIRI and cetuximab, left sided and resectable liver metastases were considered (Score 1). The additional scores considered the four clinical factors above plus the status of each biomarkers (Score 2: BRAF mutation; Score 3: PI3K mutant or PTEN <=3) and Score 4: Double-positive Phenotype (DP).

All analyses were performed using SAS V9.3 (SAS Institute, Cary, NC, USA) statistical software.

6.5.2. DETERMINATION OF SAMPLE SIZE

It was the aim of the study to identify two groups based on the well-established categories of clinical variables and the proposed biomarkers which are prognostic regarding PFS. The sample size should be sufficient detect a clinically relevant difference in PFS with a log-rank test between the two groups, given the following assumptions:

● The difference in PFS at 12 months between the two groups is at least 20% (i.e. 50% vs 30%) corresponding to a hazard ration of 1.79 assuming an exponential distribution. ● Alpha error (two-tailed): 5%
● Beta error: 20%
● Monthly recruitment: 8 patients
● Percentage of patients with classification= 0: 40%

Initially, the number of patients required to be recruited in 24 months was 155 for 115 events to occur. Assuming a 10% proportion of potentially non-assessable patients, a total of 170 patients were needed to be included. After recruiting 157 patients, it was determined that about 70 of these patients had insufficient samples for biomarker analysis preventing their inclusion into the analysis of the primary objective Therefore, in order to observe the 115 events necessary to evaluate the primary endpoint, the total number of patients was increased up to 221 recruited in 47 months (4–5 patients per month). Finally, the total study duration was 70 months (recruitment + follow-up).

7. RESULTS

Table 4 Disposition of patients

Patients enrolled	
Received at least one cycle of study treatment	
Evaluable patients	
End of study (evaluable population)	
Death	
Lost to follow-up	
Censored at database closure	
Study completion (24 months of follow-up after PD)	

Table 5 Protocol deviations

Deviation type	*Total*
Informed consent form	
Efficacy criteria	
Eligibility criteria	
Incorrect dosing regimen	
Other	
Safety	

Table 6. Baseline characteristics by biomarker

	wtBRAF (n=161)	Mutant BRAF (n=20)	p-value*	wtPI3K and PTEN >3 (n=69)	Mutant PI3K or PTEN ≤3 (n=98)	p value*	Non-DP (n=158)	DP (n=23)	p value*
Female									
Mean age (SD)									
Primary location									
Ascending colon									
Transverse colon									
Descending colon									
Sigmoid colon									
Rectum									
Stage									
I									
II									
III									
IV									
Surgery of the primary tumour									
Performance status									
0									
1									
2									
Number of metastatic organs									
0									
1									
2									
3									
4+									
Liver metastasis									
0									
≤3, ≤5 cm									
>3 or >5 cm									
Node metastases[1]									
Lung metastases[1]									
Peritoneal metastases[1]									

Patients with only resectable liver metastases[2]									
Chemotherapy									
FOLFOX+Cetuximab									
FOLFIRI+Cetuximab									
Mean Leucocytes (SD)									
Mean Haemoglobin (SD)									
Mean Platelets (SD)									
Mean ALP (SD)									
LDH >450									
Mean CEA (SD)									

[1] Fisher's exact test for categorical variables and Wilcoxon rank-sum test for ordinal or continuous data

[2] Missing in one patient The patient does not have metastasis in any other location, and those in the liver are ≤3 in number and ≤5 cm in size·

Table 7. MEASUREMENTS OF TREATMENT COMPLIANCE

	Total (n=)
Treatment disposition	
Received at least one injection (safety population)	
Efficacy population	
Received at least 12 cycles of cetuximab	
Received at least 12 cycles of chemotherapy+cetuximab	
Received less than 12 cycles of cetuximab	
Received 3 or less administrations of combination	
FOLFOX+cetuximab	
FOLFIRI+cetuximab	
End of Treatment (EOT)	
Reasons for end of treatment	
PD	
PI decision (24 due to surgery, 2 radiotherapy, 2 complete response, 49 others)	

Patient decision	
Toxicity (related to treatment)	
AE (unrelated to treatment)	
No data of EOT available	

Table 8: Patients excluded from the efficacy or safety analysis

Pat. #	Hospital	Reason for exclusion from efficacy analysis	Valid for safety

Table 9. Response Rates (efficacy population) based on BRAF status

Best Response	N= all patients (%)	N= wt-BRAF (%)	N= mutant-BRAF (%)
Complete response (CR)			
Partial response (PR)			
Stable disease (SD)			
Disease Progression (PD)			
Not evaluable (NE)			
Overall response			
Disease Control Rate			

Figure 1. Progression free-survival

Progression free survival (PFS) based on the analysis of X patients showed a median of X (95% CI: X - X) months.

Figure 2. Overall survival

When OS was evaluated, an estimated median of X (95% CI: X - X months was reported.

The median OS of wt-BRAF patients was X (95% CI: X - X) months and for that of mutant BRAF patients was X (95% CI: X - X months (adjusted HR:X, CI 95% X– X; p-value=X).

Table 10. General toxicity profile (AEs+SAEs+SUSARs)

Adverse event	Any grade	Grade 3	Grade 4	Grade 5
	N (%)	N (%)	N (%)	N (%)

Table 12. Toxicity profile post treatment beginning (AEs+SAEs+SUSARs)

Adverse event	Any grade	Grade 3	Grade 4	Grade 5
	N (%)	N (%)	N (%)	N (%)

Table 13. Toxicity profile post treatment beginning related to treatment (AEs+SAEs+SUSARs)

Adverse event	Any grade	Grade 3	Grade 4	Grade 5
	N (%)	N (%)	N (%)	N (%)

Table 14. Toxicity profile post treatment beginning related to treatment with Cetuximab (AEs+SAEs+SUSARs)

Adverse event	Any grade	Grade 3	Grade 4	Grade 5
	N (%)	N (%)	N (%)	N (%)