A study protocol for

FOLLOWING THERAPY RESPONSE THROUGH LIQUID BIOPSY IN METASTATIC COLORECTAL CANCER PATIENTS

Short title: FOLICOLOR trial

Version: 5.0 – 25-Nov-2024

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Protocol signature page

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The undersigned confirm that the following protocol has been agreed and accepted and that the Principal Investigator agrees to conduct the trial in compliance with the approved protocol and will adhere to the principles outlined in the requirements for the conduct of clinical trials in the EU as provided for in 'Directive 2001/20/EC", and any subsequent amendments, GCP guidelines, the Belgian law of May 7th 2004 regarding experiments on the human person, the Sponsor§ SOPs, and other regulatory requirements as amended. I agree to ensure that the confidential information contained in this document will not be used for any purpose other than the evaluation or conduct of the clinical investigation without the prior written consent of the Sponsor.

Name PI	Site	Signature	Date (dd/mm/yyyy)

Protocol synopsis	
Study title	FOLLOWING THERAPY RESPONSE THROUGH LIQUID BIOPSY IN METASTATIC COLORECTAL CANCER PATIENTS
Type study	Randomized, open-label, multicentric phase 3 trial
Indication	Patients with unresectable, metastatic colorectal cancer receiving first line treatment
Hypotheses	Patients who are followed up with Liquid Biopsies will have a slower decline in their quality of life than patients who are followed up using current standard follow-up techniques (CT scan/tumor marker). This implicates a longer time to deterioration (TTD) in the liquid biopsy arm, compared to the control arm. Secondary hypotheses: progressive disease will be detected earlier based on the results of liquid biopsies than based on conventional CT scans (with RECIST measurements) in 20% of patients.
Study design	Prospective, randomized, open-label, multicentric phase 3 study to evaluate the value of ctDNA in follow-up of patients treated with first line therapy for metastatic colorectal cancer. Inclusion is possible after histologically or cytologically proven colorectal adenocarcinoma with at least one measurable (metastatic) lesion at the start of first line treatment. After proven presence of methylated DNA on a first liquid biopsy sample, patients will be randomized in two study arms: • ARM 1 (control arm): Treatment decision guided by radiographic evaluation, further called the CT arm. • ARM 2 (intervention arm): Treatment decision guided by liquid biopsies, further called the LB arm. Patients will be followed by study protocol for a total of 18 months after inclusion.
Study objectives	The primary objective of this study is to determine whether the technique of monitoring patients with liquid biopsies can ensure that patients experience a slower decline in their quality of life (and can therefore maintain a good quality of life for longer). Secondary Objectives are the following: • The proportion of patients in which progressive disease can be detected earlier based on the results of liquid biopsies in comparison to conventional CT scans (with RECIST 1.1 measurements). • To evaluate time to progression and progression-free survival (PFS) in the LB-arm and the CT-arm (progression is defined as PD according to RECIST). • To evaluate the 3-year overall survival difference between both study arms. • To compare the long-term longitudinal QoL through long-term follow-up, using the same EORTC QLQ-C30 questionnaire in addition to the EORTC QLQ-CR29 to better understand the long-term trends in differences in QoL between both study arms. Exploratory Objectives will include, but will not be limited to the following: • To investigate the overall survival in both study arms. • To explore epigenetic methylation alterations in ctDNA. • To compare the use of ctDNA and CEA to predict progression. • To study the association between ctDNA levels during follow-up and progression free survival using a joint modelling approach.
Included treatment	First line therapy for metastatic CRC patients
Included dosing regimen	Chemotherapeutic agents will be given as an intravenous infusion at a dose and interval consistent with standard institutional practice.
Study population	Metastatic colorectal cancer patients starting first line treatment
Main inclusion criteria	Key Inclusion Criteria (see 5.1 for complete list of inclusion criteria): • Man or woman ≥ 18 years of age at the time the informed consent is obtained • ECOG performance status of 0-2 • Histologically or cytologically confirmed adenocarcinoma of the colon or rectum in patients with unresectable metastatic (M1) disease • At least 1 uni-dimensionally measurable lesion of at least 10 mm using conventional techniques (CT scan). Lesion must not be chosen from a previously irradiated field, unless there has been documented disease progression in that field after irradiation and prior to randomization. All sites of disease must be evaluated preferably 28 days prior to randomization but can be extended up to 90 days prior to randomization. • Adequate blood results for treatment (at treating physician's discretion) • Starting a first line treatment
Main exclusion criteria	Key Exclusion Criteria (see 5.2 for complete list of exclusion criteria): • History of prior or concurrent central nervous system metastases • History of other malignancy, except: • Malignancy treated with curative intent and with no known active disease present for ≥ 2 years prior to randomization and felt to be at low risk for recurrence by the treating physician • Adequately treated non-melanomatous skin cancer or lentigo maligna without evidence of disease • Adequately treated cervical carcinoma in situ without evidence of disease Prostatic intraepithelial neoplasia without evidence of prostate cancer

	• Prior chemotherapy or other systemic anticancer therapy for the treatment of metastatic colorectal carcinoma including but not limited to bevacizumab and anti-EGFR therapy (e.g. cetuximab, panitumumab, erlotinib, gefitinib, lapatinib) • Prior adjuvant chemotherapy (including oxaliplatin therapy) or other adjuvant systemic anticancer therapy including but not limited to bevacizumab and anti-EGFR therapy (e.g. cetuximab, panitumumab, erlotinib, gefitinib, lapatinib) for the treatment of colorectal cancer ≤ 6 months prior to randomization with the following exceptions: • Patients may have received prior fluoropyrimidine therapy if administered solely for the purpose of radio sensitization for the adjuvant or neoadjuvant treatment of rectal cancer • Radiotherapy ≤ 14 days prior to randomization. Patients must have recovered from all radiotherapy-related toxicities.
Total sample size	Sample Size: The calculated sample size is 150 patients to be randomized in a 1:1 ratio between CT arm and LB arm.
Planned study visits	No study visits will be planned. All visits will be standard of care.
Safety parameters	Clinical and laboratory events related to study (see specifications later) will be reported according to the National Cancer Institute – Common Terminology Criteria for Adverse Events (NCI-CTCAE version 5.0).
Efficacy parameters	The level of ctDNA will be assessed through NPY methylation. For patients randomized in CT-arm: progression-free survival will be assessed using standard imaging (CT scan) based on RECIST criteria 1.1.
Laboratory parameters and biomarkers	CtDNA levels and standard laboratory parameters. Additional laboratory assessments as deemed necessary by the treating physician.
Imaging parameters	Computed tomography (CT) Thorax/abdomen OR MRI
Statistics	This study aims to compare the time to deterioration (TTD) in quality of life (QoL) between patients monitored via liquid biopsies (LB-arm) versus conventional follow-up methods (CT-arm). The primary endpoint will be met if the LB-arm shows a longer TTD than the CT-arm, based on a sample size of 150 patients. Secondary analyses will assess early detection of progressive disease through liquid biopsies, progression-free survival (PFS), overall survival (OS), QoL differences, and adverse event profiles. Exploratory analyses will focus on biomarkers like ctDNA and CEA, their predictive value for progression, and correlations with PFS.

Abbreviation	Definition/explanation
UZA	Antwerp University Hospital
AE CEA	Adverse event Carcino embryonal antigeen
cfDNA	Cell free DNA
CMG	Centre of Medical Genetics
CNS	Central nervous system
CpG	Cytosin-phosphatidyc-Guanin
CR	Complete response
CRC	Colorectal cancer
CSR	Clinical study report
CTC	Common terminology criteria
CTCAE	Common terminology criteria for adverse events
ctDNA	Circulating tumor DNA
ddPCR	Digital droplet PCR
DICOM	Digital Imaging and Communications in Medicine
DMC	Data monitoring committee
DNA	Deoxyribonucleic acid
ECOG	Eastern cooperative oncology group
eCRF	Electronic case report form
EGFR	Epidermal growth factor receptor
EORTC	European Organisation for Research and Treatment of Cancer
GDP	Guanosine 5'-diphosphate
ICF	Informed consent form
ICH	International conference on harmonisation
ID	Identification number
LB	Liquid biopsy
MAP	Mitogen-activated protein kinase
MRI	Magnetic resonance imaging
mCRC	Metastatic colorectal cancer
MSI	Microsatellite instability
NCI-CTCAE	National cancer institute – common terminology criteria for adverse events
PACS	Picture Archiving and Communication System
PD	Progressive disease
PFS	Progression-free survival
PR	Partial response
QoL	Quality of life
RAF	Rapidly accelerated fibrosarcoma
RECIST	Response evaluation criteria in solid tumors
SAE	Serious adverse event
SD	Stable disease
TGF-α	Transforming growth factor alpha
TCGA	The Cancer Genome Atlas
TMG	Trial management group
TSC	Trial steering committee
TTD	Time to deterioration
USA	United States of America
VPN	Virtual private network

1. BACKGROUND AND RATIONALE

1.1. Disease and treatment

Colorectal cancer (CRC) is the third most common cancer in both men and women, comprising approximately 10% of the 766 860 new cancers in men and 11% of the 678 060 new cancers in women worldwide[1]. In 2007, an estimated 1 million deaths were projected to occur in the United States of America (USA) and Europe from colon and rectal cancer[1-2]. Of newly diagnosed patients, 15% to 25% have metastatic disease at diagnosis and up to 50% of all patients eventually develop metastatic disease[3-4].

Over the past 20 years, substantial advances have been made in the treatment of patients with metastatic colorectal cancer (mCRC) and now a wide range of options for the front-line treatment of mCRC are available. Currently, the use of combination cytotoxic therapy including 5-FU, LV, and irinotecan (FOLFIRI), 5-FU, LV, and oxaliplatin (FOLFOX), capecitabine and oxaliplatin (XELOX), or 5-FU, LV, oxaliplatin, and irinotecan (FOLFOXIRI) are typically used in the first-line setting. The addition of targeted agents against angiogenesis, in particular the vascular endothelial growth factor A (VEGF-A) inhibitor bevacizumab, to combination chemotherapy has afforded improved outcomes in mCRC. Moreover, with the availability of DNA sequencing technology, we are now able to identify novel biomarkers that are predictive for the patients benefit from other targeted agents and immune therapy. Hence, genomic testing should be performed at the time of diagnosis of metastatic disease. While no predictive biomarkers have been established for anti-VEGF (Vascular Endothelial Growth Factor) therapy, patients without alterations in KRAS, NRAS, and BRAF genes, can be treated with anti-EGFR (Epidermal Growth Factor Receptor) therapy and for patients with microsatellite unstable/mismatch repair defective tumors treatment with the immune checkpoint inhibitors (ICI) nivolumab or pembrolizumab should be considered[5].

Effective first-line therapy is therefore crucial to obtain disease control, especially for patients who may become candidates for metastatic resection. The choice of first-line therapy is mostly based on clinical factors such as the extent of disease, performance status of the patient and the primary tumor location as well as on the presence of certain molecular markers as mentioned before.

1.2. Circulating tumor DNA

During the last few years, a lot of research has been performed on liquid biopsies[6]. Different studies have described the possibility to detect tumor mutations in plasma samples of CRC patients[7-9]. This approach of detecting genomic alterations has in the meantime reached diagnostic and prognostic value in CRC. Implementation of mutation analysis (RAS, BRAF, MSI,…) in clinical practice enables personalized treatment in CRC[7].

In contrast to genetic mutations, also epigenetic changes such as DNA hypermethylation of neuropeptide Y protein (NPY), have been described in CRC[10-12]. Picoliter digital droplet PCR (ddPCR) methylation assays allows to measure NPY methylation to determine the amount of circulating tumor DNA (ctDNA) in plasma samples. In fact, NPY methylation is a surrogate marker for tumor burden in CRC patients. The main advantage of methylation assays is the applicability in almost every patient, while the use of mutation assays requires prior knowledge of mutations present in the tumor.

1.3. Exploration of epigenetic methylation alterations in ctDNA

Aberrant methylation of specific promoter regions can be a very consistent feature of cancer, in contrast to mutations, which typically occurs at a wide range of sites. Methylated ctDNA in blood is valuable in cancer prognosis and tumor response. It is important to make liquid biopsy assays more sensitive in the future to make ctDNA methylation amenable to the design of widely applicable clinical assays. To this end, additional exploration of DNA methylation in liquid biopsies is warranted.

1.4. Rationale

Many advances in systemic therapies have significantly improved the survival of patients with CRC[13]. However, there is a high variability of therapeutic responses among patients and determining the optimal personalized treatment plan is challenging.

Currently, the treatment effectiveness is generally assessed every 8 weeks at which time a decision is made to (dis)continue the current therapy. This is done using RECIST 1.1 measurements[14] from CT scans (current gold standard). However, this technique is suboptimal as it often leads to late detection of progressive disease (PD). This presents challenges for patient care, especially considering mCRC's poorer prognosis compared to other common cancer types[15]:

(1) Late detection of PD delays the decision to replace the current line with a new line of treatment. Subsequently, the ideal therapeutic window for the new line of treatment may be missed, thus impacting patient (survival) outcomes.

(2) Similar to the impact of late PD detection on survival, missing the therapeutic window for the next line of therapy can lead to prolonged exposure of the current (ineffective) therapy, resulting in

unwanted toxicities. Early detection of PD would enable timely treatment adjustments, hence reducing the risk of toxicities. Furthermore, delayed treatment adjustment can result in scenarios where patients start the new line of therapy at a worse baseline performance status (due to prolonged exposure to the current treatment, and due to the PD itself), thus making them more vulnerable to unwanted toxicities of this new line of treatment.

(3) The two previously described disadvantages can lead to worse QoL. This can be due to direct causal effects on the physical and psychological state of the patients (i.e., confronting a bad prognosis, and experiencing the unwanted toxicities), or indirect side effects like experiencing uncertainty while awaiting the results of the CT imaging, as well as waiting two months between every evaluation. All of the previously described variables lead to worse QoL.

Given the previously outlined drawbacks of delayed PD detection using current techniques, it becomes evident that a prompt identification of PD is an imperative need, even within the context of metastatic and non-curative scenarios. Emerging biomarkers like circulating tumor DNA (ctDNA) have displayed promising potential to mitigate the limitations of traditional techniques like CT imaging. These biomarkers are obtained through liquid biopsies (LBs), which operate on the principle of sampling bodily fluids (most commonly plasma) harboring ctDNA that originates directly from the tumor. The analysis of ctDNA can yield crucial insights into tumor characteristics in a minimal invasive manner. Multiple studies have shown that ctDNA analysis has utility in prognostication, therapy selection, response prediction, and follow-up[16-17]. This robust evidence spans various cancer types, establishing that the application of LBs holds great potential to improve various aspects of care for cancer patients[18]. In mCRC, pilot studies, including our research, indicate that follow-up of patients with NPY methylation in ctDNA can detect PD earlier than conventional techniques[11,19].

One such pilot study from our research group, was the lead-in study related to the current Randomized FOLICOLOR trial, where determination of the optimal cut-off value of NPY methylation for PD detection was the primary aim (NCT04735900, manuscript in preparation). This NPY methylation-based ctDNA analysis is a digital droplet PCR (ddPCR)[19] based technique where the methylation ratio of the ctDNA is determined through calculating the proportion of NPY-positive droplets compared to albumin (ALB)-positive droplets (= reference marker). This methylation ratio (plotted between 0-100%) acts as a surrogate of the level of ctDNA in the liquid biopsy, and is linearly correlated to the tumor load[11]. Hence, by measuring the methylation ratio via this technique, valuable information can be obtained on the tumor activity. In this study 16 patients were included and followed by NPY methylation based ctDNA analysis until PD. Preliminary results show that in 8 out of 13 patients with PD during study, PD could be detected earlier through LBs than CT scan with a median lead-time of 54 days (range 16-137) (presented at the EACR Conference on Liquid Biopsies 2022 in Bergamo, Italy). A receiver operating characteristic (ROC)-curve was fitted based on the combined data of the lead-in study and other studies in our center that

studied NPY methylation. Based on this ROC-curve, the optimal threshold for detection of PD was determined as an increase of 5% of NPY methylation ratio, leading to a sensitivity of 90% and specificity of 100%. Additionally, this robust technique with a validated laboratory workflow can efficiently process large number of samples with a short turnaround time. Based on this, the validated LB assay is being studied in the current Randomized FOLICOLOR trial (randomized phase II study) for follow-up of mCRC patients to determine the clinically meaningful benefit of this approach.

Hence, the application of this promising technique (NPY methylation-based ctDNA analysis) can help to detect PD earlier compared to the conventional techniques. Earlier PD detection will lead to earlier identification of patients who do not respond to the current line of therapy, prompting the treating oncologists to switch to the next line of therapy sooner in a shared decision making process along with the patients. This prevents unnecessary toxicities from the current therapy and allows the start of a new line of therapy in a more suitable therapeutic window leading to enhanced treatment efficacy, potentially improving OS and QoL. Frequent minimal invasive therapy-monitoring with LBs empowers patients as they are more aware of their disease's evolution, which can lead to true joint decision-making on therapy plans.

Despite a wealth of scientific evidence supporting liquid biopsies in mCRC[20-21], there is limited published data on (1) LBs' clinical impact on patients (e.g., QoL, toxicity), (2) patients' experiences and opinions on LBs, (3) patient knowledge about LBs, and (4) how to implement LBs in clinical practice. This knowledge gap hampers the implementation of LBs in daily practice. Therefore, the randomized FOLICOLOR trial aims to address these gaps, ensuring optimal adoption of this innovative technique for the benefit of patients, physicians, and stakeholders.

2. OBJECTIVES

This is a prospective, randomized, open-label, multicentric, phase II trial to evaluate the value of ctDNA (NPY methylation-based ctDNA analysis) in follow-up of patients treated with first-line therapy for metastatic colorectal cancer.

The overall aim of this trial is to evaluate the clinically meaningful benefit of the follow-up strategy with guiding treatment based on NPY methylation-based ctDNA analysis (see paragraph 6.1) of patients with mCRC. The clinically meaningful benefit can be obtained through improved QoL (i.e. longer TTD in the liquid biopsy arm, compared to the control arm), and several important secondary and exploratory endpoints (see below) including long-term follow-up QoL, prolonged survival (PFS and OS), and reduced toxicity.

To achieve this aim, 150 patients with mCRC starting first-line therapy from 9 centers will be randomized (1:1 ratio) in one of the two study arms:

- Control arm: Patients receive SOC follow-up with CT scans and CEA every 8 weeks. When PD on CT-scan is detected, the therapy will be adapted to second-line treatment per investigator's choice. In this arm, LB samples for NPY methylation-based ctDNA analysis will be collected, but the results will not be used for treatment decisions.
- Study arm: Patients receive follow-up based on monthly LB collection for NPY methylation-based ctDNA analysis. When PD is detected before the current SOC follow-up techniques indicate PD, the therapy will be adapted to second-line treatment per investigator's choice. In this arm, patients will also receive 8-weekly CEA measurements and CT scans to ensure they also benefit from SOC.

2.1. Primary objective

Health-related quality of life is increasingly recognized as an important endpoint in cancer clinical trials, especially in patients in advanced settings. This is also endorsed by the European Society for Medical Oncology (ESMO) through the ESMO-Magnitude of Clinical Benefit Scale[22]. Therefore, the primary objective of the randomized FOLICOLOR trial is to assess the difference in time to deterioration (TTD) in QoL between patients in which follow-up is done based on the results of LBs (LB-arm) in comparison to the patients in which follow-up is done based on the conventional follow-up techniques (CT-arm). TTD is defined as time from randomization to the first decrease from baseline on the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life questionnaire (QLQ-C30) summary score by at least 10 percent. The decline should be a sustained decline in the subsequent measurements. When evaluating changes in EORTC scores for individuals over the course of therapy, a 10% threshold is most often applied in TTD-analyses and is used to assist regulators and health authorities interpret the meaningfulness of within-patient score changes. The TTD of QoL as primary endpoint is a patient-centered outcome and an important parameter for clinical management as well as policy makers.

2.2. Secondary objective

The secondary objectives are the following:

- To determine the proportion of patients (%) in which progressive disease can be detected earlier based on the results of liquid biopsies compared to conventional CT scans.
- To evaluate time to progression and PFS in the LB-arm and the CT-arm (progression is defined as PD on cross-sectional imaging (CT or MRI scan) according to RECIST 1.1).
- To evaluate the 3-year OS difference between both study arms.
- To compare the long-term longitudinal QoL through long-term follow-up, using the same EORTC QLQ-C30 questionnaire in addition to the EORTC QLQ-CR29[23] to better understand the long-term trends in differences in QoL between both study arms.

2.3. Exploratory Objectives

The exploratory objectives will include, but will not be limited to the following:

- To investigate the long-term OS in both study arms.
- To explore and compare the use of ctDNA and CEA to predict progression.
- To explore epigenetic methylation alterations in ctDNA.
- To study the association between ctDNA levels during follow-up (= multiple points in time) and PFS (time-to-event) using a joint modelling approach.

3. STUDY ENDPOINTS

3.1. Primary endpoint

Difference in time to deterioration (TTD) in QoL, measured by the QLQ-C30 questionnaire, between patients in which follow-up, which is done based on the results of LBs (LB-arm) in comparison to the patients in which follow-up is done based on the conventional follow-up techniques (CT-arm). As stated in the objectives, the TTD is defined as time from randomization to the first decrease from baseline QLQ-C30 summary score by at least 10%. This decline should be sustained (i.e. stay lower by 10% or more) in the subsequent measurements.

3.2. Secondary endpoint

- The proportion of patients (%) in which progressive disease can be detected earlier based on the results of liquid biopsies compared to conventional cross-sectional imaging (CT or MRI scan). Progressive disease based on liquid biopsy results is defined as an increase in NPY methylation ratio of at least 5% (compared to the previous evaluation sample) and progressive disease based on cross-sectional imaging (CT or MRI scan) is defined based on RECIST 1.1.
- Progression free survival/time to progression: time from randomization to the date of first disease progression (on LB and/or cross-sectional imaging (CT or MRI scan), or death within 60 days after last evaluable tumor assessment or randomization date (whichever is later). Patients not meeting the criteria by the cutoff date are censored at the last evaluable tumor assessment date.
- Overall survival at 3 years: time from randomization date to date of death. For patients who have not died or are lost to follow-up 3 years post randomization, OS will be censored at their last known follow-up date.
- Long term overall survival: time from randomization date to date of death. Patients will be followed for survival until death or until lost to follow-up. For patients lost to follow-up OS will be censored at their last contact date.
- Difference in long-term longitudinal QoL using the EORTC QLQ-C30 and QLQ-C29 between the two arms.
- Incidence of GRADE 3-4therapy related adverse events, measured by CTCAE v5.0, to correlate with TTD as covariate.
- To explore the patient experience in both study arms with regard to the use of liquid biopsies for follow-up through questionnaires.

3.3. Exploratory endpoints

- Further exploration of DNA methylation in liquid biopsies and looking for novel biomarkers. This includes but is not limited to the exploration of epigenetic methylation alterations in ctDNA which will be performed using the newly developed IMPRESS technique (Improved Methylation Profiling using Restriction Enzymes and smMIP sequencing)[24].

- Comparison of the ctDNA measurements and CEA measurements at same timepoints to compare progression predictive value of both biomarkers.
- The association between ctDNA levels during follow-up and progression free survival using a joint modelling approach[25].

4. STUDY CENTERS

4.1. Number of centers

This study is a multicenter trial of which all mCRC patients will be recruited at approximately 9 centers in Belgium, including Antwerp University Hospital.

5. PATIENT ELIGIBILITY (INCLUSION CRITERIA)

Investigators will be expected to maintain a screening log of all potential study candidates that includes limited information about the potential candidate (age, sex), date, and outcome of the screening process (e.g., enrolled into study, reason for ineligibility, or refused to participate).

Before any study-specific procedure, the appropriate written informed consent must be obtained BASELINE (see study flowchart)).

5.1. Inclusion Criteria

5.1.1. Disease related

- Patients should have histologically or cytologically confirmed adenocarcinoma of the colon or rectum in patients with unresectable metastatic (M1) disease.
- A Next Generation Sequencing (NGS) should be done (or requested) as per standard of care. It is also acceptable to only detect RAS and BRAF mutation(s).
- There should be indication to start any kind of first-line systemic treatment for metastatic colorectal cancer.
- There should be at least 1 uni-dimensionally measurable (min. 10mm) using conventional cross-sectional imaging techniques (CT or MRI scan). Lesions must not be chosen from a previously irradiated field, unless there has been documented disease progression in that field after irradiation and prior to randomization. All disease must be evaluated preferably 28 days prior to randomization with a maximum of 90 days prior to randomization.
- There should be the presence of min. 2% of ctDNA (measured by the *NPY*-ratio) in total cfDNA on liquid biopsy, tested by the Center for Medical Genetics (CMG) during screening phase.
- The Eastern Cooperative Oncology Group (ECOG) performance status should be 0-2.

Note 1: Throughout this entire protocol, "CT scan" may be substituted for "MRI scan" in cases where CT scans are contraindicated.

5.1.2. Demographic

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

5.1.3. Laboratory

- Patients should have adequate blood results for treatment (at treating physician's discretion).

5.2. Exclusion Criteria

5.2.1. Disease Related

- Patients with a history of prior or concurrent central nervous system (CNS) metastases
- Patients with a history of other malignancy, except:
  - Malignancy treated with curative intent and with no known active disease present for ≥ 2 years prior to randomization and felt to be at low risk for recurrence by the treating physician
  - Adequately treated non-melanomatous skin cancer or lentigo maligna without evidence of disease
  - Adequately treated cervical carcinoma in situ without evidence of disease
  - Prostatic intraepithelial neoplasia without evidence of prostate cancer
- Patients with prior cancer Therapy:
  - Prior chemotherapy or other systemic anticancer therapy for the treatment of metastatic colorectal carcinoma including but not limited to bevacizumab and anti- EGFR therapy (e.g. cetuximab, panitumumab, erlotinib, gefitinib, lapatinib)
  - Prior adjuvant chemotherapy (including oxaliplatin therapy) or other adjuvant systemic anticancer therapy including but not limited to bevacizumab and anti- EGFR therapy (e.g. cetuximab, panitumumab, erlotinib, gefitinib, lapatinib) for the treatment of colorectal cancer ≤ 6 months prior to randomization with the following exceptions:
    - Patients may have received prior fluoropyrimidine therapy if administered solely for the purpose of radio-sensitization for the adjuvant or neoadjuvant treatment of rectal cancer
- Patients with radiotherapy ≤ 14 days prior to randomization. However, patients can be included if they have recovered from all radiotherapy-related toxicities.
- Patients with unresolved toxicities from prior anti-cancer therapy that, in the opinion of the investigator, excludes patient from participation.

5.2.2. General

- Patients with any significant comorbidity that in the opinion of the investigator, may increase the risks associated with study participation or treatment administration or may interfere with the conduct of the study or interpretation of study results.
- Patients who are currently enrolled in, or ≤ 30 days has passed since patient completed another investigational device or drug study(s), or patient is receiving other investigational agent(s).
- Women of child-bearing potential who is evidently pregnant (e.g. positive HCG test) or is breast feeding.

- Fertile men and women 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. Adequate contraceptive precautions include double barrier contraceptive methods (e.g. diaphragm and condom) or abstinence.
- Patients with a 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 treatment administration or may interfere with the conduct of the study or interpretation of study results
- Patients unwilling or unable to comply with study requirements (e.g. will not be available for follow-up assessment)
- Patients with any kind of disorder that compromises the ability of the patient to give written informed consent and/or to comply with study procedures (except for patients with a legally acceptable representative)

6. STUDY DESIGN

6.1. Screening period

Eligible patients are asked to sign the informed consent form (ICF). All patients who signed the informed consent form will enter into the screening period and will be assigned a unique study identification number (ID). This ID will be used to identify the patient throughout the entire clinical study.

After providing consent, a baseline liquid biopsy sample will be collected (**LB_B**) before the start of the systemic therapy (i.e. in the screening phase). This LB_B will be analyzed at the CMG (Center of Medical Genetics) to verify the presence of a measurable level (≥2%) of NPY methylation in the ctDNA.

The result of this analysis will be communicated to the investigator:

- If the analysis of LB_B shows <2% of NPY methylation in the ctDNA, the patient is not eligible for inclusion in the trial, and will be classified as screen-failure.
- If the analysis of LB_B shows ≥2% of NPY methylation in the ctDNA, the patient is eligible for inclusion (see further steps from 6.2 onwards).

In addition, after providing consent, a sample of tumor tissue (formalin-fixed paraffin-embedded for 10 sections of 6µm) can be requested. DNA extracted from the tumor tissue will then be tested for the presence of NPY methylation (tumor specific marker). Tumor material will only be provided by the local center in case enough material remains for future clinical analyses for the patient (e.g. additional histological or molecular analyses for further lines of therapy).

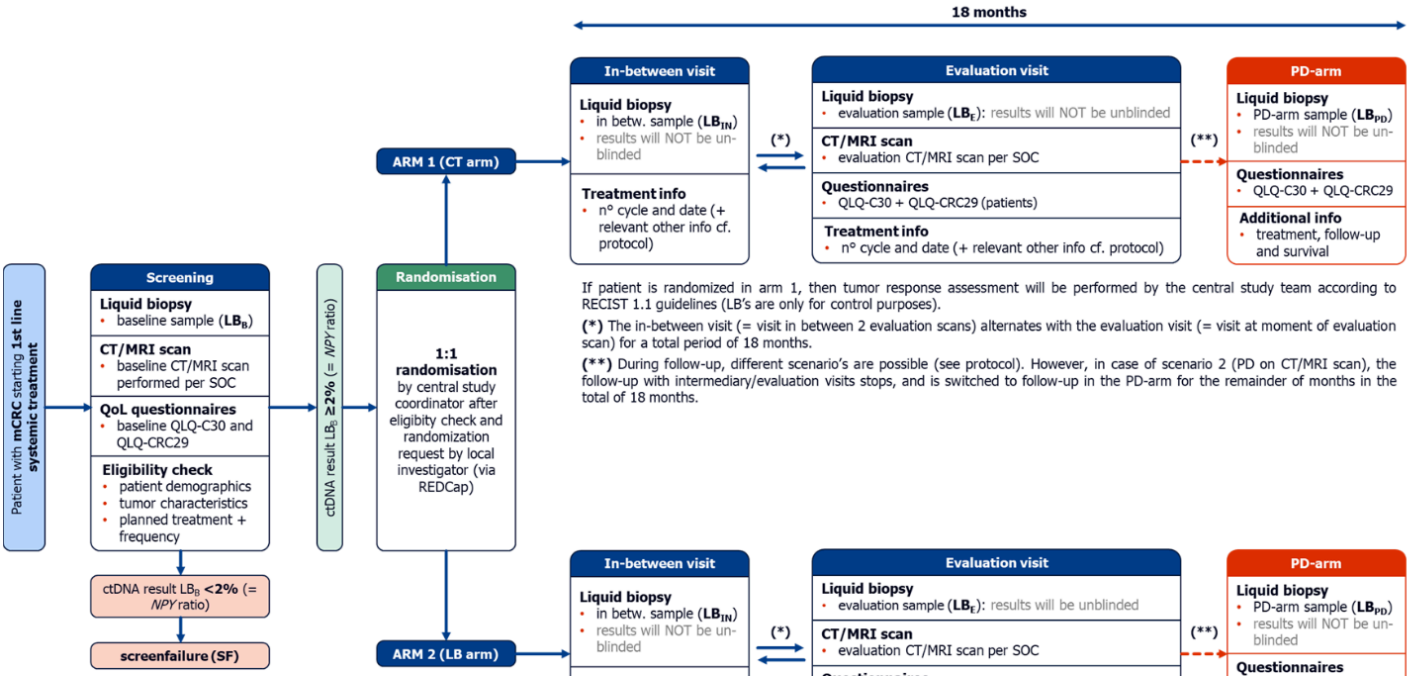
6.2. Randomization

Patients who meet all eligibility criteria during the screening period may be randomized. Patients that are determined not eligible after screening will be listed as screen failed together with the reason for screen failure.

Patients are randomized by the central study coordinator after the ctDNA result of the LB_B sample is known (and is ≥2%) and after the receipt of the randomization form (filled out by the investigator) in eCRF or send to folicolor@uza.be. Therefore, randomization will take place just after start 1st line therapy, but must be done before the 1st Follow-up. Randomization will be stratified by study site, gender and molecular tumor type (RAS wild-type vs RAS mutant type vs MSI high). Qminim, a web-based online minimization procedure will be used.

Patients will then be randomized in a 1:1 ratio to receive therapy guidance according to CT scans (ARM 1: CT arm (= control arm)), or to receive therapy guidance according to liquid biopsies (ARM 2: LB arm (= intervention arm)).

Figure 1. Flowchart with trial overview



If patient is randomized in arm 1, then tumor response assessment will be performed by the central study team according to RECIST 1.1 guidelines (LB's are only for control purposes).

(*) The in-between visit (= visit in between 2 evaluation scans) alternates with the evaluation visit (= visit at moment of evaluation scan) for a total period of 18 months.

(**) During follow-up, different scenario's are possible (see protocol). However, in case of scenario 2 (PD on CT/MRI scan), the follow-up with intermediary/evaluation visits stops, and is switched to follow-up in the PD-arm for the remainder of months in the total of 18 months.

If patient is randomized in arm 2, tumor response assessment will be performed according to the LB results (during the evaluation visits).

(*) The in-between visit (= visit in between 2 evaluation scans) alternates with the evaluation visit (= visit at moment of evaluation scan) for a total period of 18 months.

(**) During follow-up, different scenario's are possible (see protocol). However, in case of scenario 2 (PD on CT/MRI scan), the follow-up with intermediary/evaluation visits stops, and is switched to follow-up in the PD-arm for the remainder of months in the total of 18 months.

6.2.1. ARM 1 (CT arm/control arm)

If after the screening period (see 6.1), the patient is randomized (see 6.2) in the CT arm, the next steps are as follows. Within 14 days after randomization, first-line systemic treatment is planned as per standard of care (mostly 2 or 4 weekly regimen (other frequencies of regimens per SOC also possible)). Based on type of visit, study interventions are foreseen, as outlined below:

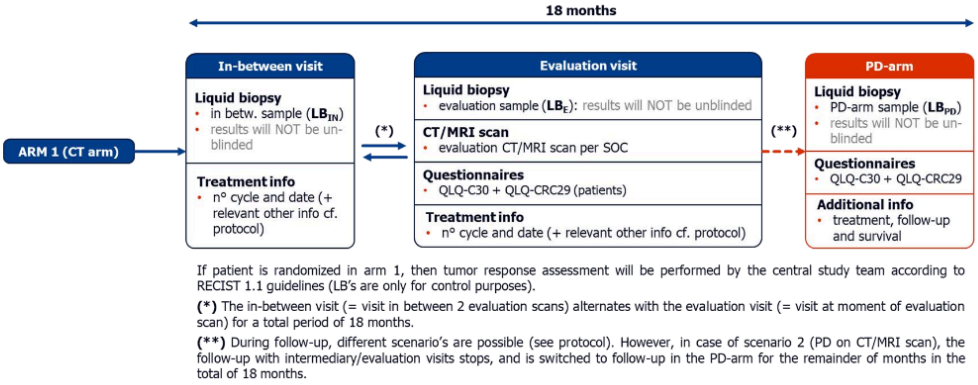


Figure 2. Overview of follow-up in arm 1 (part of figure 1)

Table 2A. Follow-up in CT arm (arm 1 / control arm)

CT	Cross-sectional imaging (CT or MRI scan)	Imaging will be performed as **per standard of care (SOC)** (in most centers after every 2 months (or 8w) of therapy (~ corresponds to 4 cycles of most two-weekly therapy regimens, or 2 cycles of four-weekly therapy regimen (e.g. Teysuno®)). However, if per SOC imaging is performed at a different frequency (e.g. every 12w), then it is also possible.
LB	Liquid biopsies (2 Streck tubes)	LB samples are obtained at the moment of every CT/MRI scan for response evaluation (= LB_E sample) and in between 2 scans (= LB_{IN} sample). This sequence is repeated until total of 18m. The LB sampling needs to be done before administration of new treatment cycle, and in case of LB_E sample additionally in a window of +/- 7 days from the CT/MRI scan.
Q	Questionnaires (QLQ-C30 + QLQ-CRC-29)	Questionnaires will be taken on a 8-weekly basis: preferably in digital format through a secured link provided via REDCap (paper option will still be available if necessary). The frequency of the questionnaires must also be aligned to the CT/MRI scan and the LB sample from that timepoint. Additionally, attempt should be made to complete these questionnaires before the consultation of the patient with the treating physician to avoid any form of bias. However, if this is practically not feasible, it must be done in a window of max. 7 days after the consultation.

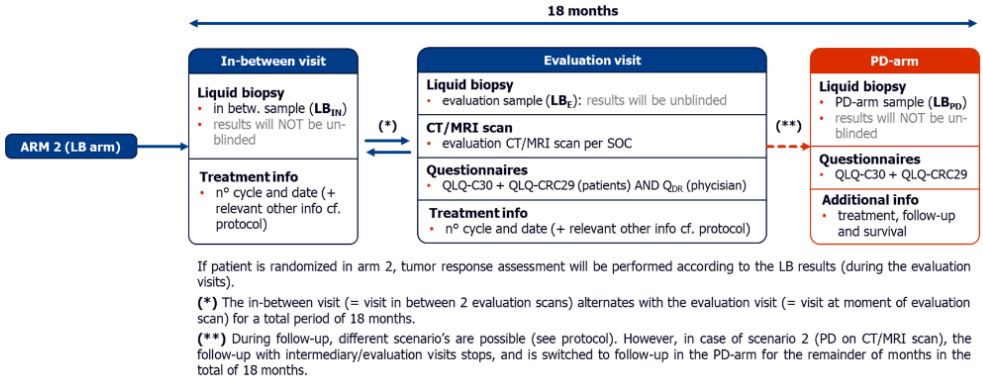
In this arm, tumor response assessment will be performed by the central study team according to RECIST 1.1 guidelines. Although LB samples are obtained and analyzed every 4 weeks, their results will NOT be communicated to the investigator as they are for control arm purposes only. The treatment for patients in the CT arm (= arm 1 or control arm) is guided by cross-sectional imaging (CT or MRI scan) results only. For cross-sectional imaging response handling, see appendix.

The following scenarios are possible:

Table 2B. Scenario's for follow-up in CT arm (arm 1 / control arm)	
In-between visit on figure 1 (= between two CT/MRI scans)	During these visits, the following study procedures are to be performed: • Corresponding **in-between LB sample = LB_{IN}** sample is obtained before start new therapy cycle → results of these LB samples will not be communicated
Evaluation visit on figure 1 (= at moment of CT/MRI scan for response evaluation)	During these visits, the following study procedures are to be performed: • **Cross-sectional imaging (CT/MRI scan)** per SOC → RECIST 1.1 evaluation will be performed • Corresponding **8-weekly QoL questionnaires** → results will not be communicated **Note**: in case of 12-weekly imaging schedule, the QoL questionnaires need to follow 8-week schedule. • Corresponding **evaluation LB-sample = LB_E** → results will not be communicated Scenario's: • **Scenario 1**: CR/PR/SD on CT/MRI scan → continue treatment or initiate therapy break = choice of treating physician based on clinical status of patient (~ standard of care): • **Scenario 1A:** in case of treatment continuation → follow the flowchart to the next month • **Scenario 1B:** in case of treatment break → continue in-between LB_{IN}-sampling (if SOC blood draw is planned) and 8-weekly questionnaire sampling (if feasible) → when the treatment is restarted, follow scenario 1C • **Scenario 1C:** when restarting treatment after a break → restart with evaluation visit where new LB_E sample is taken to determine new baseline methylation value, alongside new QoL questionnaires and imaging (as per SOC) → thereafter continue as per scenario 1A **Note:** subtract weeks/months of treatment break from total of 18 months of follow-up in the study to know remaining months of follow-up in the study. • **Scenario 2:** PD on CT/MRI scan OR clinical progression → stop current treatment → switch follow-up to PD-arm
PD-arm	Upon progression during follow-up (= scenario 2), the follow-up based on scenario 1 will be stopped. The treating physician can start the next line of treatment of choice. Although, patients must still be followed up in the study, but according to the PD-arm. In this arm, 4-weekly **LB_{PD} samples** + 8-weekly **QoL questionnaires** will be collected (if feasible) until the patient has completed a total of 18 months of follow-up in the study. If one of both is not feasible, then partial withdrawal of consent is possible.

6.2.2. ARM 2 (LB arm/intervention arm)

If after the screening period (see 6.1), the patient is randomized (see 6.2) in the LB arm, the next steps are as follows. Within 14 days after randomization, first-line systemic treatment is planned as per standard of care (mostly 2 or 4 weekly regimen (other frequencies of regimens per SOC also possible)). Based on type of visit, study interventions are foreseen, as outlined below:



If patient is randomized in arm 2, tumor response assessment will be performed according to the LB results (during the evaluation visits).

(*) The in-between visit (= visit in between 2 evaluation scans) alternates with the evaluation visit (= visit at moment of evaluation scan) for a total period of 18 months.

(**) During follow-up, different scenario's are possible (see protocol). However, in case of scenario 2 (PD on CT/MRI scan), the follow-up with intermediary/evaluation visits stops, and is switched to follow-up in the PD-arm for the remainder of months in the total of 18 months.

Figure 3. Overview of follow-up in arm 1 (part of figure 1)

Table 3A. Follow-up in LB arm (arm 2 / intervention arm)

CT	Cross-sectional imaging (CT or MRI scan)	Imaging will be performed as **per standard of care (SOC)** (in most centers after every 2 months (or 8w) of therapy (~ corresponds to 4 cycles of most two-weekly therapy regimens, or 2 cycles of four-weekly therapy regimen (e.g. Teysuno®)). However, if per SOC imaging is performed at a different frequency (e.g. every 12w), then it is also possible.
LB	Liquid biopsies	LB samples are obtained at the moment of every CT/MRI scan for response evaluation (= LB_E sample) and in between 2 scans (= LB_{IN} sample). This sequence is repeated until total of 18m. The LB sampling needs to be done before administration of new treatment cycle, and in case of LB_E sample additionally in a window of +/- 7 days from the CT/MRI scan. The evaluation LB_E samples will be analysed in real-time and communicated on time before or on the day of the consultation if feasible, otherwise shortly after the day of consultation.
Q	Questionnaires (QLQ-C30 + QLQ-CRC-29)	Questionnaires will be taken on a 8-weekly basis: preferably in digital format through a secured link provided via REDCap (paper option will still be available if necessary). The frequency of the questionnaires must also be aligned to the CT/MRI scan and the LB sample from that timepoint. Additionally, it must be completed before the consultation of the patient with the treating physician to avoid any form of bias.
Q_{DR}	Decision review questionnaires	Short questionnaires containing decision review related questions will be requested to be filled in by the treating physician at time of LB_E sample to get overview of impact of LB-results in their clinical decision-making.

In this arm, tumor response assessment will be performed according to the LB results. As outlined in

table 3A, there are two types of timepoints for the LB samples. The evaluation LB samples at the same timepoint of every standard of care CT/MRI scan for response evaluation are termed **evaluation LB samples (=LB_E)**. These LB_E results will) will be analysed and will be communicated with the local investigator (i.e. will unblinded). Treatment decisions are performed like standard of care on the base of progressive disease or response according to the NPY methylation results, according to the scenario's outlined below. On the contrary, the LB samples collected in months in between two CT/MRI scans are termed **in-between LB samples (=LB_{IN})** and will not be communicated (i.e. will be blinded) to the local investigator, except if unblinding is necessary for the treatment decisions.

Note 1: If the results of the in-between LB_{IN} samples are deemed relevant by the central study team, especially in case of inconclusive findings in the analysis of the evaluation LB_E samples, the last in-between LB_{IN} sample result previous to the respective LB_E sample result might be unblinded nevertheless upon communication of the results of the LB_E sample, in order to support the clinical decision-making.

Additionally, in this arm cross-sectional imaging (CT/MRI scan) will be performed as per standard of care and will be evaluated by the central study team according to RECIST 1.1 guidelines (ref.), and communicated alongside the LB_E results. However, the treatment for patients in the LB arm (= arm 2 or intervention arm) is guided by LB results only.

The following scenarios are possible:

Table 3B. Scenario's for follow-up in LB arm (arm 2 / intervention arm)

In-between visit on figure 1 (= in between two CT/MRI scans)	During these visits, the following study procedures are to be performed: • Corresponding **in-between LB sample = LB_{IN}** sample is obtained before start new therapy cycle between two evaluation moments with CT/MRI scan → results of these LB samples will not be communicated, unless clinically relevant or in case of inconclusive results of the evaluation LB_E samples (see note above)
Evaluation visit on figure 1 (= at moment of CT/MRI scan for response evaluation)	During these visits, the following study procedures are to be performed: • **Cross-sectional imaging (CT/MRI scan)** → RECIST 1.1 evaluation will be performed • Corresponding **8-weekly QoL questionnaires** → results will not be communicated **Note**: in case of 12-weekly imaging schedule, the QoL questionnaires need to follow 8-week schedule. • Corresponding **evaluation LB-sample = LB_E** → result will be unblinded and communicated to the investigator as treatment decisions are based on these results • Corresponding **decision review questionnaire by physician (Q_{PD})** → results will not be communicated Scenario's: • **Scenario 1:** absolute increase of <1% of NPY methylation as compared to nadir NPY methylation (= lowest NPY methylation value from baseline LB sample (LB_B) till this sample (LB_E)) → continue current treatment / initiate therapy break = choice of treating physician based on clinical status of patient (~ SOC): • **Scenario 1A:** in case of treatment continuation → follow the flowchart to the next month

	• **Scenario 1B:** in case of treatment break → continue 4 weekly LB-sampling (if SOC blood draw is planned) and 8-weekly questionnaire sampling (if feasible) → when the treatment is restarted, follow scenario 1C • **Scenario 1C:** when restarting treatment after a break → restart with evaluation visit where new LB_E sample is taken to determine new baseline methylation value, alongside new QoL questionnaires and imaging (as per SOC) → thereafter continue as per scenario 1A **Note:** subtract weeks/months of treatment break from total of 18 months of follow-up in the study to know remaining months of follow-up in the study. • **Scenario 2:** absolute increase of ≥5% of NPY methylation as compared to nadir NPY methylation (= lowest NPY methylation value from baseline LB sample (LB_B) till this sample (LB_E)) → progressive disease → stop current treatment → switch follow-up to PD-arm • **Scenario 3:** absolute increase of 1-4% of NPY methylation as compared to nadir NPY methylation (= lowest NPY methylation value from baseline LB sample (LB_B) till this sample (LB_E)) → continue current treatment, and follow the next scenario's: • **Scenario 3A:** LB sample of the next month will be unblinded (i.e. will be communicated) and confirms absolute increase in NPY methylation between 1-4% compared to the nadir → continue current treatment and follow the flowchart to the next month • **Scenario 3B:** LB sample of the next month will be unblinded (i.e. will be communicated) and shows absolute increase in NPY methylation ≥5% compared to the nadir → progressive disease → stop current treatment → switch follow-up to PD-arm • **Scenario 4:** PD on cross-sectional imaging (CT/MRI scan) OR clinical progression → needs to be considered as progression irrespective of LB results → stop current treatment → switch follow-up to PD-arm **Note:** additional patient experience questionnaire will be requested in case of progression
PD-arm	Upon progression during follow-up (= scenario 2, 3B, or 4), the follow-up based on scenario 1 will be stopped. The treating physician can start the next line of treatment of choice. Although, patients must still be followed up in the study, but according to the PD-arm. In this arm, 4-weekly **LB_{PD} samples** + 8-weekly **QoL questionnaires** will be collected (if feasible) until the patient has completed a total of 18 months of follow-up in the study. If one of both is not feasible, then partial withdrawal of consent is possible.

6.3. End of study

6.3.1. Early study termination

The Sponsor may terminate this study at any time. Reasons for termination may include but are not limited to, the following:

- Insufficient patient enrolment.
- Any information becoming available during the study that substantially changes the expected benefit of the study.

6.3.2. End of study for the patients

- **Patient completed follow-up of a total of 18 months in the study** (i.e. untill M18, either in the standard arm or in the PD-arm, see scenario's in paragraph 6.3.1 and 6.3.2)

- **Patient will undergo metastasectomy *with curative intent***
  In case no adjuvant chemotherapy is planned, the visits and collection of liquid biopsies will stop. An end of study (EoS) visit (see table 4) will be planned, and corresponding study procedures will be performed. In case adjuvant chemotherapy is planned, then follow-up in study can continue as per scenario 1A described depending in the study arm the patient was in.

- **Patient withdraws from study (discontinuing participation)**
  Patients can leave the study at any time for any reason if they wish to do so without any consequences. Should a patient decide this by withdrawal of consent, all efforts will be made to complete and report the observations as thoroughly as possible. No further data will be collected after the date of withdrawal from study. Data that was already collected will be stored and used for analysis. The Investigator should contact the patient or a responsible relative by telephone or through a personal visit to establish as completely as possible the reason for the withdrawal. A complete final evaluation at the time of the patient's withdrawal should be made with an explanation of why the patient is withdrawing from the study. Partial withdrawal of consent is also possible, if one of the following interventions is not feasible (after careful consideration): LB sampling or questionnaires during follow-up in PD-arm).

- **Patient dies**

- **Changes in a patient's condition that renders the patient not suitable for further treatment in the judgment of the investigator.** Major protocol violation or discovery of information that, if previously known, would have rendered the patient ineligible for study.

- **Withdrawn consent with data withdrawal**
  Patient withdraws informed consent and requests to delete all of his/her previously collected data from the study.

- **Lost to follow-up**

6.4. Follow-up phase after end of study after 18m

There are different reasons for a patient to proceed to the follow-up phase (after end of study), which all have a different impact on how to proceed, on data collection and study analysis:

6.4.1. Patient reaches 18 months of follow-up

The visits will stop, but patients will be followed for safety for an additional 28 days. Furthermore, information about survival status will be collected from the patient record (every 3 months) until death. After end of study, the patient will continue with standard of care, as decided by treating physician.

6.4.2. Partial withdrawal from the study, with consent to allow collection of progression and survival status information

The patient withdraws from the study but accepts to be followed for further information on further progression and survival status. It should be documented in both the medical records and in the CRF that the patient accepted to be followed for survival data. This way, survival information can be collected every 3 months until death.

6.4.3. Lost to follow-up

In the case of patients who missed scheduled visits, several attempts should be made by the site to contact these patients for follow-up information. The collection of follow-up data is extremely important in regard to the reliable estimation of study endpoints, therefore at least 3 attempts within a reasonable extent of time should be made to try to contact the patients if they do not attend clinic visits. If any of the trial patients are lost to follow-up, contact will initially be attempted through the trial research nurse or the lead investigator at each center. Where this attempt is unsuccessful, the patient's general practitioner will be contacted and asked to contact the patient or her/his family and provide follow-up information to the recruiting center. It is only after 3 attempts at contacting the patient have been unsuccessful, that a patient may be declared "Lost to follow-up".

6.4.4. Duration of the study

The inclusion of patients in this trial starts when all necessary ethical approvals have been obtained. Patient accrual period stops approximately round 27-30 months after start inclusion, or when 150 patients could be included. Considering an 18M FU, the total duration of the study will be around 4 years (excl. Survival status FU). The study ends for the patient as described in section 6.4. After end of study, information about survival status of all included patients will be followed for three years or until death. Clinical follow-up data for survival analysis (PFS and OS) will be collected from the patient record and routine clinical follow-up visit.

7. METHODOLOGY

7.1. Study schedule

Additional assessments, such as urinalysis or other laboratory assessments, can always be performed by the treating physician, if deemed necessary. Also, additional CT scans can be made, for example when the treating physician has clinical indications for progression. Results from additional tests or data from additional visits (for example if requested by the patient) will be collected for the study.

Study visits in the hospital will take place in the treatment hospital of the patient.

Table 4: Schedule of assessments

	Screening	**Inclusion**	**In-between visit** (every 4 or 6 weeks (+/- 7d) after evaluation visit)	**Evaluation visit** (every 4 or 6 weeks (+/- 7d) after in-between visit)	**PD-arm visit** (every 4 or 6 weeks +/- 7d – only after progression)	**EoS visit** (in case of completion of 18m or earlier termination in case of withdrawal or mortality)
Randomization		x				
Inclusion/exclusion criteria	x	x				
Informed consent	x					
Demographics	x					
ECOG	x	x	x	x	x	x
Medical history	x	x				
Follow-up data					(x****)	(x****)
CT / MRI scan (RECIST 1.1)	x	(x*)		x	(x****)	(x****)
CEA		x	x	x	x	x
Chemotherapy information		x	x	x	x	x
Liquid biopsy (LB) sample						
> baseline LB_B = 4 Streck	x	(x*)				
> in-between LB_{IN} = 2 Streck			x			(x**)
> evaluation LB_E = 2 Streck				x		(x**)
> progression LB_{PD} = 2 Streck (3 Streck for 1st LB_{PD} sample)					x	
FFPE samples		(x***)				
(S)AE(s)		**continuous**				
Documentation + reason EoS						x
QoL questionnaires (EORTC QLQ-C30 and QLQ-CRC29)	x		x	x	x	x
Decision review questionnaire (physicians, Q_{DR})				x		

Remarks
- (x*) = in case SOC screening CT/MRI scan and/or baseline LB_B sample is > 90 days old from date of inclusion: repeat CT/MRI scan and/or LB_B
- (x**) = 3 Streck tubes in case of EoS visit in case of completion of 18m OR earlier termination in case of withdrawal or mortality
- (x***) = optional (collection of FFPE samples: 10 sections of 6µm FFPE slides might be requested)
- (x****) = next-line treatment, imaging, and survival data can be collected every 3 months for total of 36 months after progression or EoS

7.2. Assessments and study visits

See table 4.

7.3. Data encoding

7.3.1. Study imaging data

In the CT arm, imaging data are collected in the medical file of the patient, more specifically in the Picture Archiving and Communication System (PACS), at their treating center. To enable central review of the imaging data, images will be transferred to an online platform (e.g. PACS on web) or shared by a VPN connection between a participating hospital and the central study committee.

7.3.2. Data storage and transfer

Clinical information about the patients, collected at the study visits in the hospital or by the external partner will be encoded and put in an eCRF (REDCap). UZA is the owner of the database and the information will be kept for at least 30 years after study termination.

7.4. Evaluation of data

7.4.1. Tumor response evaluation

CT arm: Image analysis are performed locally. After the CT-scan is taken, the local site will upload* a pseudomized DICOM file to the sponsor to take RECIST measurements. The sponsor will communicate the RECIST measurement within 7 working days to the local site.

LB arm: Image analysis are performed locally. After the CT-scan is taken, the local site will upload* a pseudomized DICOM file to the sponsor to take RECIST measurements. The sponsor will not communicate this measurement. In this case, the RECIST measurements are blinded for the local sites.

***Note**: See manual for uploading DICOM file. It is the local team's responsibility to check the identity (correlate with study nr.) and pseudomize before sending to the sponsor.

7.4.2. ctDNA analysis

As described, liquid biopsies contain, except for ctDNA, circulating DNA originating from non-malignant cells. One can differentiate ctDNA from non-malignant DNA by selecting on a base of NPY methylation (25).

In the LB arm, the decision about progression and potential change of therapy will be made based on ctDNA analysis. In the CT-arm, the ctDNA analysis will be blinded.

8. SAFETY REPORTING

8.1. Ethical Committee

The investigator will inform the patients and the reviewing accredited Ethical Committee if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited Ethical Committee, except insofar as suspension would jeopardize the patients' health. The investigator will take care that all patients are kept informed.

8.2. Adverse events

8.2.1. Definition Adverse Event (AE)

Adverse events are defined as any undesirable experience occurring to a patient during the study that need not be related to treatment. An adverse event is any unfavorable, unintended diagnosis, symptom, sign (including abnormal laboratory finding), syndrome, or disease that occurs during the study, having been absent at baseline, or –if present at baseline- appears to worsen. Definition Unexpected Adverse Event (UAE)

An unexpected adverse event is defined as an adverse event that is not mentioned in the protocol or consent documents, or an AE that has not been seen before. Additionally, unexpected AEs in this study can be considered as unanticipated problems involving risks to study participants and others as an event that meets all of the following criteria:

unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the protocol-related documents, such as the informed consent document; and (b) the characteristics of the patient population being studied;

related or possibly related to participation in the research (i.e. the incident, experience, or outcome may have been caused by the procedures involved in the research).

8.2.2. Recording of AEs

Only therapy related AE's (grade 3-4), will be reported and recorded on the AE form of the CRF with the following information:

The severity grade according to the NCI-CTCAE version 5.0, published November 27, 2017. The complete document can be reviewed and downloaded from the following internet site: https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_5x7.pdf (1=mild, 2=moderate, 3=severe, 4=life threatening)

Whether it constitutes an UAE or SAE

All related grade 3 and 4 AEs should be treated appropriately. Treatment may include one or more of the following: no action taken (i.e. further observation only); chemotherapy temporarily interrupted; chemotherapy permanently discontinued due to this AE; concomitant medication given; non-drug therapy given; patient hospitalized/patient's hospitalization prolonged. The action taken to treat the AE should be recorded on the AE CRF.

Once an AE is detected, it should be followed until its resolution or until it is judged to be permanent, and assessment should be made at each visit (or more frequently, if necessary) of any change in severity, the interventions required to treat it, and the outcome.

Note: Since AE's can have an impact of the QoL of patients, it is important to report these.

8.2.3. Follow-up of adverse events

All adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

8.3. Serious Adverse Events

8.3.1. Definition Serious Adverse Event

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- results in death
- is life threatening (at the time of the event)
- requires hospitalization or prolongation of existing patients' hospitalization
- results in persistent or significant disability or incapacity

Note: In this study, admission for diagnosis or treatment of recurrences are not considered a SAE

8.3.2. Recording of SAEs

All SAEs related to the extra blooddrawn (LB), with the severity of grade 3 or higher, must be reported initially in REDCap. If the SAE has to be reported to the EC, the investigator must sign the completed SAE report electronically in REDCap as soon as possible but no later than one working day from the time the local investigator has first knowledge of the SAE. REDCap will automatically send a notification to the central study coordinator when the SAE report has been created. The central Ethics Committee of UZA will inform the Medical Ethics Committee(s) of the participating centers. The (S)AE report must be updated after initial submission to REDCap as soon as final data related to the (S)AE is available.

8.4. (Suspected) Unexpected (Serious) Adverse Reactions

Since the FOLICOLOR Trial is a prospective interventional study in which there is no investigational product, (S)U(S)AR reporting does not apply.

8.5. Annual report

The sponsor will submit once a year throughout the clinical trial an annual report to the central ethical committee of the UZA for distribution to all ethical advisory boards of participating hospitals. This annual report will be adjusted (from Q1/2025) taking into account items that 'Kom Op Tegen Kanker' suggest.

This annual report consists of:

- A status update of the trial, including but not limited to the following: date of EC approval, date of inclusion of first study patient, total number of patients included, total number of patients who discontinued their participation (end of study);
- An aggregated summary table of all serious adverse events, ordered by organ system.

8.6. Data monitoring committee (DMC)

The data monitoring committee (DMC) has the role to monitor patient safety and efficacy and scientific validity of the study data while the trial is ongoing. The DMC makes recommendations to the trial steering committee (TSC).

The frequency of DMC meetings are planned as follows: once a year during the trial. Also flexibility will allow for ad hoc meetings when safety issues emerge.

The members of the DMC are independent and have no conflicts of interest with the conducted trial, principal investigator or sponsor of the study. Members of the DMC are listed in a separate file (member list).

9. STATISTICAL CONSIDERATIONS

9.1. Sample size calculation

The primary endpoint of this project is the difference in time to deterioration (TTD) in QoL between patients in which follow-up is done based on the results of LBs (LB-arm) in comparison to the patients in which follow-up is done based on the conventional follow-up techniques (CT-arm). TTD is defined as time from randomization to the first decrease from baseline on the European Organisation for Research and Treatment of Cancer (EORTC) Quality of Life questionnaire (QLQ-C30) summary score by at least 10 percent. This primary endpoint will be met if the TTD in the LB-arm is longer than the TTD in the CT-arm with a hazard ratio of 0.6 in a Cox proportional hazard model (see 7.6). Patients will be allocated in a 1:1 ratio to one of the two study arms. Qminim, a web-based online minimization tool will be used for the minimization procedure (factors include tumor type, study site, gender and age (≤ 45 year; 45-75 year; ≥ 75 year)). We would need to include 150 patients (75 patients in each arm), based on the sample size calculations described in paragraph 7.6. To achieve this projected sample size, we would need to recruit +-1 patients per site per 2 months from the 9 participating centers. We estimate that this will be feasible due to the fact that the participating centers in this trial are the same as in the FOLICOLOR lead-in study project.

In the MIROX randomized phase III trial in mCRC, the median TTD (for general health score) was 13.4 months (95% IC 6.6– 19.4) for patients treated with FOLFOX4 (6). In addition, a significantly shorter TDD (regarding pain score) was reported for patients treated with FOLFOX4 alone compared to FOLFOX7 followed by FOLFIRI, with a HR of 0.6 (95% CI 0.36-0.98 and P = 0.044). Based on the literature, we therefore assume a median TTD of 11 months in the standard of care arm (CT-arm). With the inclusion of 150 patients (75 subjects per treatment group), this would allow for detection of a HR of 0.6 in TTD using a log-rank test with 150 events achieving 80% power at a 5% significance level. For this calculation, we assumed a recruitment period of 27 months and a follow-up period of 18 months.

9.2. Primary analysis

Difference in time to deterioration (TTD) in QoL, measured by the QLQ-C30 questionnaire, between patients in which follow-up, which is done based on the results of LBs (LB-arm) in comparison to the patients in which follow-up is done based on the conventional follow-up techniques (CT-arm). For the analysis of this endpoint, a mixed model approach will be used.

9.3. Secondary analysis

For the secondary endpoints, analyses include but are not limited to the following: The proportion of patients in whom progressive disease is detected earlier using liquid biopsies compared to conventional imaging will be analyzed descriptively, with differences assessed using McNemar's test for paired data. Progression-free survival (PFS) will be compared between treatment arms using Cox proportional hazards

models, adjusting for variables such as gender and molecular subtype (RAS WT, RAS MT, or MSI high). Sensitivity analyses will include PFS evaluation within the per-protocol population and exploration of site-level differences descriptively. Overall survival at 3 years and long-term OS will be modeled similarly to PFS using Cox models, with censoring for patients alive or lost to follow-up.

Differences in quality of life (QoL), measured by EORTC QLQ-C30 and QLQ-C29, will be assessed using linear mixed models, accounting for patient-level random effects, to evaluate longitudinal changes and differences between arms at specific time points. Adverse events, including their incidence and severity, as well as on-treatment deaths (defined as deaths occurring during treatment or within 30 days after last protocol-specified treatment), will be compared using Chi-square or Fisher's exact tests as appropriate. Exploratory analyses will include patient-reported outcomes from questionnaires assessing the burden of liquid biopsies versus CT scans, confidence in liquid biopsy-guided therapy, and overall preferences between the two approaches. Descriptive statistics and appropriate statistical tests (e.g., Chi-square, t-tests) will evaluate these patient-reported experiences.

9.4. Exploratory analysis

For the exploratory endpoints, analyses include but are not limited to the following: The comparison of ctDNA and CEA measurements at corresponding timepoints will focus on evaluating their predictive value for progression. Time-dependent receiver operating characteristic (ROC) curves will be generated for each biomarker, and their predictive accuracy will be compared using the area under the curve (AUC). Correlations between ctDNA and CEA levels will also be explored descriptively and through regression models to assess agreement and complementary value.

The association between ctDNA levels during follow-up and progression-free survival (PFS) will be analyzed using a joint modeling approach. This method will incorporate longitudinal ctDNA measurements as a time-varying covariate into survival models, enabling an integrated assessment of how ctDNA trends over time relate to PFS. This approach will allow for the simultaneous estimation of ctDNA trajectory and its impact on progression risk, providing insights into its prognostic value.

For the exploration of novel methylation biomarkers in ctDNA, differences in average methylation levels will be tested using the Mann-Whitney U test.

10. STUDY MANAGEMENT

10.1 Trial Steering Committee

The trial steering committee (TSC) is a group of experts that have the task to guide the study team. The TSC is an executive decision-making group supporting actions of the Trial Management Group and providing advice. The TSC considers the recommendations from the DMC. The TSC may make recommendations to sponsor of a trial as well as to the trial team.

The TSC meets at least twice per year. Additional meetings may be planned depending on the situation. Members of the TSC are listed in a separate file (member list).

10.2 Trial Management Group (TMG)

The trial management group (TMG) is responsible for the day-to-day delivery and conduct of the trial. Members of the TMG are listed in a separate file (member list).

10.3 Futility analysis

An option for a futility analysis will be incorporated at a predefined interim timepoint to assess the probability of achieving the study's primary objective. This analysis will use pre-specified statistical criteria to evaluate whether the observed data suggests that continuing the trial as planned is unlikely to yield conclusive results. The futility analysis will focus on the primary endpoint and will be based on a comparison of the observed effect size and its confidence intervals against thresholds defined in the statistical analysis plan. While the primary purpose of the analysis is to determine whether the trial should continue, it will also provide an opportunity to assess recruitment rates, adherence to the protocol, and the consistency of data trends across treatment arms. The results of the futility analysis will be reviewed internally, and any decision to terminate or modify the study will be made in accordance with the protocol and governing ethical considerations.

11. ADMINISTRATION PROCEDURES AND PUBLICATION

11.1 Regulatory Approval

As required by local regulations, the sponsor will ensure all legal regulatory aspects are covered, and obtain approval of the appropriate regulatory bodies, prior to study initiation in regions where an approval is required.

11.2 Publication Policy

The sponsor encourages acknowledgement of all individuals/organizations involved in the funding or conduct of the study, including medical writers or statisticians patient to the consent of each individual and entity concerned, including acknowledgement of the sponsor.

The results of this study may be published or communicated to scientific meetings by the investigators involved in the study. For multicenter studies, a plan for scientific publication and presentation of the results may be agreed and implemented by the study investigators. The sponsor requires that a reasonable opportunity be given to review the content and conclusions of any abstract, presentation, or paper before the material is submitted for publication or communicated. This condition also applies to any amendments that are subsequently requested by referees or journal editors. The sponsor will undertake to comment on the draft documents within the time period agreed in the contractual arrangements, including clinical trial agreements, governing the relationship between the sponsor and authors (or the author's institution). The author will incorporate requested amendments, provided they do not alter the scientific value of the material.

The author undertakes to reasonably consider the sponsor's request for delay to the proposed publication should the sponsor reasonably deem premature to publish the results obtained at that stage of the study.

11.3 Clinical Study Report

A final clinical study report (CSR) will be prepared according to the ICH guideline on structure and contents of CSRs. Where appropriate an abbreviated report may be prepared. The CSR will be in compliance with any applicable regulatory requirements, national laws in force and will be in English. Also, during the study, reports will be made to show the progression of the study.

11.4 Contractual and Financial Details

The Investigator (and/or, as appropriate, the hospital administrative representative) and the sponsor will sign a clinical study agreement prior to the start of the study, outlining overall sponsor and investigator responsibilities in relation to the study.

11.5 Insurance, Indemnity and Compensation

The sponsor has a liability insurance which will be annually renewed.

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