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Protocol

Determination of biomarkers associated with neoadjuvant treatment response focusing on colibactin-producing *Escherichia coli* in patients with mid or low rectal cancer: a prospective cohort study (MICARE).

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Abstract

Introduction The management of mid and low rectal cancer is based on neoadjuvant chemoradiotherapy (CRT) followed by standardized surgery. There is no biomarker in rectal cancer to aid clinicians in foreseeing treatment response. The determination of factors associated with treatment response might allow the identification of patients who require tailored strategies (e.g. therapeutic de-escalation or intensification). Colibactin-producing *Escherichia coli* (CoPEC) has been associated with aggressive CRC and could be a poor prognostic factor. Currently no study has evaluated the potential association between intestinal microbiota composition and tumour response to CRT in mid and low rectal cancer. The aim of this study is to assess the association between response to neoadjuvant CRT and faecal intestinal microbiota composition and/or CoPEC prevalence in patients with mid or low rectal cancer.

Methods and analysis This is a non-randomized bicentric prospective cohort study with a recruitment capacity of 200 patients. Three stool samples will be collected from participants with histological-proven adenocarcinome of mid or low rectum who meet eligibility criteria of the study protocol: one before neoadjuvant treatment start, one in the period between CRT end and surgery, and one the day before surgery. In each sample, CoPEC will be detected by culture in special media and molecular (PCR) approaches. The global microbiota composition will be also assessed by the bacterial 16S rRNA gene sequencing. Neoadjuvant CRT response and tumour regression grade will be described using the

Dworak system at pathological examination. Clinical data and survival outcomes will also be collected and investigated.

Ethics and dissemination MICARE was approved by the local ethics committee (Comité de Protection des Personnes Sud-Est II, December 18th, 2019. Reference number 2019-A02493-54) and the institutional review board. Patients will be required to provide written informed consent. Results will be published in a peer reviewed journal.

Trial registration number NCT04103567.

Strengths and limitations of this study

- As far as we know, this is the first study to evaluate association between intestinal microbiota composition and tumour response to chemoradiotherapy in mid and low rectal cancer
- MICARE is a prospective cohort study including 200 patients
- This study is based on a non–invasive and reproductible faecal test
- Tumour response will be described at pathological examination after surgery
- The limitation of this study will include population stratification for delay between radiotherapy and surgery, and adjonction of neoadjuvant chemotherapy in tumour response evaluation

Introduction

With more than 700,000 new cases and 300,000 deaths in 2018, rectal cancer is the eighth leading cause of cancer deaths worldwide (1). The initial management of mid and low rectal cancer is based on neoadjuvant chemoradiotherapy (CRT) for locally advanced tumours. This is associated with a significant decrease of the locoregional recurrence rate, but without survival improvement (2–4). Neoadjuvant treatment is followed by standardized surgery (5). Total mesorectal excision is crucial for reducing tumour recurrence (6), but its significant morbidity can affect the patients' quality of life. Prognosis also depends on the tumour response to neoadjuvant CRT. Currently, the surgical strategy is adapted in function of the tumour response to neoadjuvant treatment, assessed by magnetic resonance

imaging (MRI) after CRT end (7). Indeed, the objective is therapeutic de-escalation with rectal preservation to decrease morbidity and functional disorders. For patients with complete response (up to 25% of patients), careful monitoring without surgery ("watch and wait" strategy) has been proposed (8,9). For small tumours with good response to CRT, transanal excision with rectal preservation seems to be feasible in terms of cancer prognosis (10). For patients with large tumours or a locally advanced disease, a tailored treatment strategy with total neoadjuvant therapy (TNT) is now a gold standard (11,12). After surgical excision, the tumour response is classified in five pathologic tumour response grades, according to the Dworak classification, on the basis of the pathology findings (13). Recent studies reported up to 30% of poor responders (grades 0 and 1) (14,15). These data emphasize the importance of the initial tumour staging and response to neoadjuvant CRT for tailoring surgical strategies. MRI is an essential tool for these two assessments (16–18). These data highlight the need of response predictive models to adapt the TNT in mid and low rectal cancer.

Gut microbiota behaves as a real organ and participates in intestinal homeostasis. An imbalance in its composition (dysbiosis) could be involved in many pathologies, including colorectal cancer (CRC) (19–21). *Escherichia coli* (E. coli) has been widely described as a bacteria which could be involved in CRC.(22,23). *E. coli* is the predominant aero-anaerobic Gram-negative specie in human colon, but it is also a pathogen involved in various intestinal diseases (24). Indeed, some *E. coli* strains have acquired the capacity to produce toxins named cyclomodulins, including colibactin that is encoded by the pks island(25). Colibactin-producing *E. coli* (CoPEC) has genotoxic effects by inducing DNA damage and chromosomal instability (25–27). CoPEC implication in CRC has been demonstrated, particularly in aggressive forms (28–34). Specifically, higher *E. coli* colonization rate and higher prevalence of CoPEC are found in patients with TNM stage III or IV tumors (29) (UICC TNM Classification, 8th Edition, 2017) (35). Moreover, CoPEC gut colonization might contribute to modulate the immunotherapy efficacy (36). Recent clinical studies discussed the prognostic role of intestinal microbiota in the tumour response following surgery and chemotherapy or immunotherapy (37), and suggested that it could be used as a biomarker to predict tumour response to neoadjuvant treatments. On the other hand, very few clinical studies have assessed the influence of gut microbiota on radiotherapy efficacy, especially in rectal cancer. Recently, a preclinical study showed that mice which survive a high dose of radiation,

harboured gut microbiota enriched with *Lachnospiraceae* and *Enterococcaceae* (38). Yet, a description of the intestinal microbiota composition before neoadjuvant therapy could allow identifying predictive bacterial markers of tumour response in rectal cancer, and to adapt TNT.

Indeed, chronic exposure of the gastrointestinal tract to genotoxins could be a prognostic marker of radiotherapy response. CoPEC colonization would start at the very beginning of life (38) and might lead to exposure of the intestinal mucosa to chronic genotoxic stress. The resulting damage could give cells the ability to resist to other genotoxic stresses, such as radiation therapy. One *in vitro* study already showed the decreased radiation sensitivity of cells incubated by colibactin (27). Therefore, developing a non-invasive method to analyse gut microbiota composition and to evaluate CoPEC implication in the response to CRT could help clinicians to tailor cancer management and to develop tools to control the pathologic microorganisms identified as new therapeutic targets.

Methods and analysis

This study protocol is written in accordance with the SPIRIT guidelines. (Supplementary file)

Objectives

Primary objective

The study primary objective is to assess the correlation between response to neoadjuvant CRT and CoPEC presence in stool samples.

Secondary objectives

- To analyse in a non-targeted manner the global microbiota composition before CRT and to evaluate the correlation between composition and response to treatment
- To study the modulation of the intestinal microbiota by CRT
- To describe the correlation between clinical data and microbiota composition modulation induced by CRT

- To determine microbiological prognostic factors of overall survival, disease-specific survival and relapse-free survival (locoregional and metastatic) in patients with low or mid rectum cancer
- To create a microbiological database for future mechanistic analyses
- To study the modulation of CoPEC colonization by CRT

Study design

The study is a non-randomized bicentric prospective cohort study. Two surgical teams will be involved - Institut du Cancer de Montpellier and CHU de Clermont-Ferrand ; and an INSERM Unit – M2iSH Clermont-Ferrand.

Patients' selection

Inclusion criteria

- Histologically-proven adenocarcinoma of low or mid rectum, of stage II or III (UICC TNM Classification, 8th Edition, 2017 (35))
- Patient eligible for neoadjuvant treatment (50 Gray radiation and capecitabine, CAP 50), according to the French national recommendations (5,39)
- Informed signed consent received
- Man or woman aged ≥18 years
- Appropriate contraceptive measures taken by men and pre-menopausal women before study entry and for at least 8 weeks after the last CRT cycle. Patients should be informed by the investigator on the contraceptive measures to use.

Exclusion criteria

- Antibiotic treatment at the time of stool sampling or in the month before.
- Presence of a derivative stoma
- Previous chemotherapy treatment for rectum cancer
- Patient not affiliated to the French social security system

- Patient with possible poor treatment compliance for psychologic, familial, social and geographic reasons
- Legal incapacity or limited legal capacity
- Pelvic radiotherapy or brachytherapy in the year before inclusion in the study
- History of other cancers in the 5 last years, except for cervical carcinoma *in situ* and skin carcinoma, but including melanoma under treatment
- Pregnant or breastfeeding woman

Study sponsor

The sponsor (Montpellier Cancer Institute, ICM) is responsible for the study design and management, and for obtaining all study authorizations (Persons Protection Committee, National Agency for Medical Security). It will also declare to these authorities the inclusion period beginning and end, produce the final study report, inform the competent authorities of the trial results, and store all study-related documents for at least 15 years after the study end.

Clinical study procedures

Inclusion in the study

The study flow diagram is presented in Figure 1.

Before study entry, all patients will receive exhaustive explanations on the study aims and procedures. A signed informed consent will be obtained from all patients before any study procedure. At baseline, demographic (sex, age), clinical (performance status, weight, height, medical history, initial diagnosis date, tumour localization, histologic type) and biological (complete blood count, carcinoembryonic antigen (CEA) level) data will be collected (Table 1). Patients will undergo rectal examination and tumour staging by computed tomography (CT), rectal MRI, and possibly rectal endoscopic ultrasound examination (depending on the centre decision).

During the surgical consultation, the first stool sample (stool sample N°1) may be collected during rectal examination (faeces left on the clinician's glove), or by proctoscopy. Otherwise, the stool sample will be collected by the patient.

Table 1: Flow chart with the clinical and radiological evaluations

Assessment	Baseline	Re-evaluation	Day before surgery	Follow-up Every 6 – 8 months
Informed consent	X			
Selection criteria validation	X			
Demographic and clinical data	X			
Physical examination	X			
Patient inclusion	X			
Stool sample	X	X	X	
Patient vital status				X
Tumour evaluation				
Rectal MRI	X	X		X
CT	X			X
Rectal examination	X	X		X

MRI: Magnetic resonance imaging; CT: computed tomography.

Neoadjuvant treatment

Patients will undergo neoadjuvant CRT in accordance with the French national guidelines (5). CRT data (dose, possible dose modifications or interruptions) and CRT complications will be recorded.

Re-evaluation

During the consultation after CRT end and before surgery, a second stool sample (stool sample N°2) will be collected, as described for the baseline sample. If the patient has received antibiotics in the month before this consultation, stool sampling will not be performed.

This second consultation will include MRI examination as during the baseline visit. The tumour response will described precisely with emphasis on the tumour regression grade according to the MERCURY experience (7).

Surgery

Surgical data (surgery type, digestive reconstruction or stoma, and surgical outcomes), anatomopathological data (histologic type, ypTN grade, Dworak grade (13), Quirke classification (40), circumferential resection, distal margins, and extramucosal vascular invasion) and biological data (RAS and BRAF mutational status, if available) will be collected. The day before surgery, before bowel mechanical preparation, the third stool sample (stool sample N°3) will be collected in hospital, as described for the previous samples. If the patient received antibiotics in the month before hospitalization, stool sampling will not be performed.

Pathologic analysis

To meet the primary objective, the pathologic analysis of the surgical specimens will describe the tumour regression grade according to the Dworak classification (13) (Table 2). Patients with grade 0 and 1 tumours will be considered poor responders, in accordance with the literature.

Table 2: Tumour Regression Grade (TRG), Dworak classification (13)

TRG	Pathology
Grade 0	No regression
Grade 1	Dominant tumour mass with obvious fibrosis and/or vasculopathy
Grade 2	Dominant fibrotic changes with few tumour cell groups (easy to find)
Grade 3	Very few (difficult to find microscopically) tumour cells in fibrotic tissue with or without mucous substance
Grade 4	No tumour cell, only fibrotic mass (total regression or response)

Safety

All adverse events will be reported following the study sponsor's pharmacovigilance procedures, and in accordance with the applicable regulation.

Follow-up and study duration

Follow-up will last 5 years from the date of surgery. The frequency of follow-up visits will be decided at each centre. Every 6 to 8 months, the disease and survival status will be assessed. Recurrence will be investigated by clinical examination with rectal MRI and CT and a tumour marker test (CEA) (Table 1). Locoregional or metastatic relapse will be reported in the case report form with the date of relapse diagnosis.

As the inclusion period will be of 36 months and the follow-up will last 5 years, the total study duration will be of 8 years.

Microbiological analyses

Sample handling

Three stool samples will be collected during the study (Figure 1): i) one at patient inclusion, before any treatment, to describe the baseline intestinal microbiota composition; ii) one during the interval between

the end of neoadjuvant CRT and surgery, at the surgical consultation for tumour reappraisal; and iii) one just before bowel preparation (mechanical or antibiotics) for surgery.

Each sample will be divided into two cryotubes: one empty and one with 15% glycerol/DMEM to preserve cell integrity. Samples will be immediately stored at -80°C until transport to the M2iSH laboratory, Clermont-Ferrand, France, which will be in charge of the molecular analysis and storage of the samples.

E. coli strain identification and CoPEC detection

All microbiological analyses will be performed as previously described (28). After thawing, samples stored in DMEM/glycerol will be crushed and diluted in sterile phosphate buffered saline pH 7.4 before plating on TBX agar and chromogenic agar chromID CPS3® plates (bioMérieux) to allow the identification and quantitation of enterobacteria. Colonies (around 48 per sample) will be collected for molecular typing, and their identification will be confirmed with the automated Vitek® II (bioMérieux) system. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR will be used as genotyping method to determine the number of *E. coli* strains per sample (28).

E. coli harbouring the colibactin-encoding *pks* island will be identified by PCR analysis of each *E.coli* isolate (41). This will allow identifying the presence of CoPEC (primary objective).

Untargeted analysis of the local microbiota composition

Global microbiota modifications will be assessed by high-throughput sequencing of the bacterial 16S rRNA gene in DNA extracted from the three stool samples using the NucleoSpin® DNA stool kit (Macherey-Nagel, Hoerdt, France), according to the manufacturer's instructions. Quantitative PCR will be performed to quantify pro-carcinogenic bacterial species, such as *Fusobacterium nucleatum, Enterococcus feacalis, bft*-positive *Bacteroides fragilis,* and CoPEC. In addition, the V4 region of the bacterial 16S rRNA gene will be amplified using the 515F/806R primer pair followed by Illumina high throughput sequencing on a MiSeq® apparatus, according to the manufacturer's guidelines. A global description of the intestinal microbiota could also be obtained by shotgun metagenomic sequencing to access the microbiota functional features after selection of the more informative samples.

Endpoints

Primary endpoint

The primary endpoint (associated with the primary objective) is the relative risk (RR) of poor response to neoadjuvant CRT in patients colonized by CoPEC ("exposed") compared to non-colonized patients ("unexposed").

Secondary endpoints

- Prevalence and CoPEC colonization rate before and after CRT
- Other bacterial strains present before CRT and relative risk of poor response to CRT in colonized and non-colonized patients
- Type, prevalence, and colonization rate of bacteria other than CoPEC in the microbiota, before and after CRT
- Percentage of colonized patients, depending on the bacterial type, according to the clinical parameters (age, sex, body mass index)
- Hazard ratio (HR) for overall survival, disease-specific survival, and relapse-free survival (locoregional or metastatic) in colonized patients, for the different bacterial types, according to the overall bacterial composition (including CoPEC), and in non-colonized patients.

Data collection and management

The database will be managed by the sponsor, and data stored at the Data processing centre, Biometrics Unit of the Montpellier Cancer Institute. Case report form design and clinical data management will be implemented using the Ennov Clinical® software. Microbiological data will be collected in a database first stored at the M2iSH laboratory, and then transferred to the sponsor database for analysis. Data and any trial documents will be made available upon reasonable request and after signature of a data access agreement.

In accordance with the General Data Protection Regulation (GDPR), a registration number will be used to identify each patient. The corresponding table will be encrypted and stored in a secure place. Special vigilance will be exercised throughout the study to maintain data anonymization.

Study monitoring, quality control, and audit

According to the sponsor's risk-based monitoring plan (study participants, logistics, resources, impact), the collection of the patient informed consents and the respect of the study protocol and procedures will be monitored.

To guarantee the originality of all data and in accordance with the Good Clinical Practices, quality control will be performed by the sponsor. The study will be managed according to the sponsor procedures and in respect of the protocol, and the quality of the data included in the report forms will be checked.

The sponsor may wish to conduct an audit at some investigating centers. Audits may be conducted by the sponsor or any duly authorized person for at least 15 years after the trial.

Statistical considerations

Sample size

The recruitment capacity for this exploratory study will be around 200 patients. For a mean rate of 30% of poor responders to the neoadjuvant treatment among the patients not colonized by CoPEC (*i.e.*, a proportion of response P2=0.30 among unexposed patients), the study will be able to estimate a relative risk of 1.7 (RR=1.7) with a 30% precision and a confidence interval at 95% (α=0.05). Patients in whom the CoPEC colonization status cannot be determined at baseline, in whom CRT must be prematurely arrested, or who cannot undergo surgery will be considered non-evaluable.

Considering a 10% rate of potentially non-evaluable patients, a total of 220 patients (20 supplementary patients) will be included in the study.

Study population

Two populations will be defined for the analysis. The intention-to-treat population will be defined as all patients included in the study, treated (patients who received complete/partial neoadjuvant treatment) and not treated (patients who did not undergo CRT), eligible (*i.e.,* all patients who were included in the study without violation of a major inclusion or exclusion criterion) or not, and with/without baseline stool sample. The per-protocol population will include all eligible patients, treated (complete or partial CRT), and with baseline stool sample.

Statistical analyses

Qualitative variables will be described by frequencies and percentages, and quantitative variables with means, standard deviations, medians, and ranges. No imputation method will be used in case of missing data. Correlations between qualitative variables will be assessed using the Chi-2 or Fisher-exact test. Quantitative variables will be compared using the Student's *t*-test or the Kruskal-Wallis test. Comparison of quantitative variables at different times (before and after CRT) will be assessed using the Wilcoxon test for matched samples. The relative risk of poor response to neoadjuvant CRT in CoPEC-colonized patients (or colonized by other bacteria) compared to non-colonized patients will be estimated using a logistic regression (univariate analysis) and will be presented with the 95% confidence interval (95% CI). Survival analyses will be performed using the Kaplan-Meier method and survival distributions compared with the log rank test. HRs and their 95% CI will be estimated with a Cox proportional risk model. A detailed statistical analysis plan (SAP) will be written before the database is locked for analysis; supplementary subgroup analyses, if appropriated, will be specified in the SAP. All analyses will be performed using the Stata version 16 software (StataCorp LP, College Station, TX).

Patient and public involvement

There was no patient or public involvement in the design of this study.

Discussion

The implication of intestinal microbiota in CRC has been widely demonstrated (42). Several recent studies suggest that different bacterial species, including CoPEC, could be used as biological biomarkers for CRC diagnosis and prognosis (29,36,41,43,44). The potential role of the gut microbiota in the modulation of the efficacy of anti-tumour treatments has been studied, with interesting results regarding chemotherapy and immunotherapy (37). However, these studies were focused on colon cancer dysbiosis and few data are available on rectal cancer and mucosa. Moreover, the correlation between gut microbiota homeostasis and radiation sensitivity remains unclear. Patients treated by pelvic radiation develop long-term complications that affect their quality of life, and have worse functional results than patients treated with surgery alone (45,46). It has been hypothesized that the intestinal microbiota has a significant impact on pelvic enteropathy (47); however, pelvic irradiation is responsible for microbiota dysbiosis (48,49). To our knowledge, no previous study has assessed the local microbiota composition and its implication in the response to CRT in rectal cancer, although treatment response is one of the key points for prognosis estimation. Biomarkers to predict tumour response in rectal cancer are still crucially needed. Imaging techniques (50) and biological markers (51,52) have been evaluated, but they are often expensive and complicated to implement. Moreover, the results are still discussed. Currently, their use seems to be limited to research and expert centres. The present study will describe the intestinal microbiota composition in patients with rectal cancer receiving neoadjuvant CRT to show its potential correlation with the tumour response, focusing on CoPEC colonization. In addition, the effect of radiotherapy on the local intestinal microbiota composition will be studied by comparing stool samples collected before and after CRT. Unlike studies on the intestinal microbiota in colon cancer in which tumour fragments are needed, in the case of mid or low rectal cancer stool samples should be representative of the local microbiota.

One of the main hypotheses to explain CoPEC effect on CRT response is based on their capacity to induce DNA damage (25–27). Besides the direct effect on the cell, radiotherapy is also cytotoxic through the production of reactive oxygen species and reactive nitrogen species (53). Chronic genotoxic stress caused by CoPEC presence in gut mucosa could lead to an adaptation of the gut mucosa to genotoxic

agents and consequently to reduced radiation sensitivity and resistance to therapy. For instance, in an *in vitro* study, Wilson et al. observed less DNA damage in colibactin-positive epithelial cells infected by CoPEC (27). Moreover, radiation sensitivity is closely linked to autophagy regulation (54,55). Recent studies showed the involvement of gut microbiota in autophagy regulation, with a link to chemoresistance (56). Ionizing radiation effects might be modified indirectly through autophagy deregulation induced by gut microbiota. In addition, radiotherapy cytotoxic effect could result in a modification of the local microenvironment with significant clinical consequences (57).

The modulation of radiotherapy efficacy by the intestinal microbiota is an emerging concept in CRC, but its study faces many obstacles, especially sample availability. In this study, we want to develop a non-invasive reproducible faecal test that could become a key biomarker to predict tumour response to CRT. Our work will help clinicians to tailor neoadjuvant therapeutic strategies with the final goal of increasing tumor response, organ preservation, and reducing surgical morbidity, while maintaining oncological safety.

Ethics and dissemination

The study protocol (version 3.0, dated on September 24th, 2019) was approved by the local ethics committee (Comité de Protection des Personnes Sud-Est II, December 18th, 2019, Reference number 2019-A02493-54) and the institutional review board COMERE. The French National Drug Agency Authority (ANSM) was informed. The study was registered on Clinicaltrials.gov, identifier NCT04103567.

All patients will be informed of the study objectives and procedures by the investigators before enrolment. A signed informed consent will be obtained from all patients before their inclusion in the study and before any study procedure is performed. All patients may end their participation in the study at any time, for whatever reason, without any consequence or prejudice concerning their care. Study participants will be able to request global results from investigators as soon as study results become available.

In the event of substantial modification, the request will be sent by the sponsor to the ethics committee for an opinion. Upon receipt of the favourable opinion, the sponsor will send the amended version of the protocol to all investigators.

The study will be conducted in accordance with the current French and European Regulatory requirements, including regulations on biomedical research from the Public Health Code, the bioethics and data protection laws and decrees, the French Jardé's law on research implicating human beings, the Good Clinical Practice, and the Helsinki Declaration.

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Authors' contributions

GC, CT, JG, CF, MJ, DP, GR, CC, PEC, PR, and MB wrote the protocol. MJ, GC, PR, CT and CF conducted statistical trial planning. PR, CT and CF handled ethics and regulatory affairs. GC, CT, HF, MB, CF, NB, PR, and MJ wrote the paper draft. GC, MB, MJ, PR, CT and CF contributed to the trial design and modifications. All authors read and approved the final manuscript.

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The funding body was not involved in the study design and will not be involved in data collection, data analysis and interpretation, and writing of the study report and publication.

Competing interests

The authors declare that they have no competing interests.

Provenance and peer review

Not commissioned; externally peer reviewed.

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Figure legends

Figure 1: MICARE flow diagram

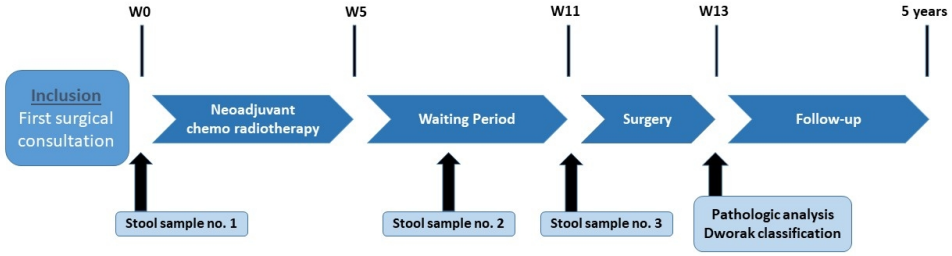

Figure 1 : MICARE flow diagram

Figure 1 : MICARE flow diagram

338x190mm (96 x 96 DPI)

SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	p1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	p3
	2b	All items from the World Health Organization Trial Registration Data Set	Protocol**
Protocol version	3	Date and version identifier	p16, paragraph1
Funding	4	Sources and types of financial, material, and other support	*Funding*, p17
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	p17
	5b	Name and contact information for the trial sponsor	p7, paragraph1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	p7, p17
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	p7, p11-12

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	p3-5
	6b	Explanation for choice of comparators	NA
Objectives	7	Specific objectives or hypotheses	*Objectives*, p5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	*Study design*, p5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	*Study design*, p5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	*Patients' selection*, p6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	*Clinical study procedures*, p7-9
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	p8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	NA
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	p8
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	p11

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	p7-8, Table1, Fig1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	*Sample size*, p12-13
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	p12-13
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	*Data collection and management*, p11

	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	NA
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	p11-12
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	p13
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	*Statistical analyses*, p13
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	*Statistical analyses*, p13
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	*Study monitoring, quality control, and* audit, p12
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	*Safety*, p9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	*Study monitoring, quality control, and* audit, p12
Ethics and dissemination			

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	*Ethics approval and consent to participate, p16*
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	p16, paragraph3
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	p16
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	p12, paragraph1
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	*Competing interests*, p16-17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	*Availability of data and materials*, p16
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	*Dissemination policy*, p13-14
	31b	Authorship eligibility guidelines and any intended use of professional writers	Protocol**
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	*Availability of data and materials*, p16

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Applicable **
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	*Sample handling*, p10

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

**More information can be provided if wished by the editor.

BMJ Open

Determination of biomarkers associated with neoadjuvant treatment response focusing on colibactin-producing Escherichia coli in patients with mid or low rectal cancer: a prospective cohort study protocol (MICARE)

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Secondary Subject Heading:	Gastroenterology and hepatology
Keywords:	ONCOLOGY, GASTROENTEROLOGY, RADIOTHERAPY, MICROBIOLOGY

Protocol

Determination of biomarkers associated with neoadjuvant treatment response focusing on colibactin-producing *Escherichia coli* in patients with mid or low rectal cancer : a prospective cohort study protocol (MICARE).

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Abstract

Introduction The management of mid and low rectal cancer is based on neoadjuvant chemoradiotherapy (CRT) followed by standardized surgery. There is no biomarker in rectal cancer to aid clinicians in foreseeing treatment response. The determination of factors associated with treatment response might allow the identification of patients who require tailored strategies (e.g. therapeutic de-escalation or intensification). Colibactin-producing *Escherichia coli* (CoPEC) has been associated with aggressive CRC and could be a poor prognostic factor. Currently no study has evaluated the potential association between intestinal microbiota composition and tumour response to CRT in mid and low rectal cancer. The aim of this study is to assess the association between response to neoadjuvant CRT and faecal intestinal microbiota composition and/or CoPEC prevalence in patients with mid or low rectal cancer.

Methods and analysis This is a non-randomized bicentric prospective cohort study with a recruitment capacity of 200 patients. Three stool samples will be collected from participants with histological-proven adenocarcinome of mid or low rectum who meet eligibility criteria of the study protocol: one before neoadjuvant treatment start, one in the period between CRT end and surgery, and one the day before surgery. In each sample, CoPEC will be detected by culture in special media and molecular (PCR) approaches. The global microbiota composition will be also assessed by the bacterial 16S rRNA gene sequencing. Neoadjuvant CRT response and tumour regression grade will be described using the Dworak system at pathological examination. Clinical data and survival outcomes will also be collected and investigated.

Ethics and dissemination MICARE was approved by the local ethics committee (Comité de Protection des Personnes Sud-Est II, December 18th, 2019. Reference number 2019-A02493-54) and the institutional review board. Patients will be required to provide written informed consent. Results will be published in a peer reviewed journal.

Trial registration number NCT04103567.

Strengths and limitations of this study

- As far as we know, this is the first study to evaluate association between intestinal microbiota composition and tumour response to chemoradiotherapy in mid and low rectal cancer
- MICARE is a prospective cohort study including 200 patients
- This study is based on a non–invasive and reproductible faecal test
- Tumour response will be described at pathological examination after surgery
- The limitation of this study will include population stratification for delay between radiotherapy and surgery, and adjonction of neoadjuvant chemotherapy in tumour response evaluation

Introduction

With more than 700,000 new cases and 300,000 deaths in 2018, rectal cancer is the eighth leading cause of cancer deaths worldwide (1). The initial management of mid and low rectal cancer is based on neoadjuvant chemoradiotherapy (CRT) for locally advanced tumours. This is associated with a significant decrease of the locoregional recurrence rate, but without survival improvement (2–4). Neoadjuvant treatment is followed by standardized surgery (5). Total mesorectal excision is crucial for reducing tumour recurrence (6), but its significant morbidity can affect the patients' quality of life. Prognosis also depends on the tumour response to neoadjuvant CRT. Currently, the surgical strategy is adapted in function of the tumour response to neoadjuvant treatment, assessed by magnetic resonance imaging (MRI) after CRT end (7). Indeed, the objective is therapeutic de-escalation with rectal preservation to decrease morbidity and functional disorders. For patients with complete response (up to 25% of patients), careful monitoring without surgery ("watch and wait" strategy) has been proposed (8,9). For small tumours with good response to CRT, transanal excision with rectal

preservation seems to be feasible in terms of cancer prognosis (10). For patients with large tumours or a locally advanced disease, a tailored treatment strategy with total neoadjuvant therapy (TNT) is now a gold standard (11,12). After surgical excision, the tumour response is classified in five pathologic tumour response grades, according to the Dworak classification, on the basis of the pathology findings (13). Recent studies reported up to 30% of poor responders (grades 0 and 1) (14,15). These data emphasize the importance of the initial tumour staging and response to neoadjuvant CRT for tailoring surgical strategies. MRI is an essential tool for these two assessments (16–18). These data highlight the need of response predictive models to adapt the TNT in mid and low rectal cancer.

Gut microbiota behaves as a real organ and participates in intestinal homeostasis. An imbalance in its composition (dysbiosis) could be involved in many pathologies, including colorectal cancer (CRC) (19–21). *Escherichia coli* (E. coli) has been widely described as a bacteria which could be involved in CRC.(22,23). *E. coli* is the predominant aero-anaerobic Gram-negative specie in human colon, but it is also a pathogen involved in various intestinal diseases (24). Indeed, some *E. coli* strains have acquired the capacity to produce toxins named cyclomodulins, including colibactin that is encoded by the pks island(25). Colibactin-producing *E. coli* (CoPEC) has genotoxic effects by inducing DNA damage and chromosomal instability (25–27). CoPEC implication in CRC has been demonstrated, particularly in aggressive forms (28–34). Specifically, higher *E. coli* colonization rate and higher prevalence of CoPEC are found in patients with TNM stage III or IV tumors (29) (UICC TNM Classification, 8th Edition, 2017) (35). Moreover, CoPEC gut colonization might contribute to modulate the immunotherapy efficacy (36). Recent clinical studies discussed the prognostic role of intestinal microbiota in the tumour response following surgery and chemotherapy or immunotherapy (37), and suggested that it could be used as a biomarker to predict tumour response to neoadjuvant treatments. On the other hand, very few clinical studies have assessed the influence of gut microbiota on radiotherapy efficacy, especially in rectal cancer. Recently, a preclinical study showed that mice which survive a high dose of radiation, harboured gut microbiota enriched with *Lachnospiraceae* and *Enterococcaceae* (38). Yet, a description of the intestinal microbiota composition before neoadjuvant therapy could allow identifying predictive bacterial markers of tumour response in rectal cancer, and to adapt TNT.

Indeed, chronic exposure of the gastrointestinal tract to genotoxins could be a prognostic marker of radiotherapy response. CoPEC colonization would start at the very beginning of life (38) and might lead to exposure of the intestinal mucosa to chronic genotoxic stress. The resulting damage could give cells the ability to resist to other genotoxic stresses, such as radiation therapy. One *in vitro* study already showed the decreased radiation sensitivity of cells incubated by colibactin (27). Therefore, developing a non-invasive method to analyse gut microbiota composition and to evaluate CoPEC implication in the response to CRT could help clinicians to tailor cancer management and to develop tools to control the pathologic microorganisms identified as new therapeutic targets.

Methods and analysis

This study protocol is written in accordance with the SPIRIT guidelines. (Supplementary file 1)

Objectives

Primary objective

The study primary objective is to assess the correlation between response to neoadjuvant CRT and CoPEC presence in stool samples.

Secondary objectives

- To analyse in a non-targeted manner the global microbiota composition before CRT and to evaluate the correlation between composition and response to treatment
- To study the modulation of the intestinal microbiota by CRT
- To describe the correlation between clinical data and microbiota composition modulation induced by CRT
- To determine microbiological prognostic factors of overall survival, disease-specific survival and relapse-free survival (locoregional and metastatic) in patients with low or mid rectum cancer

- To create a microbiological database for future mechanistic analyses
- To study the modulation of CoPEC colonization by CRT

Study design

The study is a non-randomized bicentric prospective cohort study. Two surgical teams will be involved - Institut du Cancer de Montpellier and CHU de Clermont-Ferrand ; and an INSERM Unit – M2iSH Clermont-Ferrand. The study actually started on January 2020 and the estimated study completion date is November 2027.

Patients' selection

Inclusion criteria

- Histologically-proven adenocarcinoma of low or mid rectum, of stage II or III (UICC TNM Classification, 8th Edition, 2017 (35))
- Patient eligible for neoadjuvant treatment (50 Gray radiation and capecitabine, CAP 50), according to the French national recommendations (5,39)
- Informed signed consent received
- Man or woman aged ≥18 years
- Appropriate contraceptive measures taken by men and pre-menopausal women before study entry and for at least 8 weeks after the last CRT cycle. Patients should be informed by the investigator on the contraceptive measures to use.

Exclusion criteria

- Antibiotic treatment at the time of stool sampling or in the month before.
- Presence of a derivative stoma
- Previous chemotherapy treatment for rectum cancer
- Patient not affiliated to the French social security system
- Patient with possible poor treatment compliance for psychologic, familial, social and geographic reasons

- Legal incapacity or limited legal capacity
- Pelvic radiotherapy or brachytherapy in the year before inclusion in the study
- History of other cancers in the 5 last years, except for cervical carcinoma *in situ* and skin carcinoma, but including melanoma under treatment
- Pregnant or breastfeeding woman

Study sponsor

The sponsor (Montpellier Cancer Institute, ICM) is responsible for the study design and management, and for obtaining all study authorizations (Persons Protection Committee, National Agency for Medical Security). It will also declare to these authorities the inclusion period beginning and end, produce the final study report, inform the competent authorities of the trial results, and store all study-related documents for at least 15 years after the study end.

Clinical study procedures

Inclusion in the study

The study flow diagram is presented in Figure 1.

Before study entry, all patients will receive exhaustive explanations on the study aims and procedures. A signed informed consent will be obtained from all patients before any study procedure (Supplementary file 2). At baseline, demographic (sex, age), clinical (performance status, weight, height, medical history, initial diagnosis date, tumour localization, histologic type) and biological (complete blood count, carcinoembryonic antigen (CEA) level) data will be collected (Table 1). Patients will undergo rectal examination and tumour staging by computed tomography (CT), rectal MRI, and possibly rectal endoscopic ultrasound examination (depending on the centre decision).

During the surgical consultation, the first stool sample (stool sample N°1) may be collected during rectal examination (faeces left on the clinician's glove), or by proctoscopy. Otherwise, the stool sample will be collected by the patient.

Table 1: Flow chart with the clinical and radiological evaluations

Assessment	Baseline	Re-evaluation	Day before surgery	Follow-up Every 6 – 8 months
Informed consent	X			
Selection criteria validation	X			
Demographic and clinical data	X			
Physical examination	X			
Patient inclusion	X			
Stool sample	X	X	X	
Patient vital status				X
Tumour evaluation				
Rectal MRI	X	X		X
CT	X			X
Rectal examination	X	X		X

MRI: Magnetic resonance imaging; CT: computed tomography.

Neoadjuvant treatment

Patients will undergo neoadjuvant CRT in accordance with the French national guidelines (5). The recommended regimen is a concomitant oral chemotherapy (5-FU/CAPECITABINE) and 50 Grey radiotherapy. Despite PRODIGE 23 and RAPIDO trials, it is highly recommended to add a systemic chemotherapy (FOLFIRINOX or FOLFOX) to the RCT in locally advanced rectal cancer (12). CRT data (dose, possible dose modifications or interruptions) and CRT complications will be recorded.

Re-evaluation

During the consultation after CRT end and before surgery, a second stool sample (stool sample N°2) will be collected, as described for the baseline sample. If the patient has received antibiotics in the month before this consultation, stool sampling will not be performed.

This second consultation will include MRI examination as during the baseline visit. The tumour response will described precisely with emphasis on the tumour regression grade according to the MERCURY experience (7).

Surgery

Surgical data (surgery type, digestive reconstruction or stoma, and surgical outcomes), anatomopathological data (histologic type, ypTN grade, Dworak grade (13), Quirke classification (40), circumferential resection, distal margins, and extramucosal vascular invasion) and biological data (RAS and BRAF mutational status, if available) will be collected. The day before surgery, before bowel mechanical preparation, the third stool sample (stool sample N°3) will be collected in hospital, as described for the previous samples. If the patient received antibiotics in the month before hospitalization, stool sampling will not be performed.

Pathologic analysis

To meet the primary objective, the pathologic analysis of the surgical specimens will describe the tumour regression grade according to the Dworak classification (13) (Table 2). Patients with grade 0 and 1 tumours will be considered poor responders, in accordance with the literature.

Table 2: Tumour Regression Grade (TRG), Dworak classification (13)

TRG	Pathology
Grade 0	No regression
Grade 1	Dominant tumour mass with obvious fibrosis and/or vasculopathy
Grade 2	Dominant fibrotic changes with few tumour cell groups (easy to find)
Grade 3	Very few (difficult to find microscopically) tumour cells in fibrotic tissue with or without mucous substance
Grade 4	No tumour cell, only fibrotic mass (total regression or response)

Safety

All adverse events will be reported following the study sponsor's pharmacovigilance procedures, and in accordance with the applicable regulation (Supplementary file 3).

Follow-up and study duration

Follow-up will last 5 years from the date of surgery. The frequency of follow-up visits will be decided at each centre. Every 6 to 8 months, the disease and survival status will be assessed. Recurrence will be investigated by clinical examination with rectal MRI and CT and a tumour marker test (CEA) (Table 1). Locoregional or metastatic relapse will be reported in the case report form with the date of relapse diagnosis.

As the inclusion period will be of 36 months and the follow-up will last 5 years, the total study duration will be of 8 years.

Microbiological analyses

Sample handling

Three stool samples will be collected during the study (Figure 1): i) one at patient inclusion, before any treatment, to describe the baseline intestinal microbiota composition; ii) one during the interval between the end of neoadjuvant CRT and surgery, at the surgical consultation for tumour reappraisal; and iii) one just before bowel preparation (mechanical or antibiotics) for surgery.

Each sample will be divided into two cryotubes: one empty and one with 15% glycerol/DMEM to preserve cell integrity. Samples will be immediately stored at -80°C until transport to the M2iSH laboratory, Clermont-Ferrand, France, which will be in charge of the molecular analysis and storage of the samples.

E. coli strain identification and CoPEC detection

All microbiological analyses will be performed as previously described (28). After thawing, samples stored in DMEM/glycerol will be crushed and diluted in sterile phosphate buffered saline pH 7.4 before plating on TBX agar and chromogenic agar chromID CPS3® plates (bioMérieux) to allow the identification and quantitation of enterobacteria. Colonies (around 48 per sample) will be collected for molecular typing, and their identification will be confirmed with the automated Vitek® II (bioMérieux) system. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR will be used as genotyping method to determine the number of *E. coli* strains per sample (28).

E. coli harbouring the colibactin-encoding *pks* island will be identified by PCR analysis of each *E.coli* isolate (41). This will allow identifying the presence of CoPEC (primary objective).

Untargeted analysis of the local microbiota composition

Global microbiota modifications will be assessed by high-throughput sequencing of the bacterial 16S rRNA gene in DNA extracted from the three stool samples using the NucleoSpin® DNA stool kit (Macherey-Nagel, Hoerdt, France), according to the manufacturer's instructions. Quantitative PCR will be performed to quantify pro-carcinogenic bacterial species, such as *Fusobacterium nucleatum, Enterococcus feacalis, bft*-positive *Bacteroides fragilis,* and CoPEC. In addition, the V4 region of the bacterial 16S rRNA gene will be amplified using the 515F/806R primer pair followed by Illumina high throughput sequencing on a MiSeq® apparatus, according to the manufacturer's guidelines. A

global description of the intestinal microbiota could also be obtained by shotgun metagenomic sequencing to access the microbiota functional features after selection of the more informative samples.

Endpoints

Primary endpoint

The primary endpoint (associated with the primary objective) is the relative risk (RR) of poor response to neoadjuvant CRT in patients colonized by CoPEC ("exposed") compared to non-colonized patients ("unexposed").

Secondary endpoints

- Prevalence and CoPEC colonization rate before and after CRT
- Other bacterial strains present before CRT and relative risk of poor response to CRT in colonized and non-colonized patients
- Type, prevalence, and colonization rate of bacteria other than CoPEC in the microbiota, before and after CRT
- Percentage of colonized patients, depending on the bacterial type, according to the clinical parameters (age, sex, body mass index)
- Hazard ratio (HR) for overall survival, disease-specific survival, and relapse-free survival (locoregional or metastatic) in colonized patients, for the different bacterial types, according to the overall bacterial composition (including CoPEC), and in non-colonized patients.

Data collection and management

The database will be managed by the sponsor, and data stored at the Data processing centre, Biometrics Unit of the Montpellier Cancer Institute. Case report form design and clinical data management will be implemented using the Ennov Clinical® software. Microbiological data will be

collected in a database first stored at the M2iSH laboratory, and then transferred to the sponsor database for analysis. Data and any trial documents will be made available upon reasonable request and after signature of a data access agreement.

In accordance with the General Data Protection Regulation (GDPR), a registration number will be used to identify each patient. The corresponding table will be encrypted and stored in a secure place. Special vigilance will be exercised throughout the study to maintain data anonymization.

Study monitoring, quality control, and audit

According to the sponsor's risk-based monitoring plan (study participants, logistics, resources, impact), the collection of the patient informed consents and the respect of the study protocol and procedures will be monitored.

To guarantee the originality of all data and in accordance with the Good Clinical Practices, quality control will be performed by the sponsor. The study will be managed according to the sponsor procedures and in respect of the protocol, and the quality of the data included in the report forms will be checked.

The sponsor may wish to conduct an audit at some investigating centers. Audits may be conducted by the sponsor or any duly authorized person for at least 15 years after the trial.

Statistical considerations

Sample size

The recruitment capacity for this exploratory study will be around 200 patients. For a mean rate of 30% of poor responders to the neoadjuvant treatment among the patients not colonized by CoPEC (*i.e.*, a proportion of response P2=0.30 among unexposed patients), the study will be able to estimate a relative risk of 1.7 (RR=1.7) with a 30% precision and a confidence interval at 95% (α=0.05). Patients in whom the CoPEC colonization status cannot be determined at baseline, in whom CRT must be prematurely arrested, or who cannot undergo surgery will be considered non-evaluable.

Considering a 10% rate of potentially non-evaluable patients, a total of 220 patients (20 supplementary patients) will be included in the study.

Study population

Two populations will be defined for the analysis. The intention-to-treat population will be defined as all patients included in the study, treated (patients who received complete/partial neoadjuvant treatment) and not treated (patients who did not undergo CRT), eligible (*i.e.,* all patients who were included in the study without violation of a major inclusion or exclusion criterion) or not, and with/without baseline stool sample. The per-protocol population will include all eligible patients, treated (complete or partial CRT), and with baseline stool sample.

Statistical analyses

Qualitative variables will be described by frequencies and percentages, and quantitative variables with means, standard deviations, medians, and ranges. No imputation method will be used in case of missing data. Correlations between qualitative variables will be assessed using the Chi-2 or Fisher-exact test. Quantitative variables will be compared using the Student's *t*-test or the Kruskal-Wallis test. Comparison of quantitative variables at different times (before and after CRT) will be assessed using the Wilcoxon test for matched samples. The relative risk of poor response to neoadjuvant CRT in CoPEC-colonized patients (or colonized by other bacteria) compared to non-colonized patients will be estimated using a logistic regression (univariate analysis) and will be presented with the 95% confidence interval (95% CI). Survival analyses will be performed using the Kaplan-Meier method and survival distributions compared with the log rank test. HRs and their 95% CI will be estimated with a Cox proportional risk model. A detailed statistical analysis plan (SAP) will be written before the database is locked for analysis; supplementary subgroup analyses, if appropriated, will be specified in the SAP. All analyses will be performed using the Stata version 16 software (StataCorp LP, College Station, TX).

Patient and public involvement

There was no patient or public involvement in the design of this study.

Discussion

The implication of intestinal microbiota in CRC has been widely demonstrated (42). Several recent studies suggest that different bacterial species, including CoPEC, could be used as biological biomarkers for CRC diagnosis and prognosis (29,36,41,43,44). The potential role of the gut microbiota in the modulation of the efficacy of anti-tumour treatments has been studied, with interesting results regarding chemotherapy and immunotherapy (37). However, these studies were focused on colon cancer dysbiosis and few data are available on rectal cancer and mucosa. Moreover, the correlation between gut microbiota homeostasis and radiation sensitivity remains unclear. Patients treated by pelvic radiation develop long-term complications that affect their quality of life, and have worse functional results than patients treated with surgery alone (45,46). It has been hypothesized that the intestinal microbiota has a significant impact on pelvic enteropathy (47); however, pelvic irradiation is responsible for microbiota dysbiosis (48,49). To our knowledge, no previous study has assessed the local microbiota composition and its implication in the response to CRT in rectal cancer, although treatment response is one of the key points for prognosis estimation. Biomarkers to predict tumour response in rectal cancer are still crucially needed. Imaging techniques (50) and biological markers (51,52) have been evaluated, but they are often expensive and complicated to implement. Moreover, the results are still discussed. Currently, their use seems to be limited to research and expert centers. The present study will describe the intestinal microbiota composition in patients with rectal cancer receiving neoadjuvant CRT to show its potential correlation with the tumour response, focusing on CoPEC colonization. In addition, the effect of radiotherapy on the local intestinal microbiota composition will be studied by comparing stool samples collected before and after CRT. Unlike

studies on the intestinal microbiota in colon cancer in which tumour fragments are needed, in the case of mid or low rectal cancer stool samples should be representative of the local microbiota.

One of the main hypotheses to explain CoPEC effect on CRT response is based on their capacity to induce DNA damage (25–27). Besides the direct effect on the cell, radiotherapy is also cytotoxic through the production of reactive oxygen species and reactive nitrogen species (53). Chronic genotoxic stress caused by CoPEC presence in gut mucosa could lead to an adaptation of the gut mucosa to genotoxic agents and consequently to reduce radiation sensitivity and resistance to therapy. For instance, in an *in vitro* study, Wilson et al. observed less DNA damage in colibactin-positive epithelial cells infected by CoPEC (27). Moreover, radiation sensitivity is closely linked to autophagy regulation (54,55). Recent studies showed the involvement of gut microbiota in autophagy regulation, with a link to chemoresistance (56). Ionizing radiation effects might be modified indirectly through autophagy deregulation induced by gut microbiota. In addition, radiotherapy cytotoxic effect could result in a modification of the local microenvironment with significant clinical consequences (57).

The modulation of radiotherapy efficacy by the intestinal microbiota is an emerging concept in CRC, but its study faces many obstacles, especially sample availability. In this study, we want to develop a non-invasive reproducible faecal test that could become a key biomarker to predict tumour response to CRT. Our work will help clinicians to tailor neoadjuvant therapeutic strategies with the final goal of increasing tumor response, organ preservation, and reducing surgical morbidity, while maintaining oncological safety.

Ethics and dissemination

The study protocol (version 3.0, dated on September 24th, 2019) was approved by the local ethics committee (Comité de Protection des Personnes Sud-Est II, December 18th, 2019, Reference number 2019-A02493-54) and the institutional review board COMERE. The French National Drug Agency Authority (ANSM) was informed. The study was registered on Clinicaltrials.gov, identifier NCT04103567.

All patients will be informed of the study objectives and procedures by the investigators before enrolment. A signed informed consent will be obtained from all patients before their inclusion in the study and before any study procedure is performed. All patients may end their participation in the study at any time, for whatever reason, without any consequence or prejudice concerning their care. Study participants will be able to request global results from investigators as soon as study results become available.

In the event of substantial modification, the request will be sent by the sponsor to the ethics committee for an opinion. Upon receipt of the favourable opinion, the sponsor will send the amended version of the protocol to all investigators.

The study will be conducted in accordance with the current French and European Regulatory requirements, including regulations on biomedical research from the Public Health Code, the bioethics and data protection laws and decrees, the French Jardé's law on research implicating human beings, the Good Clinical Practice, and the Helsinki Declaration.

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Authors' contributions

GC, CT, JG, CF, MJ, GR, CC, PEC, PR, and MB wrote the protocol. MJ, GC, PR, CT and CF conducted statistical trial planning. PR, CT and CF handled ethics and regulatory affairs. GC, CT, HF, MB, CF, NB, PR, and MJ wrote the paper draft. GC, MB, MJ, PR, CT and CF contributed to the trial design and modifications. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Provenance and peer review

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Figure legends

Figure 1: MICARE flow diagram

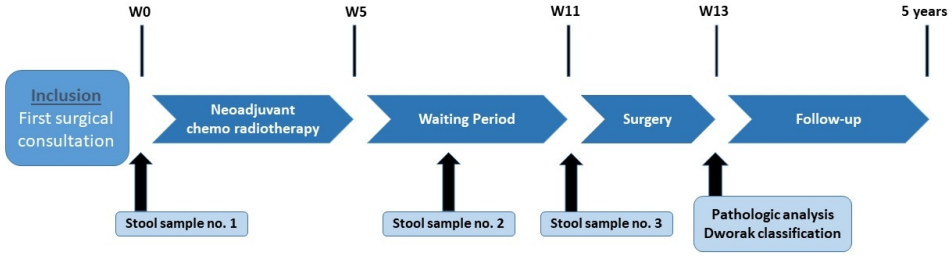

Figure 1 : MICARE flow diagram

Figure 1 : MICARE flow diagram

338x190mm (96 x 96 DPI)

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	p1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	p3
	2b	All items from the World Health Organization Trial Registration Data Set	Protocol**
Protocol version	3	Date and version identifier	p16, paragraph1
Funding	4	Sources and types of financial, material, and other support	*Funding*, p17
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	p17
	5b	Name and contact information for the trial sponsor	p7, paragraph1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	p7, p17
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	p7, p11-12

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	p3-5
	6b	Explanation for choice of comparators	NA
Objectives	7	Specific objectives or hypotheses	*Objectives*, p5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	*Study design*, p5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	*Study design*, p5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	*Patients' selection*, p6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	*Clinical study procedures*, p7-9
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	p8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	NA
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	p8
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	p11

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	p7-8, Table1, Fig1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	*Sample size*, p12-13
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	p12-13
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	*Data collection and management*, p11

	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	NA
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	p11-12
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	p13
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	*Statistical analyses*, p13
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	*Statistical analyses*, p13
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	*Study monitoring, quality control, and* audit, p12
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	*Safety*, p9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	*Study monitoring, quality control, and* audit, p12
Ethics and dissemination			

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	*Ethics approval and consent to participate, p16*
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	p16, paragraph3
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	p16
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	p12, paragraph1
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	*Competing interests*, p16-17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	*Availability of data and materials*, p16
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	*Dissemination policy*, p13-14
	31b	Authorship eligibility guidelines and any intended use of professional writers	Protocol**
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	*Availability of data and materials*, p16

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Applicable **
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	*Sample handling*, p10

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

**More information can be provided if wished by the editor.

Formulaire de consentement

Détermination de facteurs MIcrobiologiques associés à une mauvaise réponse au traitement néoadjuvant dans les CAncers du REctum : focus sur les Escherichia coli productrices de cyclomodulines
MICARE

Version 4.0 du 23/02/2021

Promoteur: Institut du Cancer de Montpellier ICM, Parc Euromédecine, 208 rue des Apothicaires, 34298 Montpellier Cedex 5

Coordonnateur de l'étude: Pr Philippe ROUANET Département de Chirurgie Oncologique, ICM

Je soussigné(e) :

Nom:…… **Prénom**:……

Date de naissance: I__I__I I__I__I I__I__I__I__I

certifie avoir lu et compris la note d'information version n°4.0 du 23/02/2021 qui m'a été remise et accepte de participer à cette recherche selon les conditions définies dans la note d'information.

J'ai bien compris que ma participation à la recherche était libre et volontaire, et que je pouvais refuser d'y participer sans avoir à me justifier, tout en continuant à bénéficier des meilleurs soins disponibles.

Je reconnais avoir pu poser toutes les questions souhaitées et avoir reçu des réponses satisfaisantes à mes questions.

Je reconnais en particulier que le droit à me faire assister par une personne de mon choix m'a été communiqué.

Je reconnais avoir disposé d'un temps de réflexion suffisant entre ces informations et le présent consentement et avoir eu si je le souhaitais l'opportunité d'en discuter avec mon médecin ou mes proches.

Les conditions de ma participation, notamment la durée de celle-ci, les contraintes, les objectifs, le déroulement de l'étude ainsi que les bénéfices et les risques éventuels, m'ont été expliqués clairement par le Dr/Pr……..

Je m'engage à suivre les contraintes expliquées dans le document d'information, à la fois pour minimiser les risques et pour la bonne réalisation de l'étude. Ma participation à l'étude pourrait être suspendue si je ne respectais pas le protocole.

J'ai compris également que je pouvais à tout moment interrompre ma participation à cette recherche, sans avoir à me justifier, sans aucun préjudice et en continuant à recevoir les meilleurs soins disponibles.
Dans ce cas, je m'engage à prévenir le médecin responsable de l'étude.

Je reconnais avoir été informé(e) que l'étude pouvait être interrompue à tout moment sur décision du promoteur ou des autorités de santé, et que toutes les mesures seraient prises dans ce cas pour assurer ma sécurité et la poursuite de ma prise en charge médicale.

J'ai bien compris que tout fait nouveau susceptible de remettre en cause mon consentement à ma participation à l'étude me serait communiqué.

J'ai bien noté que mon consentement ne dégageait pas les médecins et le promoteur de leurs responsabilités, et que je conservais tous les droits qui me sont garantis par la loi.

Formulaire de consentement

J'ai bien pris note que la lettre d'information et le consentement sont le fondement juridique pour le traitement des données dans le cadre de cette étude.

J'ai bien noté que, conformément aux dispositions de la loi relative à l'informatique, aux fichiers et aux libertés et au règlement européen 2016/679 sur la protection des données je dispose d'un droit d'accès de rectification, ainsi qu'un droit à l'effacement, à la limitation du traitement et à la portabilité des données (RGPD). Je dispose également d'un droit d'opposition à la transmission des données couvertes par le secret professionnel susceptibles d'être utilisées dans le cadre de cette recherche et d'être traitées.
J'ai bien note que, si je souhaite me retirer de l'étude, les données recueillies avant mon retrait ne pourront pas être supprimées. Par contre, aucune nouvelle donnée ne sera recueillie. Ces droits s'exercent auprès du médecin qui me suit dans le cadre de cette recherche et qui connaît mon identité.

J'ai pris connaissance que cette recherche a reçu l'avis favorable du Comité de Protection des Personnes de nom du CPP (catégories 1, 2 et 3) et l'information de l'ANSM.

Je reconnais avoir été informé(e) que le promoteur de l'étude, l'Institut régional du Cancer Montpellier a souscrit une assurance de responsabilité civile en cas de préjudice auprès de la société SHAM (contrat n° 140474).

J'autorise dans la mesure où elles sont indispensables aux fins de la recherche, l'enregistrement de données personnelles me concernant. Je sais que le promoteur s'engage à ce que ces données soient rendues confidentielles par un codage sans mention du nom et du prénom.

J'ai bien noté que j'ai le droit d'être informé(e) des résultats globaux de cette recherche selon les modalités qui ont été précisées dans le document d'information.

J'atteste être affilié(e) ou bénéficiaire d'un régime français d'assurance maladie (sécurité sociale), condition obligatoire pour pouvoir être inclus dans la recherche.

J'accepte que les prélèvements biologiques et les données associées soient traités, collectés et conservés dans une collection spécifique de l'étude et utilisés à des fins de recherche.

Je suis informé(e) de la possibilité qu'une partie des prélèvements effectués à l'occasion de ce protocole de recherche soit conservée pour une utilisation ultérieure à des fins de recherche. J'ai également été informé(e) de mon droit à m'opposer à cette conservation et l'utilisation.

☐	J'accepte que mes données cliniques soient utilisées pour des recherches ultérieures, en France ou dans l'Union Européenne
☐	J'accepte que mes prélèvements soient utilisés pour des recherches ultérieures sur le cancer, en France ou dans l'Union Européenne, ayant la même finalité

Nom du patient :

Date :

Signature :

Nom de l'investigateur :

Date :

Signature :

Je reconnais qu'un des deux exemplaires de ce formulaire attestant mon consentement m'a été remis.

TREATMENT	EVENTS	YES/NO
CHEMO-RADIOTHERAPY	Proctitis	YES/NO
	Perineal skin toxicity	YES/NO
	Weakness	YES/NO
	Nausea	YES/NO
	Diarrhea	YES/NO
	Abdominal pain	YES/NO
	Hematologic toxicity	YES/NO
	Hand-foot syndrome	YES/NO
SURGERY	**Per-operative**	
	Bleeding	YES/NO
	Intestinal perforation	YES/NO
	Vascular wound	YES/NO
	Post-operative	
	Infection	YES/NO
	Anastomotic leakage	YES/NO
	Colon ischemia	YES/NO
	Bowel obstruction	YES/NO
	Bleeding	YES/NO
	Urinary dysfunction	YES/NO

BMJ Open

Determination of biomarkers associated with neoadjuvant treatment response focusing on colibactin-producing Escherichia coli in patients with mid or low rectal cancer: a prospective clinical study protocol (MICARE)

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Manuscript ID	bmjopen-2022-061527.R2
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Primary Subject Heading:	Oncology
Secondary Subject Heading:	Gastroenterology and hepatology
Keywords:	ONCOLOGY, GASTROENTEROLOGY, RADIOTHERAPY, MICROBIOLOGY

Protocol

Determination of biomarkers associated with neoadjuvant treatment response focusing on colibactin-producing *Escherichia coli* in patients with mid or low rectal cancer : a prospective clinical study protocol (MICARE).

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Abstract

Introduction The management of mid and low rectal cancer is based on neoadjuvant chemoradiotherapy (CRT) followed by standardized surgery. There is no biomarker in rectal cancer to aid clinicians in foreseeing treatment response. The determination of factors associated with treatment response might allow the identification of patients who require tailored strategies (e.g. therapeutic de-escalation or intensification). Colibactin-producing *Escherichia coli* (CoPEC) has been associated with aggressive CRC and could be a poor prognostic factor. Currently no study has evaluated the potential association between intestinal microbiota composition and tumour response to CRT in mid and low rectal cancer. The aim of this study is to assess the association between response to neoadjuvant CRT and faecal intestinal microbiota composition and/or CoPEC prevalence in patients with mid or low rectal cancer.

Methods and analysis This is a non-randomized bicentric prospective clinical study with a recruitment capacity of 200 patients. Three stool samples will be collected from participants with histological-proven adenocarcinome of mid or low rectum who meet eligibility criteria of the study protocol: one before neoadjuvant treatment start, one in the period between CRT end and surgery, and one the day before surgery. In each sample, CoPEC will be detected by culture in special media and molecular (PCR) approaches. The global microbiota composition will be also assessed by the bacterial 16S rRNA gene sequencing. Neoadjuvant CRT response and tumour regression grade will be described using the Dworak system at pathological examination. Clinical data and survival outcomes will also be collected and investigated.

Ethics and dissemination MICARE was approved by the local ethics committee (Comité de Protection des Personnes Sud-Est II, December 18th, 2019. Reference number 2019-A02493-54) and the institutional review board. Patients will be required to provide written informed consent. Results will be published in a peer reviewed journal.

Trial registration number NCT04103567.

Strengths and limitations of this study

- As far as we know, this is the first study to evaluate association between intestinal microbiota composition and tumour response to chemoradiotherapy in mid and low rectal cancer
- MICARE is a prospective clinical study including 200 patients
- This study is based on a non–invasive and reproductible faecal test
- Tumour response will be described at pathological examination after surgery
- The limitation of this study will include population stratification for delay between radiotherapy and surgery, and adjonction of neoadjuvant chemotherapy in tumour response evaluation

Introduction

With more than 700,000 new cases and 300,000 deaths in 2018, rectal cancer is the eighth leading cause of cancer deaths worldwide (1). The initial management of mid and low rectal cancer is based on neoadjuvant chemoradiotherapy (CRT) for locally advanced tumours. This is associated with a significant decrease of the locoregional recurrence rate, but without survival improvement (2–4). Neoadjuvant treatment is followed by standardized surgery (5). Total mesorectal excision is crucial for reducing tumour recurrence (6), but its significant morbidity can affect the patients' quality of life. Prognosis also depends on the tumour response to neoadjuvant CRT. Currently, the surgical strategy is adapted in function of the tumour response to neoadjuvant treatment, assessed by magnetic resonance imaging (MRI) after CRT end (7). Indeed, the objective is therapeutic de-escalation with rectal preservation to decrease morbidity and functional disorders. For patients with complete response (up to 25% of patients), careful monitoring without surgery ("watch and wait" strategy) has been proposed (8,9). For small tumours with good response to CRT, transanal excision with rectal

preservation seems to be feasible in terms of cancer prognosis (10). For patients with large tumours or a locally advanced disease, a tailored treatment strategy with total neoadjuvant therapy (TNT) is now a gold standard (11,12). After surgical excision, the tumour response is classified in five pathologic tumour response grades, according to the Dworak classification, on the basis of the pathology findings (13). Recent studies reported up to 30% of poor responders (grades 0 and 1) (14,15). These data emphasize the importance of the initial tumour staging and response to neoadjuvant CRT for tailoring surgical strategies. MRI is an essential tool for these two assessments (16–18). These data highlight the need of response predictive models to adapt the TNT in mid and low rectal cancer.

Gut microbiota behaves as a real organ and participates in intestinal homeostasis. An imbalance in its composition (dysbiosis) could be involved in many pathologies, including colorectal cancer (CRC) (19–21). *Escherichia coli* (E. coli) has been widely described as a bacteria which could be involved in CRC.(22,23). *E. coli* is the predominant aero-anaerobic Gram-negative specie in human colon, but it is also a pathogen involved in various intestinal diseases (24). Indeed, some *E. coli* strains have acquired the capacity to produce toxins named cyclomodulins, including colibactin that is encoded by the pks island(25). Colibactin-producing *E. coli* (CoPEC) has genotoxic effects by inducing DNA damage and chromosomal instability (25–27). CoPEC implication in CRC has been demonstrated, particularly in aggressive forms (28–34). Specifically, higher *E. coli* colonization rate and higher prevalence of CoPEC are found in patients with TNM stage III or IV tumors (29) (UICC TNM Classification, 8th Edition, 2017) (35). Moreover, CoPEC gut colonization might contribute to modulate the immunotherapy efficacy (36). Recent clinical studies discussed the prognostic role of intestinal microbiota in the tumour response following surgery and chemotherapy or immunotherapy (37), and suggested that it could be used as a biomarker to predict tumour response to neoadjuvant treatments. On the other hand, very few clinical studies have assessed the influence of gut microbiota on radiotherapy efficacy, especially in rectal cancer. Recently, a preclinical study showed that mice which survive a high dose of radiation, harboured gut microbiota enriched with *Lachnospiraceae* and *Enterococcaceae* (38). Yet, a description of the intestinal microbiota composition before neoadjuvant therapy could allow identifying predictive bacterial markers of tumour response in rectal cancer, and to adapt TNT.

Indeed, chronic exposure of the gastrointestinal tract to genotoxins could be a prognostic marker of radiotherapy response. CoPEC colonization would start at the very beginning of life (38) and might lead to exposure of the intestinal mucosa to chronic genotoxic stress. The resulting damage could give cells the ability to resist to other genotoxic stresses, such as radiation therapy. One *in vitro* study already showed the decreased radiation sensitivity of cells incubated by colibactin (27). Therefore, developing a non-invasive method to analyse gut microbiota composition and to evaluate CoPEC implication in the response to CRT could help clinicians to tailor cancer management and to develop tools to control the pathologic microorganisms identified as new therapeutic targets.

Methods and analysis

This study protocol is written in accordance with the SPIRIT guidelines. (Supplementary file 1)

Objectives

Primary objective

The study primary objective is to assess the correlation between response to neoadjuvant CRT and CoPEC presence in stool samples.

Secondary objectives

- To analyse in a non-targeted manner the global microbiota composition before CRT and to evaluate the correlation between composition and response to treatment
- To study the modulation of the intestinal microbiota by CRT
- To describe the correlation between clinical data and microbiota composition modulation induced by CRT
- To determine microbiological prognostic factors of overall survival, disease-specific survival and relapse-free survival (locoregional and metastatic) in patients with low or mid rectum cancer

- To create a microbiological database for future mechanistic analyses
- To study the modulation of CoPEC colonization by CRT

Study design

The study is a non-randomized bicentric prospective clinical study. Two surgical teams will be involved - Institut du Cancer de Montpellier and CHU de Clermont-Ferrand ; and an INSERM Unit – M2iSH Clermont-Ferrand. The study actually started on January 2020 and the estimated study completion date is November 2027.

Patients' selection

Inclusion criteria

- Histologically-proven adenocarcinoma of low or mid rectum, of stage II or III (UICC TNM Classification, 8th Edition, 2017 (35))
- Patient eligible for neoadjuvant treatment (50 Gray radiation and capecitabine, CAP 50), according to the French national recommendations (5,39)
- Informed signed consent received
- Man or woman aged ≥18 years
- Appropriate contraceptive measures taken by men and pre-menopausal women before study entry and for at least 8 weeks after the last CRT cycle. Patients should be informed by the investigator on the contraceptive measures to use.

Exclusion criteria

- Antibiotic treatment at the time of stool sampling or in the month before.
- Presence of a derivative stoma
- Previous chemotherapy treatment for rectum cancer
- Patient not affiliated to the French social security system
- Patient with possible poor treatment compliance for psychologic, familial, social and geographic reasons

- Legal incapacity or limited legal capacity
- Pelvic radiotherapy or brachytherapy in the year before inclusion in the study
- History of other cancers in the 5 last years, except for cervical carcinoma *in situ* and skin carcinoma, but including melanoma under treatment
- Pregnant or breastfeeding woman

Study sponsor

The sponsor (Montpellier Cancer Institute, ICM) is responsible for the study design and management, and for obtaining all study authorizations (Persons Protection Committee, National Agency for Medical Security). It will also declare to these authorities the inclusion period beginning and end, produce the final study report, inform the competent authorities of the trial results, and store all study-related documents for at least 15 years after the study end.

Clinical study procedures

Inclusion in the study

The study flow diagram is presented in Figure 1.

Before study entry, all patients will receive exhaustive explanations on the study aims and procedures. A signed informed consent will be obtained from all patients before any study procedure (Supplementary file 2). At baseline, demographic (sex, age), clinical (performance status, weight, height, medical history, initial diagnosis date, tumour localization, histologic type) and biological (complete blood count, carcinoembryonic antigen (CEA) level) data will be collected (Table 1). Patients will undergo rectal examination and tumour staging by computed tomography (CT), rectal MRI, and possibly rectal endoscopic ultrasound examination (depending on the centre decision).

During the surgical consultation, the first stool sample (stool sample N°1) may be collected during rectal examination (faeces left on the clinician's glove), or by proctoscopy. Otherwise, the stool sample will be collected by the patient.

Table 1: Flow chart with the clinical and radiological evaluations

Assessment	Baseline	Re-evaluation	Day before surgery	Follow-up Every 6 – 8 months
Informed consent	X			
Selection criteria validation	X			
Demographic and clinical data	X			
Physical examination	X			
Patient inclusion	X			
Stool sample	X	X	X	
Patient vital status				X
Tumour evaluation				
Rectal MRI	X	X		X
CT	X			X
Rectal examination	X	X		X

MRI: Magnetic resonance imaging; CT: computed tomography.

Neoadjuvant treatment

Patients will undergo neoadjuvant CRT in accordance with the French national guidelines (5). The recommended regimen is a concomitant oral chemotherapy (5-FU/CAPECITABINE) and 50 Grey radiotherapy. Despite PRODIGE 23 and RAPIDO trials, it is highly recommended to add a systemic chemotherapy (FOLFIRINOX or FOLFOX) to the RCT in locally advanced rectal cancer (12). CRT data (dose, possible dose modifications or interruptions) and CRT complications will be recorded.

Re-evaluation

During the consultation after CRT end and before surgery, a second stool sample (stool sample N°2) will be collected, as described for the baseline sample. If the patient has received antibiotics in the month before this consultation, stool sampling will not be performed.

This second consultation will include MRI examination as during the baseline visit. The tumour response will described precisely with emphasis on the tumour regression grade according to the MERCURY experience (7).

Surgery

Surgical data (surgery type, digestive reconstruction or stoma, and surgical outcomes), anatomopathological data (histologic type, ypTN grade, Dworak grade (13), Quirke classification (40), circumferential resection, distal margins, and extramucosal vascular invasion) and biological data (RAS and BRAF mutational status, if available) will be collected. The day before surgery, before bowel mechanical preparation, the third stool sample (stool sample N°3) will be collected in hospital, as described for the previous samples. If the patient received antibiotics in the month before hospitalization, stool sampling will not be performed.

Pathologic analysis

To meet the primary objective, the pathologic analysis of the surgical specimens will describe the tumour regression grade according to the Dworak classification (13) (Table 2). Patients with grade 0 and 1 tumours will be considered poor responders, in accordance with the literature.

Table 2: Tumour Regression Grade (TRG), Dworak classification (13)

TRG	Pathology
Grade 0	No regression
Grade 1	Dominant tumour mass with obvious fibrosis and/or vasculopathy
Grade 2	Dominant fibrotic changes with few tumour cell groups (easy to find)
Grade 3	Very few (difficult to find microscopically) tumour cells in fibrotic tissue with or without mucous substance
Grade 4	No tumour cell, only fibrotic mass (total regression or response)

Safety

All adverse events will be reported following the study sponsor's pharmacovigilance procedures, and in accordance with the applicable regulation (Supplementary file 3).

Follow-up and study duration

Follow-up will last 5 years from the date of surgery. The frequency of follow-up visits will be decided at each centre. Every 6 to 8 months, the disease and survival status will be assessed. Recurrence will be investigated by clinical examination with rectal MRI and CT and a tumour marker test (CEA) (Table 1). Locoregional or metastatic relapse will be reported in the case report form with the date of relapse diagnosis.

As the inclusion period will be of 36 months and the follow-up will last 5 years, the total study duration will be of 8 years.

Microbiological analyses

Sample handling

Three stool samples will be collected during the study (Figure 1): i) one at patient inclusion, before any treatment, to describe the baseline intestinal microbiota composition; ii) one during the interval between the end of neoadjuvant CRT and surgery, at the surgical consultation for tumour reappraisal; and iii) one just before bowel preparation (mechanical or antibiotics) for surgery.

Each sample will be divided into two cryotubes: one empty and one with 15% glycerol/DMEM to preserve cell integrity. Samples will be immediately stored at -80°C until transport to the M2iSH laboratory, Clermont-Ferrand, France, which will be in charge of the molecular analysis and storage of the samples.

E. coli strain identification and CoPEC detection

All microbiological analyses will be performed as previously described (28). After thawing, samples stored in DMEM/glycerol will be crushed and diluted in sterile phosphate buffered saline pH 7.4 before plating on TBX agar and chromogenic agar chromID CPS3® plates (bioMérieux) to allow the identification and quantitation of enterobacteria. Colonies (around 48 per sample) will be collected for molecular typing, and their identification will be confirmed with the automated Vitek® II (bioMérieux) system. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR will be used as genotyping method to determine the number of *E. coli* strains per sample (28).

E. coli harbouring the colibactin-encoding *pks* island will be identified by PCR analysis of each *E.coli* isolate (41). This will allow identifying the presence of CoPEC (primary objective).

Untargeted analysis of the local microbiota composition

Global microbiota modifications will be assessed by high-throughput sequencing of the bacterial 16S rRNA gene in DNA extracted from the three stool samples using the NucleoSpin® DNA stool kit (Macherey-Nagel, Hoerdt, France), according to the manufacturer's instructions. Quantitative PCR will be performed to quantify pro-carcinogenic bacterial species, such as *Fusobacterium nucleatum, Enterococcus feacalis, bft*-positive *Bacteroides fragilis,* and CoPEC. In addition, the V4 region of the bacterial 16S rRNA gene will be amplified using the 515F/806R primer pair followed by Illumina high throughput sequencing on a MiSeq® apparatus, according to the manufacturer's guidelines. A

global description of the intestinal microbiota could also be obtained by shotgun metagenomic sequencing to access the microbiota functional features after selection of the more informative samples.

Endpoints

Primary endpoint

The primary endpoint (associated with the primary objective) is the relative risk (RR) of poor response to neoadjuvant CRT in patients colonized by CoPEC ("exposed") compared to non-colonized patients ("unexposed").

Secondary endpoints

- Prevalence and CoPEC colonization rate before and after CRT
- Other bacterial strains present before CRT and relative risk of poor response to CRT in colonized and non-colonized patients
- Type, prevalence, and colonization rate of bacteria other than CoPEC in the microbiota, before and after CRT
- Percentage of colonized patients, depending on the bacterial type, according to the clinical parameters (age, sex, body mass index)
- Hazard ratio (HR) for overall survival, disease-specific survival, and relapse-free survival (locoregional or metastatic) in colonized patients, for the different bacterial types, according to the overall bacterial composition (including CoPEC), and in non-colonized patients.

Data collection and management

The database will be managed by the sponsor, and data stored at the Data processing centre, Biometrics Unit of the Montpellier Cancer Institute. Case report form design and clinical data management will be implemented using the Ennov Clinical® software. Microbiological data will be

collected in a database first stored at the M2iSH laboratory, and then transferred to the sponsor database for analysis. Data and any trial documents will be made available upon reasonable request and after signature of a data access agreement.

In accordance with the General Data Protection Regulation (GDPR), a registration number will be used to identify each patient. The corresponding table will be encrypted and stored in a secure place. Special vigilance will be exercised throughout the study to maintain data anonymization.

Study monitoring, quality control, and audit

According to the sponsor's risk-based monitoring plan (study participants, logistics, resources, impact), the collection of the patient informed consents and the respect of the study protocol and procedures will be monitored.

To guarantee the originality of all data and in accordance with the Good Clinical Practices, quality control will be performed by the sponsor. The study will be managed according to the sponsor procedures and in respect of the protocol, and the quality of the data included in the report forms will be checked.

The sponsor may wish to conduct an audit at some investigating centers. Audits may be conducted by the sponsor or any duly authorized person for at least 15 years after the trial.

Statistical considerations

Sample size

The recruitment capacity for this exploratory study will be around 200 patients. For a mean rate of 30% of poor responders to the neoadjuvant treatment among the patients not colonized by CoPEC (*i.e.*, a proportion of response P2=0.30 among unexposed patients), the study will be able to estimate a relative risk of 1.7 (RR=1.7) with a 30% precision and a confidence interval at 95% (α=0.05). Patients in whom the CoPEC colonization status cannot be determined at baseline, in whom CRT must be prematurely arrested, or who cannot undergo surgery will be considered non-evaluable.

Considering a 10% rate of potentially non-evaluable patients, a total of 220 patients (20 supplementary patients) will be included in the study.

Study population

Two populations will be defined for the analysis. The intention-to-treat population will be defined as all patients included in the study, treated (patients who received complete/partial neoadjuvant treatment) and not treated (patients who did not undergo CRT), eligible (*i.e.,* all patients who were included in the study without violation of a major inclusion or exclusion criterion) or not, and with/without baseline stool sample. The per-protocol population will include all eligible patients, treated (complete or partial CRT), and with baseline stool sample.

Statistical analyses

Qualitative variables will be described by frequencies and percentages, and quantitative variables with means, standard deviations, medians, and ranges. No imputation method will be used in case of missing data. Correlations between qualitative variables will be assessed using the Chi-2 or Fisher-exact test. Quantitative variables will be compared using the Student's *t*-test or the Kruskal-Wallis test. Comparison of quantitative variables at different times (before and after CRT) will be assessed using the Wilcoxon test for matched samples. The relative risk of poor response to neoadjuvant CRT in CoPEC-colonized patients (or colonized by other bacteria) compared to non-colonized patients will be estimated using a logistic regression (univariate analysis) and will be presented with the 95% confidence interval (95% CI). Survival analyses will be performed using the Kaplan-Meier method and survival distributions compared with the log rank test. HRs and their 95% CI will be estimated with a Cox proportional risk model. A detailed statistical analysis plan (SAP) will be written before the database is locked for analysis; supplementary subgroup analyses, if appropriated, will be specified in the SAP. All analyses will be performed using the Stata version 16 software (StataCorp LP, College Station, TX).

Patient and public involvement

There was no patient or public involvement in the design of this study.

Discussion

The implication of intestinal microbiota in CRC has been widely demonstrated (42). Several recent studies suggest that different bacterial species, including CoPEC, could be used as biological biomarkers for CRC diagnosis and prognosis (29,36,41,43,44). The potential role of the gut microbiota in the modulation of the efficacy of anti-tumour treatments has been studied, with interesting results regarding chemotherapy and immunotherapy (37). However, these studies were focused on colon cancer dysbiosis and few data are available on rectal cancer and mucosa. Moreover, the correlation between gut microbiota homeostasis and radiation sensitivity remains unclear. Patients treated by pelvic radiation develop long-term complications that affect their quality of life, and have worse functional results than patients treated with surgery alone (45,46). It has been hypothesized that the intestinal microbiota has a significant impact on pelvic enteropathy (47); however, pelvic irradiation is responsible for microbiota dysbiosis (48,49). To our knowledge, no previous study has assessed the local microbiota composition and its implication in the response to CRT in rectal cancer, although treatment response is one of the key points for prognosis estimation. Biomarkers to predict tumour response in rectal cancer are still crucially needed. Imaging techniques (50) and biological markers (51,52) have been evaluated, but they are often expensive and complicated to implement. Moreover, the results are still discussed. Currently, their use seems to be limited to research and expert centers. The present study will describe the intestinal microbiota composition in patients with rectal cancer receiving neoadjuvant CRT to show its potential correlation with the tumour response, focusing on CoPEC colonization. In addition, the effect of radiotherapy on the local intestinal microbiota composition will be studied by comparing stool samples collected before and after CRT. Unlike

studies on the intestinal microbiota in colon cancer in which tumour fragments are needed, in the case of mid or low rectal cancer stool samples should be representative of the local microbiota.

One of the main hypotheses to explain CoPEC effect on CRT response is based on their capacity to induce DNA damage (25–27). Besides the direct effect on the cell, radiotherapy is also cytotoxic through the production of reactive oxygen species and reactive nitrogen species (53). Chronic genotoxic stress caused by CoPEC presence in gut mucosa could lead to an adaptation of the gut mucosa to genotoxic agents and consequently to reduce radiation sensitivity and resistance to therapy. For instance, in an *in vitro* study, Wilson et al. observed less DNA damage in colibactin-positive epithelial cells infected by CoPEC (27). Moreover, radiation sensitivity is closely linked to autophagy regulation (54,55). Recent studies showed the involvement of gut microbiota in autophagy regulation, with a link to chemoresistance (56). Ionizing radiation effects might be modified indirectly through autophagy deregulation induced by gut microbiota. In addition, radiotherapy cytotoxic effect could result in a modification of the local microenvironment with significant clinical consequences (57).

The modulation of radiotherapy efficacy by the intestinal microbiota is an emerging concept in CRC, but its study faces many obstacles, especially sample availability. In this study, we want to develop a non-invasive reproducible faecal test that could become a key biomarker to predict tumour response to CRT. Our work will help clinicians to tailor neoadjuvant therapeutic strategies with the final goal of increasing tumor response, organ preservation, and reducing surgical morbidity, while maintaining oncological safety.

Ethics and dissemination

The study protocol (version 3.0, dated on September 24th, 2019) was approved by the local ethics committee (Comité de Protection des Personnes Sud-Est II, December 18th, 2019, Reference number 2019-A02493-54) and the institutional review board COMERE. The French National Drug Agency Authority (ANSM) was informed. The study was registered on Clinicaltrials.gov, identifier NCT04103567.

All patients will be informed of the study objectives and procedures by the investigators before enrolment. A signed informed consent will be obtained from all patients before their inclusion in the study and before any study procedure is performed. All patients may end their participation in the study at any time, for whatever reason, without any consequence or prejudice concerning their care. Study participants will be able to request global results from investigators as soon as study results become available.

In the event of substantial modification, the request will be sent by the sponsor to the ethics committee for an opinion. Upon receipt of the favourable opinion, the sponsor will send the amended version of the protocol to all investigators.

The study will be conducted in accordance with the current French and European Regulatory requirements, including regulations on biomedical research from the Public Health Code, the bioethics and data protection laws and decrees, the French Jardé's law on research implicating human beings, the Good Clinical Practice, and the Helsinki Declaration.

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Authors' contributions

GC, CT, JG, CF, MJ, GR, CC, PEC, PR, and MB wrote the protocol. MJ, GC, PR, CT and CF conducted statistical trial planning. PR, CT and CF handled ethics and regulatory affairs. GC, CT, HF, MB, CF, NB, PR, and MJ wrote the paper draft. GC, MB, MJ, PR, CT and CF contributed to the trial design and modifications. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Provenance and peer review

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Figure legends

Figure 1: MICARE flow diagram

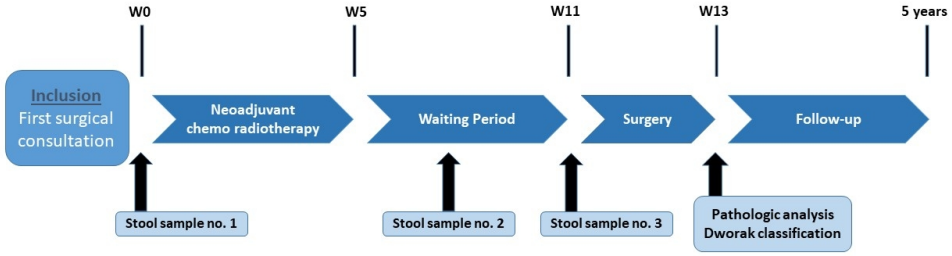

Figure 1 : MICARE flow diagram

Figure 1 : MICARE flow diagram

338x190mm (96 x 96 DPI)

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	p1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	p3
	2b	All items from the World Health Organization Trial Registration Data Set	Protocol**
Protocol version	3	Date and version identifier	p16, paragraph1
Funding	4	Sources and types of financial, material, and other support	*Funding*, p17
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	p17
	5b	Name and contact information for the trial sponsor	p7, paragraph1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	p7, p17
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	p7, p11-12

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	p3-5
	6b	Explanation for choice of comparators	NA
Objectives	7	Specific objectives or hypotheses	*Objectives*, p5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	*Study design*, p5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	*Study design*, p5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	*Patients' selection*, p6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	*Clinical study procedures*, p7-9
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	p8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	NA
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	p8
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	p11

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	p7-8, Table1, Fig1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	*Sample size*, p12-13
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	p12-13
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	*Data collection and management*, p11

	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	NA
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	p11-12
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	p13
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	*Statistical analyses*, p13
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	*Statistical analyses*, p13
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	*Study monitoring, quality control, and* audit, p12
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	*Safety*, p9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	*Study monitoring, quality control, and* audit, p12
Ethics and dissemination			

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	*Ethics approval and consent to participate, p16*
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	p16, paragraph3
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	p16
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	p12, paragraph1
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	*Competing interests*, p16-17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	*Availability of data and materials*, p16
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	*Dissemination policy*, p13-14
	31b	Authorship eligibility guidelines and any intended use of professional writers	Protocol**
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	*Availability of data and materials*, p16

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Applicable **
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	*Sample handling*, p10

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

**More information can be provided if wished by the editor.

Formulaire de consentement

Détermination de facteurs MIcrobiologiques associés à une mauvaise réponse au traitement néoadjuvant dans les CAncers du REctum : focus sur les Escherichia coli productrices de cyclomodulines
MICARE

Version 4.0 du 23/02/2021

Promoteur: Institut du Cancer de Montpellier ICM, Parc Euromédecine, 208 rue des Apothicaires, 34298 Montpellier Cedex 5

Coordonnateur de l'étude: Pr Philippe ROUANET Département de Chirurgie Oncologique, ICM

Je soussigné(e) :

Nom:…… **Prénom**:……

Date de naissance: I__I__I I__I__I I__I__I__I__I

certifie avoir lu et compris la note d'information version n°4.0 du 23/02/2021 qui m'a été remise et accepte de participer à cette recherche selon les conditions définies dans la note d'information.

J'ai bien compris que ma participation à la recherche était libre et volontaire, et que je pouvais refuser d'y participer sans avoir à me justifier, tout en continuant à bénéficier des meilleurs soins disponibles.

Je reconnais avoir pu poser toutes les questions souhaitées et avoir reçu des réponses satisfaisantes à mes questions.

Je reconnais en particulier que le droit à me faire assister par une personne de mon choix m'a été communiqué.

Je reconnais avoir disposé d'un temps de réflexion suffisant entre ces informations et le présent consentement et avoir eu si je le souhaitais l'opportunité d'en discuter avec mon médecin ou mes proches.

Les conditions de ma participation, notamment la durée de celle-ci, les contraintes, les objectifs, le déroulement de l'étude ainsi que les bénéfices et les risques éventuels, m'ont été expliqués clairement par le Dr/Pr……..

Je m'engage à suivre les contraintes expliquées dans le document d'information, à la fois pour minimiser les risques et pour la bonne réalisation de l'étude. Ma participation à l'étude pourrait être suspendue si je ne respectais pas le protocole.

J'ai compris également que je pouvais à tout moment interrompre ma participation à cette recherche, sans avoir à me justifier, sans aucun préjudice et en continuant à recevoir les meilleurs soins disponibles.
Dans ce cas, je m'engage à prévenir le médecin responsable de l'étude.

Je reconnais avoir été informé(e) que l'étude pouvait être interrompue à tout moment sur décision du promoteur ou des autorités de santé, et que toutes les mesures seraient prises dans ce cas pour assurer ma sécurité et la poursuite de ma prise en charge médicale.

J'ai bien compris que tout fait nouveau susceptible de remettre en cause mon consentement à ma participation à l'étude me serait communiqué.

J'ai bien noté que mon consentement ne dégageait pas les médecins et le promoteur de leurs responsabilités, et que je conservais tous les droits qui me sont garantis par la loi.

Formulaire de consentement

J'ai bien pris note que la lettre d'information et le consentement sont le fondement juridique pour le traitement des données dans le cadre de cette étude.

J'ai bien noté que, conformément aux dispositions de la loi relative à l'informatique, aux fichiers et aux libertés et au règlement européen 2016/679 sur la protection des données je dispose d'un droit d'accès de rectification, ainsi qu'un droit à l'effacement, à la limitation du traitement et à la portabilité des données (RGPD). Je dispose également d'un droit d'opposition à la transmission des données couvertes par le secret professionnel susceptibles d'être utilisées dans le cadre de cette recherche et d'être traitées.
J'ai bien note que, si je souhaite me retirer de l'étude, les données recueillies avant mon retrait ne pourront pas être supprimées. Par contre, aucune nouvelle donnée ne sera recueillie. Ces droits s'exercent auprès du médecin qui me suit dans le cadre de cette recherche et qui connaît mon identité.

J'ai pris connaissance que cette recherche a reçu l'avis favorable du Comité de Protection des Personnes de nom du CPP (catégories 1, 2 et 3) et l'information de l'ANSM.

Je reconnais avoir été informé(e) que le promoteur de l'étude, l'Institut régional du Cancer Montpellier a souscrit une assurance de responsabilité civile en cas de préjudice auprès de la société SHAM (contrat n° 140474).

J'autorise dans la mesure où elles sont indispensables aux fins de la recherche, l'enregistrement de données personnelles me concernant. Je sais que le promoteur s'engage à ce que ces données soient rendues confidentielles par un codage sans mention du nom et du prénom.

J'ai bien noté que j'ai le droit d'être informé(e) des résultats globaux de cette recherche selon les modalités qui ont été précisées dans le document d'information.

J'atteste être affilié(e) ou bénéficiaire d'un régime français d'assurance maladie (sécurité sociale), condition obligatoire pour pouvoir être inclus dans la recherche.

J'accepte que les prélèvements biologiques et les données associées soient traités, collectés et conservés dans une collection spécifique de l'étude et utilisés à des fins de recherche.

Je suis informé(e) de la possibilité qu'une partie des prélèvements effectués à l'occasion de ce protocole de recherche soit conservée pour une utilisation ultérieure à des fins de recherche. J'ai également été informé(e) de mon droit à m'opposer à cette conservation et l'utilisation.

☐	J'accepte que mes données cliniques soient utilisées pour des recherches ultérieures, en France ou dans l'Union Européenne
☐	J'accepte que mes prélèvements soient utilisés pour des recherches ultérieures sur le cancer, en France ou dans l'Union Européenne, ayant la même finalité

Nom du patient :

Date :

Signature :

Nom de l'investigateur :

Date :

Signature :

Je reconnais qu'un des deux exemplaires de ce formulaire attestant mon consentement m'a été remis.

TREATMENT	EVENTS	YES/NO
CHEMO-RADIOTHERAPY	Proctitis	YES/NO
	Perineal skin toxicity	YES/NO
	Weakness	YES/NO
	Nausea	YES/NO
	Diarrhea	YES/NO
	Abdominal pain	YES/NO
	Hematologic toxicity	YES/NO
	Hand-foot syndrome	YES/NO
SURGERY	**Per-operative**	
	Bleeding	YES/NO
	Intestinal perforation	YES/NO
	Vascular wound	YES/NO
	Post-operative	
	Infection	YES/NO
	Anastomotic leakage	YES/NO
	Colon ischemia	YES/NO
	Bowel obstruction	YES/NO
	Bleeding	YES/NO
	Urinary dysfunction	YES/NO