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# 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 (MICARE)

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2022-061527
Article Type:	Protocol
Date Submitted by the Author:	11-Feb-2022
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Keywords:	ONCOLOGY, GASTROENTEROLOGY, RADIOTHERAPY

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Manuscripts

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3 1 Protocol  
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6 2 **Determination of biomarkers associated with neoadjuvant treatment response focusing**  
7  
8 3 **on colibactin-producing *Escherichia coli* in patients with mid or low rectal cancer: a**  
9  
10 4 **prospective cohort study (MICARE).**  
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12

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8 27 **Funding**

9  
10  
11 28 This work was supported by the SIRIC Montpellier Cancer, grant number N/A, the Biocodex Microbiota  
12  
13 29 Foundation, grant number N/A, and La Ligue Contre le Cancer (Herauld committee), grant number N/A.

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16 30

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18  
19 31 **Abstract**

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21  
22 32 **Introduction** The management of mid and low rectal cancer is based on neoadjuvant  
23  
24 33 chemoradiotherapy (CRT) followed by standardized surgery. There is no biomarker in rectal cancer to  
25  
26 34 aid clinicians in foreseeing treatment response. The determination of factors associated with treatment  
27  
28 35 response might allow the identification of patients who require tailored strategies (e.g. therapeutic de-  
29  
30 36 escalation or intensification). Colibactin-producing *Escherichia coli* (CoPEC) has been associated with  
31  
32 37 aggressive CRC and could be a poor prognostic factor. Currently no study has evaluated the potential  
33  
34 38 association between intestinal microbiota composition and tumour response to CRT in mid and low  
35  
36 39 rectal cancer. The aim of this study is to assess the association between response to neoadjuvant CRT  
37  
38 40 and faecal intestinal microbiota composition and/or CoPEC prevalence in patients with mid or low rectal  
39  
40 41 cancer.

41  
42  
43  
44 42 **Methods and analysis** This is a non-randomized bicentric prospective cohort study with a recruitment  
45  
46 43 capacity of 200 patients. Three stool samples will be collected from participants with histological-proven  
47  
48 44 adenocarcinome of mid or low rectum who meet eligibility criteria of the study protocol: one before  
49  
50 45 neoadjuvant treatment start, one in the period between CRT end and surgery, and one the day before  
51  
52 46 surgery. In each sample, CoPEC will be detected by culture in special media and molecular (PCR)  
53  
54 47 approaches. The global microbiota composition will be also assessed by the bacterial 16S rRNA gene  
55  
56 48 sequencing. Neoadjuvant CRT response and tumour regression grade will be described using the

1  
2  
3 49 Dworak system at pathological examination. Clinical data and survival outcomes will also be collected  
4  
5 50 and investigated.  
6  
7  
8 51

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10  
11 52 **Ethics and dissemination** MICARE was approved by the local ethics committee (Comité de Protection  
12  
13 53 des Personnes Sud-Est II, December 18<sup>th</sup>, 2019. Reference number 2019-A02493-54) and the  
14  
15 54 institutional review board. Patients will be required to provide written informed consent. Results will be  
16  
17 55 published in a peer reviewed journal.  
18  
19

20 56 **Trial registration number** NCT04103567.  
21  
22  
23  
24 57

### 25 26 58 **Strengths and limitations of this study**

- 27  
28 59 • As far as we know, this is the first study to evaluate association between intestinal microbiota  
29 60 composition and tumour response to chemoradiotherapy in mid and low rectal cancer
- 30  
31 61 • MICARE is a prospective cohort study including 200 patients
- 32  
33 62 • This study is based on a non-invasive and reproducible faecal test
- 34  
35 63 • Tumour response will be described at pathological examination after surgery
- 36  
37 64 • The limitation of this study will include population stratification for delay between radiotherapy  
38 65 and surgery, and adjonction of neoadjuvant chemotherapy in tumour response evaluation  
39  
40 66

### 41 67 **Introduction**

42  
43 68 With more than 700,000 new cases and 300,000 deaths in 2018, rectal cancer is the eighth leading cause  
44  
45 69 of cancer deaths worldwide (1). The initial management of mid and low rectal cancer is based on  
46  
47 70 neoadjuvant chemoradiotherapy (CRT) for locally advanced tumours. This is associated with a  
48  
49 71 significant decrease of the locoregional recurrence rate, but without survival improvement (2–4).  
50  
51 72 Neoadjuvant treatment is followed by standardized surgery (5). Total mesorectal excision is crucial for  
52  
53 73 reducing tumour recurrence (6), but its significant morbidity can affect the patients' quality of life.  
54  
55 74 Prognosis also depends on the tumour response to neoadjuvant CRT. Currently, the surgical strategy is  
56  
57 75 adapted in function of the tumour response to neoadjuvant treatment, assessed by magnetic resonance  
58  
59  
60

1  
2  
3 76 imaging (MRI) after CRT end (7). Indeed, the objective is therapeutic de-escalation with rectal  
4  
5 77 preservation to decrease morbidity and functional disorders. For patients with complete response (up to  
6  
7 78 25% of patients), careful monitoring without surgery ("watch and wait" strategy) has been proposed  
8  
9 79 (8,9). For small tumours with good response to CRT, transanal excision with rectal preservation seems  
10  
11 80 to be feasible in terms of cancer prognosis (10). For patients with large tumours or a locally advanced  
12  
13 81 disease, a tailored treatment strategy with total neoadjuvant therapy (TNT) is now a gold standard  
14  
15 82 (11,12). After surgical excision, the tumour response is classified in five pathologic tumour response  
16  
17 83 grades, according to the Dworak classification, on the basis of the pathology findings (13). Recent  
18  
19 84 studies reported up to 30% of poor responders (grades 0 and 1) (14,15). These data emphasize the  
20  
21 85 importance of the initial tumour staging and response to neoadjuvant CRT for tailoring surgical  
22  
23 86 strategies. MRI is an essential tool for these two assessments (16–18). These data highlight the need of  
24  
25 87 response predictive models to adapt the TNT in mid and low rectal cancer.

26  
27  
28 88 Gut microbiota behaves as a real organ and participates in intestinal homeostasis. An imbalance in its  
29  
30 89 composition (dysbiosis) could be involved in many pathologies, including colorectal cancer (CRC) (19–  
31  
32 90 21). *Escherichia coli* (*E. coli*) has been widely described as a bacteria which could be involved in  
33  
34 91 CRC.(22,23). *E. coli* is the predominant aero-anaerobic Gram-negative specie in human colon, but it is  
35  
36 92 also a pathogen involved in various intestinal diseases (24). Indeed, some *E. coli* strains have acquired  
37  
38 93 the capacity to produce toxins named cyclomodulins, including colibactin that is encoded by the pks  
39  
40 94 island(25). Colibactin-producing *E. coli* (CoPEC) has genotoxic effects by inducing DNA damage and  
41  
42 95 chromosomal instability (25–27). CoPEC implication in CRC has been demonstrated, particularly in  
43  
44 96 aggressive forms (28–34). Specifically, higher *E. coli* colonization rate and higher prevalence of CoPEC  
45  
46 97 are found in patients with TNM stage III or IV tumors (29) (UICC TNM Classification, 8<sup>th</sup> Edition,  
47  
48 98 2017) (35). Moreover, CoPEC gut colonization might contribute to modulate the immunotherapy  
49  
50 99 efficacy (36). Recent clinical studies discussed the prognostic role of intestinal microbiota in the tumour  
51  
52 100 response following surgery and chemotherapy or immunotherapy (37), and suggested that it could be  
53  
54 101 used as a biomarker to predict tumour response to neoadjuvant treatments. On the other hand, very few  
55  
56 102 clinical studies have assessed the influence of gut microbiota on radiotherapy efficacy, especially in  
57  
58 103 rectal cancer. Recently, a preclinical study showed that mice which survive a high dose of radiation,  
59  
60

1  
2  
3 104 harboured gut microbiota enriched with *Lachnospiraceae* and *Enterococcaceae* (38). Yet, a description  
4  
5 105 of the intestinal microbiota composition before neoadjuvant therapy could allow identifying predictive  
6  
7 106 bacterial markers of tumour response in rectal cancer, and to adapt TNT.

8  
9 107 Indeed, chronic exposure of the gastrointestinal tract to genotoxins could be a prognostic marker of  
10  
11 108 radiotherapy response. CoPEC colonization would start at the very beginning of life (38) and might lead  
12  
13 109 to exposure of the intestinal mucosa to chronic genotoxic stress. The resulting damage could give cells  
14  
15 110 the ability to resist to other genotoxic stresses, such as radiation therapy. One *in vitro* study already  
16  
17 111 showed the decreased radiation sensitivity of cells incubated by colibactin (27). Therefore, developing  
18  
19 112 a non-invasive method to analyse gut microbiota composition and to evaluate CoPEC implication in the  
20  
21 113 response to CRT could help clinicians to tailor cancer management and to develop tools to control the  
22  
23 114 pathologic microorganisms identified as new therapeutic targets.  
24  
25  
26  
27 115

## 30 116 **Methods and analysis**

31  
32  
33 117 This study protocol is written in accordance with the SPIRIT guidelines. (Supplementary file)

### 34 35 36 118 ***Objectives***

#### 37 38 39 119 *Primary objective*

40  
41  
42 120 The study primary objective is to assess the correlation between response to neoadjuvant CRT and  
43  
44 121 CoPEC presence in stool samples.

#### 45 46 47 122 *Secondary objectives*

- 48  
49  
50 123 - To analyse in a non-targeted manner the global microbiota composition before CRT and to  
51  
52 124 evaluate the correlation between composition and response to treatment
- 53  
54 125 - To study the modulation of the intestinal microbiota by CRT
- 55  
56 126 - To describe the correlation between clinical data and microbiota composition modulation  
57  
58 127 induced by CRT  
59  
60

- 1  
2  
3 128 - To determine microbiological prognostic factors of overall survival, disease-specific survival and  
4  
5 129 relapse-free survival (locoregional and metastatic) in patients with low or mid rectum cancer  
6  
7 130 - To create a microbiological database for future mechanistic analyses  
8  
9 131 - To study the modulation of CoPEC colonization by CRT  
10  
11

12 132 ***Study design***  
13  
14

15 133 The study is a non-randomized bicentric prospective cohort study. Two surgical teams will be involved  
16  
17 134 - Institut du Cancer de Montpellier and CHU de Clermont-Ferrand ; and an INSERM Unit – M2iSH  
18  
19 135 Clermont-Ferrand.  
20  
21

22 136 ***Patients' selection***  
23  
24

25 137 ***Inclusion criteria***  
26  
27

- 28 138 - Histologically-proven adenocarcinoma of low or mid rectum, of stage II or III (UICC TNM  
29  
30 139 Classification, 8<sup>th</sup> Edition, 2017 (35))  
31  
32 140 - Patient eligible for neoadjuvant treatment (50 Gray radiation and capecitabine, CAP 50),  
33  
34 141 according to the French national recommendations (5,39)  
35  
36 142 - Informed signed consent received  
37  
38 143 - Man or woman aged  $\geq 18$  years  
39  
40 144 - Appropriate contraceptive measures taken by men and pre-menopausal women before study  
41  
42 145 entry and for at least 8 weeks after the last CRT cycle. Patients should be informed by the  
43  
44 146 investigator on the contraceptive measures to use.  
45  
46  
47

48 147 ***Exclusion criteria***  
49  
50

- 51 148 - Antibiotic treatment at the time of stool sampling or in the month before.  
52  
53 149 - Presence of a derivative stoma  
54  
55 150 - Previous chemotherapy treatment for rectum cancer  
56  
57 151 - Patient not affiliated to the French social security system  
58  
59  
60



- 1  
2  
3 152 - Patient with possible poor treatment compliance for psychologic, familial, social and geographic  
4  
5 153 reasons  
6  
7 154 - Legal incapacity or limited legal capacity  
8  
9 155 - Pelvic radiotherapy or brachytherapy in the year before inclusion in the study  
10  
11 156 - History of other cancers in the 5 last years, except for cervical carcinoma *in situ* and skin  
12  
13 157 carcinoma, but including melanoma under treatment  
14  
15  
16 158 - Pregnant or breastfeeding woman  
17  
18  
19 159  
20  
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22 160

### 160 ***Study sponsor***

23  
24 161 The sponsor (Montpellier Cancer Institute, ICM) is responsible for the study design and management,  
25  
26 162 and for obtaining all study authorizations (Persons Protection Committee, National Agency for Medical  
27  
28 163 Security). It will also declare to these authorities the inclusion period beginning and end, produce the  
29  
30 164 final study report, inform the competent authorities of the trial results, and store all study-related  
31  
32 165 documents for at least 15 years after the study end.  
33  
34  
35

### 36 166 ***Clinical study procedures***

#### 37 38 39 167 *Inclusion in the study*

40  
41  
42 168 The study flow diagram is presented in Figure 1.  
43  
44

45 169 Before study entry, all patients will receive exhaustive explanations on the study aims and procedures.

46  
47 170 A signed informed consent will be obtained from all patients before any study procedure. At baseline,  
48  
49 171 demographic (sex, age), clinical (performance status, weight, height, medical history, initial diagnosis  
50  
51 172 date, tumour localization, histologic type) and biological (complete blood count, carcinoembryonic  
52  
53 173 antigen (CEA) level) data will be collected (Table 1). Patients will undergo rectal examination and  
54  
55 174 tumour staging by computed tomography (CT), rectal MRI, and possibly rectal endoscopic ultrasound  
56  
57 175 examination (depending on the centre decision).  
58  
59  
60

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

179 **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

180 MRI: Magnetic resonance imaging; CT: computed tomography.

181 *Neoadjuvant treatment*

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

184

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3 188 *Re-evaluation*  
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6 189 During the consultation after CRT end and before surgery, a second stool sample (stool sample N°2)  
7  
8 190 will be collected, as described for the baseline sample. If the patient has received antibiotics in the month  
9  
10 191 before this consultation, stool sampling will not be performed.  
11  
12

13 192 This second consultation will include MRI examination as during the baseline visit. The tumour  
14  
15 193 response will be described precisely with emphasis on the tumour regression grade according to the  
16  
17 194 MERCURY experience (7).  
18  
19

20 195 *Surgery*  
21  
22

23 196 Surgical data (surgery type, digestive reconstruction or stoma, and surgical outcomes),  
24  
25 197 anatomopathological data (histologic type, ypTN grade, Dworak grade (13), Quirke classification (40),  
26  
27 198 circumferential resection, distal margins, and extramucosal vascular invasion) and biological data (RAS  
28  
29 199 and BRAF mutational status, if available) will be collected. The day before surgery, before bowel  
30  
31 200 mechanical preparation, the third stool sample (stool sample N°3) will be collected in hospital, as  
32  
33 201 described for the previous samples. If the patient received antibiotics in the month before hospitalization,  
34  
35 202 stool sampling will not be performed.  
36  
37

38  
39 203 *Pathologic analysis*  
40  
41

42 204 To meet the primary objective, the pathologic analysis of the surgical specimens will describe the tumour  
43  
44 205 regression grade according to the Dworak classification (13) (Table 2). Patients with grade 0 and 1  
45  
46 206 tumours will be considered poor responders, in accordance with the literature.  
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211 **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)

212

213 ***Safety***

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

216 ***Follow-up and study duration***

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

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

224 ***Microbiological analyses***

225 ***Sample handling***

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

1  
2  
3 228 the end of neoadjuvant CRT and surgery, at the surgical consultation for tumour reappraisal; and iii) one  
4  
5 229 just before bowel preparation (mechanical or antibiotics) for surgery.  
6  
7

8 230 Each sample will be divided into two cryotubes: one empty and one with 15% glycerol/DMEM to  
9  
10 231 preserve cell integrity. Samples will be immediately stored at -80°C until transport to the M2iSH  
11  
12 232 laboratory, Clermont-Ferrand, France, which will be in charge of the molecular analysis and storage of  
13  
14 233 the samples.  
15

#### 16 234 *E. coli* strain identification and CoPEC detection

17  
18  
19 235 All microbiological analyses will be performed as previously described (28). After thawing, samples  
20  
21 236 stored in DMEM/glycerol will be crushed and diluted in sterile phosphate buffered saline pH 7.4 before  
22  
23 237 plating on TBX agar and chromogenic agar chromID CPS3<sup>®</sup> plates (bioMérieux) to allow the  
24  
25 238 identification and quantitation of enterobacteria. Colonies (around 48 per sample) will be collected for  
26  
27 239 molecular typing, and their identification will be confirmed with the automated Vitek<sup>®</sup> II (bioMérieux)  
28  
29 240 system. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR will be used as genotyping  
30  
31 241 method to determine the number of *E. coli* strains per sample (28).  
32  
33

34  
35 242 *E. coli* harbouring the colibactin-encoding *pks* island will be identified by PCR analysis of each *E. coli*  
36  
37 243 isolate (41). This will allow identifying the presence of CoPEC (primary objective).  
38  
39

#### 40 244 *Untargeted analysis of the local microbiota composition*

41  
42  
43 245 Global microbiota modifications will be assessed by high-throughput sequencing of the bacterial 16S  
44  
45 246 rRNA gene in DNA extracted from the three stool samples using the NucleoSpin<sup>®</sup> DNA stool kit  
46  
47 247 (Macherey-Nagel, Hoerd, France), according to the manufacturer's instructions. Quantitative PCR will  
48  
49 248 be performed to quantify pro-carcinogenic bacterial species, such as *Fusobacterium nucleatum*,  
50  
51 249 *Enterococcus faecalis*, *bft*-positive *Bacteroides fragilis*, and CoPEC. In addition, the V4 region of the  
52  
53 250 bacterial 16S rRNA gene will be amplified using the 515F/806R primer pair followed by Illumina high  
54  
55 251 throughput sequencing on a MiSeq<sup>®</sup> apparatus, according to the manufacturer's guidelines. A global  
56  
57 252 description of the intestinal microbiota could also be obtained by shotgun metagenomic sequencing to  
58  
59 253 access the microbiota functional features after selection of the more informative samples.  
60

1  
2  
3 254 **Endpoints**  
4

5  
6 255 *Primary endpoint*  
7

8  
9 256 The primary endpoint (associated with the primary objective) is the relative risk (RR) of poor response  
10  
11 257 to neoadjuvant CRT in patients colonized by CoPEC ("exposed") compared to non-colonized patients  
12  
13 258 ("unexposed").  
14

15  
16 259

17  
18  
19 260 *Secondary endpoints*  
20

21  
22 261 - Prevalence and CoPEC colonization rate before and after CRT  
23

24  
25 262 - Other bacterial strains present before CRT and relative risk of poor response to CRT in colonized  
26  
27 263 and non-colonized patients  
28

29 264 - Type, prevalence, and colonization rate of bacteria other than CoPEC in the microbiota, before  
30  
31 265 and after CRT  
32

33 266 - Percentage of colonized patients, depending on the bacterial type, according to the clinical  
34  
35 267 parameters (age, sex, body mass index)  
36

37 268 - Hazard ratio (HR) for overall survival, disease-specific survival, and relapse-free survival  
38  
39 269 (locoregional or metastatic) in colonized patients, for the different bacterial types, according to  
40  
41 270 the overall bacterial composition (including CoPEC), and in non-colonized patients.  
42  
43

44  
45 271 **Data collection and management**  
46

47  
48 272 The database will be managed by the sponsor, and data stored at the Data processing centre, Biometrics  
49  
50 273 Unit of the Montpellier Cancer Institute. Case report form design and clinical data management will be  
51  
52 274 implemented using the Ennov Clinical® software. Microbiological data will be collected in a database  
53  
54 275 first stored at the M2iSH laboratory, and then transferred to the sponsor database for analysis. Data and  
55  
56 276 any trial documents will be made available upon reasonable request and after signature of a data access  
57  
58 277 agreement.  
59  
60

1  
2  
3 278 In accordance with the General Data Protection Regulation (GDPR), a registration number will be used  
4  
5 279 to identify each patient. The corresponding table will be encrypted and stored in a secure place. Special  
6  
7 280 vigilance will be exercised throughout the study to maintain data anonymization.  
8  
9

### 10 281 ***Study monitoring, quality control, and audit***

11  
12  
13 282 According to the sponsor's risk-based monitoring plan (study participants, logistics, resources, impact),  
14  
15 283 the collection of the patient informed consents and the respect of the study protocol and procedures will  
16  
17 284 be monitored.

18  
19  
20 285 To guarantee the originality of all data and in accordance with the Good Clinical Practices, quality  
21  
22 286 control will be performed by the sponsor. The study will be managed according to the sponsor  
23  
24 287 procedures and in respect of the protocol, and the quality of the data included in the report forms will  
25  
26 288 be checked.

27  
28  
29 289 The sponsor may wish to conduct an audit at some investigating centers. Audits may be conducted by  
30  
31 290 the sponsor or any duly authorized person for at least 15 years after the trial.  
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### 34 291 ***Statistical considerations***

#### 35 36 37 292 *Sample size*

38  
39  
40 293 The recruitment capacity for this exploratory study will be around 200 patients. For a mean rate of 30%  
41  
42 294 of poor responders to the neoadjuvant treatment among the patients not colonized by CoPEC (*i.e.*, a  
43  
44 295 proportion of response  $P_2=0.30$  among unexposed patients), the study will be able to estimate a relative  
45  
46 296 risk of 1.7 (RR=1.7) with a 30% precision and a confidence interval at 95% ( $\alpha=0.05$ ). Patients in whom  
47  
48 297 the CoPEC colonization status cannot be determined at baseline, in whom CRT must be prematurely  
49  
50 298 arrested, or who cannot undergo surgery will be considered non-evaluable.  
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54 299 Considering a 10% rate of potentially non-evaluable patients, a total of 220 patients (20 supplementary  
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56 300 patients) will be included in the study.  
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3 302 *Study population*  
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6 303 Two populations will be defined for the analysis. The intention-to-treat population will be defined as all  
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8 304 patients included in the study, treated (patients who received complete/partial neoadjuvant treatment)  
9  
10 305 and not treated (patients who did not undergo CRT), eligible (*i.e.*, all patients who were included in the  
11  
12 306 study without violation of a major inclusion or exclusion criterion) or not, and with/without baseline  
13  
14 307 stool sample. The per-protocol population will include all eligible patients, treated (complete or partial  
15  
16 308 CRT), and with baseline stool sample.  
17

18  
19 309 *Statistical analyses*  
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21  
22 310 Qualitative variables will be described by frequencies and percentages, and quantitative variables with  
23  
24 311 means, standard deviations, medians, and ranges. No imputation method will be used in case of missing  
25  
26 312 data. Correlations between qualitative variables will be assessed using the Chi-2 or Fisher-exact test.  
27  
28 313 Quantitative variables will be compared using the Student's *t*-test or the Kruskal-Wallis test.  
29  
30 314 Comparison of quantitative variables at different times (before and after CRT) will be assessed using  
31  
32 315 the Wilcoxon test for matched samples. The relative risk of poor response to neoadjuvant CRT in  
33  
34 316 CoPEC-colonized patients (or colonized by other bacteria) compared to non-colonized patients will be  
35  
36 317 estimated using a logistic regression (univariate analysis) and will be presented with the 95% confidence  
37  
38 318 interval (95% CI). Survival analyses will be performed using the Kaplan-Meier method and survival  
39  
40 319 distributions compared with the log rank test. HRs and their 95% CI will be estimated with a Cox  
41  
42 320 proportional risk model. A detailed statistical analysis plan (SAP) will be written before the database is  
43  
44 321 locked for analysis; supplementary subgroup analyses, if appropriated, will be specified in the SAP. All  
45  
46 322 analyses will be performed using the Stata version 16 software (StataCorp LP, College Station, TX).  
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50 323 **Patient and public involvement**  
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52  
53 324 There was no patient or public involvement in the design of this study.  
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## 327 **Discussion**

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

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

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3 354 agents and consequently to reduced radiation sensitivity and resistance to therapy. For instance, in an *in*  
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5 355 *vitro* study, Wilson et al. observed less DNA damage in colibactin-positive epithelial cells infected by  
6  
7 356 CoPEC (27). Moreover, radiation sensitivity is closely linked to autophagy regulation (54,55). Recent  
8  
9 357 studies showed the involvement of gut microbiota in autophagy regulation, with a link to  
10  
11 358 chemoresistance (56). Ionizing radiation effects might be modified indirectly through autophagy  
12  
13 359 deregulation induced by gut microbiota. In addition, radiotherapy cytotoxic effect could result in a  
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15 360 modification of the local microenvironment with significant clinical consequences (57).

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18 361 The modulation of radiotherapy efficacy by the intestinal microbiota is an emerging concept in CRC,  
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20 362 but its study faces many obstacles, especially sample availability. In this study, we want to develop a  
21  
22 363 non-invasive reproducible faecal test that could become a key biomarker to predict tumour response to  
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24 364 CRT. Our work will help clinicians to tailor neoadjuvant therapeutic strategies with the final goal of  
25  
26 365 increasing tumor response, organ preservation, and reducing surgical morbidity, while maintaining  
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28 366 oncological safety.

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### 32 368 **Ethics and dissemination**

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35 369 The study protocol (version 3.0, dated on September 24<sup>th</sup>, 2019) was approved by the local ethics  
36  
37 370 committee (Comité de Protection des Personnes Sud-Est II, December 18<sup>th</sup>, 2019, Reference number  
38  
39 371 2019-A02493-54) and the institutional review board COMERE. The French National Drug Agency  
40  
41 372 Authority (ANSM) was informed. The study was registered on Clinicaltrials.gov, identifier  
42  
43 373 NCT04103567.

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46 374 All patients will be informed of the study objectives and procedures by the investigators before  
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48 375 enrolment. A signed informed consent will be obtained from all patients before their inclusion in the  
49  
50 376 study and before any study procedure is performed. All patients may end their participation in the study  
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52 377 at any time, for whatever reason, without any consequence or prejudice concerning their care. Study  
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54 378 participants will be able to request global results from investigators as soon as study results become  
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56 379 available.

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3 380 In the event of substantial modification, the request will be sent by the sponsor to the ethics committee  
4  
5 381 for an opinion. Upon receipt of the favourable opinion, the sponsor will send the amended version of  
6  
7 382 the protocol to all investigators.  
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10 383 The study will be conducted in accordance with the current French and European Regulatory  
11  
12 384 requirements, including regulations on biomedical research from the Public Health Code, the bioethics  
13  
14 385 and data protection laws and decrees, the French Jardé's law on research implicating human beings, the  
15  
16 386 Good Clinical Practice, and the Helsinki Declaration.  
17  
18

### 19 387 **Acknowledgements**

20  
21  
22 388 The authors thank the Clinical Research and Innovation Department of the Montpellier Cancer Institute  
23  
24 389 for help with regulatory and administrative aspects of the study, Dr. Stéphanie Delaine for her help in  
25  
26 390 obtaining funding for the study, and Pierre Sauvanet and Michael Rodrigues for technical advice.  
27  
28

### 29 391 **Authors' contributions**

30  
31  
32 392 GC, CT, JG, CF, MJ, DP, GR, CC, PEC, PR, and MB wrote the protocol. MJ, GC, PR, CT and CF  
33  
34 393 conducted statistical trial planning. PR, CT and CF handled ethics and regulatory affairs. GC, CT, HF,  
35  
36 394 MB, CF, NB, PR, and MJ wrote the paper draft. GC, MB, MJ, PR, CT and CF contributed to the trial  
37  
38 395 design and modifications. All authors read and approved the final manuscript.  
39  
40

### 41 396 **Funding**

42  
43  
44 397 This work was supported by the SIRIC Montpellier Cancer, the Biocodex Microbiota Foundation and  
45  
46 398 La Ligue Contre le Cancer (Herauld committee).  
47  
48

49  
50 399 The funding body was not involved in the study design and will not be involved in data collection, data  
51  
52 400 analysis and interpretation, and writing of the study report and publication.  
53  
54

### 55 401 **Competing interests**

56  
57  
58 402 The authors declare that they have no competing interests.  
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3 403 **Provenance and peer review**  
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6 404 Not commissioned; externally peer reviewed.  
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16 radiotherapy: mechanisms of resistance and recurrence. *Nat Rev Cancer*. 2015 Jul;15(7):409–25.  
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23 **Figure legends**

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25 577 Figure 1: MICARE flow diagram  
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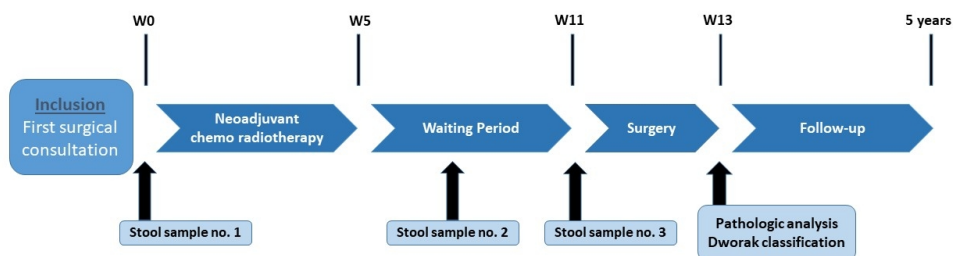


Figure 1 : MICARE flow diagram

Figure 1 : MICARE flow diagram

338x190mm (96 x 96 DPI)



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
<b>Administrative information</b>			
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	<i>Funding</i> , 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

1 **Introduction**

2

3 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

4

5

6 6b Explanation for choice of comparators NA

7

8 Objectives 7 Specific objectives or hypotheses *Objectives*, p5

9

10 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

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14 **Methods: Participants, interventions, and outcomes**

15

16 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

17

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19 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

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24 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

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27 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

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30 11c Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) NA

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34 11d Relevant concomitant care and interventions that are permitted or prohibited during the trial p8

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36 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

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1	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
2				
3				
4	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	<i>Sample size</i> , p12-13
5				
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7	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	p12-13
8				
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### 10 **Methods: Assignment of interventions (for controlled trials)**

#### 11 Allocation:

12				
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14	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
15				
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19	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
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24	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
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27	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
28				
29				
30		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
31				
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### 34 **Methods: Data collection, management, and analysis**

35				
36	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	<i>Data collection and management</i> , p11
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1		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
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4	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
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8	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
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11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	<i>Statistical analyses,</i> p13
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14		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)	<i>Statistical analyses,</i> p13
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19	<b>Methods: Monitoring</b>			
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21	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	<i>Study monitoring,</i> <i>quality control, and</i> <i>audit, p12</i>
22				
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26		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
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29	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	<i>Safety, p9</i>
30				
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33	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	<i>Study monitoring,</i> <i>quality control, and</i> <i>audit, p12</i>
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38	<b>Ethics and dissemination</b>			
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1	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	<i>Ethics approval and consent to participate, p16</i>
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4				
5	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
6				
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9	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	p16
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13		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
14				
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16	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
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19	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	<i>Competing interests, p16-17</i>
20				
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22	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	<i>Availability of data and materials, p16</i>
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25	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
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29	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	<i>Dissemination policy, p13-14</i>
30				
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33		31b	Authorship eligibility guidelines and any intended use of professional writers	Protocol**
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35		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	<i>Availability of data and materials, p16</i>
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39 **Appendices**

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1	Informed consent	32	Model consent form and other related documentation given to participants and authorised surrogates	Applicable **
2	materials			
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4	Biological	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular	<i>Sample handling,</i>
5	specimens		analysis in the current trial and for future use in ancillary studies, if applicable	p10
6				

7 \*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.  
 8 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons  
 9 "[Attribution-NonCommercial-NoDerivs 3.0 Unported](#)" license.  
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11 \*\*More information can be provided if wished by the editor.  
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# 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)

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2022-061527.R1
Article Type:	Protocol
Date Submitted by the Author:	03-Aug-2022
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<b>Primary Subject Heading</b>:	Oncology
Secondary Subject Heading:	Gastroenterology and hepatology
Keywords:	ONCOLOGY, GASTROENTEROLOGY, RADIOTHERAPY, MICROBIOLOGY

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6 2 **Determination of biomarkers associated with neoadjuvant treatment response focusing**  
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8 3 **on colibactin-producing *Escherichia coli* in patients with mid or low rectal cancer : a**  
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10 4 **prospective cohort study protocol (MICARE).**  
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16 6 Christophe Taoum<sup>1\*</sup>, Guillaume Carrier<sup>1, 2</sup>, Marta Jarlier<sup>3</sup>, Gwenaëlle Roche<sup>2, 4</sup>, Johan Gagnière<sup>2, 4</sup>,  
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11 28 **Abstract**

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14 29 **Introduction** The management of mid and low rectal cancer is based on neoadjuvant  
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16 30 chemoradiotherapy (CRT) followed by standardized surgery. There is no biomarker in rectal cancer to  
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18 31 aid clinicians in foreseeing treatment response. The determination of factors associated with treatment  
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20 32 response might allow the identification of patients who require tailored strategies (e.g. therapeutic de-  
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22 33 escalation or intensification). Colibactin-producing *Escherichia coli* (CoPEC) has been associated  
23  
24 34 with aggressive CRC and could be a poor prognostic factor. Currently no study has evaluated the  
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26 35 potential association between intestinal microbiota composition and tumour response to CRT in mid  
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28 36 and low rectal cancer. The aim of this study is to assess the association between response to  
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30 37 neoadjuvant CRT and faecal intestinal microbiota composition and/or CoPEC prevalence in patients  
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32 38 with mid or low rectal cancer.

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36 39 **Methods and analysis** This is a non-randomized bicentric prospective cohort study with a recruitment  
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38 40 capacity of 200 patients. Three stool samples will be collected from participants with histological-  
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40 41 proven adenocarcinome of mid or low rectum who meet eligibility criteria of the study protocol: one  
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42 42 before neoadjuvant treatment start, one in the period between CRT end and surgery, and one the day  
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44 43 before surgery. In each sample, CoPEC will be detected by culture in special media and molecular  
45  
46 44 (PCR) approaches. The global microbiota composition will be also assessed by the bacterial 16S  
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48 45 rRNA gene sequencing. Neoadjuvant CRT response and tumour regression grade will be described  
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50 46 using the Dworak system at pathological examination. Clinical data and survival outcomes will also be  
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52 47 collected and investigated.

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3 49 **Ethics and dissemination** MICARE was approved by the local ethics committee (Comité de  
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5 50 Protection des Personnes Sud-Est II, December 18<sup>th</sup>, 2019. Reference number 2019-A02493-54) and  
6  
7 51 the institutional review board. Patients will be required to provide written informed consent. Results  
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9 52 will be published in a peer reviewed journal.

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12 53 **Trial registration number** NCT04103567.

### 13 14 15 16 54 17 18 55 **Strengths and limitations of this study**

- 19  
20 56 • As far as we know, this is the first study to evaluate association between intestinal microbiota  
21 57 composition and tumour response to chemoradiotherapy in mid and low rectal cancer
- 22  
23 58 • MICARE is a prospective cohort study including 200 patients
- 24  
25 59 • This study is based on a non-invasive and reproducible faecal test
- 26  
27 60 • Tumour response will be described at pathological examination after surgery
- 28  
29 61 • The limitation of this study will include population stratification for delay between  
30 62 radiotherapy and surgery, and adjonction of neoadjuvant chemotherapy in tumour response  
31 63 evaluation

### 32 33 64 34 65 **Introduction**

35  
36 66 With more than 700,000 new cases and 300,000 deaths in 2018, rectal cancer is the eighth leading  
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38 67 cause of cancer deaths worldwide (1). The initial management of mid and low rectal cancer is based  
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40 68 on neoadjuvant chemoradiotherapy (CRT) for locally advanced tumours. This is associated with a  
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42 69 significant decrease of the locoregional recurrence rate, but without survival improvement (2–4).  
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44 70 Neoadjuvant treatment is followed by standardized surgery (5). Total mesorectal excision is crucial for  
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46 71 reducing tumour recurrence (6), but its significant morbidity can affect the patients' quality of life.  
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48 72 Prognosis also depends on the tumour response to neoadjuvant CRT. Currently, the surgical strategy  
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50 73 is adapted in function of the tumour response to neoadjuvant treatment, assessed by magnetic  
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52 74 resonance imaging (MRI) after CRT end (7). Indeed, the objective is therapeutic de-escalation with  
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54 75 rectal preservation to decrease morbidity and functional disorders. For patients with complete response  
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56 76 (up to 25% of patients), careful monitoring without surgery ("watch and wait" strategy) has been  
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58 77 proposed (8,9). For small tumours with good response to CRT, transanal excision with rectal

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3 78 preservation seems to be feasible in terms of cancer prognosis (10). For patients with large tumours or  
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5 79 a locally advanced disease, a tailored treatment strategy with total neoadjuvant therapy (TNT) is now a  
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7 80 gold standard (11,12). After surgical excision, the tumour response is classified in five pathologic  
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9 81 tumour response grades, according to the Dworak classification, on the basis of the pathology findings  
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11 82 (13). Recent studies reported up to 30% of poor responders (grades 0 and 1) (14,15). These data  
12  
13 83 emphasize the importance of the initial tumour staging and response to neoadjuvant CRT for tailoring  
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15 84 surgical strategies. MRI is an essential tool for these two assessments (16–18). These data highlight  
16  
17 85 the need of response predictive models to adapt the TNT in mid and low rectal cancer.  
18  
19 86 Gut microbiota behaves as a real organ and participates in intestinal homeostasis. An imbalance in its  
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21 87 composition (dysbiosis) could be involved in many pathologies, including colorectal cancer (CRC)  
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23 88 (19–21). *Escherichia coli* (*E. coli*) has been widely described as a bacteria which could be involved in  
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25 89 CRC.(22,23). *E. coli* is the predominant aero-anaerobic Gram-negative specie in human colon, but it is  
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27 90 also a pathogen involved in various intestinal diseases (24). Indeed, some *E. coli* strains have acquired  
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29 91 the capacity to produce toxins named cyclomodulins, including colibactin that is encoded by the pks  
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31 92 island(25). Colibactin-producing *E. coli* (CoPEC) has genotoxic effects by inducing DNA damage and  
32  
33 93 chromosomal instability (25–27). CoPEC implication in CRC has been demonstrated, particularly in  
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35 94 aggressive forms (28–34). Specifically, higher *E. coli* colonization rate and higher prevalence of  
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37 95 CoPEC are found in patients with TNM stage III or IV tumors (29) (UICC TNM Classification, 8<sup>th</sup>  
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39 96 Edition, 2017) (35). Moreover, CoPEC gut colonization might contribute to modulate the  
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41 97 immunotherapy efficacy (36). Recent clinical studies discussed the prognostic role of intestinal  
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43 98 microbiota in the tumour response following surgery and chemotherapy or immunotherapy (37), and  
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45 99 suggested that it could be used as a biomarker to predict tumour response to neoadjuvant treatments.  
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47 100 On the other hand, very few clinical studies have assessed the influence of gut microbiota on  
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49 101 radiotherapy efficacy, especially in rectal cancer. Recently, a preclinical study showed that mice which  
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51 102 survive a high dose of radiation, harboured gut microbiota enriched with *Lachnospiraceae* and  
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53 103 *Enterococcaceae* (38). Yet, a description of the intestinal microbiota composition before neoadjuvant  
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55 104 therapy could allow identifying predictive bacterial markers of tumour response in rectal cancer, and  
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57 105 to adapt TNT.



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3 106 Indeed, chronic exposure of the gastrointestinal tract to genotoxins could be a prognostic marker of  
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5 107 radiotherapy response. CoPEC colonization would start at the very beginning of life (38) and might  
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7 108 lead to exposure of the intestinal mucosa to chronic genotoxic stress. The resulting damage could give  
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9 109 cells the ability to resist to other genotoxic stresses, such as radiation therapy. One *in vitro* study  
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11 110 already showed the decreased radiation sensitivity of cells incubated by colibactin (27). Therefore,  
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13 111 developing a non-invasive method to analyse gut microbiota composition and to evaluate CoPEC  
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15 112 implication in the response to CRT could help clinicians to tailor cancer management and to develop  
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17 113 tools to control the pathologic microorganisms identified as new therapeutic targets.  
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## 22 23 24 115 **Methods and analysis**

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27 116 This study protocol is written in accordance with the SPIRIT guidelines. (Supplementary file 1)  
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### 30 117 **Objectives**

#### 31 32 33 118 *Primary objective*

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36 119 The study primary objective is to assess the correlation between response to neoadjuvant CRT and  
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38 120 CoPEC presence in stool samples.  
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#### 41 121 *Secondary objectives*

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44 122 - To analyse in a non-targeted manner the global microbiota composition before CRT and to  
45  
46 123 evaluate the correlation between composition and response to treatment  
47  
48 124 - To study the modulation of the intestinal microbiota by CRT  
49  
50 125 - To describe the correlation between clinical data and microbiota composition modulation  
51  
52 126 induced by CRT  
53  
54 127 - To determine microbiological prognostic factors of overall survival, disease-specific survival  
55  
56 128 and relapse-free survival (locoregional and metastatic) in patients with low or mid rectum  
57  
58 129 cancer  
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- 1  
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3 130 - To create a microbiological database for future mechanistic analyses  
4  
5 131 - To study the modulation of CoPEC colonization by CRT  
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7

8 132 ***Study design***  
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10  
11 133 The study is a non-randomized bicentric prospective cohort study. Two surgical teams will be  
12  
13 134 involved - Institut du Cancer de Montpellier and CHU de Clermont-Ferrand ; and an INSERM Unit –  
14  
15 135 M2iSH Clermont-Ferrand. The study actually started on January 2020 and the estimated study  
16  
17 136 completion date is November 2027.  
18  
19

20 137 ***Patients' selection***  
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22

23 138 ***Inclusion criteria***  
24  
25

- 26 139 - Histologically-proven adenocarcinoma of low or mid rectum, of stage II or III (UICC TNM  
27  
28 140 Classification, 8<sup>th</sup> Edition, 2017 (35))  
29  
30 141 - Patient eligible for neoadjuvant treatment (50 Gray radiation and capecitabine, CAP 50),  
31  
32 142 according to the French national recommendations (5,39)  
33  
34 143 - Informed signed consent received  
35  
36 144 - Man or woman aged  $\geq 18$  years  
37  
38 145 - Appropriate contraceptive measures taken by men and pre-menopausal women before study  
39  
40 146 entry and for at least 8 weeks after the last CRT cycle. Patients should be informed by the  
41  
42 147 investigator on the contraceptive measures to use.  
43  
44  
45

46 148 ***Exclusion criteria***  
47  
48

- 49 149 - Antibiotic treatment at the time of stool sampling or in the month before.  
50  
51 150 - Presence of a derivative stoma  
52  
53 151 - Previous chemotherapy treatment for rectum cancer  
54  
55 152 - Patient not affiliated to the French social security system  
56  
57 153 - Patient with possible poor treatment compliance for psychologic, familial, social and  
58  
59 154 geographic reasons  
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3 155 - Legal incapacity or limited legal capacity  
4  
5 156 - Pelvic radiotherapy or brachytherapy in the year before inclusion in the study  
6  
7 157 - History of other cancers in the 5 last years, except for cervical carcinoma *in situ* and skin  
8  
9 158 carcinoma, but including melanoma under treatment  
10  
11 159 - Pregnant or breastfeeding woman  
12  
13  
14  
15 160  
16  
17 161 ***Study sponsor***  
18  
19  
20 162 The sponsor (Montpellier Cancer Institute, ICM) is responsible for the study design and management,  
21  
22 163 and for obtaining all study authorizations (Persons Protection Committee, National Agency for  
23  
24 164 Medical Security). It will also declare to these authorities the inclusion period beginning and end,  
25  
26 165 produce the final study report, inform the competent authorities of the trial results, and store all study-  
27  
28 166 related documents for at least 15 years after the study end.  
29  
30  
31 167 ***Clinical study procedures***  
32  
33  
34 168 ***Inclusion in the study***  
35  
36  
37 169 The study flow diagram is presented in Figure 1.  
38  
39  
40 170 Before study entry, all patients will receive exhaustive explanations on the study aims and procedures.  
41  
42 171 A signed informed consent will be obtained from all patients before any study procedure  
43  
44 172 (Supplementary file 2). At baseline, demographic (sex, age), clinical (performance status, weight,  
45  
46 173 height, medical history, initial diagnosis date, tumour localization, histologic type) and biological  
47  
48 174 (complete blood count, carcinoembryonic antigen (CEA) level) data will be collected (Table 1).  
49  
50 175 Patients will undergo rectal examination and tumour staging by computed tomography (CT), rectal  
51  
52 176 MRI, and possibly rectal endoscopic ultrasound examination (depending on the centre decision).  
53  
54  
55  
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177 During the surgical consultation, the first stool sample (stool sample N°1) may be collected during  
 178 rectal examination (faeces left on the clinician's glove), or by proctoscopy. Otherwise, the stool sample  
 179 will be collected by the patient.

180 **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

181 MRI: Magnetic resonance imaging; CT: computed tomography.

182 *Neoadjuvant treatment*

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

188

189

190

191

### 192 *Re-evaluation*

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

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

### 199 *Surgery*

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

### 207 *Pathologic analysis*

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

211

212

213

214

215 **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)

216

217 ***Safety***

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

220 ***Follow-up and study duration***

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

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

228 ***Microbiological analyses***229 ***Sample handling***

1  
2  
3 230 Three stool samples will be collected during the study (Figure 1): i) one at patient inclusion, before  
4  
5 231 any treatment, to describe the baseline intestinal microbiota composition; ii) one during the interval  
6  
7 232 between the end of neoadjuvant CRT and surgery, at the surgical consultation for tumour reappraisal;  
8  
9 233 and iii) one just before bowel preparation (mechanical or antibiotics) for surgery.

11  
12 234 Each sample will be divided into two cryotubes: one empty and one with 15% glycerol/DMEM to  
13  
14 235 preserve cell integrity. Samples will be immediately stored at -80°C until transport to the M2iSH  
15  
16 236 laboratory, Clermont-Ferrand, France, which will be in charge of the molecular analysis and storage of  
17  
18 237 the samples.

#### 20 238 *E. coli* strain identification and CoPEC detection

22  
23  
24 239 All microbiological analyses will be performed as previously described (28). After thawing, samples  
25  
26 240 stored in DMEM/glycerol will be crushed and diluted in sterile phosphate buffered saline pH 7.4  
27  
28 241 before plating on TBX agar and chromogenic agar chromID CPS3® plates (bioMérieux) to allow the  
29  
30 242 identification and quantitation of enterobacteria. Colonies (around 48 per sample) will be collected for  
31  
32 243 molecular typing, and their identification will be confirmed with the automated Vitek® II  
33  
34 244 (bioMérieux) system. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR will be used as  
35  
36 245 genotyping method to determine the number of *E. coli* strains per sample (28).

37  
38  
39 246 *E. coli* harbouring the colibactin-encoding *pks* island will be identified by PCR analysis of each *E. coli*  
40  
41 247 isolate (41). This will allow identifying the presence of CoPEC (primary objective).

#### 43 248 *Untargeted analysis of the local microbiota composition*

44  
45  
46  
47 249 Global microbiota modifications will be assessed by high-throughput sequencing of the bacterial 16S  
48  
49 250 rRNA gene in DNA extracted from the three stool samples using the NucleoSpin® DNA stool kit  
50  
51 251 (Macherey-Nagel, Hoerd, France), according to the manufacturer's instructions. Quantitative PCR  
52  
53 252 will be performed to quantify pro-carcinogenic bacterial species, such as *Fusobacterium nucleatum*,  
54  
55 253 *Enterococcus faecalis*, *bft*-positive *Bacteroides fragilis*, and CoPEC. In addition, the V4 region of the  
56  
57 254 bacterial 16S rRNA gene will be amplified using the 515F/806R primer pair followed by Illumina  
58  
59 255 high throughput sequencing on a MiSeq® apparatus, according to the manufacturer's guidelines. A

1  
2  
3 256 global description of the intestinal microbiota could also be obtained by shotgun metagenomic  
4  
5 257 sequencing to access the microbiota functional features after selection of the more informative  
6  
7 258 samples.  
8  
9

## 10 259 ***Endpoints***

### 11 12 13 260 *Primary endpoint*

14  
15  
16 261 The primary endpoint (associated with the primary objective) is the relative risk (RR) of poor response  
17  
18 262 to neoadjuvant CRT in patients colonized by CoPEC ("exposed") compared to non-colonized patients  
19  
20 263 ("unexposed").  
21  
22

23 264

### 24 25 26 265 *Secondary endpoints*

- 27  
28  
29 266 - Prevalence and CoPEC colonization rate before and after CRT  
30  
31  
32 267 - Other bacterial strains present before CRT and relative risk of poor response to CRT in  
33  
34 268 colonized and non-colonized patients  
35  
36 269 - Type, prevalence, and colonization rate of bacteria other than CoPEC in the microbiota, before  
37  
38 270 and after CRT  
39  
40 271 - Percentage of colonized patients, depending on the bacterial type, according to the clinical  
41  
42 272 parameters (age, sex, body mass index)  
43  
44 273 - Hazard ratio (HR) for overall survival, disease-specific survival, and relapse-free survival  
45  
46 274 (locoregional or metastatic) in colonized patients, for the different bacterial types, according to  
47  
48 275 the overall bacterial composition (including CoPEC), and in non-colonized patients.  
49  
50  
51

### 52 276 ***Data collection and management***

53  
54  
55 277 The database will be managed by the sponsor, and data stored at the Data processing centre,  
56  
57 278 Biometrics Unit of the Montpellier Cancer Institute. Case report form design and clinical data  
58  
59 279 management will be implemented using the Ennov Clinical® software. Microbiological data will be  
60



1  
2  
3 280 collected in a database first stored at the M2iSH laboratory, and then transferred to the sponsor  
4  
5 281 database for analysis. Data and any trial documents will be made available upon reasonable request  
6  
7 282 and after signature of a data access agreement.  
8  
9

10 283 In accordance with the General Data Protection Regulation (GDPR), a registration number will be  
11  
12 284 used to identify each patient. The corresponding table will be encrypted and stored in a secure place.  
13  
14 285 Special vigilance will be exercised throughout the study to maintain data anonymization.  
15  
16

### 17 286 ***Study monitoring, quality control, and audit***

18  
19  
20 287 According to the sponsor's risk-based monitoring plan (study participants, logistics, resources,  
21  
22 288 impact), the collection of the patient informed consents and the respect of the study protocol and  
23  
24 289 procedures will be monitored.  
25  
26

27 290 To guarantee the originality of all data and in accordance with the Good Clinical Practices, quality  
28  
29 291 control will be performed by the sponsor. The study will be managed according to the sponsor  
30  
31 292 procedures and in respect of the protocol, and the quality of the data included in the report forms will  
32  
33 293 be checked.  
34  
35

36 294 The sponsor may wish to conduct an audit at some investigating centers. Audits may be conducted by  
37  
38 295 the sponsor or any duly authorized person for at least 15 years after the trial.  
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40

### 41 296 ***Statistical considerations***

#### 42 43 44 297 *Sample size*

45  
46  
47 298 The recruitment capacity for this exploratory study will be around 200 patients. For a mean rate of  
48  
49 299 30% of poor responders to the neoadjuvant treatment among the patients not colonized by CoPEC  
50  
51 300 (*i.e.*, a proportion of response  $P_2=0.30$  among unexposed patients), the study will be able to estimate a  
52  
53 301 relative risk of 1.7 ( $RR=1.7$ ) with a 30% precision and a confidence interval at 95% ( $\alpha=0.05$ ). Patients  
54  
55 302 in whom the CoPEC colonization status cannot be determined at baseline, in whom CRT must be  
56  
57 303 prematurely arrested, or who cannot undergo surgery will be considered non-evaluable.  
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3 304 Considering a 10% rate of potentially non-evaluable patients, a total of 220 patients (20 supplementary  
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5 305 patients) will be included in the study.  
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8 306  
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11 307 *Study population*  
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13  
14 308 Two populations will be defined for the analysis. The intention-to-treat population will be defined as  
15  
16 309 all patients included in the study, treated (patients who received complete/partial neoadjuvant  
17  
18 310 treatment) and not treated (patients who did not undergo CRT), eligible (*i.e.*, all patients who were  
19  
20 311 included in the study without violation of a major inclusion or exclusion criterion) or not, and  
21  
22 312 with/without baseline stool sample. The per-protocol population will include all eligible patients,  
23  
24 313 treated (complete or partial CRT), and with baseline stool sample.  
25

26  
27 314 *Statistical analyses*  
28

29  
30 315 Qualitative variables will be described by frequencies and percentages, and quantitative variables with  
31  
32 316 means, standard deviations, medians, and ranges. No imputation method will be used in case of  
33  
34 317 missing data. Correlations between qualitative variables will be assessed using the Chi-2 or Fisher-  
35  
36 318 exact test. Quantitative variables will be compared using the Student's *t*-test or the Kruskal-Wallis test.  
37  
38 319 Comparison of quantitative variables at different times (before and after CRT) will be assessed using  
39  
40 320 the Wilcoxon test for matched samples. The relative risk of poor response to neoadjuvant CRT in  
41  
42 321 CoPEC-colonized patients (or colonized by other bacteria) compared to non-colonized patients will be  
43  
44 322 estimated using a logistic regression (univariate analysis) and will be presented with the 95%  
45  
46 323 confidence interval (95% CI). Survival analyses will be performed using the Kaplan-Meier method  
47  
48 324 and survival distributions compared with the log rank test. HRs and their 95% CI will be estimated  
49  
50 325 with a Cox proportional risk model. A detailed statistical analysis plan (SAP) will be written before  
51  
52 326 the database is locked for analysis; supplementary subgroup analyses, if appropriated, will be specified  
53  
54 327 in the SAP. All analyses will be performed using the Stata version 16 software (StataCorp LP, College  
55  
56 328 Station, TX).  
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3 329 **Patient and public involvement**  
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6 330 There was no patient or public involvement in the design of this study.  
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15 333 **Discussion**  
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18 334 The implication of intestinal microbiota in CRC has been widely demonstrated (42). Several recent  
19  
20 335 studies suggest that different bacterial species, including CoPEC, could be used as biological  
21  
22 336 biomarkers for CRC diagnosis and prognosis (29,36,41,43,44). The potential role of the gut microbiota  
23  
24 337 in the modulation of the efficacy of anti-tumour treatments has been studied, with interesting results  
25  
26 338 regarding chemotherapy and immunotherapy (37). However, these studies were focused on colon  
27  
28 339 cancer dysbiosis and few data are available on rectal cancer and mucosa. Moreover, the correlation  
29  
30 340 between gut microbiota homeostasis and radiation sensitivity remains unclear. Patients treated by  
31  
32 341 pelvic radiation develop long-term complications that affect their quality of life, and have worse  
33  
34 342 functional results than patients treated with surgery alone (45,46). It has been hypothesized that the  
35  
36 343 intestinal microbiota has a significant impact on pelvic enteropathy (47); however, pelvic irradiation is  
37  
38 344 responsible for microbiota dysbiosis (48,49). To our knowledge, no previous study has assessed the  
39  
40 345 local microbiota composition and its implication in the response to CRT in rectal cancer, although  
41  
42 346 treatment response is one of the key points for prognosis estimation. Biomarkers to predict tumour  
43  
44 347 response in rectal cancer are still crucially needed. Imaging techniques (50) and biological markers  
45  
46 348 (51,52) have been evaluated, but they are often expensive and complicated to implement. Moreover,  
47  
48 349 the results are still discussed. Currently, their use seems to be limited to research and expert centers.  
49  
50 350 The present study will describe the intestinal microbiota composition in patients with rectal cancer  
51  
52 351 receiving neoadjuvant CRT to show its potential correlation with the tumour response, focusing on  
53  
54 352 CoPEC colonization. In addition, the effect of radiotherapy on the local intestinal microbiota  
55  
56 353 composition will be studied by comparing stool samples collected before and after CRT. Unlike  
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3 354 studies on the intestinal microbiota in colon cancer in which tumour fragments are needed, in the case  
4  
5 355 of mid or low rectal cancer stool samples should be representative of the local microbiota.  
6  
7 356 One of the main hypotheses to explain CoPEC effect on CRT response is based on their capacity to  
8  
9 357 induce DNA damage (25–27). Besides the direct effect on the cell, radiotherapy is also cytotoxic  
10  
11 358 through the production of reactive oxygen species and reactive nitrogen species (53). Chronic  
12  
13 359 genotoxic stress caused by CoPEC presence in gut mucosa could lead to an adaptation of the gut  
14  
15 360 mucosa to genotoxic agents and consequently to reduce radiation sensitivity and resistance to therapy.  
16  
17 361 For instance, in an *in vitro* study, Wilson et al. observed less DNA damage in colibactin-positive  
18  
19 362 epithelial cells infected by CoPEC (27). Moreover, radiation sensitivity is closely linked to autophagy  
20  
21 363 regulation (54,55). Recent studies showed the involvement of gut microbiota in autophagy regulation,  
22  
23 364 with a link to chemoresistance (56). Ionizing radiation effects might be modified indirectly through  
24  
25 365 autophagy deregulation induced by gut microbiota. In addition, radiotherapy cytotoxic effect could  
26  
27 366 result in a modification of the local microenvironment with significant clinical consequences (57).  
28  
29 367 The modulation of radiotherapy efficacy by the intestinal microbiota is an emerging concept in CRC,  
30  
31 368 but its study faces many obstacles, especially sample availability. In this study, we want to develop a  
32  
33 369 non-invasive reproducible faecal test that could become a key biomarker to predict tumour response to  
34  
35 370 CRT. Our work will help clinicians to tailor neoadjuvant therapeutic strategies with the final goal of  
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37 371 increasing tumor response, organ preservation, and reducing surgical morbidity, while maintaining  
38  
39 372 oncological safety.

373

#### 374 **Ethics and dissemination**

375 The study protocol (version 3.0, dated on September 24<sup>th</sup>, 2019) was approved by the local ethics  
376 committee (Comité de Protection des Personnes Sud-Est II, December 18<sup>th</sup>, 2019, Reference number  
377 2019-A02493-54) and the institutional review board COMERE. The French National Drug Agency  
378 Authority (ANSM) was informed. The study was registered on Clinicaltrials.gov, identifier  
379 NCT04103567.

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3 380 All patients will be informed of the study objectives and procedures by the investigators before  
4  
5 381 enrolment. A signed informed consent will be obtained from all patients before their inclusion in the  
6  
7 382 study and before any study procedure is performed. All patients may end their participation in the  
8  
9 383 study at any time, for whatever reason, without any consequence or prejudice concerning their care.  
10  
11 384 Study participants will be able to request global results from investigators as soon as study results  
12  
13 385 become available.

14  
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16 386 In the event of substantial modification, the request will be sent by the sponsor to the ethics committee  
17  
18 387 for an opinion. Upon receipt of the favourable opinion, the sponsor will send the amended version of  
19  
20 388 the protocol to all investigators.

21  
22  
23 389 The study will be conducted in accordance with the current French and European Regulatory  
24  
25 390 requirements, including regulations on biomedical research from the Public Health Code, the bioethics  
26  
27 391 and data protection laws and decrees, the French Jardé's law on research implicating human beings,  
28  
29 392 the Good Clinical Practice, and the Helsinki Declaration.

### 33 393 **Acknowledgements**

34  
35  
36 394 The authors thank the Clinical Research and Innovation Department of the Montpellier Cancer  
37  
38 395 Institute for help with regulatory and administrative aspects of the study, Dr. Stéphanie Delaine for her  
39  
40 396 help in obtaining funding for the study, and Pierre Sauvanet and Michael Rodrigues for technical  
41  
42 397 advice.

### 45 398 **Authors' contributions**

46  
47  
48 399 GC, CT, JG, CF, MJ, GR, CC, PEC, PR, and MB wrote the protocol. MJ, GC, PR, CT and CF  
49  
50 400 conducted statistical trial planning. PR, CT and CF handled ethics and regulatory affairs. GC, CT, HF,  
51  
52 401 MB, CF, NB, PR, and MJ wrote the paper draft. GC, MB, MJ, PR, CT and CF contributed to the trial  
53  
54 402 design and modifications. All authors read and approved the final manuscript.

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3 **405 Funding**  
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5

6 406 This work was supported by the SIRIC Montpellier Cancer, the Biocodex Microbiota Foundation and  
7  
8 407 La Ligue Contre le Cancer (Herauld committee).  
9

10  
11 408 The funding body was not involved in the study design and will not be involved in data collection,  
12  
13 409 data analysis and interpretation, and writing of the study report and publication.  
14  
15

16 410  
17  
18

19 **411 Competing interests**  
20  
21

22 412 The authors declare that they have no competing interests.  
23  
24

25 **413 Provenance and peer review**  
26  
27

28 414 Not commissioned; externally peer reviewed.  
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31 415  
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47 586 **Figure legends**

48  
49 587 Figure 1: MICARE flow diagram  
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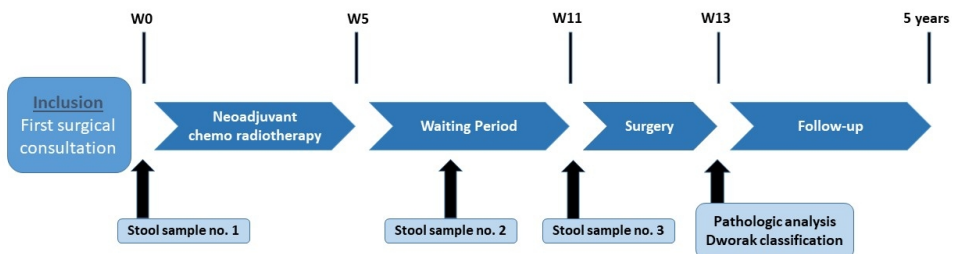


Figure 1 : MICARE flow diagram

Figure 1 : MICARE flow diagram

338x190mm (96 x 96 DPI)



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

Section/item	Item No	Description	Addressed on page number
<b>Administrative information</b>			
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	<i>Funding</i> , 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

1	<b>Introduction</b>			
2				
3	Background and	6a	Description of research question and justification for undertaking the trial, including summary of relevant	p3-5
4	rationale		studies (published and unpublished) examining benefits and harms for each intervention	
5				
6		6b	Explanation for choice of comparators	NA
7				
8	Objectives	7	Specific objectives or hypotheses	<i>Objectives</i> , p5
9				
10	Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group),	
11			allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	<i>Study design</i> , p5
12				
13				
14	<b>Methods: Participants, interventions, and outcomes</b>			
15				
16	Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will	<i>Study design</i> , p5
17			be collected. Reference to where list of study sites can be obtained	
18				
19	Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and	<i>Patients' selection</i> ,
20			individuals who will perform the interventions (eg, surgeons, psychotherapists)	p6
21				
22				
23				
24	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be	<i>Clinical study</i>
25			administered	<i>procedures</i> , p7-9
26				
27		11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose	p8
28			change in response to harms, participant request, or improving/worsening disease)	
29				
30		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence	NA
31			(eg, drug tablet return, laboratory tests)	
32				
33		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	p8
34				
35	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood	
36			pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg,	p11
37			median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen	
38			efficacy and harm outcomes is strongly recommended	
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1	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
2				
3				
4	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	<i>Sample size</i> , p12-13
5				
6				
7	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	p12-13
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9				

### 10 **Methods: Assignment of interventions (for controlled trials)**

#### 11 Allocation:

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13				
14	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
15				
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19	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
20				
21				
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23				
24	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
25				
26				
27	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
28				
29				
30		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
31				
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### 34 **Methods: Data collection, management, and analysis**

35				
36	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	<i>Data collection and management</i> , p11
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1		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
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3				
4	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
5				
6				
7				
8	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
9				
10				
11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	<i>Statistical analyses,</i>
12				p13
13				
14		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)	<i>Statistical analyses,</i>
15				p13
16				
17				

**Methods: Monitoring**

19				
20				
21	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	<i>Study monitoring, quality control, and audit, p12</i>
22				
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25				
26		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
27				
28				
29	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	<i>Safety, p9</i>
30				
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33	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	<i>Study monitoring, quality control, and audit, p12</i>
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**Ethics and dissemination**

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1	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	<i>Ethics approval and consent to participate, p16</i>
2				
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4				
5	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
6				
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8				
9	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	p16
10				
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12				
13		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
14				
15				
16	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
17				
18				
19	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	<i>Competing interests, p16-17</i>
20				
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22	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	<i>Availability of data and materials, p16</i>
23				
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25	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
26				
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29	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	<i>Dissemination policy, p13-14</i>
30				
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33		31b	Authorship eligibility guidelines and any intended use of professional writers	Protocol**
34				
35		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	<i>Availability of data and materials, p16</i>
36				
37				
38				

## Appendices

1	Informed consent	32	Model consent form and other related documentation given to participants and authorised surrogates	Applicable **
2	materials			
3				
4	Biological	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular	<i>Sample handling,</i>
5	specimens		analysis in the current trial and for future use in ancillary studies, if applicable	p10
6				

7 \*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.  
8 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons  
9 "[Attribution-NonCommercial-NoDerivs 3.0 Unported](#)" license.  
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11 \*\*More information can be provided if wished by the editor.  
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# 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:** |\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|

**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 V 4.0 du 23/02/2021 du protocole MICARE

# 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.

<input type="checkbox"/>	J'accepte que mes données cliniques soient utilisées pour des recherches ultérieures, en France ou dans l'Union Européenne
<input type="checkbox"/>	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 :

Nom de l'investigateur :

Date :

Date :

Signature :

Signature :

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

Formulaire de consentement V 4.0 du 23/02/2021 du protocole MICARE

TREATMENT	EVENTS	YES/NO
<b>CHEMO-RADIOTHERAPY</b>	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
<b>SURGERY</b>	<b><u>Per-operative</u></b>	
	Bleeding	YES/NO
	Intestinal perforation	YES/NO
	Vascular wound	YES/NO
	<b><u>Post-operative</u></b>	
	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)

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2022-061527.R2
Article Type:	Protocol
Date Submitted by the Author:	09-Sep-2022
Complete List of Authors:	<p>Taoum, Christophe; Institut régional du Cancer de Montpellier, Surgical Oncology  Carrier, Guillaume; Institut régional du Cancer de Montpellier, Surgical Oncology; Clermont Auvergne University, Microbes, Intestin, Inflammation et Susceptibilité de l'Hôte (M2iSH)  Jarlier, Marta; Regional Cancer Centre Val d'Aurelle - Paul Lamarque, Biometrics unit  Roche, Gwenaëlle; Clermont Auvergne University, Microbes, Intestin, Inflammation et Susceptibilité de l'Hôte (M2iSH)  Gagniere, Johan; University Hospital of Clermont-Ferrand, Digestive and Hepatobiliary Surgery  Fiess, Catherine; Regional Cancer Centre Val d'Aurelle - Paul Lamarque, Clinical Research and Innovation Department  De forges, Helene; Regional Cancer Centre Val d'Aurelle - Paul Lamarque, Clinical Research and Innovation Department, Institut du Cancer de Montpellier  Chevarin, Caroline; Clermont Auvergne University  Colombo, Pierre-Emmanuel; Regional Cancer Centre Val d'Aurelle - Paul Lamarque, Surgical Oncology  Barnich, Nicolas; Clermont Auvergne University, Microbes, Intestin, Inflammation et Susceptibilité de l'Hôte (M2iSH)  Rouanet, Philippe; Regional Cancer Centre Val d'Aurelle - Paul Lamarque, Surgical Oncology  Bonnet, Mathilde; Clermont Auvergne University, Microbes, Intestin, Inflammation et Susceptibilité de l'Hôte (M2iSH)</p>
<b>Primary Subject Heading</b>:	Oncology
Secondary Subject Heading:	Gastroenterology and hepatology
Keywords:	ONCOLOGY, GASTROENTEROLOGY, RADIOTHERAPY, MICROBIOLOGY



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6 2 **Determination of biomarkers associated with neoadjuvant treatment response focusing**  
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8 3 **on colibactin-producing *Escherichia coli* in patients with mid or low rectal cancer : a**  
9  
10 4 **prospective clinical study protocol (MICARE).**  
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13 5

16 6 Christophe Taoum<sup>1\*</sup>, Guillaume Carrier<sup>1, 2</sup>, Marta Jarlier<sup>3</sup>, Gwenaëlle Roche<sup>2, 4</sup>, Johan Gagnière<sup>2, 4</sup>,  
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11 28 **Abstract**  
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14 29 **Introduction** The management of mid and low rectal cancer is based on neoadjuvant  
15  
16 30 chemoradiotherapy (CRT) followed by standardized surgery. There is no biomarker in rectal cancer to  
17  
18 31 aid clinicians in foreseeing treatment response. The determination of factors associated with treatment  
19  
20 32 response might allow the identification of patients who require tailored strategies (e.g. therapeutic de-  
21  
22 33 escalation or intensification). Colibactin-producing *Escherichia coli* (CoPEC) has been associated  
23  
24 34 with aggressive CRC and could be a poor prognostic factor. Currently no study has evaluated the  
25  
26 35 potential association between intestinal microbiota composition and tumour response to CRT in mid  
27  
28 36 and low rectal cancer. The aim of this study is to assess the association between response to  
29  
30 37 neoadjuvant CRT and faecal intestinal microbiota composition and/or CoPEC prevalence in patients  
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32 38 with mid or low rectal cancer.  
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36 39 **Methods and analysis** This is a non-randomized bicentric prospective clinical study with a  
37  
38 40 recruitment capacity of 200 patients. Three stool samples will be collected from participants with  
39  
40 41 histological-proven adenocarcinome of mid or low rectum who meet eligibility criteria of the study  
41  
42 42 protocol: one before neoadjuvant treatment start, one in the period between CRT end and surgery, and  
43  
44 43 one the day before surgery. In each sample, CoPEC will be detected by culture in special media and  
45  
46 44 molecular (PCR) approaches. The global microbiota composition will be also assessed by the bacterial  
47  
48 45 16S rRNA gene sequencing. Neoadjuvant CRT response and tumour regression grade will be  
49  
50 46 described using the Dworak system at pathological examination. Clinical data and survival outcomes  
51  
52 47 will also be collected and investigated.  
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3 49 **Ethics and dissemination** MICARE was approved by the local ethics committee (Comité de  
4  
5 50 Protection des Personnes Sud-Est II, December 18<sup>th</sup>, 2019. Reference number 2019-A02493-54) and  
6  
7 51 the institutional review board. Patients will be required to provide written informed consent. Results  
8  
9 52 will be published in a peer reviewed journal.

10  
11  
12 53 **Trial registration number** NCT04103567.  
13  
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16 54

### 17 18 55 **Strengths and limitations of this study**

- 19  
20 56 • As far as we know, this is the first study to evaluate association between intestinal microbiota  
21 57 composition and tumour response to chemoradiotherapy in mid and low rectal cancer
- 22  
23 58 • MICARE is a prospective clinical study including 200 patients
- 24  
25 59 • This study is based on a non-invasive and reproducible faecal test
- 26  
27 60 • Tumour response will be described at pathological examination after surgery
- 28  
29 61 • The limitation of this study will include population stratification for delay between  
30 62 radiotherapy and surgery, and adjonction of neoadjuvant chemotherapy in tumour response  
31 63 evaluation

### 32 33 64 34 65 **Introduction**

35  
36 66 With more than 700,000 new cases and 300,000 deaths in 2018, rectal cancer is the eighth leading  
37  
38 67 cause of cancer deaths worldwide (1). The initial management of mid and low rectal cancer is based  
39  
40 68 on neoadjuvant chemoradiotherapy (CRT) for locally advanced tumours. This is associated with a  
41  
42 69 significant decrease of the locoregional recurrence rate, but without survival improvement (2–4).  
43  
44 70 Neoadjuvant treatment is followed by standardized surgery (5). Total mesorectal excision is crucial for  
45  
46 71 reducing tumour recurrence (6), but its significant morbidity can affect the patients' quality of life.  
47  
48 72 Prognosis also depends on the tumour response to neoadjuvant CRT. Currently, the surgical strategy  
49  
50 73 is adapted in function of the tumour response to neoadjuvant treatment, assessed by magnetic  
51  
52 74 resonance imaging (MRI) after CRT end (7). Indeed, the objective is therapeutic de-escalation with  
53  
54 75 rectal preservation to decrease morbidity and functional disorders. For patients with complete response  
55  
56 76 (up to 25% of patients), careful monitoring without surgery ("watch and wait" strategy) has been  
57  
58 77 proposed (8,9). For small tumours with good response to CRT, transanal excision with rectal

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2  
3 78 preservation seems to be feasible in terms of cancer prognosis (10). For patients with large tumours or  
4  
5 79 a locally advanced disease, a tailored treatment strategy with total neoadjuvant therapy (TNT) is now a  
6  
7 80 gold standard (11,12). After surgical excision, the tumour response is classified in five pathologic  
8  
9 81 tumour response grades, according to the Dworak classification, on the basis of the pathology findings  
10  
11 82 (13). Recent studies reported up to 30% of poor responders (grades 0 and 1) (14,15). These data  
12  
13 83 emphasize the importance of the initial tumour staging and response to neoadjuvant CRT for tailoring  
14  
15 84 surgical strategies. MRI is an essential tool for these two assessments (16–18). These data highlight  
16  
17 85 the need of response predictive models to adapt the TNT in mid and low rectal cancer.  
18  
19 86 Gut microbiota behaves as a real organ and participates in intestinal homeostasis. An imbalance in its  
20  
21 87 composition (dysbiosis) could be involved in many pathologies, including colorectal cancer (CRC)  
22  
23 88 (19–21). *Escherichia coli* (*E. coli*) has been widely described as a bacteria which could be involved in  
24  
25 89 CRC.(22,23). *E. coli* is the predominant aero-anaerobic Gram-negative specie in human colon, but it is  
26  
27 90 also a pathogen involved in various intestinal diseases (24). Indeed, some *E. coli* strains have acquired  
28  
29 91 the capacity to produce toxins named cyclomodulins, including colibactin that is encoded by the pks  
30  
31 92 island(25). Colibactin-producing *E. coli* (CoPEC) has genotoxic effects by inducing DNA damage and  
32  
33 93 chromosomal instability (25–27). CoPEC implication in CRC has been demonstrated, particularly in  
34  
35 94 aggressive forms (28–34). Specifically, higher *E. coli* colonization rate and higher prevalence of  
36  
37 95 CoPEC are found in patients with TNM stage III or IV tumors (29) (UICC TNM Classification, 8<sup>th</sup>  
38  
39 96 Edition, 2017) (35). Moreover, CoPEC gut colonization might contribute to modulate the  
40  
41 97 immunotherapy efficacy (36). Recent clinical studies discussed the prognostic role of intestinal  
42  
43 98 microbiota in the tumour response following surgery and chemotherapy or immunotherapy (37), and  
44  
45 99 suggested that it could be used as a biomarker to predict tumour response to neoadjuvant treatments.  
46  
47 100 On the other hand, very few clinical studies have assessed the influence of gut microbiota on  
48  
49 101 radiotherapy efficacy, especially in rectal cancer. Recently, a preclinical study showed that mice which  
50  
51 102 survive a high dose of radiation, harboured gut microbiota enriched with *Lachnospiraceae* and  
52  
53 103 *Enterococcaceae* (38). Yet, a description of the intestinal microbiota composition before neoadjuvant  
54  
55 104 therapy could allow identifying predictive bacterial markers of tumour response in rectal cancer, and  
56  
57 105 to adapt TNT.

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2  
3 106 Indeed, chronic exposure of the gastrointestinal tract to genotoxins could be a prognostic marker of  
4  
5 107 radiotherapy response. CoPEC colonization would start at the very beginning of life (38) and might  
6  
7 108 lead to exposure of the intestinal mucosa to chronic genotoxic stress. The resulting damage could give  
8  
9 109 cells the ability to resist to other genotoxic stresses, such as radiation therapy. One *in vitro* study  
10  
11 110 already showed the decreased radiation sensitivity of cells incubated by colibactin (27). Therefore,  
12  
13 111 developing a non-invasive method to analyse gut microbiota composition and to evaluate CoPEC  
14  
15 112 implication in the response to CRT could help clinicians to tailor cancer management and to develop  
16  
17 113 tools to control the pathologic microorganisms identified as new therapeutic targets.  
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## 22 23 24 115 **Methods and analysis**

25  
26  
27 116 This study protocol is written in accordance with the SPIRIT guidelines. (Supplementary file 1)  
28  
29

### 30 117 ***Objectives***

#### 31 32 33 118 *Primary objective*

34  
35  
36 119 The study primary objective is to assess the correlation between response to neoadjuvant CRT and  
37  
38 120 CoPEC presence in stool samples.  
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#### 41 121 *Secondary objectives*

- 42  
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44 122 - To analyse in a non-targeted manner the global microbiota composition before CRT and to  
45  
46 123 evaluate the correlation between composition and response to treatment  
47  
48 124 - To study the modulation of the intestinal microbiota by CRT  
49  
50 125 - To describe the correlation between clinical data and microbiota composition modulation  
51  
52 126 induced by CRT  
53  
54 127 - To determine microbiological prognostic factors of overall survival, disease-specific survival  
55  
56 128 and relapse-free survival (locoregional and metastatic) in patients with low or mid rectum  
57  
58 129 cancer  
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3 130 - To create a microbiological database for future mechanistic analyses  
4  
5 131 - To study the modulation of CoPEC colonization by CRT  
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7

8 132 ***Study design***  
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11 133 The study is a non-randomized bicentric prospective clinical study. Two surgical teams will be  
12  
13 134 involved - Institut du Cancer de Montpellier and CHU de Clermont-Ferrand ; and an INSERM Unit –  
14  
15 135 M2iSH Clermont-Ferrand. The study actually started on January 2020 and the estimated study  
16  
17 136 completion date is November 2027.  
18  
19

20 137 ***Patients' selection***  
21  
22

23 138 ***Inclusion criteria***  
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- 26 139 - Histologically-proven adenocarcinoma of low or mid rectum, of stage II or III (UICC TNM  
27  
28 140 Classification, 8<sup>th</sup> Edition, 2017 (35))  
29  
30 141 - Patient eligible for neoadjuvant treatment (50 Gray radiation and capecitabine, CAP 50),  
31  
32 142 according to the French national recommendations (5,39)  
33  
34 143 - Informed signed consent received  
35  
36 144 - Man or woman aged  $\geq 18$  years  
37  
38 145 - Appropriate contraceptive measures taken by men and pre-menopausal women before study  
39  
40 146 entry and for at least 8 weeks after the last CRT cycle. Patients should be informed by the  
41  
42 147 investigator on the contraceptive measures to use.  
43  
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46 148 ***Exclusion criteria***  
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- 49 149 - Antibiotic treatment at the time of stool sampling or in the month before.  
50  
51 150 - Presence of a derivative stoma  
52  
53 151 - Previous chemotherapy treatment for rectum cancer  
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55 152 - Patient not affiliated to the French social security system  
56  
57 153 - Patient with possible poor treatment compliance for psychologic, familial, social and  
58  
59 154 geographic reasons  
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3 155 - Legal incapacity or limited legal capacity  
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5 156 - Pelvic radiotherapy or brachytherapy in the year before inclusion in the study  
6  
7 157 - History of other cancers in the 5 last years, except for cervical carcinoma *in situ* and skin  
8  
9 158 carcinoma, but including melanoma under treatment  
10  
11 159 - Pregnant or breastfeeding woman  
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15 160  
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17 161 ***Study sponsor***  
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20 162 The sponsor (Montpellier Cancer Institute, ICM) is responsible for the study design and management,  
21  
22 163 and for obtaining all study authorizations (Persons Protection Committee, National Agency for  
23  
24 164 Medical Security). It will also declare to these authorities the inclusion period beginning and end,  
25  
26 165 produce the final study report, inform the competent authorities of the trial results, and store all study-  
27  
28 166 related documents for at least 15 years after the study end.  
29  
30  
31 167 ***Clinical study procedures***  
32  
33  
34 168 ***Inclusion in the study***  
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36  
37 169 The study flow diagram is presented in Figure 1.  
38  
39  
40 170 Before study entry, all patients will receive exhaustive explanations on the study aims and procedures.  
41  
42 171 A signed informed consent will be obtained from all patients before any study procedure  
43  
44 172 (Supplementary file 2). At baseline, demographic (sex, age), clinical (performance status, weight,  
45  
46 173 height, medical history, initial diagnosis date, tumour localization, histologic type) and biological  
47  
48 174 (complete blood count, carcinoembryonic antigen (CEA) level) data will be collected (Table 1).  
49  
50 175 Patients will undergo rectal examination and tumour staging by computed tomography (CT), rectal  
51  
52 176 MRI, and possibly rectal endoscopic ultrasound examination (depending on the centre decision).  
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177 During the surgical consultation, the first stool sample (stool sample N°1) may be collected during  
 178 rectal examination (faeces left on the clinician's glove), or by proctoscopy. Otherwise, the stool sample  
 179 will be collected by the patient.

180 **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

181 MRI: Magnetic resonance imaging; CT: computed tomography.

### 182 *Neoadjuvant treatment*

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

188

189

190

191

### 192 *Re-evaluation*

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

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

### 199 *Surgery*

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

### 207 *Pathologic analysis*

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

211

212

213

214

215 **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)

216

217 ***Safety***

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

220 ***Follow-up and study duration***

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

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

228 ***Microbiological analyses***229 ***Sample handling***

1  
2  
3 230 Three stool samples will be collected during the study (Figure 1): i) one at patient inclusion, before  
4  
5 231 any treatment, to describe the baseline intestinal microbiota composition; ii) one during the interval  
6  
7 232 between the end of neoadjuvant CRT and surgery, at the surgical consultation for tumour reappraisal;  
8  
9 233 and iii) one just before bowel preparation (mechanical or antibiotics) for surgery.

11  
12 234 Each sample will be divided into two cryotubes: one empty and one with 15% glycerol/DMEM to  
13  
14 235 preserve cell integrity. Samples will be immediately stored at -80°C until transport to the M2iSH  
15  
16 236 laboratory, Clermont-Ferrand, France, which will be in charge of the molecular analysis and storage of  
17  
18 237 the samples.

#### 20 238 *E. coli* strain identification and CoPEC detection

21  
22  
23  
24 239 All microbiological analyses will be performed as previously described (28). After thawing, samples  
25  
26 240 stored in DMEM/glycerol will be crushed and diluted in sterile phosphate buffered saline pH 7.4  
27  
28 241 before plating on TBX agar and chromogenic agar chromID CPS3® plates (bioMérieux) to allow the  
29  
30 242 identification and quantitation of enterobacteria. Colonies (around 48 per sample) will be collected for  
31  
32 243 molecular typing, and their identification will be confirmed with the automated Vitek® II  
33  
34 244 (bioMérieux) system. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR will be used as  
35  
36 245 genotyping method to determine the number of *E. coli* strains per sample (28).

37  
38  
39 246 *E. coli* harbouring the colibactin-encoding *pks* island will be identified by PCR analysis of each *E. coli*  
40  
41 247 isolate (41). This will allow identifying the presence of CoPEC (primary objective).

#### 42 43 44 248 *Untargeted analysis of the local microbiota composition*

45  
46  
47 249 Global microbiota modifications will be assessed by high-throughput sequencing of the bacterial 16S  
48  
49 250 rRNA gene in DNA extracted from the three stool samples using the NucleoSpin® DNA stool kit  
50  
51 251 (Macherey-Nagel, Hoerd, France), according to the manufacturer's instructions. Quantitative PCR  
52  
53 252 will be performed to quantify pro-carcinogenic bacterial species, such as *Fusobacterium nucleatum*,  
54  
55 253 *Enterococcus faecalis*, *bft*-positive *Bacteroides fragilis*, and CoPEC. In addition, the V4 region of the  
56  
57 254 bacterial 16S rRNA gene will be amplified using the 515F/806R primer pair followed by Illumina  
58  
59 255 high throughput sequencing on a MiSeq® apparatus, according to the manufacturer's guidelines. A

1  
2  
3 256 global description of the intestinal microbiota could also be obtained by shotgun metagenomic  
4  
5 257 sequencing to access the microbiota functional features after selection of the more informative  
6  
7 258 samples.  
8  
9

## 10 259 ***Endpoints***

### 11 12 13 260 *Primary endpoint*

14  
15  
16 261 The primary endpoint (associated with the primary objective) is the relative risk (RR) of poor response  
17  
18 262 to neoadjuvant CRT in patients colonized by CoPEC ("exposed") compared to non-colonized patients  
19  
20 263 ("unexposed").  
21  
22

23 264

### 24 25 26 265 *Secondary endpoints*

- 27  
28  
29 266 - Prevalence and CoPEC colonization rate before and after CRT  
30  
31  
32 267 - Other bacterial strains present before CRT and relative risk of poor response to CRT in  
33  
34 268 colonized and non-colonized patients  
35  
36 269 - Type, prevalence, and colonization rate of bacteria other than CoPEC in the microbiota, before  
37  
38 270 and after CRT  
39  
40 271 - Percentage of colonized patients, depending on the bacterial type, according to the clinical  
41  
42 272 parameters (age, sex, body mass index)  
43  
44 273 - Hazard ratio (HR) for overall survival, disease-specific survival, and relapse-free survival  
45  
46 274 (locoregional or metastatic) in colonized patients, for the different bacterial types, according to  
47  
48 275 the overall bacterial composition (including CoPEC), and in non-colonized patients.  
49  
50  
51

### 52 276 ***Data collection and management***

53  
54  
55 277 The database will be managed by the sponsor, and data stored at the Data processing centre,  
56  
57 278 Biometrics Unit of the Montpellier Cancer Institute. Case report form design and clinical data  
58  
59 279 management will be implemented using the Ennov Clinical® software. Microbiological data will be  
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3 280 collected in a database first stored at the M2iSH laboratory, and then transferred to the sponsor  
4  
5 281 database for analysis. Data and any trial documents will be made available upon reasonable request  
6  
7 282 and after signature of a data access agreement.  
8  
9

10 283 In accordance with the General Data Protection Regulation (GDPR), a registration number will be  
11  
12 284 used to identify each patient. The corresponding table will be encrypted and stored in a secure place.  
13  
14 285 Special vigilance will be exercised throughout the study to maintain data anonymization.  
15  
16

### 17 286 ***Study monitoring, quality control, and audit***

18  
19  
20 287 According to the sponsor's risk-based monitoring plan (study participants, logistics, resources,  
21  
22 288 impact), the collection of the patient informed consents and the respect of the study protocol and  
23  
24 289 procedures will be monitored.  
25  
26

27 290 To guarantee the originality of all data and in accordance with the Good Clinical Practices, quality  
28  
29 291 control will be performed by the sponsor. The study will be managed according to the sponsor  
30  
31 292 procedures and in respect of the protocol, and the quality of the data included in the report forms will  
32  
33 293 be checked.  
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36 294 The sponsor may wish to conduct an audit at some investigating centers. Audits may be conducted by  
37  
38 295 the sponsor or any duly authorized person for at least 15 years after the trial.  
39  
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### 41 296 ***Statistical considerations***

#### 42 297 *Sample size*

43  
44  
45 298 The recruitment capacity for this exploratory study will be around 200 patients. For a mean rate of  
46  
47 299 30% of poor responders to the neoadjuvant treatment among the patients not colonized by CoPEC  
48  
49 300 (*i.e.*, a proportion of response  $P_2=0.30$  among unexposed patients), the study will be able to estimate a  
50  
51 301 relative risk of 1.7 ( $RR=1.7$ ) with a 30% precision and a confidence interval at 95% ( $\alpha=0.05$ ). Patients  
52  
53 302 in whom the CoPEC colonization status cannot be determined at baseline, in whom CRT must be  
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55 303 prematurely arrested, or who cannot undergo surgery will be considered non-evaluable.  
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3 304 Considering a 10% rate of potentially non-evaluable patients, a total of 220 patients (20 supplementary  
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5 305 patients) will be included in the study.  
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11 307 *Study population*  
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13  
14 308 Two populations will be defined for the analysis. The intention-to-treat population will be defined as  
15  
16 309 all patients included in the study, treated (patients who received complete/partial neoadjuvant  
17  
18 310 treatment) and not treated (patients who did not undergo CRT), eligible (*i.e.*, all patients who were  
19  
20 311 included in the study without violation of a major inclusion or exclusion criterion) or not, and  
21  
22 312 with/without baseline stool sample. The per-protocol population will include all eligible patients,  
23  
24 313 treated (complete or partial CRT), and with baseline stool sample.  
25

26  
27 314 *Statistical analyses*  
28

29  
30 315 Qualitative variables will be described by frequencies and percentages, and quantitative variables with  
31  
32 316 means, standard deviations, medians, and ranges. No imputation method will be used in case of  
33  
34 317 missing data. Correlations between qualitative variables will be assessed using the Chi-2 or Fisher-  
35  
36 318 exact test. Quantitative variables will be compared using the Student's *t*-test or the Kruskal-Wallis test.  
37  
38 319 Comparison of quantitative variables at different times (before and after CRT) will be assessed using  
39  
40 320 the Wilcoxon test for matched samples. The relative risk of poor response to neoadjuvant CRT in  
41  
42 321 CoPEC-colonized patients (or colonized by other bacteria) compared to non-colonized patients will be  
43  
44 322 estimated using a logistic regression (univariate analysis) and will be presented with the 95%  
45  
46 323 confidence interval (95% CI). Survival analyses will be performed using the Kaplan-Meier method  
47  
48 324 and survival distributions compared with the log rank test. HRs and their 95% CI will be estimated  
49  
50 325 with a Cox proportional risk model. A detailed statistical analysis plan (SAP) will be written before  
51  
52 326 the database is locked for analysis; supplementary subgroup analyses, if appropriated, will be specified  
53  
54 327 in the SAP. All analyses will be performed using the Stata version 16 software (StataCorp LP, College  
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56 328 Station, TX).  
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3 **329 Patient and public involvement**  
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6 330 There was no patient or public involvement in the design of this study.  
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15 **333 Discussion**  
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18 334 The implication of intestinal microbiota in CRC has been widely demonstrated (42). Several recent  
19  
20 335 studies suggest that different bacterial species, including CoPEC, could be used as biological  
21  
22 336 biomarkers for CRC diagnosis and prognosis (29,36,41,43,44). The potential role of the gut microbiota  
23  
24 337 in the modulation of the efficacy of anti-tumour treatments has been studied, with interesting results  
25  
26 338 regarding chemotherapy and immunotherapy (37). However, these studies were focused on colon  
27  
28 339 cancer dysbiosis and few data are available on rectal cancer and mucosa. Moreover, the correlation  
29  
30 340 between gut microbiota homeostasis and radiation sensitivity remains unclear. Patients treated by  
31  
32 341 pelvic radiation develop long-term complications that affect their quality of life, and have worse  
33  
34 342 functional results than patients treated with surgery alone (45,46). It has been hypothesized that the  
35  
36 343 intestinal microbiota has a significant impact on pelvic enteropathy (47); however, pelvic irradiation is  
37  
38 344 responsible for microbiota dysbiosis (48,49). To our knowledge, no previous study has assessed the  
39  
40 345 local microbiota composition and its implication in the response to CRT in rectal cancer, although  
41  
42 346 treatment response is one of the key points for prognosis estimation. Biomarkers to predict tumour  
43  
44 347 response in rectal cancer are still crucially needed. Imaging techniques (50) and biological markers  
45  
46 348 (51,52) have been evaluated, but they are often expensive and complicated to implement. Moreover,  
47  
48 349 the results are still discussed. Currently, their use seems to be limited to research and expert centers.  
49  
50 350 The present study will describe the intestinal microbiota composition in patients with rectal cancer  
51  
52 351 receiving neoadjuvant CRT to show its potential correlation with the tumour response, focusing on  
53  
54 352 CoPEC colonization. In addition, the effect of radiotherapy on the local intestinal microbiota  
55  
56 353 composition will be studied by comparing stool samples collected before and after CRT. Unlike  
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1  
2  
3 354 studies on the intestinal microbiota in colon cancer in which tumour fragments are needed, in the case  
4  
5 355 of mid or low rectal cancer stool samples should be representative of the local microbiota.  
6  
7 356 One of the main hypotheses to explain CoPEC effect on CRT response is based on their capacity to  
8  
9 357 induce DNA damage (25–27). Besides the direct effect on the cell, radiotherapy is also cytotoxic  
10  
11 358 through the production of reactive oxygen species and reactive nitrogen species (53). Chronic  
12  
13 359 genotoxic stress caused by CoPEC presence in gut mucosa could lead to an adaptation of the gut  
14  
15 360 mucosa to genotoxic agents and consequently to reduce radiation sensitivity and resistance to therapy.  
16  
17 361 For instance, in an *in vitro* study, Wilson et al. observed less DNA damage in colibactin-positive  
18  
19 362 epithelial cells infected by CoPEC (27). Moreover, radiation sensitivity is closely linked to autophagy  
20  
21 363 regulation (54,55). Recent studies showed the involvement of gut microbiota in autophagy regulation,  
22  
23 364 with a link to chemoresistance (56). Ionizing radiation effects might be modified indirectly through  
24  
25 365 autophagy deregulation induced by gut microbiota. In addition, radiotherapy cytotoxic effect could  
26  
27 366 result in a modification of the local microenvironment with significant clinical consequences (57).  
28  
29 367 The modulation of radiotherapy efficacy by the intestinal microbiota is an emerging concept in CRC,  
30  
31 368 but its study faces many obstacles, especially sample availability. In this study, we want to develop a  
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33 369 non-invasive reproducible faecal test that could become a key biomarker to predict tumour response to  
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35 370 CRT. Our work will help clinicians to tailor neoadjuvant therapeutic strategies with the final goal of  
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37 371 increasing tumor response, organ preservation, and reducing surgical morbidity, while maintaining  
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39 372 oncological safety.

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#### 374 **Ethics and dissemination**

375 The study protocol (version 3.0, dated on September 24<sup>th</sup>, 2019) was approved by the local ethics  
376 committee (Comité de Protection des Personnes Sud-Est II, December 18<sup>th</sup>, 2019, Reference number  
377 2019-A02493-54) and the institutional review board COMERE. The French National Drug Agency  
378 Authority (ANSM) was informed. The study was registered on Clinicaltrials.gov, identifier  
379 NCT04103567.

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3 380 All patients will be informed of the study objectives and procedures by the investigators before  
4  
5 381 enrolment. A signed informed consent will be obtained from all patients before their inclusion in the  
6  
7 382 study and before any study procedure is performed. All patients may end their participation in the  
8  
9 383 study at any time, for whatever reason, without any consequence or prejudice concerning their care.  
10  
11 384 Study participants will be able to request global results from investigators as soon as study results  
12  
13 385 become available.

14  
15  
16 386 In the event of substantial modification, the request will be sent by the sponsor to the ethics committee  
17  
18 387 for an opinion. Upon receipt of the favourable opinion, the sponsor will send the amended version of  
19  
20 388 the protocol to all investigators.

21  
22  
23 389 The study will be conducted in accordance with the current French and European Regulatory  
24  
25 390 requirements, including regulations on biomedical research from the Public Health Code, the bioethics  
26  
27 391 and data protection laws and decrees, the French Jardé's law on research implicating human beings,  
28  
29 392 the Good Clinical Practice, and the Helsinki Declaration.

### 33 393 **Acknowledgements**

34  
35  
36 394 The authors thank the Clinical Research and Innovation Department of the Montpellier Cancer  
37  
38 395 Institute for help with regulatory and administrative aspects of the study, Dr. Stéphanie Delaine for her  
39  
40 396 help in obtaining funding for the study, Pierre Sauvanet and Michael Rodrigues for technical advice,  
41  
42 397 and Maxime Tressol for the microbiological analyses.

### 45 398 **Authors' contributions**

46  
47  
48 399 GC, CT, JG, CF, MJ, GR, CC, PEC, PR, and MB wrote the protocol. MJ, GC, PR, CT and CF  
49  
50 400 conducted statistical trial planning. PR, CT and CF handled ethics and regulatory affairs. GC, CT, HF,  
51  
52 401 MB, CF, NB, PR, and MJ wrote the paper draft. GC, MB, MJ, PR, CT and CF contributed to the trial  
53  
54 402 design and modifications. All authors read and approved the final manuscript.

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3 **405 Funding**  
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5

6 406 This work was supported by the SIRIC Montpellier Cancer, the Biocodex Microbiota Foundation and  
7  
8 407 La Ligue Contre le Cancer (Herauld committee).  
9

10  
11 408 The funding body was not involved in the study design and will not be involved in data collection,  
12  
13 409 data analysis and interpretation, and writing of the study report and publication.  
14  
15

16 410  
17

18  
19 **411 Competing interests**  
20

21  
22 412 The authors declare that they have no competing interests.  
23  
24

25 **413 Provenance and peer review**  
26

27  
28 414 Not commissioned; externally peer reviewed.  
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31 415  
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47 586 **Figure legends**

48  
49 587 Figure 1: MICARE flow diagram  
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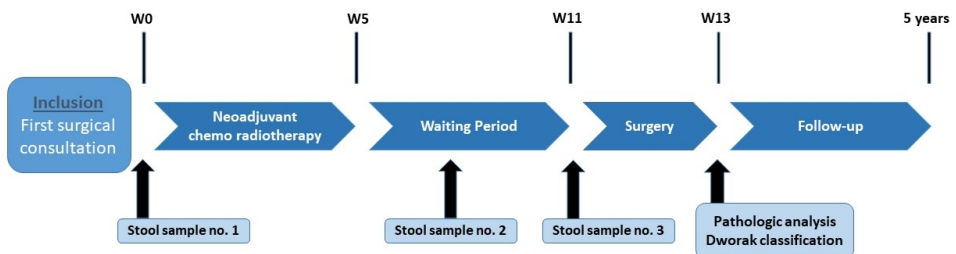


Figure 1 : MICARE flow diagram

Figure 1 : MICARE flow diagram

338x190mm (96 x 96 DPI)



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

Section/item	Item No	Description	Addressed on page number
<b>Administrative information</b>			
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	<i>Funding</i> , 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

1	<b>Introduction</b>			
2				
3	Background and	6a	Description of research question and justification for undertaking the trial, including summary of relevant	p3-5
4	rationale		studies (published and unpublished) examining benefits and harms for each intervention	
5				
6		6b	Explanation for choice of comparators	NA
7				
8	Objectives	7	Specific objectives or hypotheses	<i>Objectives</i> , p5
9				
10	Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group),	
11			allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	<i>Study design</i> , p5
12				
13				
14	<b>Methods: Participants, interventions, and outcomes</b>			
15				
16	Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will	<i>Study design</i> , p5
17			be collected. Reference to where list of study sites can be obtained	
18				
19	Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and	<i>Patients' selection</i> ,
20			individuals who will perform the interventions (eg, surgeons, psychotherapists)	p6
21				
22				
23				
24	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be	<i>Clinical study</i>
25			administered	<i>procedures</i> , p7-9
26				
27		11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose	p8
28			change in response to harms, participant request, or improving/worsening disease)	
29				
30		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence	NA
31			(eg, drug tablet return, laboratory tests)	
32				
33		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	p8
34				
35	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood	
36			pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg,	p11
37			median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen	
38			efficacy and harm outcomes is strongly recommended	
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1	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
2				
3				
4	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	<i>Sample size</i> , p12-13
5				
6				
7	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	p12-13
8				
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### 10 **Methods: Assignment of interventions (for controlled trials)**

#### 11 Allocation:

12				
13				
14	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
15				
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19	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
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24	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
25				
26				
27	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
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30		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
31				
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### 34 **Methods: Data collection, management, and analysis**

35				
36	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	<i>Data collection and management</i> , p11
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1		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
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4	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
5				
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8	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
9				
10				
11		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	<i>Statistical analyses,</i>
12				p13
13				
14		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)	<i>Statistical analyses,</i>
15				p13
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**Methods: Monitoring**

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21	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	<i>Study monitoring, quality control, and audit, p12</i>
22				
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26		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
27				
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29	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	<i>Safety, p9</i>
30				
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33	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	<i>Study monitoring, quality control, and audit, p12</i>
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**Ethics and dissemination**

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1	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	<i>Ethics approval and consent to participate, p16</i>
2				
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4				
5	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
6				
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8				
9	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	p16
10				
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13		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
14				
15				
16	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
17				
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19	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	<i>Competing interests, p16-17</i>
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22	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	<i>Availability of data and materials, p16</i>
23				
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25	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
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29	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	<i>Dissemination policy, p13-14</i>
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33		31b	Authorship eligibility guidelines and any intended use of professional writers	Protocol**
34				
35		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	<i>Availability of data and materials, p16</i>
36				
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## Appendices



1	Informed consent	32	Model consent form and other related documentation given to participants and authorised surrogates	Applicable **
2	materials			
3				
4	Biological	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular	<i>Sample handling,</i>
5	specimens		analysis in the current trial and for future use in ancillary studies, if applicable	p10
6				

7 \*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.  
8 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons  
9 "[Attribution-NonCommercial-NoDerivs 3.0 Unported](#)" license.  
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11 \*\*More information can be provided if wished by the editor.  
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# 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:** |\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|\_|

**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 V 4.0 du 23/02/2021 du protocole MICARE

# 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.

<input type="checkbox"/>	J'accepte que mes données cliniques soient utilisées pour des recherches ultérieures, en France ou dans l'Union Européenne
<input type="checkbox"/>	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 :

Nom de l'investigateur :

Date :

Date :

Signature :

Signature :

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

Formulaire de consentement V 4.0 du 23/02/2021 du protocole MICARE

TREATMENT	EVENTS	YES/NO
<b>CHEMO-RADIOTHERAPY</b>	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
<b>SURGERY</b>	<b><u>Per-operative</u></b>	
	Bleeding	YES/NO
	Intestinal perforation	YES/NO
	Vascular wound	YES/NO
	<b><u>Post-operative</u></b>	
	Infection	YES/NO
	Anastomotic leakage	YES/NO
	Colon ischemia	YES/NO
	Bowel obstruction	YES/NO
	Bleeding	YES/NO
Urinary dysfunction	YES/NO	