TOTAL NEOADJUVANT TREATMENT WITHOUT SURGERY FOR LOCALLY ADVANCED RECTAL CANCER: PROSPECTIVE CLINICAL TRIAL TO ASSESS TUMOR COMPLETE RESPONSE, CIRCULATING TUMOR GENETIC AND EPIGENETIC BIOMARKERS, AND STROMAL TRANSCRIPTOME TO INTERPRET CLINICAL OUTCOME

(NO-CUT TRIAL)

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List of abbreviations:

CBCT: cone beam computed tomography

cCR clinical complete response

CCT consolidation chemotherapy

CRM circumferential resection margins

CRT chemoradiation

CT Computed Tomography

ctDNA cell-free DNA

DFS disease free survival

DRE Digital Rectal Exam

EUS Endorectal ultrasound

ICT induction chemotherapy

IGRT: image guided radiation therapy

LR local recurrence

MRI Magnetic Resonance Imaging

NOM Non-operative Management

OS overall survival

pCR complete pathologic response

RT radiotherapy

SIB: simultaneous integrated boost

TME total mesorectal excision

TNT total neoadjuvant therapy

VMAT: volumetric arc therapy

W&W watch-and-wait

1 Background and rationale of the study

Colorectal cancer is the third most common cancer worldwide and the second leading cause of cancer death in Europe. Rectal cancer accounts for about 25-30% of all colorectal cancer diagnoses (1). Five-year survival rates depend on stage at diagnosis, about 92% for stage I, 87% for stage IIA, 63% for stage IIB, 89% for stage IIIA, 69% for stage IIIB, and for stage IIIC cancers the survival rate is about 53%; stage IV rectal cancers have a 5-year relative survival rate of about 11% (2)

1.1 Locally advanced rectal cancer standard management

The rectum is located within the pelvis, extending from the transitional mucosa of the anal dentate line to the sigmoid colon at the peritoneal reflection. The location of a rectal tumor is usually indicated by the distance between either the anal verge, the dentate line, or the anorectal ring and the lower edge of the tumor: thus, cancers of the upper (10-15 cm), medium (5-10 cm), and lower rectum are identified (3). The management of rectal cancers varies from that of colon cancer because of the increased risk of local recurrence and a poorer overall prognosis. This is particularly true for medium and lower rectal cancer, due to the close proximity of the rectum to pelvic structures and organs, the absence of a perithoneum serosa surrounding this part of the rectum, and the technical difficulties associated with obtaining wide surgical margins at resection.

In order to achieve improved local control, the most effective diagnostic/staging and therapeutic approaches to the management of rectal cancer are multimodal with dedication of multidisciplinary teams of cancer specialists with expertise in surgical oncology, medical oncology, radiation therapy, and diagnostic imaging including endoscopy and radiology. The introduction of the **total mesorectal excision** (**TME**) technique has represented a remarkable historical improvement in the surgical treatment of rectal cancer, resulting in decrease of local recurrence (LR) rates and improvement of overall survival (OS). TME with either **low anterior resection** or **abdominoperineal resection** is presently indicated and usually performed for stages II and III rectal cancer [LR rate <5%, 5-year OS rate 76-90%, peri-operative mortality 1-13%] (4,5). Long-term morbidities include altered bladder and bowel function (mainly incontinence and urgency),

colostomy, sexual dysfunction as detailed elsewhere (6). Moreover, anastomotic leak is a common complication, reported in up to 12% of cases. TME is the mainstay of therapy for resectable rectal cancers of the middle and lower rectum according to National Comprehensive Cancer Network (NCCN), Associazione Italiana Oncologia Medica (AIOM), and Associazione Italiana Radioterapia Oncologica (AIRO) current guidelines. TME is performed after chemoradiation therapy for selected stage II and stage III disease (staging of rectal cancer according to **Table 1**). In particular, pre-operative or neoadjuvant therapy consisting of 5-fluorouracil based **chemoradiation** (CRT) (local 45-50 Gy to the pelvis in 25-28 fractions combined with systemic intravenous or oral chemotherapy with a fluoropyrimidine), is the preferred treatment option for patients with selected stages II (cT3b,c,d N0 and cT4 N0) and stage III (any cT N+) disease (NCCN Rectal Cancer Guide Lines 2016; AIOM Colorectal Cancer Guide Lines 2015). This indication is based on the results of multiple studies and on a Cochrane Systematic Review comparing radiotherapy with CRT as well as preoperative versus postoperative treatment, all favoring preoperative CRT both in term of better long term local control and tolerability (7–9). The advantages of pre-operative CRT in locally advanced rectal cancer management are: tumor down-staging, with safer surgical resectability; higher probability of tumor-free circumferential resection margins (CRM) and, in some cases, of sphincter-preserving surgery; improved local control with lower incidence of LR; finally, an improved systemic control on micrometastases has been hypothesized with chemotherapy use, but no significant benefit in OS has been shown to date.

At the present time, post-operative adjuvant treatment with 5-fluorouracil is guided by the initial T and N stages (10,11). The question of whether oxaliplatin should be added to adjuvant 5-fluorouracil/leucovorin (5-FU/LV) for the management of resected stages II and III rectal cancer is an ongoing debate. However, the addition of 4 months oxaliplatin to 5-FU/LV for the adjuvant treatment of colon cancer is now considered standard care and is somewhat extended to rectal cancer patients (12). To date, the trimodality treatment (pre-operative CRT and TME followed by adjuvant chemotherapy) is the standard of care for locally advanced rectal cancer patients, providing high rates of local control. Overall approximately one-third of the patients with stage II/III rectal cancer are expected to die from progression of distant metastases and the remaining survivors experience significant consequences mainly due to local therapies including radiation therapy or surgery.

Table 1. TNM and stages for rectal cancer from AJCC 8th edition.

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the pericolorectal tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum
T4b	Tumor directly invades or is adherent to other organs or structures
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to 1 organ or site (eg, liver, lung, ovary, nonregional node)
M1b	Metastases in more than 1 organ/site or the peritoneum

Stage	T	N	M	Dukes	MAC
0	Tis	N0	M0	--	--
I	T1	N0	M0	A	A
	T2	N0	M0	A	B1
IIA	T3	N0	M0	B	B2
IIB	T4a	N0	M0	B	B2
IIC	T4b	N0	M0	B	B3
IIIA	T1-T2	N1/N1c	M0	C	C1
	T1	N2a	M0	C	C1
IIIB	T3-T4a	N1/N1c	M0	C	C2
	T2-T3	N2a	M0	C	C1/C2
	T1-T2	N2b	M0	C	C1
IIIC	T4a	N2a	M0	C	C2
	T3-T4a	N2b	M0	C	C2
	T4b	N1-N2	M0	C	C3
IVA	Any T	Any N	M1a	--	--
IVB	Any T	Any N	M1b	--	--

1.2 Non-operative Management (NOM) of rectal cancer

With the **chemoradiation therapy** (CRT), the resulting pathologic complete response (pCR) across all stages has been documented in up to 30% of patients (9,13). Most importantly, patients achieving pCR have lower rates of tumor recurrence, and improved survival, compared to those who do not achieve pCR (14,15). Moreover, data from the National Surgical Quality Improvement Project document a 35% risk of morbidity associated with both low anterior and abdominoperineal resection (16). Long-term morbidity includes bowel and bladder incontinence, sexual dysfunction, and complications associated with temporary and permanent stomas (6,17,18).

Due to the observation of the absence of residual tumor in the pathological specimens of a significant proportion of patients treated with CRT for local or locally advanced rectal cancer, in the early-2000s, two clinical issues arose: firstly, if pCR could be predicted after CRT with clinical, radiological, or endoscopic restaging assessment thus defining clinical complete response (cCR); and secondly if patients with cCR should necessarily undergo radical surgery to achieve cure at the cost of morbidity, mortality, and functional consequences associated with radical rectal surgery. Consequently, an increasing number of reports suggested that non-operative management (NOM), consisting of close surveillance of patients with cCR, could be an acceptable alternative to rectal surgery (proctectomy). Led by small prospective series published since the late 90's by Habr-Gama and colleagues, several small international series have reported similar oncologic outcomes in cCR patients followed by close active surveillance (the so-called watch-and-wait (W&W) or NOM approach) compared to those treated with radical surgery (13,19) (**Table 1**).

In particular, Habr-Gama et al., reported several pioneering institutional-level series with cCR ranging from 26% to 38% (20–24). Despite the very interesting pivotal concept of NOM of locally advanced rectal cancer, the major limitation of such reports is that a sizeable proportion of early stage rectal cancer patients were included. In the largest series of 99 patients managed by NOM, 6% had LR within the rectal lumen (25).

Appelt and colleagues reported the results of an observational study of 55 patients with T2 or T3 N0–N1 adenocarcinomas treated by high-dose chemoradiotherapy (60 Gy in 30 fractions) for 6 weeks (26). They reported that an extraordinarily high proportion (40 of 51 patients, 78%) achieved a cCR. These patients were managed by NOM, and at 2-year 22% of patients had local regrowth, all managed with delayed complete resection. Between 2011 and 2013, Renehan et al, managed with a NOM approach 31 patients

achieving cCR out of 259 (12%). In their analysis, a further 98 patients, selected from a UK regional registry, similarly managed from 2005 to 2015, were added to the NOM group (129 patients) (27). After a median follow-up of 33 months from start of CRT, 44 (34%) of the 129 patients with a cCR managed by NOM had local regrowth. Interestingly, 42 (95%) of these 44 regrowths were mucosal lesions; two (5%) had submucosal or mesorectal lesions. Of the 41 patients managed by NOM with non-metastatic local regrowth, 36 (88%) had salvage therapy: 31 (76%) of 41 underwent subsequent salvage surgery (30 with R0 resections and one with an R1 resection); and five (12%) patients underwent radiotherapy.

In all the above mentioned reports, 2 to 5-year OS and DFS rates resulted at least comparable to that of patients treated with standard surgery following neo-adjuvant CRT.
On the other hand, these small single institution pilot studies have been conducted enrolling small cohorts of patients with less than 500 patients having been evaluated worldwide. A high variability in stage at diagnoses, local recurrence rate, distant recurrence rate (0-60% and 0-17%, respectively) and type and outcome of salvage therapy (0 to 100%) have been reported and no reliable data on long term outcomes are available. Based on these limitations, the NOM of rectal cancer deserves consideration within purposely designed clinical trials (28).

Table 1. Current data on non-operative management (NOM) of rectal cancer from literature

	N° pts	Distance from ARJ(cm)	N°cCR tot	N°cCRst I	N°cCRst II	N°cCRst III	Median FU (months)	Radiosensitising CT	Adjuvant CT	Response evaluation (weeks)	Local recurrences	Recurrences complete resection	Distance recurrences	DFS	OS
Habr-Gama 2004[1]	265	0-7	71 (26.8%)	NS	NS	16 (22.5%)	57	FUFA	-	8	2 (2.8%)	2 (100%)	3 (4.2%)	92% (5y)	100% (5y)
Habr-Gama 2006[2]	361	0-7	99 (27.4%)	10*	46*	22*	60	FUFA	-	8	5 (5%)	3 (60%)	8 (8%)	85% (5y)	93% (5y)
Lim 2007[3]	48	0-12	27 (56%)	NS	NS	NS	49	5Fu based	-	4-6	11 (41%)	NS	11 (41%)	NS	NS
Hughes 2010[4]	58	2-9	10 (17%)	Ns	NS	NS	unclear	5Fu based	-	6-8	6 (60%)	NS	1 (10%)	NS	NS
Habr-Gama 2011[5]	173	0-7	67 (39%)	NS	NS	NS	65	5Fu based	-	8	8 (12%)	8 (100%)	7 (10%)	72% (5y)	96% (5y)
Maas 2011[6]	192	0-10	21 (11%)	2 (9.5%)	4 (19%)	15 (71.5%)	25	Capecitabine 825mg/m2 bid	XELOX x 6 (if N+)	6-8	1 (5%)	1 (100%)	-	89% (2y)	100% (2y)
Smith 2012[7]	265	0.5-12	32 (12%)	8 (25%)	6 (19%)	18 (56%)	28	5Fu/cape	FOLFOX/ XELOX/5Fu	4-10	6 (19%)	6 (100%)	3 (9%)	88% (2y)	96% (2y)
Dalton 2012[8]	49	5	6 (12%)	0	1 (17%)	5 (83%)	25.5	Capecitabine 825mg/m2 bid	-	6-8	NS	NS	NS	NS	NS
Habr-Gama 2013[9]	69	0-7	47 (68%)	14 (30%)	19 (40%)	14 (30%)	60	FUFA	-	10	12 (25.5%)	11 (91.6%	8 (17%)	72% (3y)	90% (3y)
Appelt 2015[10]	51	0-6	40 (78%)	16 (40%)	7 (17.5%)	17 (42.5%)	23.9	UFT	-	6	9 (22.5%)	9 (100%)	3 (7.5%)	NS	NS
Renehan 2016[11]	259	4-8	31 (12%) + 98 129 tot	NS	NS	84 (65%)	33	5Fu based	-	≥8	41 (32%)	31 (76%)	7 (5.5%)	88% (3y)	96% (3y)

ARJ anorectal junction, NS not stated, *pretreatment staging available for only 78 pts.

1 Habr-Gama A, Perez RO, Nadalin W, et al. *AnnSurg*. 2004; 2 Habr-Gama A, Perez RO, Proscurshim I, et al. *J GastrointestSurg*. 2006; 3 Lim L, Chao M, Shapiro J, et al. *Dis Colon Rectum* 2007; 4 Hughes R, Harrison M, Glynne-Jones R. *Acta Oncol* 2010; 5 Habr-Gama A, Perez RO, Sao Juliao GP, et al. *Semin Radiat Oncol*. 2011; 6 Maas M, Beets-Tan RG, Lambregts DM, et al. *J ClinOncol*. 2011;7 Smith JD, Ruby JA, Goodman KA, et al. *AnnSurg*. 2012; 8 Dalton R, Velineni R, Osborne M, et al. *ColorectalDis*. 2012; 9 Habr-Gama A, Sabbaga J, Gama-Rodrigues J, São Julião GP, et al. *Dis Colon Rectum.* 2013; 10 Appelt AL, Ploen J, Harling H, et al *Lancet Oncol* 2015; Renehan AG, Malcomson L, Emsley R, Gollins S, Maw A, et al. *Lancet Oncol.* 2016.

1.3 Role of induction chemotherapy (ICT) and consolidation chemotherapy (CCT)

A plethora of evidences suggests, with the recent advances of preoperative strategies (CRT) and surgery of rectal cancer, that rate of local recurrence decreased significantly. Distant metastases nonetheless continue to represent a major problem for rectal cancer patients. A pooled analysis of five European randomized controlled trials (RCTs) demonstrated that the 5-year distant metastasis rate was 30.8% in 2759 recruited patients (29). Furthermore, in a study conducted using the NCCN Colorectal Cancer Database, patients with rectal cancer were evaluated on the frequency of receiving neoadjuvant and postoperative systemic chemotherapy. Results of that study indicated that the number of patients who completed postoperative treatment was significantly lower than anticipated (30). From these observations, a shift is emerging towards administering full-dose systemic treatment in the neoadjuvant setting with the promise to improve compliance rates, reduce toxicity, and decrease distant relapse rates. Multiple prospective trials have reported on the use of this strategy, also referred to as **total neoadjuvant therapy** (TNT) for patients with LARC, incorporating both ChT and CRT in the neoadjuvant setting (48).

A phase II study evaluated neoadjuvant capecitabine/oxaliplatin (**CAPOX**) before CRT and surgery in newly diagnosed patients with magnetic resonance imaging (MRI) defined poor-risk rectal cancer that included tumors with a threatened circumferential resection margin, T3 tumors at or below elevators muscles, tumors beyond 5 mm into perirectal fat, T4 tumors and T1-4N2 tumors (31). Patients received 12 weeks of neoadjuvant capecitabine/oxaliplatin followed by concomitant capecitabine and radiotherapy. TME was planned 6 weeks after CRT. Postoperatively, patients received another 12 weeks of capecitabine. This study demonstrated that the radiologic response rate after CAPOX was 88% and increased to 97% at the completion of CRT (31). More recently, a single-center pilot trial from Memorial Sloan Kettering Cancer Center evaluated the concept of selective use of chemoradiation for patients with intermediate risk rectal cancer as determined by MRI. This phase II study enrolled patients with tumors 5–12 cm from the anal verge with no threatened radial margin. Patients received induction **FOLFOX-bevacizumab** for 6 cycles followed by restaging (32). Those who had a clinical response from the induction regimen did not receive any further preoperative treatment and proceeded to TME surgery. Patients who did not obtain an adequate response received additional CRT prior to surgery. Of the 32 patients enrolled, 30 patients achieved R0 resection with induction chemotherapy alone. The remaining two patients were intolerant to FOLFOX-bevacizumab, received CRT instead and also subsequently underwent successful R0

resection (32). This pilot study demonstrated that chemotherapy alone is sufficient for local and distant disease control in carefully selected patients and provided the background to support the currently ongoing PROSPECT study available across the United States. This is a phase II/III randomized trial evaluating the impact of selective use of RT in contrast to standard neoadjuvant CRT for locally advanced rectal cancer. Patients in the intervention arm receive 6 cycles of **FOLFOX** chemotherapy followed by careful restaging with either pelvic MRI or endoscopic ultrasonography (EUS). Those patients who achieve objective tumor response (as estimated based on a clinical response 20%) proceed directly to rectal cancer resection followed by postoperative systemic therapy at the discretion of the primary provider (NCT01515787). For those who do not achieve tumor response, CRT is administered. The study control arm is standard CRT followed by TME surgery and adjuvant chemotherapy.

Since a still unexplored concern of the NOM strategy regards a potentially increased risk of distant relapses, we hypothesize that an optimal upfront neoadjuvant systemic therapy (**Table 2 and 3**) is advisable in order to lower as much as possible the risk of failure due to distant relapse.

Table 2 - Studies of neoadjuvant chemotherapy alone in rectal cancer

Study	Key inclusion criteria	#pts	Treatment	pCR rate	Outcomes
Ishii, et al.	T3 or T4	26	Irinotecan, 5-FU, Leucovorin x 8 weeks	3.8%	5-year DFS 74% 5-year OS 84%
Uehara, et al.	MRI-defined poor risk: T4, N2, CRM ≤1mm, extramural invasion >5mm	32	CAPOX, bevacizumab x 12 weeks	13%	R0 resection rate 90%
Hasegawa, et al.	T4 or N+	25	CAPOX, bevacizumab x 12 weeks	4%	R0 resection rate 92% DFS at 31 months 68%
Cercek, et al.	No radiation, resected primary	20	FOLFOX +/- bevacizumab	35%	N/A
Schrag, et al.	T3	32	FOLFOX + bevacizumab x 8 weeks	25%	R0 resection rate 100% 4-year LR 0% 4-year DFS 84%
pCR, pathologic complete response; DFS, disease free survival; OS, overall survival; CRM, circumferential resection margin; LR, local recurrence.					

Table 3 - Studies of neoadjuvant chemotherapy followed by chemoradiation

Study	Key inclusion criteria	#pts	Treatment	pCR rates	Outcomes
EXPERT (Chau et al, 2006)	MRI-defined poor risk: T4, T3 at or below levators, N2, CRM ≤1 mm, extramural invasion >5 mm	77	CAPOX ×12 weeks → chemoRT with capecitabine → adjuvant capecitabine ×12 weeks	24% (16/67)	R0 resection rate 99% ORR 97% 1 year DFS 87% 1 year OS 93%
GCR-3 (Fernández-Martos C, et al, 2010)	Tumor within 2 mm of CRM, T3 ≤6 cm from anal verge, T3N+, resectable T4	108	ChemoRT with capecitabine and oxaliplatin → surgery → adjuvant CAPOX CAPOX → chemoRT with capecitabine and oxaliplatin → surgery	13% 14%	R0 resection 87% Downstaging 58% 18 months DFS 82% 18 months OS 89% R0 resection 86% Downstaging 43% 18 months DFS 76% 18 months OS 91%
CONTRE (Perez K, et al, 2013)	T3, T4 or N+	36	FOLFOX ×16 weeks → chemoRT with capecitabine or 5-FU	29% (6/21)	R0 resection—100%
Maréchal, et al, 2012	T2-T4N+	57	Chemoradiation with 5-FU FOLFOX ×4 weeks → Chemoradiation with 5-FU	28% 25%	ypT0-1 34.5% Downstaging 72% CRM + (≤1 mm) 14% ypT0-1 32.1% Downstaging 61% CRM + (≤1 mm) 4%
EXPERT-C (Dewdney A, et al, 2012)	T3 at or below levators, T4, CRM ≤1 mm, extramural extension ≥5 mm, extramural venous invasion	165	CAPOX + cetuximab × 12 weeks → chemoRT with capecitabine + cetuximab CAPOX ×12 weeks → chemoRT with capecitabine	11%* 9%*	R0 resection 92%* Response rate 84% (93%*) R0 resection 92%* Response rate 76% (75%*) *, results for analysis of KRAS wild-type population
AVACROSS (Noguét et al, 2011)	T3 low rectal, mid rectum with CRM ≤2 mm, N+ with CRM ≤2 mm, operable T4, T3N+	47	CAPOX + bevacizumab × 12 weeks → chemoRT with capecitabine + bevacizumab	35% (16/45)	R0 resection 98% DFS at 32 months 84%

Pts, patients; pCR, pathologic complete response; MRI, magnetic resonance imaging; CRM, circumferential resection margin; RT, radiotherapy; ORR, objective response rate; DFS, disease free survival; OS, overall survival.

1.4 Liquid biopsy in colorectal cancer

We and others have recently documented that solid tumors including colorectal cancers shed cell-free tumor DNA circulating in the blood (ctDNA) that can be detected by techniques referred to as *liquid biopsy* (33). The latter allows to generate molecular profiles which capture the heterogeneity of the malignancy more comprehensively then tumor tissue biopsy and it can be applied to monitor response of colorectal cancers to therapy (34,35). To date, studies investigating ctDNA detection in colorectal cancer did not discriminate results according to tumor location, so that application of liquid biopsy can be assimilated as for either colon or rectal origin of the tumor.

Several approaches including colonoscopy, evaluation of serum markers (CEA and Ca19-9) (36), together with Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) scans (37) are used to diagnose and stage rectal cancer. The use of CEA or CA19-9, however, is limited by the lack of sensitivity and specificity (38), whereas ctDNA, being tumor specific, is expected to reduce or abrogate false-positivity issues associated with commonly used cancer biomarkers. Many studies demonstrated how plasma ctDNA levels can be exploited to closely monitor colorectal cancer patients and to readily recognize individuals with high-risk of recurrence (39). In particular, several reports showed how ctDNA levels in plasma decrease after surgery and their monitoring during their follow-up predict tumor recurrence (40). In rectal cancer, possible opportunities for development of liquid biopsy include detection of minimal residual disease after CT-RT and, in the context of NOM, potential detection of early recurrence during follow-up. Possible drawbacks in this tumor type are the paucity of tumor tissue available for molecular diagnosis to drive detection of ctDNA in plasma, since diagnosis is achieved mainly by tissue biopsy, and the possibly lower rate of detection of ctDNA by liquid biopsy in non-stage IV cancer (33).

1.5 Blood-based methylation markers in colorectal cancer

Methylation markers are leading candidates for non-invasive cancer detection, diagnosis and prognosis (41). They can be used instead of ctDNA to circumvent the absence of patient specific mutations for monitoring tumor burden dynamics via liquid biopsy. Recent evidences about the performance of methylated ctDNA in CRC screening (42) compelled the FDA to approve the first blood test exclusively based on Septin 9 (SEPT9) methylation detection.

We recently developed a six-methylated genes panel (6-MGP) to longitudinally follow-up metastatic CRC patients (Barault et al, Gut 2017 *in press*). The 6-MGP showed sensitivity and specificity of 90.4 and 94.0% respectively in a cohort of 232 individuals (182 samples from patients affected by metastatic CRC and 50 self-declared healthy donors). As shown in **Figure 1**, healthy donors and mCRC patients were analyzed for six selected markers (SEPT9, C9ORF50, GRIA4, EYA4, MSC, MAP3K14-AS1). We found that all six markers showed strong significant differences in methylation distribution, discriminating between healthy and mCRC patients (u-test: p<0.0001).

Based on these preliminary findings and the notion that RC does not genetically differ from others cancer of the colon (43), we reasoned to apply the 6-MGP to the setting of locally advanced rectal cancer to distinguish between those patients that after chemo-radiation therapy have achieved or not durable and true tumor ablation.

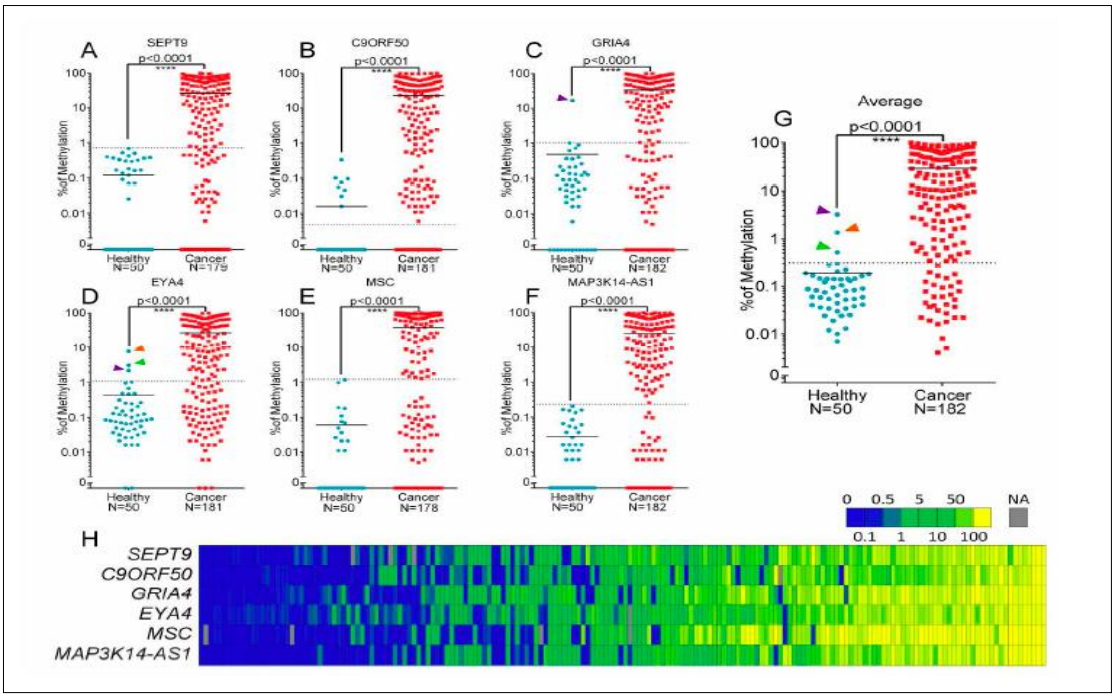


Figure 1 - Prevalence of methylated markers in cfDNA. Fifty self-declared healthy donors (blue) and 182 mCRC patients (red) were analyzed for the six selected markers. Group mean is represented by a horizontal bar. Mann-Whitney U test was performed to compare distribution in healthy and cancer patients which were all significantly different (with p-value<0.0001). Representation of individual markers: A: SEPT9, B: C9ORF50, C: GRIA4, D: EYA4, E: MSC, F: MAP3K14-AS1. G: Representation of average methylation signal. Three healthy donors presented an average methylation value above positivity threshold (purple, orange and green arrow), which was due to high positivity in GRIA4 and EYA4 for the first case, and EYA4 in the other two individuals. H: Heatmap of methylation values in mCRC cases sorted by average methylation.

1.6 Transcriptional stromal score in rectal cancer

Recently, Medico et al, developed transcriptional signatures correlating the abundance of cancer-associated fibroblasts (CAFs), leukocytes or endothelial cells in human colon and rectal cancer samples with sensitivity to preoperative (chemo) radiotherapy (44). In particular, they exploited transcriptional profiles of sorted CRC cell populations and defined three signatures, composed of 131, 47 and 35 genes, specifically expressed by CAFs, leukocytes and endothelial cells, respectively. Thus they obtained three “stromal scores” (CAF, leukocyte and endothelial) quantifying stromal cell population and assessed their clinical correlation by generating global gene expression profiles from 52 biopsies of rectal cancer taken before preoperative (chemo) radiotherapy. They then compared the stromal scores calculated in the pretreatment samples with the radiotherapy response evaluated in post-treatment surgical samples, by the Mandard scoring system. For each of the three stromal scores, first-quartile samples were called “low score” and fourth-quartile samples “high score”, finding low values to be associated with increased sensitivity to radiotherapy and high values associated with resistance. Finally, they defined a ‘compound stromal score’ as the median of the three percentiles. This compound score was found to significantly discriminate sensitive samples (with low score) from resistant ones (with high score).

1.7 Effects on quality of life

Current challenges in the treatment of LARC include the preservation of QoL in surviving patients undergoing standard surgery with TME, since all patients eventually develop functional *sequelae* due to removal of the rectum (low anterior resection syndrome) which significantly impairs their quality of life (QoL) (45). The NOM approach, by avoiding TME, is meant to preserve QoL; however following pelvic chemo-RT functional *sequelae* can develop as well and therefore rigorous measurement of patient-reported outcome measures [PROM] are warranted.

1.8 Aims of the study

We aim to assess whether an oxaliplatin-enhanced neoadjuvant chemo-radiotherapy, followed by an imaging-intensive, liquid biopsy-enriched surveillance, can spare stage II-III rectal cancer from undergoing up-front demolitive radical surgery with a clinically acceptable rate of distant relapse. Intensive imaging surveillance will allow timing salvage surgery of local disease without impinging on survival .

Within the translational component of the study we will establish by retrospective correlative analysis of contextual imaging and blood molecular findings whether circulating mutated a/o methylated tumoral DNA is a predictive marker for residual disease, whether transcriptional stromal scores predict response to preoperative treatment, and whether a combination of tissue and blood markers can identify early distant relapses.

Finally, since NOM offers to patients a more acceptable alternative to radical surgery, we will used patients related outcome measures to evaluate quality of life and perception of clinical benefit

2 Objectives of the trial

2.1 Primary objective

- To assess if the risk of distant relapse in patients managed with Induction ChemoTherapy (ICT) and Chemo-RadioTherapy (CRT) followed by NOM and intensive follow-up is clinically acceptable

2.2 Translational Objectives

To determine association of:

- 6-methylated gene panel (6-MGP) in liquid biopsy with local a/o relapse free survival
- ctDNA in liquid biopsy with local a/o relapse free survival
- stromal score in baseline tumor tissue biopsy with local response

2.3 Secondary Objectives

- To assess whether the anticipation of standard adjuvant oxaliplatin-based chemotherapy prior to CRT increases the rate of clinical complete responses
- To assess the Local Recurrence (LR) rate, organ (rectum) preservation rate, and colostomy-free survival
- To assess Overall Survival (OS)
- To assess the outcome of NOM in terms of patient-reported outcome measures [PROM]

2.4 End-points

Primary end points:

- Distant Relapse-Free Survival (DRFS) rate at 2.5 year

Translational end points:

- Association between 6-methylated gene panel and hazard ratio (**HR**) for relapse free survival
- Association between ctDNA and relative hazard per standard deviation unit (**HRσ**)
- Association between stromal score and odds ratio of response to treatment

Secondary end points:

- Clinical complete response rate
- Local recurrence and organ preservation rate, colostomy-free survival
- Overall survival
- Patient reported outcomes (European Organization for Research and Treatment of Cancer [EORTC] QLQ-C30 and its colorectal cancer specific module QLQ-38)

3 Patient selection criteria

3.1 Inclusion criteria

- Histologically confirmed diagnosis of adenocarcinoma of the medium/lower rectum
- Patients must have Stage II (cT3-4 N0) or Stage III (cT1-4, N1-3) tumor
- Locally advanced rectal cancer amenable to Total Mesorectal Excision (TME)/Abdominal-Perineal Amputation
- No evidence of distant metastases by chest, abdomen, and pelvis contrast enhanced CT scan (TC-PET WB is acceptable alternative in patient allergic to iodate contrast medium)
- No prior pelvic radiation therapy
- No prior oncologic medical therapy or surgery for rectal cancer
- Age >18 years
- No infections requiring systemic antibiotic treatment
- Performance status 0-1 (ECOG Scale)

- ANC > 1.5 cell/mm3, Hb>8.0 g/ dL, PLT>150,000/mm3, total bilirubin < or equal or 1.5 x upper limit of normal, AST < or equal to three times upper limit of normal, ALT< or equal to three times upper limit of normal; Serum creatinine level < or equal to 1.5 times the upper limit of normal
- Patients must read, agree to, and sign a statement of Informed Consent prior to participation
- Women with childbearing potential who are negative for pregnancy test (urine or blood) and who agree to use effective contraceptive methods
- Male subjects must also agree to use effective contraception

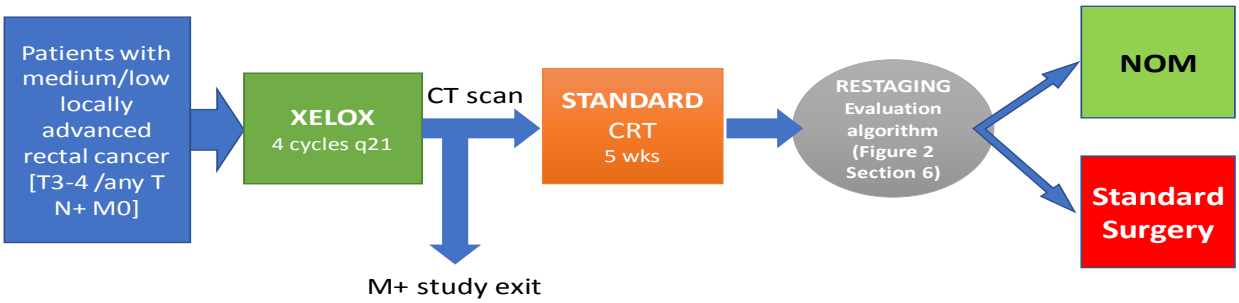
3.2 Exclusion criteria

- Recurrent rectal cancer
- Patients with a history of any arterial thrombotic event within the past 6 months, including angina (stable or unstable), MI, or CVA
- Intolerance or contraindication to MR procedure
- Patients with any other concurrent medical or psychiatric condition
- Gastro-intestinal abnormalities, inability to take oral medication, any condition affecting absorption
- Patients with a history of a prior malignancy within the past 5 years, except for adequately treated basal cell or squamous cell skin cancer, or in situ cervical cancer.
- Patients with a history of thrombotic episodes, such as deep venous thrombosis, pulmonary embolus, MI, or CVA occurring more than 6 months prior to enrollment may be considered for protocol participation, provided they are on stable doses of anticoagulant therapy. Patients who are anticoagulated for atrial fibrillation or other conditions may participate, provided they are on stable doses of anticoagulant therapy.
- Patients receiving other anticancer or experimental therapy.

4 Trial Design

NO-CUT is a one-stage phase II trial seeking to establish whether an oxaliplatin-based chemotherapy preceding standard neo-adjuvant fluropyrimidines-based chemo radiotherapy, can safely spare demolitive surgical intervention in patients with operable rectal cancer, without increasing the risk of distant relapse. The trial also has a translational component aimed at establishing whether selected genomic, epigenetic, and transcriptomic markers are predictive of tumor and patient outcome.

Figure 2 - Study design



The study design (**Figure 2**) require a rectal tumor biopsy at screening phase (**Baseline Biopsy**, n=180 eligible), followed by induction treatment (4 cycles of XELOX q21 and 5 weeks of pelvic CT-RT), then restaging with radiological imaging (MRI, CT-scan and endoscopy) including tumor biopsy (**Post-induction Biopsy**, n=180 eligible). According to the algorithm defining tumor response (NOM triage algorithm, see **Figure 3** in section 6 - Criteria of evaluation and definition of cCR), patients will enter NOM Cohort or Standard Cohort (standard surgery with tumor resection **Surgical Tumor Specimen**, expected n=136).

During the follow-up phase of the trial, NOM Cohort will be followed with intensive local imaging (NOM protocol for follow-up) and periodic blood and urine samplings (**Liquid Biopsies**, four samples at baseline, pre-surgery, at time of enrollment in NOM Cohort and at time of any tumor relapse, either distant or local) for 5 years or until death, tumor

relapse or withdrawal from the study. Patients in the **NOM Cohort** experiencing local tumor relapse, if feasible, undergo standard surgery (**NOM Surgical Tumor specimen**).
In **Surgery Cohort** along with standard imaging procedures, the protocol requires a liquid biopsy at the same time points of **NOM Cohort (Liquid Biopsies**, four samples at baseline, pre-surgery, post-surgery and at time of any tumor relapse, either distant or local**)**, for 5 years until death, tumor relapse or withdrawal from the study.

The biopsy and surgical specimens will be used for pathologic diagnosis at Niguarda Cancer Center and University of Milan, and for research purposes aimed at discriminating those cases who are resistant or sensitive to the protocol treatment, at Candiolo Cancer Center and University of Turin.
In particular, evaluable samples will be analyzed for DNA sequencing, to evaluate the performance in terms of sensitivity and specificity of a stromal gene signature (44), and to correlate genetic and epigenetic markers with the clinical endpoints of the study (34,47). The correlation between ctDNA and cancer relapse, along with the clinical endpoints of tumor response, will be evaluated on liquid biopsies. All translational analyses will be performed in a double blind fashion.

4.1 Sample size

A Brookmeyer and Crowley approach (46) will be used to test the null hypothesis that the true distant relapse-free survival (DRFS) at 2.5 years rate is ≤75% against a one-sided alternative. The NOM cohort of the trial need to accrue 44 evaluable patients over 2.5 years and follow them up for at least 2.5 years. Such design yields a type I error rate of 10% and power of 80% when the true 2.5 years DRFS proportion is 87%. Since it is assumed that the proportion of patients entering in the NOM phase of the trial is at least 25% (32) of the treated patients, a total of approximately 180 patients need to be enrolled in the study.

4.2 Analysis

Statistical analysis of DRFS predictors will be conducted using the Cox proportional hazard model. Analysis of factors associated with response will be performed by mean of logistic regression analysis. Concordance of continuous and dichotomous biomarkers between pre- and post-CR-RT will be estimated using respectively the Pearson's correlation coefficient and the proportion of concordant pairs. The McNemar's test will be

used to statistically detect difference of biomarkers prevalence between pre and post CT-RT.

4.3 Accrual and Duration of Study

This study is multicentric and involves selected centers with experience in clinical research in the field of colorectal cancer. The estimated accrual for this study is 1.8 patients per month. Thus, patient accrual is expected to be completed within 24 months. Additional time is required to allow the response data to mature (2.5 years from last patient in).

All of the patients registered in the study will be accounted for. The number of patients who were not evaluable, who died or withdrew before treatment began will be specified. The distribution of follow-up time will be described and the number of patients lost to follow-up will be given.

Study initiation is expected in October 2017 and study end in October 2022 (30 months of accrual and 30 months of follow-up).

4.4 Safety Monitoring

Adverse events will be monitored on an ongoing basis and their frequencies reported annually. Toxic effects will be categorized using the NCI Common Terminology Criteria for Adverse Events, Version 4.3. The worst event for each patient will be described. Both events related and unrelated to treatment will be captured.

Clinical and laboratory data will be tabulated and compared to normal ranges for the institution.

5 Study procedures

5.1 Induction chemotherapy and chemo-radiotherapy Drug Administration

The **induction chemo-therapy (ICT)** is prescribed as 4 cycles of standard dose XELOX (Capecitabine 1000 mg/m2 BID days 1-14 every 3 weeks; oxaliplatin 130 mg/m2 day 1 every 3 weeks) before radiotherapy start.

The **CRT regimen** will start after 3-4 weeks from start of 4th cycle of ICT and it consists of: 50-54 Gy [46Gy to the pelvis, with an integrated boost of 4Gy to the primary tumor and mesorectum, in 25 fractions over a 5-week period (a second integrated boost of 4Gy will be given to the primary tumor in case of T4 staging)]. Technique: VMAT (SIB) plus set up online control using CBCT (IGRT). Starting on the first day of RT, patients receive standard dose of capecitabine 825 mg/m2 po BID, for the duration of radiotherapy.

5.1.1 Premedication

Standard anti-emetics will be prescribed during the ICT chemotherapy administration phase, according to the center clinical practice.

5.1.2 Dose Adjustments

Doses will be reduced for haematological and other adverse events. Dose adjustments are to be made according to the greatest degree of toxicity. Adverse events will be graded using the NCI Common Terminology Criteria for Adverse Events Version 4.03 (CTCAE).

5.2 Tumor assessment

5.2.1 Baseline tumor staging:

Digital Rectal Exam (DRE)

Colonoscopy with tumor biopsies

Endorectal ultrasound (EUS)

Pelvic MRI with contrast medium

Thorax, abdomen, and pelvis CT scan with contrast medium

5.2.2 After induction chemotherapy (Restaging #1):

These procedures must be performed at the end of ICT (i.e. during the 3rd or 4th week after start of the 4th cycle of ICT):

Thorax, abdomen, and pelvis CT scan with contrast medium

Pelvic MRI

5.2.3 At the end of chemo-radiotherapy (Restaging #2):

These procedures must be performed between 11-12 weeks after CRT completion:

Digital Rectal Exam

Endorectal ultrasound with tumor biopsies

Pelvic MRI with contrast medium

Thorax, abdomen, and pelvis CT scan with contrast medium

5.2.4 Restaging #3

In selected patients (see algorithm in **Figure 3 –** Section 6 - Criteria of evaluation and definition of cCR), 4-5 weeks after restaging #2 a further *Pelvic MRI with contrast medium* should be performed.

5.3 Follow-up for local relapse in NOM cohort

To protect patients treated non-operatively against the risk of local tumor progression, we will monitor them intensively during follow-up.

NOM cohort patients will be followed by surgeon physicians in the Department of Surgery for all study procedures and visits. Patients will perform **physical examination including DRE** after 2 and 4 months and every 4 months thereafter; **endoscopic evaluation** (proctoscopy or EUS if clinically indicated) every 4 months for the first 2 years and then every 6 months until the fifth year; **pelvic MRI with contrast medium** every 4 months for the first two years and then every 6 months up to the third year included and then every 12 months until the fifth year (see **Table** in **5.5 Study Procedures**).

5.4 Follow-up for distant relapse (NOM and standard surgery cohort)

All patients will be followed-up for distant relapse according to current guidelines. Patient will be followed up by a medical oncology physician in the Department of Oncology for systemic assessments by CT scan as *per* protocol (CT scan every 6 months for the 5 years, with a mandatory CT scan at 30 months); laboratory, ctDNA and quality of life assessment will be performed at every follow-up visit.

5.5 Study procedures

	Screening	Treatment Phase				Follow up Phase (NOM)			Follow up Phase (Standard surgery)		
		Before every ICT cycle	Restaging #1: After cycle 6 of ICT	Restaging #2: 11-12 weeks after the end of CRT	Restaging #3: 4-5 weeks after Restaging #2[$]	1st year	2nd year	3rd to 5th year	1st year	2nd year	3rd to 5th year
Window visit	</= 28 days	± 3 days	± 3 days	± 3 days	± 3 days	± 3 days	± 3 days	± 3 days	± 3 days	± 3 days	± 3 days
Informed Consent	X										
Confirmation of eligibility criteria	X										
Medical History and Demographics	X										
Physical examamination	X	X	X	X	X	Every 4 months	Every 4 months	Every 6 months	Every 4 months	Every 4 months	Every 6 months
Vital signs (PA, FC, SAT.)	X	X									
Weight	X										
Height	X										
Concomitant Medication	X										
ECOG Performance Status	X			X	X	Every 4 months	Every 4 months	Every 6 months	Every 4 months	Every 4 months	Every 6 months
Adverse Events assessment	X	X	X	X	X	Every 4 months	Every 4 months	Every 6 months	Every 4 months	Every 4 months	Every 6 months
ECG	X										
Laboratory											
Hematology§	X	X		X	X	Every 4 months	Every 4 months	Every 6 months	Every 4 months	Every 4 months	Every 6 months
Blood chemistry#	X	X		X	X	Every 4 months	Every 4 months	Every 6 months	Every 4 months	Every 4 months	Every 6 months
CEA	X		X	X	X	Every 4 months	Every 4 months	Every 6 months	Every 4 months	Every 4 months	Every 6 months
Pregnancy Test (serum or urine)	X										
Disease Assessments											
DRE	X		X	X	X	After 2 and 4 months, and then every 4 months	Every 4 months	**Every 6 months**			
Endoscopic evaluation (colonoscopy° or proctoscopy	X°			X*	X*	Every 4 months*	Every 4 months*	**Every 6 months**			
Endorectal ultrasound -EUS	X			X	X						
Pelvic MRI with (*) or without contrast medium	X*		X	X*	X*	Every 4 months*	Every 4 months*	Every 6 months for the 3rd year, then every 12 months			
CT scan (Thorax, abdomen, pelvis)	X		X	X		Every 6 months	Every 6 months	**Every 6 months (mandatory CT scan at 30 months)**	Every 6 months	Every 6 months	**Every 6 months (mandatory CT scan at 30 months)**
Other investigations											
Liquid Biopsy	X			X	X	Every 4 months	Every 6 months		Every 4 months	Every 6 months	
Tumor Biopsy	X			X	X						
Quality of Life Assessment	X		X	X	X						

Notes: [$]Only for patients meeting criteria of algorithm reported in Figure 3; [§]Hematology: Hematology: hemoglobin, hematocrit, platelet count, total white blood cell count (WBC) and differential (neutrophil count, lymphocyte, monocyte, eosinophil, and basophil counts), red blood cell count (RBC). [#]Chemistry: sodium, potassium, chloride, bicarbonate, creatinine, calcium, albumin, total bilirubin, total protein, glucose, alkaline phosphatase, AST, ALT urea or BUN. ICT: induction chemiotherapy CRT: chemioradiotherapy DRE: digital rectal examination MR: magnetic resonance imaging CT: computed tomography

6 Criteria of evaluation and definition of cCR

One of the main challenges to a multi-centric NOM approach is the development of uniform and reproducible criteria for tumor response. To this end we have devise a specific algorithm to define clinical response. All patients will undergo mandatory endoscopy with biopsy and MRI. The combined results of these three procedures will inform the triage of each single patient according to the algorithm below (**Figure 3)**.

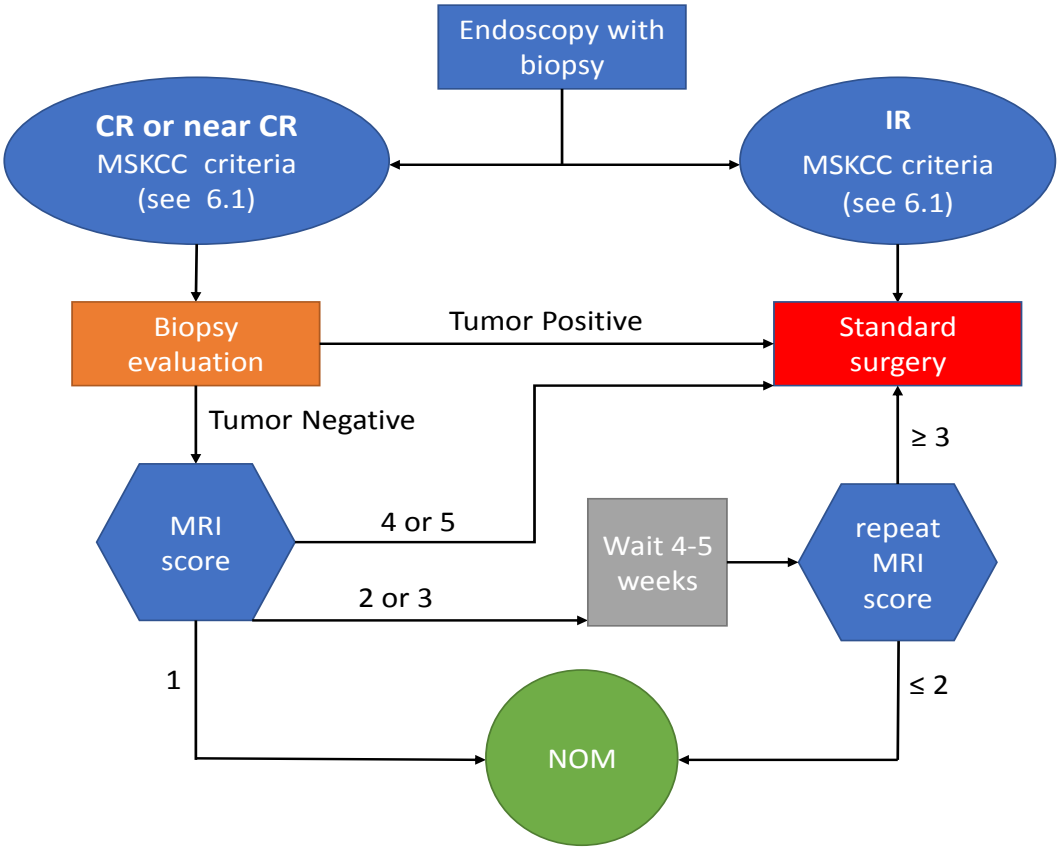


Figure 3 – Decision algorithm for NOM triage

Patients with residual macroscopic disease at endoscopy will undergo immediate surgery. Treatments of patients with complete or near complete response at endoscopy will be determined by the results of biopsy. Patient with positive biopsy for residual tumor will be directed to immediate surgery. MRI will guide the subsequent triage of patients with negative biopsies according to **Figure 3** and section 6.2.2.

6.1 Endoscopic criteria of response

*Endoscopic Memorial Sloan Kettering Cancer Center (MSKCC) criteria for **complete response (CR)**:*

- Flat, white scar
- Teleangectasia
- No ulcer
- No nodularity

*Endoscopic MSKCC criteria for near **complete response (nCR)**:*

- Irregular mucosa
- Small mucosal nodules or minor mucosal abnormality
- Superficial ulceration
- Mild persisting eritema of the scar

*Endoscopic MSKCC criteria for incomplete **response (IR):***

- Visible tumor

6.2 MRI criteria of response

The primary tumor and the regional lymph nodes will be evaluated at Restage #2 (11-12 weeks after CRT completion) and Restage #3 (4-5 weeks after Restage #2) by digital rectal exam (DRE), endoscopic exam and rectal MRI, according to the algorithm in **Fig.2.**

6.2.1 MRI procedures

Baseline and follow up T staging of rectal tumors using MRI must be done with high-resolution technique for optimal visualization of rectal and mesorectal anatomy and for characterization of mesorectal lymph nodes. Comparison of post-treatment MR images with pretreatment MR images is essential and ideally both should be acquired using the same angles. Pretreatment images are used to help locate the treated tumor, which may be difficult to visualize in patients who have had a good response to CRT. The rectum should be distended with 100-150 ml of sonographic gel. After initial localization imaging, large-FOV sagittal and axial images are acquired. These first two sequences allow an overview of the treated tumor, potentially involved lymph nodes, and direction of the rectal wall. This overview enables the planning of the following three high-spatial-resolution sequences that are vital for visualization of the tumor and post-treatment fibrosis. The first

sequence planned is axial to the plane of the tumor and rectal wall. Thin-section (maximum, 3 mm) axial T2-weighted images through the rectal cancer area are planned using the sagittal T2-weighted images. These images are obtained perpendicular to the long axis of the rectum using a 16-cm FOV. The second sequence is axial imaging for evaluation of the lymph node drainage territory. The third sequence is in the coronal plane for low rectal cancers. Relying on oblique axial imaging alone can be limiting at the level of the anorectal junction. Therefore, high-resolution coronal imaging, which will show the relationship between the rectal wall and the levator muscles and between the anal sphincter complex and the intersphincteric plane, is useful for tumors in the lower one third of the rectum. After the acquisition of T2w sequences, DWI and contrast enhanced images are acquired in the axial plane. Morphologic changes seen in surgical specimens after CRT include collagen, fibrosis, desmoplasia, mucin, inflammatory change resulting in submucosal edema, and necrosis. On post-CRT T2-weighted MRI areas of fibrosis have very low signal intensity, whereas areas of residual tumor have intermediate signal-intensity. Restricted diffusion and residual enhancement. The signal intensity of fibrosis is similar to that of the muscularis propria, and signal intensity of residual tumor is similar to that of baseline tumor. Review of high-resolution images will enable delineation of small foci of intermediate-signal-intensity tumor within areas of low-signal-intensity fibrosis.

6.2.2 MRI response criteria

An MRI-based tumor regression grading system will be applied. The entire tumor is assessed to determine if fibrous signal intensity or if tumor signal intensity is present. The radiologic interpretation requires comparison of high-resolution oblique images with baseline scans to determine the proportion of tumor that has become fibrotic (low T2w signal intensity, low contrast enhancement) and the proportion of remaining residual intermediate signal intensity.

The following score will be used to assess response:

1. Predominance of fibrosis with no residual intermediate tumor signal
2. Predominance of fibrosis with minimal residual intermediate tumor signal
3. Substantial tumor signal-intensity present but that signal-intensity does not predominate the fibrosis

4. Predominance of tumor with minimal low-signal-intensity fibrosis
5. Tumor unchanged from baseline

For the differentiation of grade 1 and 2 response, after the initial reading of PI radiologist, a further independent reading by 2 radiologists will be obtained and documented, and in cases of discrepancy the final judgment will be established by majority.

6.3 Overall assessment of clinical response

Overall assessment of clinical Complete Response (cCR) or near Complete Response (nCR) will be carried out according to MSKCC criteria showed in **Table** 5 and results interpreted according to algorithm in **Figure 3**. Patients with cCR or nCR can enter the **NOM Cohort**. Patients with nCR are eligible for enrollment in NOM Cohort if the tumor biopsy after CT-RT is negative.

Table 5. Memorial Sloan Kettering Cancer Center (MSKCC) Definition of Complete, near Complete and Incomplete Response

	Complete Response	Near Complete Response	Incomplete Response
Endoscopy	Flat, white scar Telangiectasia No ulcer No nodularity	Irregular mucosa Small mucosal nodules or minor mucosal abnormality Superficial ulceration Mild persisting erythema of the scar	Visible tumor
Digital Rectal Exam	Normal	Smooth induration or minor mucosal abnormalities	Palpable tumor nodules
MRI-T2W	Only dark T2 signal, no intermediate T2 signal	Mostly dark T2 signal, some remaining intermediate signal	More intermediate than dark T2 signal, no T2 scar
	AND	AND/OR	AND/OR
	No visible lymph nodes	Partial regression of lymph nodes	No regression of lymph nodes
MRI-DW	No visible tumor on B800-B1000 signal	Significant regression of signal on B800-B1000	Insignificant regression of signal on B800-B1000
	AND/OR	AND/OR	AND/OR
	Lack of or low signal on ADC map Uniform, linear signal in wall above tumor is ok	Minimal or low residual signal on ADC map	Obvious low signal on ADC map

7 Forms and procedures for collecting data

7.1 Data handling and record keeping

7.1.1 Case Report Form (CRF)

An electronic Case Report Form will be completed for each enrolled subject. The language used must be English. The completed original Case Report Forms are the sole property of Sponsor and should not be made available in any form to third parties, except for authorized representatives of appropriate regulatory authorities, without written permission from Sponsor.

The Investigator or an authorized staff member (medically qualified) has the responsibility to ensure completion and to review and sign all Case Report Forms.

However, the Investigator has final personal responsibility for the accuracy and authenticity of all clinical and laboratory data entered on the Case Report Form.

Subject source documents are the hospital subject records maintained at the study site. In case where the source documents are the hospital chart, the information collected on the Case Report Form must match with those charts. In some cases a portion of the source documents are not the hospital subject records. The investigator and Sponsor must agree which items will be recorded in the source documents and for which items the Case Report Form will stand as the source document. This must be stated in the “Data Location List” (filed in the Investigator File).

7.1.2 Data Handling

Medical terms are coded according to the MedDRA dictionary. Data will be analyzed using SAS® System or other available statistical software. Data cleaning will include both visual and computer-driven procedures in order to minimize logical inconsistencies and errors within the collected data. The data are checked for completeness, accuracy and consistency. The errors detected will be rectified by means of Data Clarification List (DCL) that will be used by the monitor for resolution of queries. The original DCL will be kept together with the patient CRF.

8 Reporting adverse events

8.1 Definitions

An **Adverse Event (AE)** is defined as any untoward medical occurrence or experience in a patient or clinical investigation subject which occurs following the administration of the trial medication regardless of the dose or causal relationship. This can include any unfavorable and unintended signs (such as rash or enlarged liver), or symptoms (such as nausea or chest pain), an abnormal laboratory finding (including blood tests, x-rays or scans) or a disease temporarily associated with the use of the protocol treatment.

A **Serious Adverse Event (SAE)** is defined as any undesirable experience occurring to a patient, whether or not considered related to the protocol treatment.

Adverse events and adverse drug reactions which are considered as **serious** are those which result in:

- death
- a life threatening event (i.e. the patient was at immediate risk of death at the time the reaction was observed)
- hospitalization or prolongation of hospitalization
- persistent or significant disability/incapacity
- a congenital anomaly/birth defect
- any other medically important condition (i.e. important adverse reactions that are not immediately life threatening or do not result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed above)

8.2 Reporting procedure

Each adverse event is to be classified by the investigator as SERIOUS or NON-SERIOUS. This classification of the seriousness of the event determines the reporting procedures to be followed. If a serious adverse event occurs, the Niguarda Pharmacovigilance (Fax Number: + 39 0264444981 or mailbox: farmacia@ospedaleniguarda.it) is to be notified, using the SAE report form, within 24 hours of awareness of the event by the investigator. If the initial report is incomplete or the event is still ongoing at the time of reporting or if new significant information

becomes available, this report is to be followed by submission of follow-up information within 5 calendar days after the initial notification. Reporting requirements for adverse events are summarized in the following **Table 6**.

Table 6. SAE reporting.

Gravity	Reporting Time	Type of Report
SERIOUS	Within 24 hours from awareness by the investigator	Initial report on SAE report form + case report form
	Within 5 calendar days from initial report	Follow-up/Final report on SAE report form
NON SERIOUS	Per case report form submission procedure	Case report form

If for any reason the SAE form transmission is not possible, Niguarda Pharmacovigilance should be informed by phone (+ 39 0264443637 or + 39 0264443288 or + 39 0264442144) of the occurrence of the event. In this exceptional case, Niguarda Pharmacovigilance will complete a SAE form with information received, which will be sent to the investigator for confirmation, and in the meanwhile pharmacovigilance procedures will be initiated. Serious adverse events should also be reported on the adverse event case report form. The form to be used for serious adverse event expedited reporting is not the same as the adverse event case report form, but where the same data are collected, the forms must be completed in a consistent manner. For example, the same adverse event term should be used on both forms. All SAE will be handled according to GCP and Italian laws.

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9 Quality assurance

9.1 Monitoring

Monitoring visits to the trial site will be made periodically during the trial by a qualified CRO to verify that the trial is conducted according to study protocol, GCP principles and regulatory requirements. The monitor will verify the accurate and complete recording of data on CRFs, source documents, Investigators File and drug accountability records.

The investigator/institution guarantees direct access to source documents of the study patients and to any other trial related documentation.

It is important that the investigator(s) and/or their relevant personnel are available during the monitoring visits.

9.2 Auditing

Representative members of Sponsor Quality Assurance may conduct an on site audit. The investigator will be informed if an audit is to take place.

Representative of Regulatory Agencies may also conduct an inspection of the study. If informed of such an inspection, the Investigator should notify Sponsor immediately. The investigator will ensure that the auditors/ inspectors have access to the clinical supply, study site facilities, source documents and all study files.

10 Ethical considerations

10.1 Patient protection

The responsible investigator will ensure that this study is conducted in agreement with either the Declaration of Helsinki (Tokyo, Venice, Hong Kong, Somerset West and Edinburgh amendments) or the laws and regulations of the country, whichever provides the greatest protection of the patient.

The protocol has been written, and the study will be conducted according to the ICH Harmonized Tripartite Guideline for Good Clinical Practice (ref: http://www.ifpma.org/-pdfifpma/e6.pdf).

The protocol will be approved by the Ethics Committees of Milan, Area 3 ASST Grande Ospedale Metropolitano Niguarda.

10.2 Subject identification

To enable evaluation and/or audits and/or regulatory authorities inspections, the Investigator agrees to keep records, including the identity of all participating subjects (“Subject identification code list”), all original signed informed consent forms, copies of all case report forms, source documents, detailed records of treatment disposition as well as the documentation included in the Investigator Trial File according to local regulations.

If the Investigator relocates, retires, or for any reason withdraws from the study, Sponsor should be prospectively notified. The study records must be transferred to an acceptable designee, such as another investigator or another institution. The investigator must obtain Sponsor's written permission before disposing of any records.

10.3 Informed consent

It is the responsibility of the investigator to give each patient (or the patient acceptable representative) full and adequate verbal and written information regarding the objective and procedures of the trial and the possible risks involved. The patient must be informed about his/her right to withdraw from trial at any time. The patient should have time and opportunity to enquire about details of the trial and to decide whether or not to participate in the trial.

Written subject information must be approved by an independent ethic committee (IEC) and must be given to each patient before any trial-related procedure is undertaken.

It is responsibility of the investigator to obtain informed consent signed and dated by the patient and by the medical person conducting the informed consent discussion, prior to undertaken any trial-related procedure. One copy of the signed and dated Informed Consent Form should be given to the patient. The originally signed document should be archived in the confidential section of the Investigator File.

The approved patient information sheet must not be changed without prior approval by Sponsor and by the IEC.

When new study information arise during the study, the patients still on treatment must be informed and a new Informed Consent form or an addendum to the already signed Informed Consent form must be signed and dated by the patients.

If a patient becomes incompetent during the course of a trial where it was not anticipated, legally acceptable representative authorization should be obtained for a subject's continued participation.

10.4 Study discontinuation criteria

This study may be prematurely terminated or suspended, if in the opinion of Sponsor there is sufficient reasonable cause. Written notification documenting the reason for study termination will be promptly provided to the investigator. Circumstances that may warrant termination include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to patients

- Insufficient adherence to protocol requirements

- Insufficient complete and/or evaluable data

After such a decision, the investigator must promptly contact all participating patients to inform them about the decision taken.

Should the study be closed prematurely or suspended the IEC should also be informed promptly and provided with the reason for termination or suspension. In case of termination the study materials must be collected and returned to the Sponsor and all Case Report Forms must be completed to the greatest extent possible.

11 Trial sponsorship and financing

The responsible Investigator will ensure that this study is conducted in compliance with:

- The clinical trial protocol, following the instructions and procedures described in it
- ICH Harmonized Tripartite Guidelines for Good Clinical Practice
- Declaration of Helsinki concerning medical research in humans (Helsinki 1964, amended

Current and applicable local regulatory requirements and laws. The name, address, telephone and fax number of the study “sponsor” (according to GCP definition) must be included in the protocol

Financial support is being applied for to non-profit research agencies such as Associazione Italiana Ricerca Cancro (AIRC) (Investigator Grant 2017 and AIRC 5x1000 2017) and Fondazione Oncologia Niguarda that require ethical approval prior to granting.

12 Trial insurance

The involved parties will be insured in accordance with the applicable laws and regulation for injuries and/or damages that may arise as a consequence of this trial.

13 Publication policy

At trial conclusion, the Investigator shall have the right to use the data arising from enrolled patients for congress communications and scientific publications. The Promoter of the study shall have full access to all data of the study and has full and total responsibility of:

1. the preparation of the manuscript (s) and of collected data in this study;

2. the final decision on the number, order, and the names of the contributing authors.

Generally, it will be considered co-authored the institution principal investigator or one representative, which have participated in the study. The number and order of co-authors will be established according to the proportion of patients actually employed by each representative and from the guidelines supplied by the principal investigator.

14 References

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