STATISTICAL ANALYSIS PLAN
NO-CUT TRIAL (EU-CT 2017-003671-60)

Administrative information:

Sponsor name	Grande Ospedale Metropolitano Niguarda
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EU CT number / REC no	2017-003671-60
Trial title	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)
Trial ID	NO-CUT trial
Trial registration number	EU-CT 2017-003671-60, NCT03565029

SAP and protocol version:

SAP version and date:	This SAP is version 1, dated 17 March 2025
Protocol version	This document has been written based on information contained in the study protocol version 1.0, dated 23 October 2017

SAP revision history:

Protocol version	SAP version	Section number changed	Description and reason for change	Date changed
[1.0]	[1.0]	[NA]	[NA]	[17 March 2025]

SIGNATURE PAGE

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ABBREVIATIONS

AE	Adverse Event
ATC	Anatomical/Therapeutic/Chemical
BMI	Body Mass Index
BPM	Beats Per Minute
cCR	clinical Complete Response
cfDNA	cell-free DNA
CI	Confidence Interval
cm	contrast media
cmDNA	circulating methylated DNA
CRM	Circumferential Resection Margin
CRT	Chemo-RadioTherapy
CT	Computed Tomography
ctDNA	Circulating Tumor DNA
DRFS	Distant Relapse-Free Survival
DMC	Data Monitoring Committee
DRE	Digital Rectal Exam
ECG	Electrocardiogram
eCRF	electronic Case Report Form
EDC	Electronic Data Capture
EMVI	ExtraMural Vein Invasion
ERUS	EndoRectal UltraSound
ES	Enrolment Set
H0	Null Hypothesis
ICT	Induction Chemotherapy

FAS	Full Analysis Set
LB	Liquid biopsy
LR	Local Relapse/Regrowth
LRFS	Local Relapse/Regrowth-Free Survival
MedDRA	Medical Dictionary for Regulatory Activities
MRI	Magnetic Resonance Imaging
NOM	Non-Operative Management
OS	Overall Survival
pMMR	Proficient MisMatch Repair
PFS	Progression-Free Survival
PROM	Patient Reported Outcome Measures
PS	Performance Status
SAE	Serious Adverse Event
SAS	Statistical Analysis Set
SD	Standard Deviation
SOC	System Organ Class
TNT	Total Neoadjuvant Therapy

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1 Introduction

1.1 Background and Rationale

See **Study Protocol** v1.0 for Background and Rationale of the NO-CUT trial in patients with proficient mismatch repair (pMMR), locally advanced, low-to-mid, rectal cancer.

1.2 Intervention(s)

1.2.1 Brief description of the study intervention(s)

Total neoadjuvant therapy (TNT) with CAPOX Induction Chemotherapy (ICT) for 4 cycles every q3weeks followed by long course Chemo-RadioTherapy (CRT) over 5 weeks. Eleven-12 weeks after CRT completion, surgery or non-operative management (NOM) according to the tumour response. See **Study Protocol** v1.0 for details.

1.2.2 Control settings (if applicable)

NA

1.3 Trial Objectives

1.3.1 Primary Objective

To assess if the risk of distant relapse in patients managed with TNT followed by NOM and intensive follow-up is clinically acceptable.

1.3.2 Secondary Objectives

The secondary objectives of this study are to assess:

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

1.3.3 Exploratory Objectives

The exploratory objective of this study is to investigate the association of:

- 6-methylated gene panel (6-MGP) in liquid biopsy with local and/or distant relapse-free survival (LRFS and DRFS, respectively).
- Circulating tumor DNA (ctDNA) in liquid biopsy with LRFS and/or DRFS
- Stromal (transcriptomic) score in baseline tumor tissue biopsy with local response

2 Trial Methods

2.1 Trial Design

The NO-CUT study is designed as a single-arm, open-label, multi-centre, single-country, phase II trial. Patients are assigned to NOM or surgery according to response to TNT. The trial is seeking to establish whether an oxaliplatin-based ICT preceding standard neoadjuvant fluropyrimidines-based CRT, 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 tumour and patient outcome. The primary endpoint is the distant relapse free survival at 30 months in patients assigned to NOM.

2.2 Randomisation

NA

2.3 Statistical Framework

2.3.1 Hypothesis Test

A Brookmeyer and Crowley approach will be used to test the null hypothesis (H0) that the true 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% of the treated patients, total sample size will be of approximately 180 patients. There is only one identified primary analysis in this trial. All other efficacy analyses will be regarded as supportive or exploratory.

2.3.2 Confidence Intervals and p-values

All calculated p-values will be two-sided and compared to a 5% significance level. If a p-value is less than 0.05, the corresponding treatment group difference will be denoted as statistically significant. All efficacy estimates will be presented with two-sided 95% confidence intervals. As there is only one primary H0 to be tested in this trial, there will be no adjustments for multiplicity.

2.3.3 Decision Rule

This trial is designed to address a single primary outcome. A safe DRFS is claimed if the primary H0 is rejected on the significance level (alpha) of 0.1 (one-sided). That is, if the lower limit of the 95% two-sided confidence interval for the DRFS rate at 30 months is greater than 75%.

2.4 Timing of Outcome Assessments

Defined in the study procedures table in the protocol v1.0, here reported:

	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

2.5 Statistical Interim Analyses and Stopping Guidance

There will be no interim analyses in this trial.

2.6 Timing of Main Analysis

The main analysis is planned after all patients have been enrolled, all data available have been entered, verified and validated and the primary database has been locked.

3 Trial Population

3.1 Screening Data, Eligibility and Recruitment

The total number of screened patients and reasons for not entering the trial will be summarised and tabulated. A CONSORT flow diagram will be used to summarise the number of patients who were:

- assessed for eligibility at screening
- eligible at screening
- ineligible at screening*
- included in the efficacy evaluable population*
- discontinued the intervention (TNT) *
- lost to follow-up before assignment to NOM or surgery*
- assigned to NOM and included in the primary analysis
- assigned to NOM and excluded from the primary analysis*

*reasons will be provided.

3.2 Baseline Patient Characteristics

Each subject is uniquely identified by a patient code at the enrollment center. At baseline, the following variables are collected in the electronic Case Report Form (eCRF):

- Demographics and physical status: date of birth, age, sex, height, weight, ECOG performance status (PS), and comorbidities.
- Oncological Characteristics and Disease Stage: Clinical T and N stage, along with the aggregated TNM stage as classified according to the TNM/AJCC 8th edition and based on staging procedures including digital rectal exam (DRE), pelvic magnetic resonance imaging (MRI) with contrast medium (cm), chest-abdomen computed tomography (CT) scan with cm, endoscopy with endorectal ultrasound (ERUS); histology and microsatellites status retrieved from the baseline tumor biopsy performed during colonoscopy or ERUS.
- Imaging and Laboratory: Additional radiological and laboratory parameters include MRI findings such as presence of mesorectal fascial invasion (CRM), extramural venous invasion (EMVI), baseline primary tumor size, and distance from anal verge defining the tumor location (mid or low), according to ESMO guidelines; serum CEA levels at baseline.

Trial variables include:

- Treatment and Response: Patients are grouped by clinical response to TNT (cCR or incomplete response) and assignment to NOM or surgery, based on the NO-CUT decision algorithm including DRE, MRI, CT, and ERUS with biopsy as described in the **Study Protocol** v1.0. TNT is reported as % of ICT administered (completion of 4 CAPOX cycles) and % of chemoradiotherapy (CRT) administered (% of total radiation dose).
- Restaging Evaluations: Dates and results of selected variables for each restaging (intermediate post-ICT – restaging #1; after TNT, 11-12 weeks post CRT completion – restaging #2; delayed, 4-5 weeks post restaging #2 in selected cases with near cCR – restaging #3) allow tracking of disease status over longitudinal time points, integrating clinic-radiological variables like RECIST for progression, imaging findings through MRI (tumor regression grade, TRG), ERUS, DRE and biopsy results, and laboratory values (CEA).
- Surgical Outcomes and Follow-Up: Surgery date and pathological response to treatment was assessed according to Mandard TRG score. Other surgical variables (pTNM and pathology Quirke status) are included in the postoperative pathology reports. Follow-up care visits and exams are recorded (date of last follow-up and vital status) to establish OS, DRFS (the primary endpoint of the study), LRFS, and progression-free survival (PFS) (as defined below). In case of distant recurrence, the site of metastasis is documented. If patients receive adjuvant chemotherapy, this is recorded. Causes of death were recorded when known.
- Adverse Events and Additional Details: The presence of common Adverse Events (AE) and their relative grade of severity according to the CTCAE classification v5, in addition to the resulting action and relationship with study treatment, are collected. Information of study discontinuation dates and reasons for withdrawal are also collected.
- Biomarkers and Molecular Analyses: Measurements of blood cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), and circulating methylated DNA (cmDNA) at various time

points (baseline, restaging #2, restaging #3, during follow-up, at relapse) are recorded when available. The overall status of ctDNA and cmDNA, referred to as liquid biopsy (LB), is considered positive if any of the two variables is detected in blood, and negative otherwise. The final dataset comprises molecular data such as mutation status of key genes (*APC*, *KRAS*, *TP53*, *NRAS*, *BRAF*, *PIK3CA*, and *ERRB2*); RNA sequencing data derived from baseline tissue biopsies are available as classifications into molecular subtypes (CMS, CRIS, leukocyte score, other stromal scores, etc.).

3.3 Withdrawal/Follow-up

See **Study Protocol** v1.0 for details.

3.4 Adherence and Protocol Deviations

3.4.1 Adherence to Allocated Treatment

NA

3.4.2 Protocol Deviations

The following are pre-defined major protocol deviations regarded to affect the efficacy of the intervention:

- Entering the trial when the eligibility criteria should have prevented trial entry
- Non completion of protocol treatment for reasons other than tumour progression, death, adverse event, or early surgery

Patients not completing TNT are included in the analysis dataset and will be used as the denominator to calculate the percentages of baseline characteristics. No formal statistical testing will be undertaken.

3.5 Analysis Populations

The Enrolled Set (ES) will include all patients who provide informed consent and are included into the study database.

The Full Analysis Set (FAS) will be defined as all patients eligible and having received at least one study treatment infusion.

The Safety Analysis Set (SAS) will include all patients having received at least one study treatment infusion.

4 Outcome Definitions

4.1 General Definitions and Derived Variables

The following variables were derived from existing data captured in the eCFR.

4.1.1 Body Mass Index (BMI)

BMI is calculated as body weight in kilograms divided by the square of the height in meters. Factorized BMI was also summarised as:

- Underweight: BMI <18.5 kg/m²
- Normal weight: BMI 18.5 – 24.9 kg/m²
- Overweight: BMI 25.0 – 29.9 kg/m²
- Obesity: BMI ≥30.0 – 34.9 kg/m²

4.1.2 Clinical complete response (cCR)

cCR corresponds to the assignment to NOM according to the algorithm in the **Study Protocol** v1.0. All others will be considered as clinical incomplete response (IR).

4.1.3 Pathological Complete response (pCR)

pCR response is defined as the pathological TRG = 1 according to Mandard score (complete fibrosis). All others will be considered as pathological IR.

4.1.4 Complete response (CR)

CR is defined as either cCR or pCR. All others will be considered as IR.

4.1.5 Factorized CEA

CEA was considered elevated if >5 ng/ml, as per clinical practice, or normal otherwise.

Some variables, as PS or clinical TNM staging for the primary tumor, were categorized with aggregated levels when certain categories were underrepresented for statistical analysis, while always maintaining biological relevance.

4.2 Primary Outcome Definition: Distant relapse free survival

The primary outcome is DRFS at 2.5 years (30 months, $DRFS_{30}$). Distant relapse is defined as the occurrence of metastases at any time after the completion TNT. LR is not included as an event in the definition of DRFS, which exclusively considers distant metastases. DRFS is computed from start of TNT to the occurrence of distant relapse or cancer-related death. Non-cancer-related death are censored for DRFS analysis. Patients experiencing LR are still considered at risk for distant relapse and censored at the last follow-up visit.

4.3 Secondary Outcomes Definitions

4.3.1 Clinical complete response rate

Rate of cCR after TNT, calculated on all patient in the FAS.

4.3.2 Local recurrence rate, organ preservation rate, colostomy-free survival

Local recurrence and organ preservation rates are defined as the 3-year rate of LR and organ preservation in NOM patients, respectively. Colostomy-free survival is defined as the time from the start of TNT to surgery with colostomy or cancer or treatment-related death. Death from other causes

and other events that preclude these analyses (i.e., distant relapse, adverse events) were considered for censoring.

4.3.3 Local relapse-free survival (LRFS)

For LRFS, the event date is the date of first LR or cancer-related death. Events of LR after surgery or NOM are recorded from time to decision to the event or last follow-up.

4.3.4 Overall survival (OS)

OS is defined as the time from the start of TNT to death for any cause.

4.3.5 Patient reported outcome measures (PROM)

PROMs are QoL scores from the European Organization for Research and Treatment of Cancer [EORTC] QLQ-C30 questionnaire and its rectal cancer specific module QLQ-29 (wrongly referred in the protocol as QLQ-38).

4.3.6 Disease-free survival (DFS)

Despite not being part of the original secondary endpoints of the NO-CUT study, we will also analyse DFS, where the event date is the date of first occurrence of distant or local relapse or death (whichever occurs first) and is calculated from start of TNT.

5 Analysis Methods

5.1 Methods for Primary Outcome

5.1.1 Descriptive Statistics

Baseline patient characteristics will be summarised for the whole population (180 patients). Continuous variables will be presented as means and standard deviations (SD), or medians and interquartile ranges (IQR), depending on their distribution. Maximum and minimum may be reported. Categorical variables will be presented as frequencies and percentages. Confidence intervals (CI) will be 95%.

5.1.2 Primary Inferential Analysis

5.1.2.1 Definitions of Events and Censoring

Event Date: For DRFS, the event date is the date of first documented distant relapse or cancer-related death (whichever occurs first). For other endpoints please refer to section 4.3.

Censoring: Patients who do not experience the event of interest by the analysis cutoff date (or who are lost to follow-up prior to an event) will be censored at the date of last adequate disease assessment or contact.

5.1.2.2 Statistical Methods for Survival Endpoints

Kaplan-Meier (KM) curves will be used to estimate the survival functions for DRFS, LRFS, PFS and OS. Median survival times (if reached) and time-specific survival rates (e.g., 30-month DRFS rate) will be reported with 95% confidence intervals (CI). Log-rank tests will be used to compare survival distributions between relevant subgroups, such as LB-positive vs. LB-negative after TNT.

5.1.2.3 Cox Proportional Hazards Models

Univariate Analyses: A Cox proportional hazards model will be fitted for each variable of interest (e.g., LB, CMS, etc). Unadjusted hazard ratios (HR) with 95% CI and corresponding p-values will be reported.

Multivariate Analyses: Variables with clinical relevance or those achieving a prespecified p-value threshold ($p < 0.10$) in univariate analyses are prioritized for inclusion in a multivariate Cox model. The final model will estimate adjusted HRs with 95% CI for each covariate. The assumption of proportional hazards will be tested (e.g., using Schoenfeld residuals), and if violated, alternative modeling approaches (e.g., time-dependent covariates or accelerated failure time models) will be considered.

5.1.3 Assumption Checks and Alternative Analyses

Where applicable, assumption checks will be performed for exploratory analyses such as Cox regression to check for proportional hazards.

5.1.4 Subgroup Analyses

NA

5.2 Methods for Dichotomous Secondary Outcomes

Patient characteristics will be investigated according to their association with cCR and CR, as defined in section 4.1.2 and 4.1.4. Data will be presented as proportions with 95% CI, with appropriate statistical tests (Chi square, Wilcoxon, McNemar's test) to assess the significant factors affecting cCR vs IR, and CR vs IR. We will define logistic regression models for both cCR and CR, to estimate and describe the impact of selected features on response to TNT, and subsequently propose a prediction nomogram. In both cCR models, the control group will consist of patients experiencing IR The model will be calibrated and internally validated. External validation is out of scope of present work but can be considered after publication.

5.3 Missing Data

For all time-to-event analyses (cox regression) and response analyses (logistic regression), if there is significant missingness in baseline covariates (e.g., LB), we will consider:

- Complete-case analysis: Only patients with non-missing covariates are included.
- Multiple imputation: If clinically appropriate and feasible.

5.4 Sensitivity Analyses

Additional analyses may be conducted to assess the robustness of results to:

1. Different definitions of baseline time (e.g., date of randomization vs. date of NOM assignment).
2. Adjusted/unadjusted model analysis (for instance, cox regression models)
3. Missing data handling (e.g., imputation scenarios).

5.5 Additional Analyses

Performance of the logistic regression models will be reported using metrics such as area under the receiver operating characteristic curve (AUC-ROC) and concordance index (c-index). Calibration of the logistic model for nomogram will be done using calibration plots.

5.6 Sample size

Sample size calculation has been based on the primary endpoint as reported in section 2.3.1.

6 Safety Analyses and Adverse Events

Adverse events will be reported, 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.

7 Statistical Software

All statistical analyses will be done in R version 4.4.1 (R Core Team 2024) and the relative R packages used with version number will be reported.

8 References

8.1 Literature References

Guidelines for the Content of Statistical Analysis Plans in Clinical Trials. 2017;318(23):2337-2343. doi:10.1001/jama.2017.18556.

8.2 Reference to Data Handling Plan

NA

8.3 Reference to the Trial Master File and Statistical Documentation

NA

8.4 Reference to other Standard Operating Procedures or Documents

NA