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Standard (8 weeks) vs long (12 weeks) timing to minimally-invasive surgery after NeoAdjuvant Chemoradiotherapy for rectal cancer: a multicenter randomized controlled parallel group trial (TiMiSNAR)

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Abstract

Background: The optimal timing of surgery in relation to chemoradiation is still controversial. Retrospective analysis has demonstrated in the recent decades that the regression of adenocarcinoma can be slow and not complete until after several months. More recently, increasing pathologic Complete Response rates have been demonstrated to be correlated with longer time interval. The purpose of the trial is to demonstrate if delayed timing of surgery after neoadjuvant chemoradiotherapy actually affects pathologic Complete Response and reflects on disease-free survival and overall survival rather than standard timing.

Methods: The trial is a multicenter, prospective, randomized controlled, unblinded, parallel-group trial comparing standard and delayed surgery after neoadjuvant chemoradiotherapy for the curative treatment of rectal cancer. Three-hundred and forty patients will be randomized on an equal basis to either robotic-assisted/standard laparoscopic rectal cancer surgery after 8 weeks or robotic-assisted/standard laparoscopic rectal cancer surgery after 12 weeks.

Discussion: To date, it is well-know that pathologic Complete Response is associated with excellent prognosis and an overall survival of 90%. In the Lyon trial the rate of pCR or near pathologic Complete Response increased from 10.3 to 26% and in retrospective studies the increase rate was about 23–30%. These results may be explained on the relationship between radiation therapy and tumor regression: DNA damage occurs during irradiation, but cellular lysis occurs within the next weeks. Study results, whether confirmed that performing surgery after 12 weeks from neoadjuvant treatment is advantageous from a technical and oncological point of view, may change the current pathway of the treatment in those patient suffering from rectal cancer.

Trial registration: ClinicalTrials.gov NCT3465982.

Keywords: Radiation therapy, Minimally invasive surgery, Rectal cancer, Neoadjuvant treatment, Robotic surgery, TaTME, Timing to surgery,

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Background

Chemoradiotherapy is a well-known risk reducing treatment of local recurrence in the treatment of rectal cancer, followed by total mesorectal excision (TME). In low rectal tumors, surgery alone has the 30% overall survival and a local recurrence rate of about 55–65%, with a disease-free survival of 30–35% [1]. Preoperative administration of fluorouracil-based chemotherapy improved local recurrence rates to 7% [2]. The optimal timing of surgery in relation to chemoradiation is still controversial. Retrospective analysis has demonstrated in the recent decades that the regression of adenocarcinoma can be slow and not complete until after several months [3]. More recently, increasing pCR (pathological complete response) rates have been demonstrated to be correlated with longer time interval [4–6]. Conversely, several reports have shown no impact of the interval after chemoradiation on pCR and technical performance [7, 8]. In the Lyon trial the rate of pCR or near pCR increased from 10.3 to 26% [9] and in retrospective studies the increase rate was about 23–30%. These results may be explained on the relationship between radiation therapy and tumor regression: DNA (DeoxyriboNucleic Acid) damage occurs during irradiation, but cellular lysis occurs within the next weeks [10]. A recent pilot study on comparison of resonance imaging and histopathological responses at two times, has suggested that volume reduction and down-staging occur between week 9 and week 14 after neoadjuvant treatment, with a 23% pCR rate at longer time [11]. In the Stockholm III trial, a significantly lower frequency of postoperative complications was reported, even though not described in the other studies where morbidity and complications were the same. All of these studies, however, presented some biases, such as absence of randomization, the choice of surgical timing made arguably by the surgeon, tumor size and response to RCT (radiochemotherapy), different cut-off period and a limited number of recruited patients, that may have negatively or positively influenced these results [12, 13]. Delaying surgery with the aim to detect excellent responders for organ preservation, eventually, may be legitimate, even though the start of adjuvant therapy, whose advantage in pretreated rectal cancer patients is still controversial, would be delayed, and this may negatively affect survival [14, 15]. A recent meta-analysis on thirteen reports has been published, showing rates of 14 and 20% in the shorter and longer group, respectively. This meta-analysis has some biases: the pCR correlation with surgical delay could not be adjusted in a multivariate analysis with other clinicopathological variables, the outcome (DFS and OS) of pCR, even if likely better than those without pCR as literature demonstrates, could not be directly assessed due to lack of individual patient data, the number of patients operated on in the delayed group could have been chosen using a surgical decision, different time intervals were grouped all together, no randomized trial were included in the meta-analysis, and the relevance of the reports included in was assessed by NOS scale (Newcastle–Ottawa scale), that is quite arbitrary, several reports on observation, demonstrating a higher percentage of pCR, were not included, but it is quite relevant to consider also these studies. TiMiSNAR has been developed to improve and define previous results from retrospective and review analyses.

Methods/design

The trial is a multicenter, prospective, randomized controlled, unblinded, parallel-group trial comparing standard and delayed surgery after neoadjuvant chemoradiotherapy for the curative treatment of rectal cancer. Three-hundred and forty patients will be randomized on an equal basis to either robotic-assisted/standard laparoscopic rectal cancer surgery after 8 weeks or robotic-assisted/standard laparoscopic rectal cancer surgery after 12 weeks (Fig. 1). Eight weeks are the current standard interval to surgery after neodjuvant treatment, while 12 weeks represent the “minimum” longer time interval to determine further tumor modifications and the “a priori” choice to avoid hypothetic surgical detrimental effect (postoperative complications related to radiation therapy). The recruiting interval will be of 5 years and the follow-up period will end 5 years after the last patient is randomized.

The trial has been held in Alessandria at SS. Antonio e Biagio e Cesare Arrigo Community Hospital, Italy and in others National Academic and not-Academic Centers, list of which is available at https://www.timisnar.it.

The Primary Endpoint is pCR; secondary endpoints are: DFS (disease-free survival), OS (overall survival), postoperative complications (Clavien-Dindo classification), reintervention, late complications (Clavien-Dindo classification), radiation toxicity, chemotherapy toxicity, QoL (quality of life), Functional status.

Inclusion Criteria are: age > 18 years, cT3/4 N0/+M0 confirmed on CT-scan (Computed Tomography Scan), MRI (Magnetic Resonance Imaging - stratification for T3a-b-c-d), tumor starting from the distal or medium rectum (even those crossing the peritoneal reflection at distal margin, within 15 cm from the anal margin), histologically-proven adenocarcinoma of the rectum, eligible for a resective surgery with TME (low anterior resection, intersphyncteric resection, abdominoperineal resection), eligible for resection by minimally-invasive surgery (standard or robotic-assisted laparoscopic procedure, all robotic systems will be accepted), eligible for chemoradiation treatment, able to give written informed

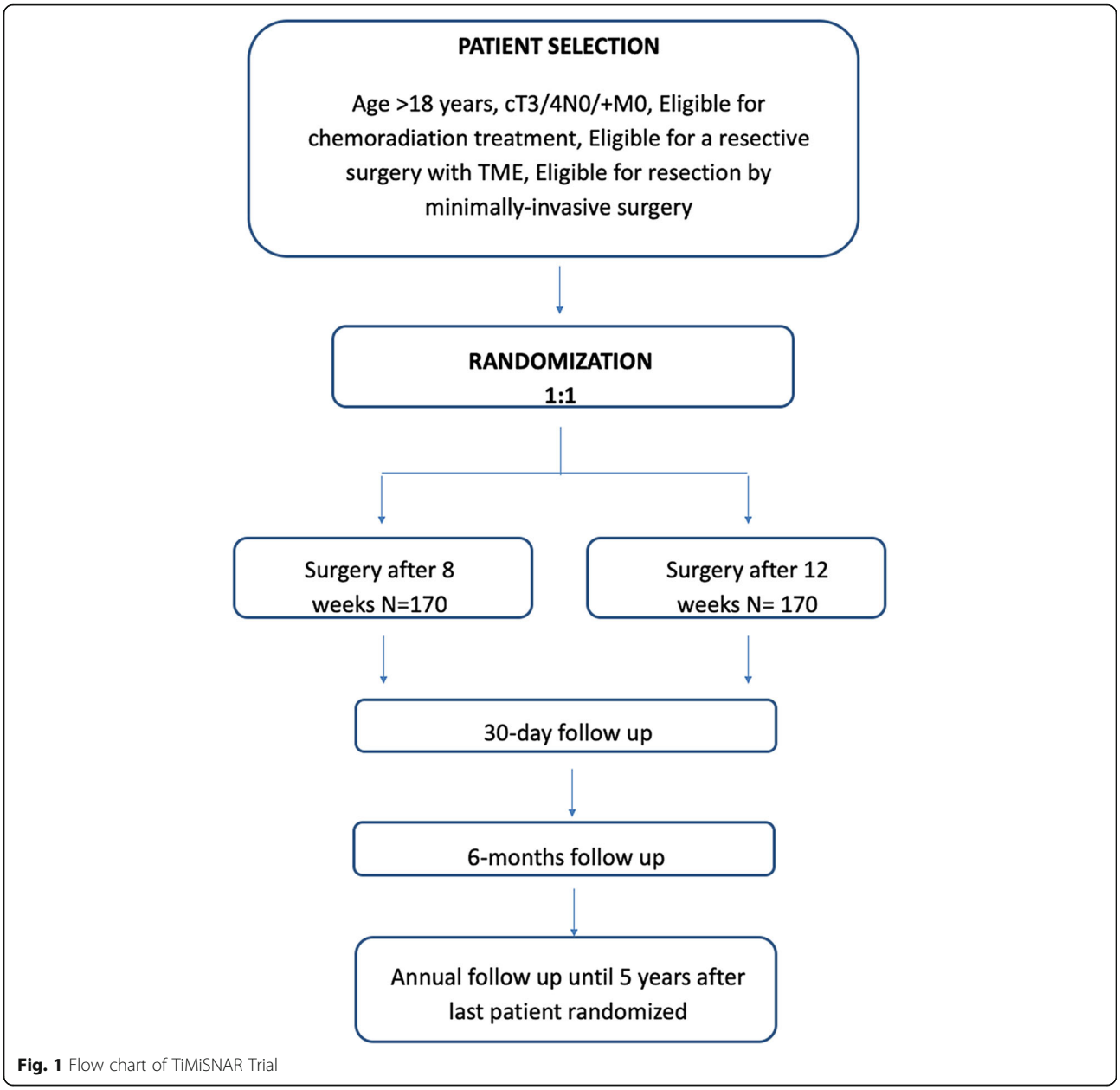

Fig. 1 Flow chart of TiMiSNAR Trial

consent, capable of completing required questionnaires at time of consent (provided questionnaires are available in a language spoke fluently by the participant).

Main exclusion Criteria are: metastatic disease, squamous carcinoma of the anal canal, unable to complete neoadjuvant treatment.

Patients will be randomized on a 1:1 basis to receive minimally-invasive rectal cancer surgery 8 or 12 weeks after neoadjuvant treatment and will be allocated a unique trial number.

Participants will be randomized using Sealed Envelope Ltd. 2017 Online Simple randomization service. Allocation concealment will be ensured, as the service will not release the randomization code until the patient has been recruited into the trial, which takes place after all baseline measurements have been completed.

An informed consent to participate has been prepared and will be obtained by all the participants.

All patients who give consent for participation and who fulfil the inclusion criteria will be randomized. Randomization will be requested by the staff member responsible for recruitment and clinical interviews from all participating centers. Due to the nature of the intervention neither participants nor staff can be blinded to allocation, but are strongly inculcated not to disclose the allocation status of the participant at the follow up assessments.

All the involved centers have to respect the following criteria: site able to perform robotic-assisted and standard laparoscopic rectal cancer surgery and TaTME (transanal total mesorectal excision); site able to provide standard neoadjuvant treatment, both chemo and radiation therapy; predicted capability to recruit a minimum of 15 patients per year to the trial.

Neoadjuvant treatment consists in long course radiation therapy with IMRT (Intensity Modulated Radio-Therapy – 50-54 Gy in 25–28 fractions; an optional boost is suggested) associated to concomitant chemotherapy treatment (Capecitabine 825 mg /m2/ twice daily during radiation therapy).

Several studies have compared IMRT of rectal cancer to 3D Conformal Radiotherapy. Although results from comparative randomized clinical trial are not available yet, IMRT is usually associated with less dose to organ at risk, such as urinary bladder, small bowel and anal sphincters (in selected cases). This is translated into better clinical outcomes, in terms of gastrointestinal toxicity, genitourinary toxicity and skin side effects [16–20].

Restaging and treatment-efficacy assessment after Neoadjuvant therapy

The MERCURY study group has developed an MRI-based tumor regression grading (ymrTRG) system by applying the principles of histopathological tumor regression grade (ypTRG) [21].

Recently, a pilot study from UK has defined two groups of patients divided into favourable vs unfavourable responders based on the following three factors:

- ymrT
- ymrTRG
- Change in volume

ymrT is based on the interpretation of local extent of persistent tumor signal intensity relative to the layers of bowel wall on T2-weighted images. Tumor response is evaluated as either replacement of tumor signal by low signal intensity fibrosis (dark stroma) or the development of high signal intensity mucin pools, that are not considered to be tumor.

ymrTRG is based on principles similar to the pathological ypTRG system described by Dworak and subsequently modified by Mandard.

Change in volume, better defined as percentage volume reduction is calculated multiplying tumor length, width and height, using the following formula:

$$100*\{(\text{Volume at baseline})-(\text{Volume post}-\text{CRT})\}/(\text{Volume at baseline})$$

Time interval to surgery in this trial are 8 weeks and 12 weeks after treatment, that are the standard and the expected “minimum” longer time interval to determine further tumor modifications. Post-treatment staging for evaluation of postneoadjuvant treatment response, eventually, will depend on MRI evaluation at week 7 for patients in both the two arms; a MRI evaluation will be repeated at week 11 for patients randomized in the delayed arm.

A Thoraco-abdominal CT-Scan with and without contrast enhancement will be performed at week 6 after neoadjuvant surgery, for restaging of potential disseminated disease.

All MRI exams are collected and sent to the Promoting Center for final revision by a well-trained Pelvic MRI expert radiologist. Every participating center must fill in a structured MRI form according to the fac-simile provided by the ESGAR (European Society of Gastrointestinal and Abdominal Radiology) [22].

Surgery

Minimally-invasive mesorectal resection is required: both robotic or standard laparoscopic approach or TaTME will be accepted, in accordance with each surgeon’s usual practice. The specifics of each operation will be at the discretion of the operating surgeon (e.g. port-site placement, mobilization of the splenic flexure, inferior mesenteric artery/vein division, high versus low vascular division etc.), as well as the decision to convert to an open operation. Conversion to open operation is defined as the use of a laparotomy wound for any part of the mesorectal dissection. All participating centers are allowed and suggested to use Indocyanine Green test (ICG), wherever available, but it is not mandatory. Several studies have shown that ICG test could reduce anastomotic leakage and thus postoperative complications, that are important in light of the secondary endpoints. A recent systematic review and meta-analysis by Blanco-Colino et al. has shown that ICG fluorescence imaging seems to reduce AL rates following colorectal surgery for cancer [23].

Post-operative care and follow up

Post-operative care and follow up will be as per institutional protocol, but patients must be reviewed at 30 days, and 6 months post-operatively at a minimum. Any further visits will be according to local standard clinical practice. All patients will be followed up as per protocol until 5 years after the last patient has been randomized.

Statistical evaluation

Sample size

The primary endpoint is the pCR rate. Based on the published results from prospective studies on delayed time interval or observation only and on retrospective study for standard time interval, we assume that the

mean rate of pCR in the standard treatment is about 15%, while the mean pCR rate in the observation treatment or longer time interval is 30%. To determine this difference, 270 patients are required, using a two-group continuity corrected χ^2 test of equal proportions, assuming an α error of 4.9% and a power of 80% (MedCalc Version 17.9.7); an interim analysis on efficacy will be performed when half of events will be observed. The conservative Haybittle-Peto [24] boundary will be used as a stopping guidance in order to perform the final analysis at the significance level of 4.9%, two sides. Considering results from the pilot study reported on section 1, the percentage of unfavourable patients is 20% (favourable MRI tumor regression grade is defined as grades 1, 2 and 3; unfavourable MRI regression as grades 4 and 5). In addition, a meta-analysis on results from five randomized European clinical trials for locally advanced rectal cancer, has confirmed this rate of "poor" responders subgroup, identified by having no pCR and no DFS within 2 years [25]. In computing the sample size, we assume that the percentage of missing data will be 5%. A total of 340 patients, 170 for each arm, is intended to be enrolled, eventually. Patients will be randomized on a 1:1 basis to receive minimally-invasive rectal cancer surgery 8 or 12 weeks after neoadjuvant treatment and will be allocated a unique trial number. A computer-generated software with block randomization criteria will be used to ensure treatment groups are well-balanced for timing of surgery. All enrolled patients' data will be registered in a prospective electronic database (ACCESS, MICROSOFT OFFICE Professional Plus 2010, regular licensed).

All data will be entered by means of case report forms. Original study forms will be entered and kept on file at the Coordinator site (SS. Antonio e Biagio e Cesare Arrigo Hospital). When a form is selected, the participating site staff will pull that form, copy it, and sent the copy to the DCC (Data Coordinating Center) for re-entry. Participant files are to be stored in numerical order and stored in a secure and accessible place and manner. Participant files will be maintained in storage for a period of 5 years after completion of the study.

The DCC will send monthly email reports with information on missing data, missing forms, and missing visits. Personnel at the Core Coordinating Center and the Participating Sites should review these reports for accuracy and report any discrepancies to the DCC.

Statistical analysis

All efficacy outcomes will be assessed in the intention-to-treat population, which includes all enrolled patients who did not violate the eligibility criteria. pCR, OS and DFS will be assessed from the time of treatment allocation to local progression, death or disease progression. Patients who will not die and will not experience local of distant disease progression at the date of study cutoff will be censored at the last available information on status.

Time-to-event data will be analyzed by the Kaplan-Meier method and compared with the log-rank test. Cox proportional hazards model will be used to adjust the treatment effect for baseline prognostic factors.

Serious adverse events reporting (SAE)

Any SAE considered to be reasonably related to the investigational treatment or study participation, have to be promptly notified.

This must be done by email within 24 h of the initial observation of the event. The principal investigator will decide if these events are related to the trial treatment (i.e. unrelated, likely related, and not assessable) and the decision will be recorded on the Serious Adverse Event form, if necessary with the reasoning of the principal investigator.

The investigator is obligated to assess the relationship between investigational treatment and the occurrence of each AE/SAE. A "reasonable possibility" is meant to convey that there are facts/evidence or arguments to suggest a causal relationship, rather than a relationship cannot be ruled out. The investigator will use clinical judgement to determine the relationship. Alternative causes, such as natural history of the underlying diseases, concomitant therapy, other risk factors, and the temporal relationship of the event to the investigational product will be considered and investigated.

End of the study

The end of the study is defined as 5 years after the date that the last patient has been randomized to the trial.

Research ethics approval

The protocol, site-specific informed consent forms, participant education and recruitment materials, and other requested documents — and any subsequent modifications — also has been reviewed and approved by SS. Antonio e Biagio e Cesare Arrigo Hospital Ethical Committee on 31 May 2018.

Discussion

To date, it is well-know that pCR is associated with excellent prognosis and an overall survival of 90% [1]. In the Lyon trial the rate of pCR or near pCR increased from 10.3 to 26% [2] and in retrospective studies the increase rate was about 23–30%. These results may be explained on the relationship between radiation therapy and tumor regression: DNA damage occurs during irradiation, but cellular lysis occurs within the next weeks [3]. In the Stockholm III trial, a significantly lower

frequency of postoperative complications was reported, even though not described in the other studies where morbidity and complications were the same.

There are several audiences for this trial: Oncologists, Surgeons, Radiation oncologists, Patients and the public, Academia, General Practitioners.

Another crucial point of the trial is the use of a structured MRI report, as recommended by the European Society of Gastrointestinal and Abdominal Radiology (ESGAR) [22], for primary staging and for restaging after neoadjuvant treatment. One of the goals of the trial is to determine whether MRI can specifically depict cancer local diffusion and predict downstaging and be used as a good prognostic instrument. High quality MRI, indeed, allows further subclassification of cT3, which is recommended by European Society for Medical Oncology (ESMO) guidelines and it is useful in stratifying and selecting patients with indication to neoadjuvant treatment before surgery.

In summary, the optimal interval between adjuvant chemoradiation and surgery may give the opportunity to optimize patients, initiate an individualized and "targeted" treatment, and favor organ preservation.

TiMiSNAR (NCT3465982 – https://www.timisnar.it) results, whether confirmed that performing surgery after 12 weeks from neoadjuvant treatment is advantageous from a technical and oncological point of view, may change the current pathway of the treatment in those patient suffering from rectal cancer.

Abbreviations

CT: Computed Tomography; DFS: Disease-free survival; DNA: DeoxyriboNucleic Acid; ESGAR: European Society of Gastrointestinal and Abdominal Radiology; ICG: Indocyanine Green test; IMRT: Intensity Modulated Radiotherapy; MRI: Magnetic Resonance Imaging; NOS: The Newcastle-Ottawa Scale; OS: Overall Survival; pCR: pathologic Complete Response; QoL: Quality of Life; RCT: radiochemotherapy; TaTME: transanal total mesorectal excision; TME: Total Mesorectal Excision; ymrTRG: MRI-based tumor regression grading; ypTRG: pathologic tumor regression grading; ESMO: European Society for Medical Oncology

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Not Applicable
Administrative Information
Organisational structure and responsibilities
Principal Investigator
Design and conduct of TiMiSNAR
Preparation of protocol and revisions
Preparation of investigators brochure (IB) and CRFs [Case Report Forms]
Organising steering committee meetings
Managing CTO [Clinical Trials Office]
Publication of study reports
Members of TMC [Trial Management Committee]
Steering committee (SC)
Agreement of final protocol
All lead investigators will be steering committee members
Recruitment of patients and liasing with principal investigator
Reviewing progress of study and if necessary agreeing changes to the protocol and/or investigators brochure to facilitate the smooth running of the study
Trial Management Committee (TMC)
(Principle investigator, Administrator (D.ssa Marinella Bertolotti))
Study planning
Organisation of steering committee meetings
Provide annual SUSAR [Serious unexpected suspected adverse events] reporting
Responsible for trial master file
Advice for lead investigators
Data verification
Randomization
Data Manager
Maintenance of trial IT system and data entry
Data verification
Lead Investigators
In each participating center a lead investigator (senior surgeon) will be identified, to be responsible for identification, recruitment, data collection and completion of CRFs, along with follow up of study patients and adherence to study protocol and investigators brochure. Lead investigators will be steering committee members

Primary sponsor

Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo di Alessandria, Italy.

Authors' contributions

IM, FP1, EB, SG1, GR, EC, LB1, LB2, GP, FP2, CND, RP, EM1, TC, EM2, UE, RD, RR, FP3, AC, BM, SG2, PB1, PB2, RB, CC, VT, ET, VF, MR, FP4, GN, PF have made substantial contributions to the conception or design of the work. VT is the statistician involved in the analysis and interpretation of data. All authors read and approved the final manuscript.

Funding

No funds have been utilized for this trial.

Availability of data and materials

NOT APPLICABLE (the current manuscript doesn't contain any data related to patients; it's only a draft).

Ethics approval and consent to participate

The present study has obtained Ethical Approval by SS. Antonio e Biagio e Cesare Arrigo Hospital Ethical Committee on 31 May 2018 and informed consent to participate in the study has been elaborated and will be obtained from participants.

Consent for publication

NOT APPLICABLE.

Competing interests

The authors declare that they have no competing interests.

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Trials

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Expression levels of circulating miRNAs as biomarkers during multimodal treatment of rectal cancer - TiMiSNAR-mirna: a substudy of the TiMiSNAR Trial (NCT03962088)

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Abstract

Background: Neoadjuvant chemoradiotherapy followed by surgery is the mainstay treatment for locally advanced rectal cancer, leading to significant decrease in tumor size (downsizing) and a shift towards earlier disease stage (downstaging). Extensive histopathological work-up of the tumor specimen after surgery including tumor regression grading and lymph node status helped to visualize individual tumor sensitivity to chemoradiotherapy, retrospectively. As the response to neoadjuvant chemoradiotherapy is heterogeneous, however, valid biomarkers are needed to monitor tumor response. A relevant number of studies aimed to identify molecular markers retrieved from tumor tissue while the relevance of blood-based biomarkers is less stringent assessed. MicroRNAs are currently under investigation to serve as blood-based biomarkers. To date, no screening approach to identify relevant miRNAs as biomarkers in blood of patients with rectal cancer was undertaken. The aim of the study is to investigate the role of circulating miRNAs as biomarkers in those patients included in the TiMiSNAR Trial (NCT 03465982). This is a biomolecular substudy of TiMiSNAR Trial (NCT03962088).

Methods: All included patients in the TiMiSNAR Trial are supposed to undergo blood collection at the time of diagnosis, after neoadjuvant treatment, after 1 month from surgery, and after adjuvant chemotherapy whenever indicated.

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Discussion: TiMiSNAR-MIRNA will evaluate the association of variation between preneoadjuvant and postneoadjuvant expression levels of miRNA with pathological complete response. Moreover, the study will evaluate the role of liquid biopsies in the monitoring of treatment, correlate changes in expression levels of miRNA following complete surgical resection with disease-free survival, and evaluate the relation between changes in miRNA during surveillance and tumor relapse.

Trial registration: Clinicaltrials.gov NCT03962088. Registered on 23 May 2019.

Keywords: Colorectal cancer, miRNA, Neoadjuvant treatment, Biomarkers, Translational research

Background

Neoadjuvant chemoradiotherapy (nCHT) followed by surgery is the main treatment for locally advanced rectal cancer, leading to significant decrease in tumor size (downsizing) and a shift towards earlier disease stage (primary tumor and lymph nodes involvement—downstaging) [1]. As the response to nCHT is heterogeneous, however, valid biomarkers are needed to monitor tumor response [2–4]. Therefore, it is of high importance to stratify and identify those patients, who can benefit from an individualized targeted therapy. To date, a significant number of studies aims to identify molecular markers retrieved from tumor tissue while the relevance of blood-based biomarkers is less stringent assessed.

Blood samples, i.e., liquid biopsy, indeed, offer several advantages [5, 6]:

1. Taking blood samples is less invasive, less expensive, easy to schedule, and nearly without any severe complications.
2. Blood samples are a source of fresh DNA and RNA, without modifications due to preservatives; especially in the case of rectal cancer, beyond intratumoral heterogeneity, tumor biopsies are in general accompanied by normal, adenomatous, or stromal tissue. This contamination may affect results of molecular analyses
3. Investigating blood from patients can account for molecular heterogeneity and surrogate for tumor burden since tumor-derived fragments or biomarkers are collected from all tumor cells in a patients' body through circulation.
4. Liquid biopsy may offer both the possibility of dynamic monitoring under treatment and the possibility to assess disease activity even after pathologic complete response (pCR) or after resection of the tumor when no tissue is left for molecular analyses.

Carcinoembryonic antigen (CEA) is, to date, established as a colorectal cancer (CRC)-related tumor marker, but its unsuitability as a screening and prognostic marker has been demonstrated [7]. Circulating tumor DNA (ctDNA) represents, nowadays, the main approach to monitor tumor burden and therapy resistance, to evaluate the presence of residual disease after potentially curative treatment, and to monitor disease recurrence with high sensitivity and specificity [8].

MicroRNAs (miRNAs) are currently under investigation to serve as blood-based biomarkers as a potential alternative to CEA and ctDNA. miRNAs are small, noncoding RNAs that regulate gene expression by post-transcriptional mRNA binding, which promotes the destabilization of target miRNAs. They are highly conserved between species, stable, and easily detectable even in small concentrations and have been widely analyzed in physiological and pathological processes, and their expression is tissue specific [9–11]. miRNA genes often have multiple transcription start sites, and the promoters of intronic miRNAs are sometimes distinct from the promoters of their host genes [11, 12]. miRNA biogenesis process follows two steps: a nucleic and a cytoplasmatic phase. In nucleus, miRNAs are transcribed in primary-microRNAs by RNA polymerase II and this process is controlled by RNA Pol II-associated transcription factors and epigenetic regulators [12, 13]. Further, they are processed by Drosha RNase III endonuclease in shorter stem loops of about 60–70 nucleotides in length, called pre-miRNAs [13]. Pre-miRNAs are then transported from the nucleus to the cytoplasm via Exportin 5 and processed in mature miRNAs by RNase III endonuclease Dicer [14–17]. Further, maturation of miRNAs is carried out by the RISC-loading complex (RNA-induced silencing complex) [18]. miRNAs constitute the largest class of gene regulation and are involved in all developmental processes, including stem cell and germline maintenance, development and differentiation, transcriptional and post-transcriptional gene silencing, and subcellular localization [19, 20]. miRNAs regulate gene expression through the degradation of mRNA transcripts of their target genes and the translation regulation of mRNA transcript without RNA degradation [21].

Expression patterns of miRNA can be developmental stage specific or, in other circumstances, tissue and site

specific. The target specificity of miRNAs is largely predetermined by their so-called seed-sequence (containing nucleotides at positions 2–7 of the miRNA).

It is well-known that miRNA is present in blood, but its lability and the presence of ribonuclease in the plasma raised some questions about how miRNA is carried in the blood flow and its detectability [22, 23], suggesting a mechanism of protection against ribonuclease degradation.

One of the protecting mechanisms that have been suggested is that extracellular RNA is bound with DNA [24], but it has been excluded afterwards [25]. Another mechanism of RNA release that has been at first postulated was cell death like apoptosis or mechanical stress in which apoptotic bodies [26] containing miRNAs are released in the blood flow, thus protecting them from Rnase degradation [27]. It is well-known also that most cell types release continuously soluble factors and exfoliate membrane-derived vesicles into the extracellular space [28]. These kind of vesicles are called exosomes and are distinctly different from apoptotic bodies. Exosomes are nanovesicles that are involved in cell-to-cell communication and regulation of different biological processes [29]. In recent years, exosomes have emerged being involved in both physiological processes, such as immune response and neuronal function, and also in the development and progression of disease, such as cancer [30–32].

Exosomes can facilitate intercellular communication through transportation of grown factors and miRNAs and other small molecules, constituting the probable mechanism of miRNA transportation and protection against degradation [33, 34]. RNA has been found, indeed, on cancer cell surface, and it has been also found in vesicles shed in vitro from a human colon adenocarcinoma cell line [35]. Cancer cells have been demonstrated to secrete high quantity of exosomes than normal cells [33, 34], and exosomal miRNAs are supposed to play an important role in cancer cell proliferation, angiogenesis, metastasis, drug resistance, and tumor inhibition [36–41]; some studies have shown the role of exosomal miRNAs in cellular pathways from life to death, from metabolism to communication [35]. Phenotypes of tumors, indeed, have been demonstrated depending not only on cancer cells but also on surrounding tumor microenvironment [42]. Cancer-cell derived exosomes-miRNAs contribute to the recruitment and reprogramming of constituents associated with tumor environment, modifying the extracellular matrix, reprogram functions of immunologically active factor and immune target cells [43]. To date, four mechanisms are known through which miRNAs influence tumor microenvironment: (1) self-modulation through which less aggressive cancer cells receive exosomal miRNAs delivered by more aggressive cancer cells [44, 45]; (2) distant communication with other cells in the tumor microenvironment for preparing a distant site of tumor proliferation (metastasis-inducing mechanism by down-regulation of tight junctions and endothelial monolayers destruction) [46, 47]; (3) miRNAs from normal cells that can alter the behavior of tumor cells [48]; and (4) viral infection that stimulates secretion of exosomes with aberrant miRNAs inducing normal cells in a pre-tumoral condition [49].

Based on these findings, miRNA detection in plasma can play a crucial prognostic role from initial to developmental phase of tumorigenesis and tumor progression, with a fascinating possibility for personalized tumor therapy [50, 51].

To date, no screening approach to identify relevant miRNAs as biomarkers in blood of patients with rectal cancer has been undertaken.

Methods/design

The Timing To Minimally Invasive Surgery After Neoadjuvant Chemoradiotherapy For Rectal Cancer: A Multicenter Randomized Controlled Trial - Biomarkers Substudy is an observational prospectically design study on the evaluation of the circulating miRNA in serum.

All included patients in the TiMiSNAR Trial (already approved by local Ethical Committees on 8/5/2018) are supposed to undergo blood collection at the time of diagnosis, 1 month after neoadjuvant treatment, 1 month after surgery, and at 1, 3, 6, and 12 months during adjuvant chemotherapy (based on therapy protocol), whenever indicated or at 1, 3, 6 and 12 months during surveillance (Fig. 1).

An informed consent to participate has been prepared and will be obtained by all the participants and collected by the Principal investigator (Dr. Igor Monsellato). Vacutainer tube will be addressed by a unique code offered by a computer software. The study will take place in community clinics and academic hospitals.

miR-17, miR-18b, miR-20a, miR-31, and miR-193a_3p, miR-125b, miR-345, miR-154, miR-409-3p, miR-127-3p, miR-214, miR-299-5p and miR-125b, miR-33a, miR-30e, miR-338-3p, miR-200a and miR-378 expression levels will be evaluated during multimodal therapy [1, 2, 52, 53].

Plasma sample collection

Fifteen milliliters of whole blood samples are collected in Vacutainer tubes with spray-coated K2EDTA and stored at room temperature. Blood undergoes centrifugation for plasma separation within 2 h, to minimize the hemolysis and nucleic acid degradation.

Tubes are subjected within 1 h to a first centrifugation step at 2200×*g* for 15 min at room temperature. Plasma supernatants are transferred to 15-mL tubes, carefully avoiding contact with the lymphocytic ring, and tubes

	STUDY PERIOD					
	Enrolment	Post-Enrolment				Close-out
TIMEPOINT		*Baseline*	*Post-neoad*[1]	*Post-Surgery*	*Post-CHT*[2]	*End of Follow up*
ENROLMENT:						
Eligibility screen	X					
Informed consent	X					
INTERVENTIONS:						
Liquid Biopsy		X	X	X	X	
ASSESSMENTS:						
Variation between preneoadjuvant and postneoadjuvant expression levels of miRNA and pCR		X	X			
Evaluation of the role of liquid biopsies in the monitoring of treatment			X	X	X	X
Correlation of changes in expression levels of miRNA following complete surgical resection with disease-free survival				X	X	X

[1]neoad: neoadjuvant chemoradiotherapy
[2]CHT: chemotherapy; Whenever adjuvant chemotherapy is requested

Fig. 1 SPIRIT figure

are centrifuged a second time at 3000×*g* and RT for 10 min to remove cellular debris.

Plasma samples are then collected into 1.5-mL cryovials, and all the aliquots are stored at − 80 °C.

Plasma RNA extraction

Total RNA, including miRNAs, is isolated using a commercial kit (miRNeasy Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration is assessed using a spectrophotometer. Adequate RNA concentration for mRNA expression is ≥ 30 ng/μL, and its quality is acceptable if the ratio between the value of the absorbance (*A*) at 260 nm and the absorbance at 280 nm is ≥ 1.8 and the ratio between the value of absorbance (*A*) at 260 nm and the one at 230 nm is ≥ 2.

miRNAs expression assay

The nCounter miRNA Expression Assay (NanoString Technologies, Seattle, WA, USA) is designed to provide an ultra-sensitive, reproducible, and highly multiplexed method for detecting miRNAs in total RNA across all biological levels of expression. The assay provides a method for detecting miRNAs without the use of reverse transcription or amplification by using molecular barcodes called nCounter Reporter Probes. The assay can be run on total RNA isolated from liquid biopsy.

Sample preparation involves a multiplexed annealing of the specific tags to their target miRNA, a ligation reaction, and an enzymatic purification to remove the unligated tags. Sequence specificity between each miRNA and its appropriate tag is ensured by careful, stepwise control of annealing and ligation temperatures. Control RNA included in the nCounter miRNA Sample Preparation Kit allows the user to monitor the ligation efficiency and specificity through each step of the reaction.

NanoString technology is based on the direct molecular barcoding and digital detection of target miRNAs using a color-coded probe pair. The probe pair consists of a Reporter Probe, which carries the signal on its 5′ end, and a Capture Probe, which carries a biotin on its 3′ end. The complexity of the color codes, comprised of four colors in six positions, allows a large diversity of targets present in the same sample to be individually resolved and identified during data collection.

After hybridization, excess probes are washed away using a two-step magnetic bead-based purification.

Magnetic beads derivatized with short nucleic acid sequences that are complementary to the Capture Probe and the Reporter Probes are used sequentially. First, the hybridization mixture containing target/probe complexes is allowed to bind to magnetic beads complementary to sequences on the Capture Probe. Wash steps are

performed to remove excess Reporter Probes and non-target cellular transcripts. After washing, the Capture Probes and target/probe complexes are eluted off the beads and are hybridized to magnetic beads complementary to sequences on the Reporter Probe. An additional wash is performed to remove excess Capture Probes. Finally, the purified target/probe complexes are eluted off the beads and immobilized on the cartridge for data collection. Data are analyzed using the nSolver™ software or other analysis programs.

Primary endpoint

To evaluate the association of variation between preneoadjuvant and postneoadjuvant expression levels of miRNA with response to treatment.

Secondary endpoints

a) To evaluate the role of liquid biopsies in the monitoring of treatment
b) To correlate changes in expression levels of miRNA following complete surgical resection with disease-free survival
c) To evaluate the relation between changes in miRNA during surveillance and tumor relapse

Data analysis

Patient subpopulation for the analysis is formed by the eligible patients with surgical evaluation and availability of plasma sample for the requested RNA analyses.

Baseline characteristics will be described for overall sample population by means of standard summary statistics (absolute frequencies, mean median and extreme values for continuous data, percentage for categorical data).

The association of variation between preneoadjuvant and postneoadjuvant expression levels of miRNA with treatment response will be presented with contingency tables and analyzed by mean of a logistic model. The odds ratio for the association and the AUC will be calculated, together with the corresponding 95% confidence intervals.

For secondary objectives (a) and (c), the role of liquid biopsies in the monitoring of treatment will be investigated by using a semi-parametric survival model (Cox model) with time-dependent variables, in order to incorporate modifications in the plasma measurements over time and their association with outcome, while for objective (b) the same analysis will be applied only on the subgroup of patients achieving pCR.

Discussion

Mechanisms behind the recurrence/metastatic process in CRC are still not fully understood [52]. An important challenge in medical oncology is to identify patient or tumor characteristics to be correlated to the response to neoadjuvant and adjuvant treatment. Response variety of that implies an individualized treatment approach [52–54]. A new targeted approach to disease has been advocated for prevention and treatment based on individual characteristics regarding the environment, genes, lifestyle, and individual risk factors [4, 5, 53, 54].

MicroRNAs (miRNAs) are small, noncoding sequences that are post-transcriptional regulators of gene expression; depending on the genes they regulate, miRNAs can function as either oncogenes or tumor suppressors. In 2011, Della Vittoria Scarpati et al., showed their first results on miRNA evaluation in tissues as biomarkers for tumor response after neoadjuvant treatment on 38 patients. They found that two miRNAs (miR-630 and miR-622) were upregulated in all patients of group A (pathologic complete response) and downregulated in all patients of group B (all responses except complete) (sensitivity and specificity: 100%) [55].

In 2017, Jo et al. published their results of the analysis on circulant miRNA on 17 rectal cancer affected patients. All miRNAs that were retrieved from the group of upregulated miRNAs in the tumor showed a trend towards a reduced expression in the plasma of rectal cancer patients compared to the control samples. Expression levels of miRNAs in the plasma that were selected based on a decreased expression in the tumor compared to the mucosa were irregularly up- or downregulated miRNAs. They concluded that miR-30c and 31 may have a potential relevance as biomarker in rectal cancer to distinguish between cancer and non-cancer patients in the plasma [2]. As we stated before, based on findings by Jo et al., D'Angelo et al., and Yu et al., miR-17, miR-18b, miR-20a, miR-31, and miR-193a_3p, miR-125b, miR-345, miR-154, miR-409-3p, miR-127-3p, miR-214, miR-299-5p and miR-125b, miR-33a, miR-30e, miR-338-3p, miR-200a and miR-378 expression levels will be evaluated during multimodality therapy [1, 2, 51].

Comparing miRNA levels in all steps of the treatment with tumor response and finally with disease relapse and postoperative and oncologic outcome, we argue that miRNA could help to select patients who can or cannot benefit from surgery or from neoadjuvant alone in a setting of organ preservation, or from adjuvant treatment.

Trial status

Protocol Version: V3.18 11/28/2018. Recruitment starting date: 3/25/2019. Recruitment ending date: 3/25/2022.

Abbreviations
nCHT: Neoadjuvant chemoradiotherapy; TRG: Tumor regression grading; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; miRNA: Microribonucleic acid; pCR: Pathologic complete response; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; ctDNA: Circulant deoxyribonucleic acid; RNase: Ribonuclease; RISC: RNA-induced silencing complex

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Not applicable.

Dissemination and publication policy
To ensure that the outcomes from the research inform practice and thereby maximize the benefit to patients and the National Health System (NHS), we will promote the dissemination of the research to academic and non-academic audiences, who will advise and support dissemination to the public. Additionally, information will be collected and networks established throughout this study to further inform and strengthen the strategy. The principal way of dissemination will be the publication of a research report in a remarkable medical journal. To maintain the scientific integrity of the trial, data will not be released prior to the first publication of the analysis of the primary endpoint, either for trial publication or oral presentation purposes, without the permission. A second dissemination channel would be press release. The media is a crucial audience for research findings because it is both a target for and disseminator of research evidence.
Other dissemination activities will include the use of electronic media such as websites and social media, interactive workshops across the country on implementation of good practice guidelines, oral presentation, or poster in National and International congresses. We will begin to disseminate findings within 12 months of starting the project.

Authors' contributions
IM conceptualization, methodology, design, writing and editing. VT statistical analysis. SO methodology and design. All the other authors have made substantial contribution on validation and preparation of the work.

Funding
No funds have been utilized for this trial.

Availability of data and materials
Not applicable (the current manuscript does not contain any data related to patients; it is only a draft).

Ethics approval and consent to participate
The present study has obtained Central Ethical Approval by SS. Antonio e Biagio e Cesare Arrigo Hospital Ethical Committee on 21 March 2019 and from all CE of participating centers. Informed consent to participate in the study has been elaborated and will be obtained from participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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