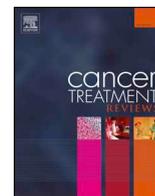




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Systematic or Meta-analysis Studies

Liquid biopsy for rectal cancer: A systematic review

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ABSTRACT

Background: The management of locally advanced rectal cancer (RC) is an evolving clinical field where the multidisciplinary approach can reach its best, and liquid biopsy for obtaining tumor-derived component such as circulating tumor DNA (ctDNA) might provide complementary informations.

Methods: A systematic review of studies available in literature of liquid biopsy in non-metastatic RC has been performed according to PRISMA criteria to assess the role of ctDNA as a diagnostic, predictive and prognostic biomarker in this setting.

Results: Twenty-five publications have been retrieved, of which 8 full-text articles, 7 abstracts and 10 clinical trials. Results have been categorized into three groups: diagnostic, predictive and prognostic. Few but promising data are available about the use of liquid biopsy for early diagnosis of RC, with the main limitation of sensitivity due to low concentrations of ctDNA in this setting. In terms of prediction of response to chemoradiation, still inconclusive data are available about the utility of a pre-treatment liquid biopsy, whereas some studies report a positive correlation with a dynamic (pre/post-treatment) monitoring. The presence of minimal residual disease by ctDNA was consistently associated with worse prognosis across studies.

Conclusions: The use of liquid biopsy for monitoring response to chemoradiation and assess the risk of disease recurrence are the most advanced potential applications for liquid biopsy in RC, with implications also in the context of non-operative management strategies.

Background

Colorectal cancer (CRC) is one of the most diagnosed cancers worldwide, with 1.84 million estimated new cases in 2018 [1]. About 25–30% of all colorectal cancer diagnoses accounts for rectal cancer (RC) [2]. In the European Union, the incidence of RC is ~125,000 per year, i.e. ~35% of the total colorectal cancer incidence, reflecting 15–25 cases/100 000 population per year and is predicted to further increase in both genders. The mortality is 4–10/100 000 population per year, with a median age at diagnosis of ~70 years [3].

The rectum and colon have a different embryological origin, anatomy and function [4]. RC has thus distinctive clinical features as compared to colon cancer, with an increased risk of local spread and recurrence. As a consequence, the treatments for primary rectal and colon cancer are different [5]. The incidence of RC has been decreasing as the increasingly

spread use of screening allows for identification and endoscopic removal of premalignant lesions [6]; however, several recent studies have shown an increase in incidence of rectal cancers among young people [7].

The treatment landscape in RC paralleled that of colon cancer and has evolved over the last decade following the approval of several targeted therapies for the advanced disease, leading to improvements in tumor response rates and patient survival [8,9]. However, progresses in medical treatment in the metastatic setting have been mainly incremental despite considerable advances in the knowledge of tumor biology [10]. In this regard, primary tumor location (right-sided or left-sided of the colorectum) has been identified as a surrogate marker for underlying molecular classification, with differences in a continuum spectrum between colon and rectal carcinomas [11].

In the non-metastatic setting, operative approaches such as transanal endoscopic microsurgery, open and laparoscopic proctectomy

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[12] are effective in earlier stages, while a trimodality treatment (pre-operative chemoradiation therapy (CRT), surgery with total mesorectal excision (TME) followed by adjuvant chemotherapy when recommended) is the standard of care for locally advanced RC patients. A significant risk of distal recurrence is present in rectal tumors radically operated in particular within the first 5 years for stages II and III of Dukes at around 30% and 50%, respectively, probably caused by the presence of micrometastatic spread [6]. A pooled analysis of five European randomized controlled trials demonstrated that the 5-year distant metastasis rate was 30.8% in 2,759 recruited patients [3]. An increasing number of reports suggested that a non-operative management (NOM), consisting of close surveillance of patients with clinical complete response (cCR) after chemoradiotherapy, 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 [13,14], several 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 [15,16]. More recently, the International Watch & Wait Database (IWWDD) described the outcome of the W&W strategy in a large-scale registry of more than 1000 patients, reporting excellent survival and small risk of local unsalvageable disease recurrence [17]. Despite the body of retrospective literature is greatly increasing, key knowledge gaps limiting widespread use of W&W/NOM remain, and clinical studies aimed at identifying patients who are good candidates for this approach are ongoing [18].

Follow-up supported by clinical examinations, imaging and endoscopies aims to improve prognosis by early detection of local or distant recurrence. Isolated carcinoembryonic antigen (CEA) monitoring is insufficiently sensitive. The analysis of serum protein levels, such as CEA, allows a fast and cost-effective method to quantify cancer progress, but it's distorted by limited sensitivity and specificity, in particular during treatment courses due to inflammation and discharge of protein in the bloodstream. Moreover, a portion of patients with metastatic RC does not show visible plasmatic CEA levels during the disease [19,20].

The management of RC is an evolving clinical field where the multidisciplinary approach can reach its best and the ability to discern patients at low risk from those at high risk of recurrence is the prerequisite for the most appropriate treatment choice. With this regard, liquid biopsy for obtaining tumor-derived component such as circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs) might provide crucial complementary information at the diagnosis of RC and in different moments during its treatment. Liquid biopsies may indeed represent a precious basin of new generation biomarkers [21,22] and are being evaluated also in RC for diagnosis, treatment choice, monitoring disease response, tracking acquired mutations linked to targeted therapy resistance, and detecting minimal residual disease.

The aim of this systematic review is to assess the role of ctDNA as a diagnostic, predictive and prognostic biomarker in non-metastatic RC.

Methods

Definition of the outcome

The purpose is to evaluate the current clinical potential of liquid biopsy, in particular cell-free DNA (cfDNA)/circulating tumor DNA (ctDNA), in non-metastatic RC patients.

Data source and search strategy

A systematic literature review was performed according to PRISMA Statement Criteria [23,24] in June 2019. The PubMed database was systematically reviewed as of June 11th, 2019 and all retrieved studies were manually screened for relevant references missed in the primary search. Unpublished data presented as abstract in relevant international

congresses [American Society of Clinical Oncology (ASCO), European Society of Medical Oncology (ESMO)] were also systematically searched for. Furthermore, ongoing clinical trial exploring the value of liquid biopsy in non-metastatic rectal cancer were searched on clinical-trial.gov. The decision to include a study for review was made by consensus between two authors (EGP and DM). The research criteria were limited to human studies published only in English language. The Medical Subject Heading terms used for the search were ("rectal" or "rectum" or "LARC") and ("liquid biopsy" or "ctDNA" or "cfDNA" or "circulating tumor DNA" or "circulating free DNA" or "methylated DNA" or "DNA methylation").

Main study inclusion criteria:

- Involved the measurement of cfDNA/ctDNA in plasma/serum in patients with RC;
- Diagnosis, treatment response and/or survival data collected and correlated with cf/ctDNA.

Study exclusion criteria:

- Involved patients with metastatic RC;
- Involved patients with CRC without mention about the tumor location.

Results

A total of 838 records were screened to be included in the systematic review (Fig. 1). According to selection criteria, we identified 8 records found through database searching (PUBMED) and 17 through other sources (ESMO, ASCO, Clinicaltrial.gov).

As a result, 25 records were eligible and included in the systematic review: 8 full-text articles studies, 7 abstracts presented at international congresses, and 10 ongoing ctDNA clinical trials.

We subdivided our results into 3 categories according to the investigated role of ctDNA/cfDNA or methylated DNA: diagnostic, predictive of treatment response and prognostic (in terms of disease recurrence or survival) (Fig. 2). Finally, we provide a summary of published or presented works (Table 1) and of ongoing trials (Table 2).

cfDNA and ctDNA as diagnostic tool

In 2011 an Italian group evaluated the ability of cfDNA to discriminate healthy patients (plasma samples collected after a negative colonoscopy) from patients with RC. Through quantitative PCR (using Alu 115, Alu 147 and β -globin gene), they found that the baseline level of cfDNA was significantly higher in RC patients than in healthy individuals [25].

A Chinese group also observed higher concentration of cfDNA in RC than in healthy individuals, where mutated *KRAS* and methylated *MGMT* were not detected. Moreover, the ratio of 400-/100-bp DNA fragments (an index of cfDNA integrity) was higher in RC patients than in healthy controls, in which cfDNA is considered to originate mainly from apoptotic process of normal cells [26].

Shalaby et al. highlighted the capacity of *MGMT* and *ERCC1* methylation status to distinguish benign and malignant rectal tumors. The study was performed in blood and tissue of 43 benign and 50 malignant rectal tumors patients. They observed a significant higher frequency of *MGMT* and *ERCC1* methylation in RC patients than in cases with benign tumors, both in tissue and blood samples (sensitivity around 60% and specificity of 93–95% for each gene on plasma). The combination of *MGMT* and *ERCC1* methylation reached a specificity for differentiation between benign and malignant rectum tumor of 100% in blood samples, with a sensitivity of 32% [27].

More recently, Zhang et al. observed that both colon and RC could be detected by ctDNA, with the latter having lower median plasma cfDNA concentrations than colon cancer patients (14.2 ng/ml vs.

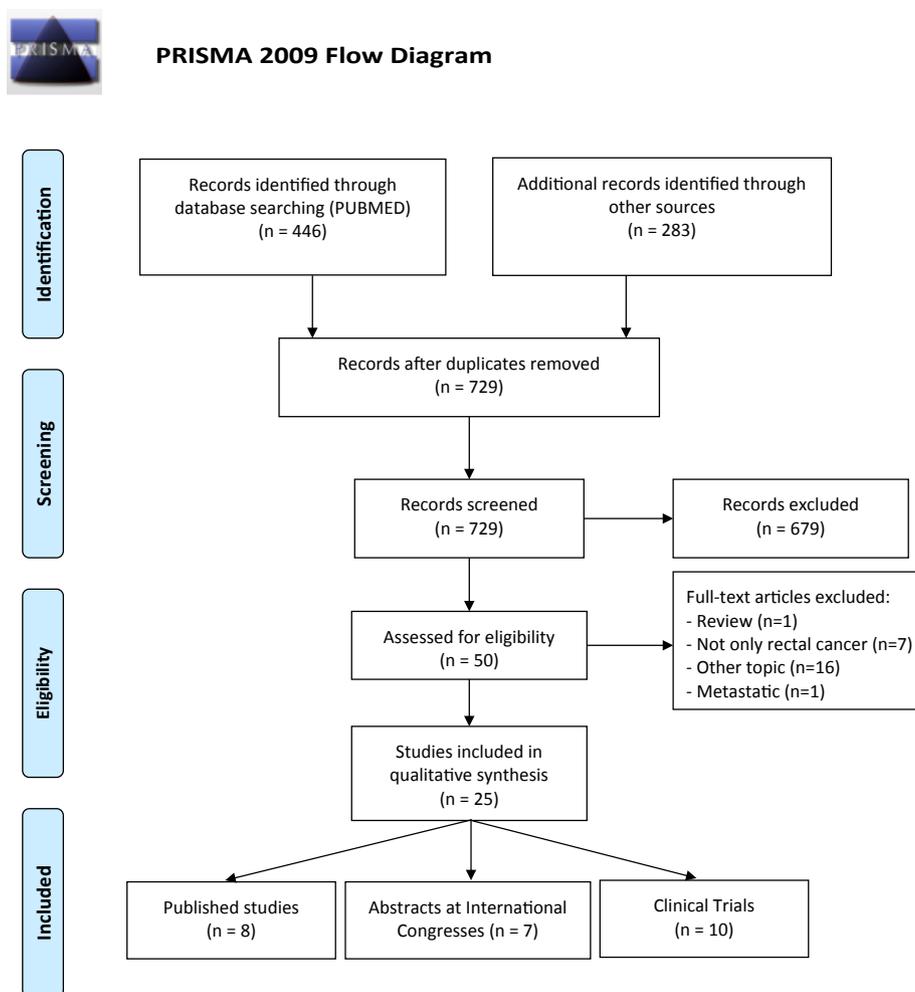


Fig. 1. Flow diagram representing the systematic review process performed according to PRISMA Statement.

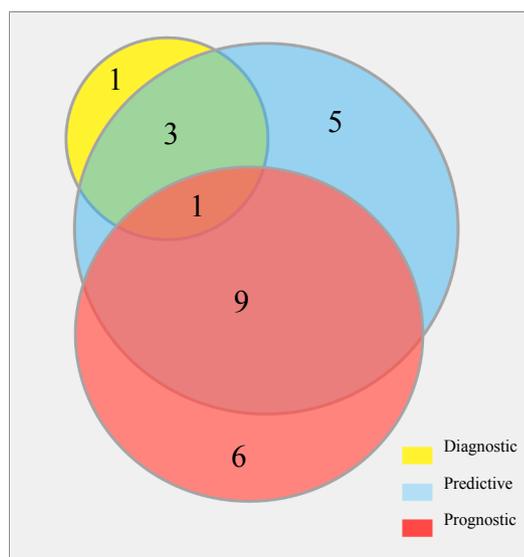


Fig. 2. Euler-Venn diagram representing the results of our systematic research according to the investigated role of ct/cf DNA in rectal cancer.

8.94 ng/ml). The study was conducted on 29 patients, including 10 with RC. For each patient, a freshly frozen tissue sample was collected during surgery and the plasma obtained was analyzed using an 85-gene panel, with a mutation concordance rate between ctDNA and tissue of

70% in all patients, lower in the subgroup of RC patients (5/10, 50%) [28].

At the present time, one clinical trial is exploring the feasibility of ctDNA as a detection index for RC (Table 2).

cfDNA and ctDNA as tool to predict treatment response

Different groups analyzed the role of cf/ctDNA as a tool to monitor treatment response after CRT in locally-advanced rectal cancer (LARC). In 2008, Zitt and colleagues [29] observed for the first time a correlation between the concentration of cfDNA and clinical response after surgery (not after neoadjuvant CRT) in 26 patients: responders exhibited a reduction in cfDNA level, while non-responders showed an incremented cfDNA [29].

Agostini et al. analyzed cfDNA on 67 LARC patients before and after neoadjuvant CRT, before surgery. Levels of longer fragments of cfDNA were reduced in responsive patients (tumor regression grade (TRG) 1–2, according to the Mandard score) compared to non-responsive (TRG 3–5). In particular the post-CRT cfDNA integrity (the ratio of 400-/100-bp DNA fragments) was associated with response (P = 0.0009), confirming that cfDNA long fragments are more tumor-specific than short fragments. Baseline levels of cfDNA were not correlated with tumor response [25].

The relationship between cfDNA concentration and TRG score was also observed by a Chinese group who focused also on different aspects of cfDNA: the 400-/100-bp ratio of DNA fragments, the methylation status of *MGMT* and the mutational status of *KRAS*. They treated 34

Table 1
Studies exploring the role of liquid biopsy in non-metastatic rectal cancer. * Available as an abstract.

Reference	Year	N. patients	Country	Assay	Main findings
<i>Diagnostic</i>					
Agostini et al.	2011	67	Italy	qPCR	cfDNA levels (using Alu 115, 247 and β globin gene) were higher in RC than in healthy group ($P < 0.0001$).
Sun et al.	2014	34	China	qPCR	Concentrations of 100 bp and 400 bp fragments and the ratio of 400-/100-bp DNA were higher in RC than in healthy group ($p < 0.01$). Mutated KRAS and methylated MGMT were not found in cfDNA of healthy controls.
Shalaby et al.	2017	93	Egypt	PCR	MGMT or ERCC1 were methylated for 4.7% and 7% in the blood of patients with benign lesions and for 58% and 60% in RC patients ($p < 0.001$). Mutation concordance rate among ctDNA and tissue was 50% in RC patients.
Zhang et al.*	2019	10	China/USA	NGS	
<i>Predict/Monitor Treatment Response</i>					
Zitt. et al.	2008	26	Austria	qPCR	Post surgery cfDNA responders: 2.2 ng/ml; cfDNA non responders: 5.1 ng/ml ($p = 0.006$).
Agostini et al.	2011	67	Italy	qPCR	Baseline cfDNA levels not correlated with tumor response.
Sun et al.	2014	34	China	PCR	In responders, the median levels of Alu 247 and the cfDNA integrity index (the ratio of 400-/100-bp DNA fragments) were significantly lower after CRT compared to baseline ($p = 0.0048$ and 0.0005 , respectively). The good response group had significantly higher baseline 400-bp DNA levels and DNA integrity index. Good response group had lower cfDNA integrity after CRT compared before CRT. MGMT promoter methylation at baseline was higher in responders, with no reduction after treatment, while the rate of KRAS mutation decreased in both groups after CRT.
Shalaby et al.	2017	93	Egypt	PCR	Significant correlation between baseline MGMT and ERCC1 methylation and response to CRT.
Schou et al.	2018	123	Denmark	Direct fluorescent assay	No differences in cfDNA levels between before and after CRT.
Carpinetti et al.	2015	4	Brazil	Whole genome sequencing	ctDNA levels decreased in RC achieving response to CRT.
Tie et al.	2018	159	Australia	NGS	No association between post-CRT ctDNA status and pCR. Postoperative ctDNA detection was associated with high-risk pathological factors such as ypT3-4 and ypN1-2 stage.
Li et al.*	2017	30	China	NGS	ctDNA predicts change in tumor burden better than CEA.
McDuff et al.*	2019	31	USA	NGS	The rate of R0-NN resection was higher among pts with undetectable preoperative ctDNA compared to pts with a detectable ctDNA.
Chen et al.*	2019	9	USA	qPCR	Methylated BCAT1 or IKZF1 genes were found in 5/9 patients.
Zhou et al.*	2019	61	China	NGS	Correlation between decrease of methylation and partial/complete response.
Yang et al.*	2019	119	China	NGS	Correlation between undetectable preoperative ctDNA status and pathological downstage ($p = 0.02$). Correlation between preoperative ctDNA positivity and the persistently involved lymph node ($p = 0.02$). TP53 and APC gene in pre-treatment samples negatively correlated with response to nCRT. Detection of pre-treatment mutations in any time points during nCRT was significantly ($P = 0.03$) decreased from TRG3 to TRG0 group.
<i>Predicting disease recurrence</i>					
Tie et al.	2018	159	Australia	NGS	Worse RFS if ctDNA was detectable after CRT or after surgery (estimated 3-year recurrence-free survival was 33% for postoperative ctDNA-positive patients vs 87% for ctDNA-negative).
Schou et al.	2018	123	Denmark	Direct fluorescent assay	High risk of recurrence pts with baseline cfDNA levels above 75th quartile (HR = 2.48, 95% P = 0.007).
Carpinetti et al.	2015	4	Brazil	Whole genome sequencing	Changes of ctDNA levels after surgery predict tumour recurrence.
McDuff et al.*	2019	31	USA	NGS	Patients with detectable postoperative ctDNA had worse RFS.
Khakoo et al.*	2018	47	UK	Sequencing	ctDNA level was higher in pts who showed metastases (64%) related to pts that did not (8.3% P = 0.0005).
Chen et al.*	2019	9	USA	qPCR	Patients with high levels of methylated IKZF1 and BCAT1 in post-treatment ctDNA recurred 2 months after surgery.
Selafani et al.	2018	97	UK	ddPCR	No difference in outcome between patients with or without detectable ctDNA after CRT.
Yang et al.*	2019	119	China	NGS	Detection of pre-treatment mutations in ctDNA after completion of nCRT was significantly associated with worse DFS.

cfDNA: circulating free DNA; RC: rectal cancer; ctDNA: circulating tumor DNA; CRT: chemoradiotherapy; pCR: pathologic complete response; NN: node negative; TRG: tumor regression grade; RFS: relapse-free survival; DFS: disease-free survival.

Table 2
Ongoing studies investigating the role of cf/ct-DNA in non-metastatic rectal cancer.

Study (ClinicalTrials.gov identifier)	Country	Phase	Pts	cf/ct DNA related outcomes
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) (NCT03565029)	Italy	Phase II	180	PROGNOSTIC: local a/o relapse-free survival
Circulating Tumour DNA (ctDNA) Rectal Cancer and the Relationship to Extramural Venous Invasion (NCT02579278)	UK	Observational, Prospective	40	PREDICTIVE: presence or absence of ctDNA post CRT in EMVI-positive rectal cancer
Application of Circulating Tumor DNA Test in the Diagnosis and Treatment of Patients With Advanced Rectal Cancer (NCT03615170)	China	Observational, Prospective	200	DIAGNOSTIC: explore the feasibility of ctDNA as a detection index for rectal cancer; PREDICTIVE: evaluation of preoperative concurrent chemoradiotherapy, so as to provide guidance for subsequent treatment;
Observational Study on Rectal Cancer to Verify if Response After Chemo-radiotherapy Can be Predicted With a Particular Blood Test. (LiBRcCa) (NCT03699410)	Switzerland	Observational, Prospective	35	PROGNOSTIC: search for possible recurrence related mutations
A Study of the Role of Circulating Tumor DNA in Predicting the Likelihood of Organ Preservation After Clinical Complete Response to Neoadjuvant Therapy for Rectal Cancer (NCT03749083)	USA	Observational, Prospective	55	PREDICTIVE: negative prognostic value of ctDNA drawn from the mesenteric and peripheral blood to investigate if can predict the response after chemo-radiotherapy and before surgery
Investigation of the Value of ctDNA in Diagnosis, Treatment, and Surveillance of Surgically Resectable Colorectal Cancer – Cohorts for T1-2 NO rectal cancer who undergo local or radical resection (NCT03038217)	China	Observational, Prospective	300	PROGNOSTIC: local recurrence rate
MRI Simulation-guided Boost in Short-course Preoperative Radiotherapy for Unresectable Rectal Cancer (SUNRISE) (NCT03714490)	China	Phase II	200	PROGNOSTIC: disease free survival, local recurrence rate, overall survival
Multicenter, Prospective, RCT: Investigation of Combined Modality Therapy for Locally Advanced Mid/Low Rect (NCT03042000)	China	Prospective, observational	1200	PREDICTIVE: predicting of treatment response; PROGNOSTIC: survival
Totally Neoadjuvant FOLFOXIRI + Short-course Radiation + XELOX in Patients With Locally Advanced Rectal Cancer (NCT03484221)	China	Phase II	30	PREDICTIVE: predicting the therapeutic effects of NCRT; PROGNOSTIC: disease free survival
Preoperative Chemoradiotherapy With Raltitrexed for Intermediate or Locally Advanced Rectal Cancer in the Fit Elderly (NCT02992886)	China	Phase II	68	PROGNOSTIC: survival
				PREDICTIVE: predictive treatment response; PROGNOSTIC: disease-free survival

LARC patients with CRT followed by surgery. The good response group of patients had a significantly higher baseline 400-bp plasma cfDNA levels and showed a significant decrease of these fragments in plasma after CRT. On the contrary, no difference was observed regard the level of 100-bp fragments before and after CRT both in responders and non-responders. In addition, the rate of *MGMT* promoter methylation at baseline was higher in responders, with no reduction after treatment, while the rate of *KRAS* mutation decreased in both groups after CRT [26].

Shalaby et al. described the same correlation between methylated status of the promoter of *MGMT* and *ERCC1* genes with response to CRT. A higher methylation status was associated with a better tumor response after preoperative CRT [27].

A Danish group quantified the total level of cfDNA by fluorescence assay, using 40 μ L of plasma of 123 LARC patients. They observed no differences either between baseline and post-treatment (CRT preceded by induction chemotherapy in 42% of cases) levels of cfDNA or between patients achieved a pathological complete response (pCR) and poor responders [30].

Carpinetti et al. firstly observed a decrease in ctDNA (detected due to tumor-specific chromosomal rearrangements) in 4 patients that achieved a response to CRT. By the way, cfDNA was negative both in patients with partial and complete pathological response [31].

Tie et al. analyzed tissue and multiple plasma samples of 159 LARC patients treated with neoadjuvant CRT through next generation sequencing (NGS). ctDNA was detectable in 77% of plasma samples before treatment, 8% during CRT and 12% at a postoperative stage. Detectable ctDNA after surgery was associated with known high risk pathological features (i.e. ypT3-4, node positive), but there was no statistically meaningful association between reduction or negativization of ctDNA after CRT and pCR [32].

Li et al. observed in a small study of 30 patients that ctDNA level variations (somatic mutations identified by NGS of 61-gene panels) can predict pathological response to neoadjuvant CRT, better than classic markers as CEA or CA 19.9 [33].

McDuff et al. reported a higher rate of R0-node negative resections after CRT among 17 patients with undetectable preoperative ctDNA, compared to 10 patients with detectable ctDNA. The former group had a higher pCR rate (24% vs. 10%) [34].

Chen et al. identified ctDNA analyzing the methylation status of *BCAT1* or *IKZF1* gene through qPCR assay in 9 LARC patients. Five patients showed positivity for one or both of methylated genes before CRT, four of them exhibited a decrease in detection after treatment, consistent with partial or complete responses [35].

Two Chinese prospective cohort studies, presented at 2019 ASCO meeting, recruited 180 LARC patients, overall, with serial plasma collection analyzed through NGS gene panels to detect mutations in ctDNA [36,37]. Yang et al. reported a negative correlation between presence of *TP53* and *APC* gene in pre-treatment samples and response to nCRT, and detectability of pre-treatment mutations during nCRT significantly decreased from TRG3 to TRG0 group [37]. Zhou et al. observed a significant predictive role of pre-surgery ctDNA levels, where its persistency was linked with pathological N+, while an undetectable preoperative ctDNA correlated with pathological downstaging [36].

At the present time, six clinical trials are exploring the possibility to use ctDNA as a predictive tool (Table 2).

cfDNA and ctDNA as a prognostic tool for disease recurrence or survival

In a Danish study of 123 LARC patients, a solid association of baseline cfDNA level (measured through a fluorescence assay) with disease free survival (DFS) was found. High levels of cfDNA were correlated with higher risk of local or distant recurrence and with shorter time to recurrence. A non-statistically significant trend for overall survival (OS) was also observed [30].

At a median follow up of 24 months, Tie and colleagues noticed an

increased risk of recurrence in patients with ctDNA persistence after CRT or surgery. This risk of recurrence was irrespective either of pathological risk level (ypT3-4N+ vs ypT1-2N0 vs pCR) or of adjuvant therapy, with an estimate 3y RFS of 33% vs 87%. Post-operative ctDNA detection was a stronger prognostic biomarker than CEA levels. Moreover, 74% of patients recurred within 12 months after surgery, 9/19 had persistent ctDNA in plasma.

As already said, Carpinetti et al. analysed in 4 LARC patients the use of ctDNA to monitor disease response and recurrence. Two patients with persistent positive level of ctDNA, during their follow up, developed liver metastasis concomitantly with an incremental in ctDNA level. Other 2 patients showed a drop in ctDNA levels after CRT, with negative follow up for recurrence and no more evidence of ctDNA in plasma [31].

Four studies, presented at last ASCO and ESMO congresses, have explored the use of ctDNA as a tool to assess response and predict surgical outcome in LARC [34,35,38,39]. McDuff et al., among 22 patients treated with preoperative CRT, reported a shorter recurrence free survival in cases with detectable post-operative ctDNA [34]. Khakoo showed that persistence of ctDNA at mid CRT or detection of ctDNA at the end of CRT were associated with development of metastasis [39]. In the work presented by Chen, one patient that showed persistent high level of methylated genes after CRT, recurred in two months after surgery [35].

Conversely, in a study performed on 97 LARC patients receiving induction chemotherapy with CAPOX followed by CRT and then adjuvant CAPOX with or without cetuximab (EXPERT-C trial), Scalfani et al. did not found a significant association between ctDNA positivity/negativity and progression free survival (PFS) or OS using qPCR [40].

At the 2019 ASCO meeting, Yang et al. described a significant association between persistence of pre-treatment mutations in ctDNA after completion of CRT and worse DFS [37].

Eight clinical trials are exploring the possibility to use cf/ctDNA as a prognostic tool (Table 2).

Discussion

In present review several studies supported the use of liquid biopsy in RC as an innovative, minimally invasive procedure that could assist either the diagnostic/staging process and the assessment of treatment response. The limitations of data retrieved are mostly related to the relatively small sample size of the studies, heterogeneity of techniques used for liquid biopsy and timing of plasma samples (eg. after CRT or after surgery), and differences in treatment context (e.g. induction chemotherapy or not) for patients with different stages of non-metastatic RC.

We found 4 studies (overall 204 patients) which explored the role of liquid biopsy as a diagnostic tool in RC. Measurement of cfDNA levels was performed with different techniques: ALU-based quantitative-PCR, tracking mutations of *KRAS*, or, more recently, tracking of several genes with NGS and assessing the methylation status of *MGMT* and *ERCC1* in ctDNA. These reports, although heterogeneous, suggest that measuring cfDNA levels or detecting ctDNA might discriminate RC patients from healthy controls and from individuals harbouring rectal adenoma. In particular, a high specificity in discriminating RC was reported for *MGMT* and *ERCC1* methylation or *KRAS* mutation detection.

These data are consistent with results obtained in CRC patients [42–43]. However, it should be taken into account that in this setting of early detection of cancer, the sensibility of liquid biopsy is limited by low concentrations of circulating DNA, that have been reported to be even lower in RC [28].

Contrasting data have been retrieved about the reliability of circulating DNA as a tool to predict treatment response in RC. The clinical value of baseline levels of cf/ctDNA is not clear. Only one small study showed a correlation between higher levels of longer cfDNA fragments (index of DNA integrity) and response [26], but these data were not

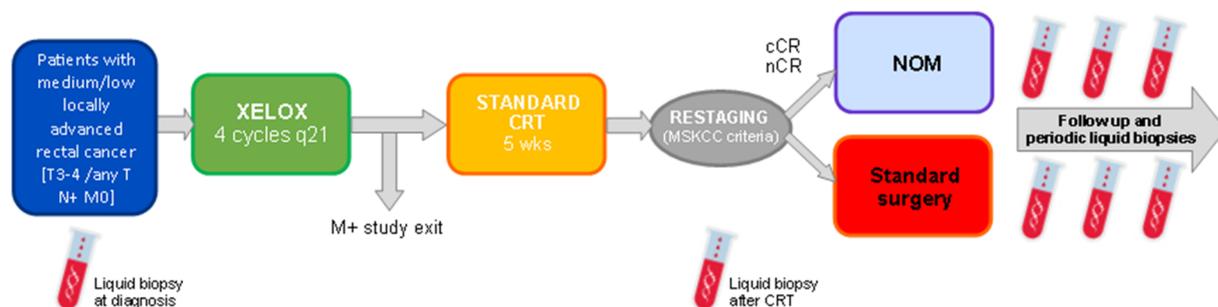


Fig. 3. Study design of the NO-CUT trial (NCT03565029) investigating as translational endpoint the potential role of liquid biopsy in the non-operative management of rectal cancer.

consistent with results of a previous wider study [25]. Nevertheless, a strong methylation of *MGMT* or *ERCC1* genes at baseline might better predict a tumor response after preoperative CRT [26,27]; on the contrary, detection of *TP53* and *APC* gene in ctDNA of pre-treatment samples has been negatively associated with response to CRT [37]. Notably, in a previous presentation of the same study, Yang et al. reported no difference in baseline ctDNA levels between responders and non-responders [38].

A noteworthy evidence, observed by most groups with different assays, consists in the reduction in cf/ctDNA levels in responders, while non-responders often show an incremented circulating DNA [25,26,29,31–33,35–37,44]. The timing of plasma collection represents a crucial aspect in this setting: at baseline, after induction chemotherapy, after neoadjuvant CRT or after surgery. Zitt et al. observed a reduction of DNA levels after CRT in all 26 patients but it was not predictive of pathological downstaging. A significant difference between responders and non-responders was found only in plasma samples collected after surgery [29].

In this same setting, a DNA integrity index (a ratio between long and short DNA fragments) has been proposed to be a useful guide to discriminate responding and non-responding patients even with plasma analysis conducted after neoadjuvant CRT [25,26].

More recent studies, adopting NGS assays, have demonstrated the potential of post-CRT ctDNA samples to predict tumor response, enhancing the confidence in ctDNA as a tool to guide patient selection for watch and wait strategy. Different groups observed a correlation between undetectable preoperative ctDNA status and pathological downstage [32,33,36,37,44]. A weaker methylation of *BCAT1* or *IKZF1* has been observed after CRT in good responders by Chen et al. [35].

Finally, we found inconclusive data about the association between reduction or total clearance of circulating DNA after CRT and pCR. Likely, circulating DNA has no sufficient sensitivity to rule out the presence of minimal residual disease [32].

In terms of survival and disease recurrence, almost all studies have shown a correlation between persistence of ctDNA after treatment and disease recurrence during follow up [30,32,35–37,39,44]. One group also observed an association between high baseline cfDNA level and local or distant recurrence, with a trend for shorter OS [30].

Tie et al., in a recent prospective study of LARC patient, detected ctDNA in 77%, 8.3% and 12% of pretreatment, postchemoradiotherapy and post-surgery plasma samples, respectively. On the basis of ctDNA levels, they were able to stratify patients at very high risk of recurrence (ctDNA detectable after CRT (HR 6,6) or after surgery (HR 13,0)), estimating a 3-year recurrence-free survival of 33% vs 87% in positive/negative ctDNA patients. Postoperative ctDNA status remained an independent predictor of RFS irrespective of clinicopathological risk factor or adjuvant chemotherapy [32].

These results are in line with the conclusions of studies conducted in the setting of resected colon cancer, where evidence of ctDNA after surgery or after adjuvant chemotherapy were linked with shorter recurrence-free survival [45,46].

In contrast, among patients treated in the EXPERT-C trial (induction CAPOX, CRT, surgery, adjuvant CAPOX +/- cetuximab) a significant association between ctDNA positivity/negativity and PFS or OS was not observed. However, the plasma sample in this study was collected before surgery and all patients received both neoadjuvant and adjuvant chemotherapy [40].

The potential role of liquid biopsy in RC is also currently being explored as translational endpoints in numerous clinical trials and can find an important application in the setting of NOM. The ongoing NO-CUT study (NCT03565029), a phase 2 clinical trial, will assess whether an oxaliplatin-enhanced neoadjuvant CRT, followed by an imaging-intensive, liquid biopsy-enriched surveillance, can spare stage II-III rectal cancers from undergoing up-front demolitive radical surgery with a clinically acceptable rate of distant relapse (Fig. 3). The translational component of the study will establish, by retrospective correlative analysis of contextual imaging and blood molecular findings, whether circulating mutated and/or methylated tumoral DNA is a predictive marker for residual disease, and whether there is a correlation between ctDNA and cancer relapse.

Ethical approval and consent to participate

Not applicable because the article is a review.

Contributors

- Daniela Massihnia: conception and design of the review, acquisition, analysis and interpretation of data, drafting of the article.
- Elio Gregory Pizzutilo: conception and design of the review, acquisition, analysis and interpretation of data, drafting of the article.
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Declaration of Competing Interest

A.S-B. has acted as a consultant/advisory member for Amgen, Bayer, Lilly and Merck-Serono. S.S is advisory board member for Amgen, Bayer, BMS, Celgene, Incyte, Merck, Novartis, Roche, Seattle Genetics. A.A. is advisory board member for Amgen and Bayer.

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