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Circulating tumor DNA in patients with locally advanced rectal cancer treated with multimodal treatment

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Abstract

Background: The management of locally advanced rectal cancer (LARC) relies on a multimodal approach. Neither instrumental work-up nor molecular biomarkers are currently available to identify a risk-adapted strategy.

Objectives: We aim to investigate the role of circulating tumor DNA (ctDNA) and its clearance at different timepoints during chemo-radiotherapy (CRT) and correlate them with clinical outcomes.

Design: Between November 2014 and November 2019, we conducted a monocentric prospective observational study enrolling consecutive patients with LARC managed with neoadjuvant standard CRT (capecitabine and concomitant pelvic long-course radiotherapy), followed by consolidation capecitabine in selected cases and surgery.

Methods: Blood samples for ctDNA were obtained at pre-planned timepoints. We evaluated the correlation of baseline variant allele frequency (VAF) with pathologic complete response (pCR) down-staging, node regression (pN0), event-free survival (EFS), and overall survival (OS).

Results: Among 112 screened patients, 61 were enrolled. In all, 38 (62%) had a positive ctDNA at baseline with VAF >0 and 23 had negative ctDNA (VAF $=0$). Among patients with negative ctDNA, 30% had a complete response, while only 13% of positive ctDNA patients had pCR [odds ratio (OR) 0.35 (95% confidence interval (CI): 0.10–1.26), $p=0.11$]. Similarly, 96% and 74% of pN0 were observed among negative and positive ctDNA patients, respectively [OR 0.13 (95% CI: 0.02–1.07), $p=0.058$]. The presence of a baseline VAF >0 was associated with a trend toward a lower EFS compared with VAF $=0$ patients [hazard ratio (HR) $=2.30$, 95% CI: 0.63–8.36, $p=0.21$]. Within the limitations of small sample size, no difference in OS was observed according to the baseline ctDNA status (HR $=1.18$, 95% CI: 0.35–4.06, $p=0.79$).

Conclusion: Within the limitations of a reduced number of patients, patients with baseline negative ctDNA seem to show a higher probability of pN0 status and a trend toward improved EFS. Prospective translational studies are required to define the role of ctDNA analysis in the multimodal treatment of LARC.

Keywords: chemoradiotherapy, circulating tumor DNA, ctDNA, liquid biopsy, rectal cancer

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Background

Treatment of rectal cancer has evolved over the years thanks to improvements in the multidisciplinary approach.[1] For almost two decades, short-course radiotherapy or long-course radiotherapy combined with radio-sensitizing chemotherapy (chemo-radiotherapy, CRT) represented the standard of care for locally advanced rectal cancer (LARC).[2,3]

To date, the therapeutic approach of microsatellite stable LARC has changed integrating the use of induction or consolidation chemotherapy with standard CRT, which is called total neoadjuvant treatment (TNT). This strategy improved rates of pathologic complete response (pCR) and, furthermore, provided the possibility to move for non-operative management for patients with a complete response (CR) that could avoid surgery.[4–6] Therefore, within this more complex scenario, a major challenge is the optimization of efficacy and the reduction of toxicity. Although most of the patients experience tumor regression, the percentage of pCR remains around 10–30%.[2–5] In addition, occurrence is observed in 30% of the cases.

Therefore, the identification of reliable biomarkers to guide and personalize the treatment for each patient represents an unmet need. In this regard, emerging data support the role of liquid biopsy as a noninvasive tool to evaluate treatment response, identify resistance alterations, and capture tumor heterogeneity and minimal residual disease (MRD) in colorectal cancer.[7–12] The detection of circulating tumor DNA (ctDNA) in patients with localized colon cancer after radical surgery is associated with a really high risk of recurrence and identifies patients who take advantage of adjuvant chemotherapy.[11,12] Similarly, the detection of ctDNA after neoadjuvant treatment in LARC is correlated to an increased probability of tumor relapse.[10] However, the role of ctDNA in evaluating response to CRT or TNT in LARC is still debated and under investigation.[13–17]

Even before the use of TNT, we sought to investigate the role of ctDNA in patients with rectal cancer treated with CRT and to correlate it with clinical outcomes.

Materials and methods

Study population and procedures

We conducted a prospective observational study enrolling 112 patients with histologically confirmed locally advanced rectal adenocarcinoma (stages T3, T4, or N+) managed with long-course neoadjuvant CRT and surgery at European Institute of Oncology between November 2014 and November 2019. CRT consisted of capecitabine 1650 mg/m^2 concomitant with a total dose of 50.4 Gy divided into 25 fractions on the mesorectum. Based on our previous experience, consolidation capecitabine for 1–2 cycles in case of T4 and/or N+ baseline clinical stage was allowed.[18] All patients received surgery within 8–12 weeks since the end of CRT. Adjuvant chemotherapy with fluoropyrimidine ± oxaliplatin was admitted. We included in the current study 61 tumors that displayed *KRAS*/*NRAS*/*BRAF*/*PIK3CA* alterations that were evaluable for liquid biopsy analysis.

Clinicopathological characteristics were collected at baseline and during the follow-up period. Serial blood samples were obtained at specific preplanned time points: at baseline (within 2 weeks from treatment start, T0), after CRT (2–6 weeks since the end of radiotherapy, T1), after surgery (within 3–5 weeks, T2), and after adjuvant chemotherapy (4–6 weeks after the end of treatment, T3). The ctDNA clearance was defined as a 100% decrease of variant allele frequency (VAF) in a target mutation and was assessed at T1, T2, and T3 [clearance 1 (C1), C2, and C3, respectively]; down-staging was defined as the decrease of the stage from clinical assessment at diagnosis to the pathological stage on the pathology report. Pelvic magnetic resonance imaging (MRI) and chest–abdominal–pelvic computed tomography (CT) scans were performed at baseline and then 6–8 weeks after radiotherapy. After surgery follow-up assessments were conducted according to clinical practice, including CT scan, carcinoembryonic antigen (CEA), and CA 19.9 measurement, physical examination every 6 months for the first 3 years, then every 12 months for the next 2 years. Total colonoscopy was performed 12 months after surgery, if negative after 2–3 years, then every 5 years. The study endpoint was to evaluate the role of baseline ctDNA and ctDNA

dynamics as a potential biomarker in patients with LARC who undergo neoadjuvant CRT. The reporting of this study conforms to the STROBE guidelines.

Liquid biopsy analysis

For translational analyses, 20 ml of peripheral blood samples was collected. The process for ctDNA preparation should have been performed within 5 h from the blood draw. For plasma preparation, blood must be collected in a tube treated with an anticoagulant, preferably ethylenediaminetetraacetic acid. The supernatant or plasma was removed by centrifugation. ctDNA was extracted from 2 ml of plasma using QIAamp Circulating Nucleic Acid-QIAGEN following the manufacturer's instructions. Mutations detected on tumor biopsy (*KRAS* in exons 2, 3, and 4; *NRAS* in exons 2, 3, and 4; *BRAF* in exons 11 and 15; *PIK3CA* in exons 9 and 20) were evaluated on circulating-free DNA. The analysis was performed with the Droplet Digital protein chain reaction (PCR) (QX200 Biorad), using the appropriate ddPCR Mutation Detection Assays (Biorad).

Statistical analysis

Continuous data were reported as median and ranges or interquartile ranges. Categorical data were reported as counts and percentages.

Univariable logistic regression models were performed to assess the association between positive VAF at baseline with pCR, downstaging, and pN0.

Overall survival (OS) was defined as the time from diagnosis until death or last contact. Event-free survival (EFS) was defined as the time from the date of diagnosis to subsequent recurrence, death, or last contact, whichever occurred first. OS and EFS were estimated using the Kaplan–Meier method. The log-rank test was used to assess differences between groups. The association between VAF with death or EFS events was evaluated using univariable Cox proportional hazards regression models.

A p value less than 0.05 was considered statistically significant.

All analyses were performed with the statistical software SAS 9.4 (SAS Institute, Cary, NC, USA).

Results

Patients' characteristics

Table 1 summarizes the main clinical characteristics of the entire population. In all, 40 patients were male (66%) and the median age at diagnosis

Table 1. Distribution of patients, tumor, and treatment characteristics.

Variable	Level	Overall (*N* = 61)
Age at diagnosis, median (range)		61 (25–79)
Sex	Male	40 (66)
	Female	21 (34)
Tumor site	Proximal	10 (16)
	Medium	23 (38)
	Distal	28 (46)
cT	1	1 (2)
	2	4 (7)
	3	56 (92)
cN	0	8 (13)
	Positive (number NA)	2 (3)
	1	24 (39)
	2	25 (41)
	NA	2 (3)
cM	0	61 (100)
Mutation on biopsy tissue	*KRAS* mt	44 (72)
	NRAS mt	8 (13)
	PIK3CA mt	6 (10)
	BRAF mt	1 (2)
	KRAS + *PIK3CA* mt	1 (2)
	KRAS + *BRAF* mt	1 (2)
Microsatellite status	MSS	52 (95)
	MSI	3 (5)
	Missing	6
Capecitabine plus radiotherapy	No	0 (0)
	Yes	61 (100)

(Continued)

Table 1. (Continued)

Variable	Level	Overall (*N* = 61)
Consolidation chemotherapy with capecitabine after CRT	No	33 (54)
	Yes	28 (46)
pT	0	11 (18)
	is	2 (3)
	1	4 (7)
	2	16 (26)
	3	26 (43)
	4	2 (3)
pN	0	60 (82)
	1	8 (13)
	2	3 (5)
pCR	No	49 (80)
	Yes	12 (20)
Downstaging	No	12 (20)
	Yes	49 (80)
TRG	TRG1	10 (19)
	TRG2	6 (11)
	TRG3	22 (41)
	TRG4	15 (28)
	TRG5	1 (2)
	Missing	7
Radicality of surgery	R0	59 (97)
	R1	2 (3)
Adjuvant treatment	Capecitabine	27 (43.5)
	CAPOX	6 (9.7)
	FOLFOX	3 (5)
	No	25 (40)
	Missing	1

CRT, chemoradiation; is, *in situ*; MSI, microsatellite instable, MSS, microsatellite stable; mt, mutant; pCR, pathologic complete response; TRG, tumor regression grade; NA, not available.

was 61 years (range: 25–79). Around half of the population was diagnosed with distal tumors ($n = 28$, 46%). Most of the patients were diagnosed with T3 (92%) and/or node-positive disease (83%). Median preoperative CEA was 2.6 ng/ml (range: 0.5–227.9). All patients received neoadjuvant long-course chemoradiation, with no disease progression during treatment and underwent curative surgery. Between the end of CRT and surgery, 28 (46%) patients received consolidation with 1–2 courses of capecitabine. In all, 12 (20%) patients achieved the pCR and 49 (80%) had a downstaging on the pathology report. Only two patients (3%) had an R1 surgery. In total, 36 (60%) received adjuvant chemotherapy (27 capecitabine, 6 CAPOX, and 3 FOLFOX).

Analysis of ctDNA and tumor markers level over time

We evaluated the dynamics of ctDNA at different timepoints. Liquid biopsy at T0, T1, T2, and T3 was available for 61 (100%), 58 (95%), 23 (38%), and 5 (8%) patients, respectively (Figure 1). The median ctDNA value was 0.10 ng/2 ml (range: 0.00–17.40) at T0. The distribution of VAF is reported in Figure 2 and Supplemental Figure 1. Of the 61 patients, 38 (62%) had a positive ctDNA at baseline with VAF > 0 while 23 (38%) had a negative ctDNA (VAF = 0) (Figure 1). The distribution of CEA and CA 19.9 levels at different timepoints is reported in Supplemental Figure 1 and Supplemental Table 2.

Predictors of clinical outcomes

Among patients with VAF = 0 at baseline, 30% achieved a pCR, compared to 13% of patients with VAF > 0 [OR 0.35 (95% confidence interval (CI): 0.10–1.26), $p = 0.11$] (Table 2). A numerically higher tumor downstaging was reported for patients with VAF = 0 compared with VAF > 0 (86% *versus* 76%) [OR 0.48 (95% CI: 0.12–2.01), $p = 0.32$]. Consistently, a pN0 status was observed in 96% and 74% of patients with negative and positive ctDNA, respectively [OR 0.13 (95% CI: 0.02–1.07), $p = 0.058$].

After a median follow-up of 68 months, the 5-year EFS for the study population was 81% (95% CI: 68–89) and the 5-year OS was 85% (95% CI: 72–92) (Supplemental Figure 2).

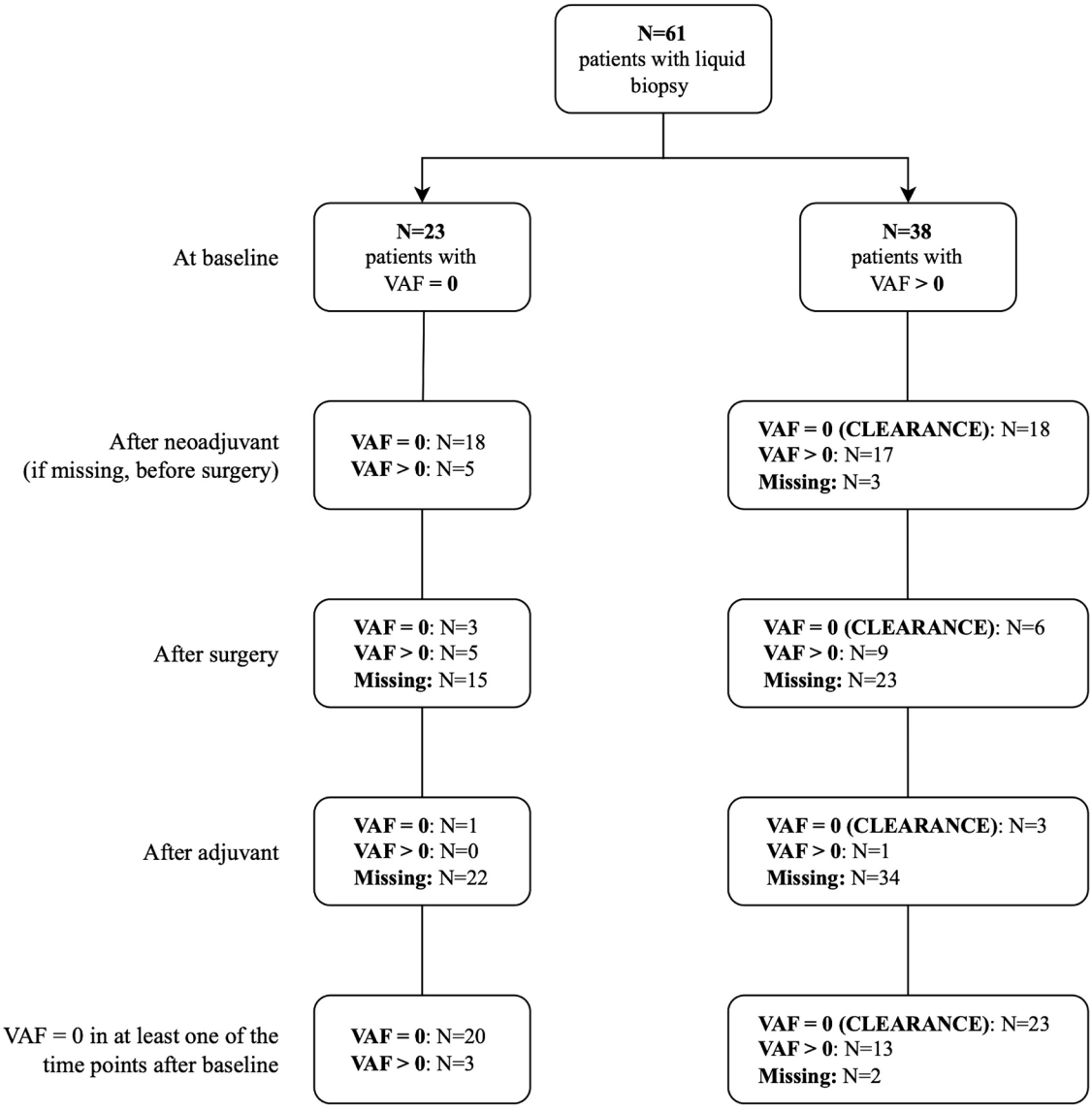

Figure 1. Study diagram.
VAF, variant allele frequency.

The presence of a baseline VAF > 0 compared with VAF = 0 was correlated with a twofold higher risk of recurrence with a trend toward a lower EFS [hazard ratio (HR) = 2.30, 95% CI: 0.63–8.36, $p = 0.21$]. Within the limitations of small sample size, no difference in OS was observed according to the baseline ctDNA status (HR = 1.18, 95% CI: 0.35–4.06, $p = 0.79$).

Lastly, for the subset of patients with available information on VAF after surgery, EFS and OS were calculated and stratified by VAF = 0 *versus* VAF > 0 (Figure 3). Five-year EFS was 88% (39–98) for VAF = 0, and 73% (37–91) for VAF > 0 (p: 0.509). Consistently, 5-year OS was 70% (23–92) and 77% (44–92) for VAF = 0 and VAF > 0, respectively ($p = 0.752$).

Exploratory analysis of VAF variation and correlation with recurrence and survival

We investigated the impact of VAF variation over time on clinical outcomes. In our cohort, 18 patients remained VAF negative (31%) at T1, 32 patients (55%) experienced a decrease in VAF level, but still detectable, at T1 and 8 an increase

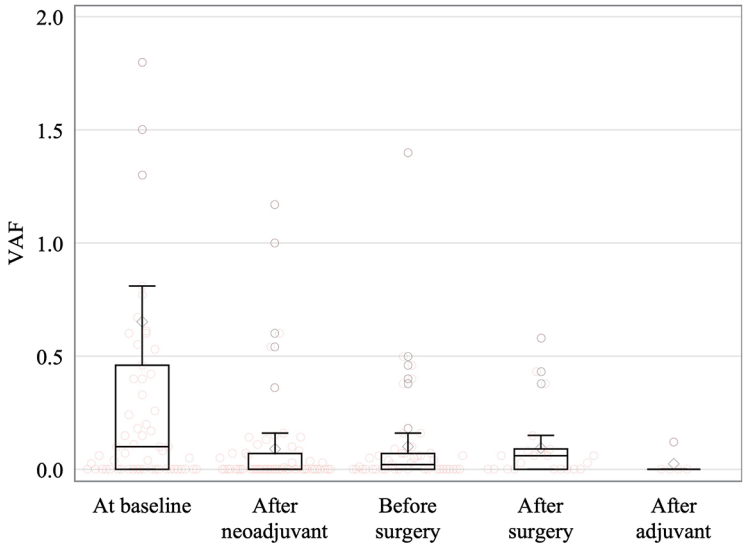

Figure 2. Distribution of VAF at different timepoints.
VAF, variant allele frequency.

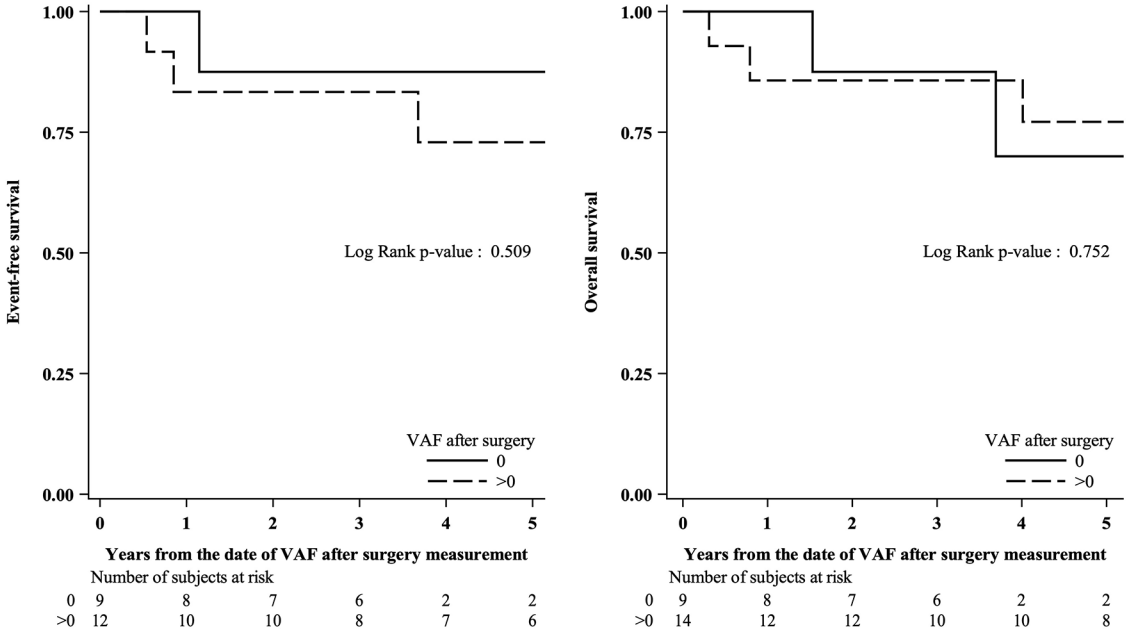

Figure 3. EFS and OS in population with VAF > 0 or VAF = 0 after surgery. Two patients had recurrence before the date of VAF determination and have been excluded from event-free survival analysis.
EFS, event-free survival; OS, overall survival; VAF, variant allele frequency.

(14%) (for 3 patients, there was no information about VAF value at T1) (Table 4). According to these subgroups, 5-year EFS was 89% (62–97), 74% (54–86), and 86% (33–98) for stable, decreased, and increased VAF, respectively, and 5-year OS was 93% (61–99), 80% (60–90), and 88% (39–98), respectively. No significant difference was observed for changes in VAF levels in relation to death or recurrence (Table 3).

Discussion

We conducted an observational prospective study to investigate the potential role of serial liquid

Table 2. Correlation between variant allele fraction at baseline and pCR, downstaging, and pN0.

Variable	Level	Outcome/Tot (%)	Univariable analysis		
			OR	95% CI	*p* Value
		pCR/Tot (%)			
VAF at baseline	0	7/23 (30)	Ref.	–	–
	>0	5/38 (13)	0.35	0.10–1.26	0.11
		Downstaging/Tot (%)			
VAF at baseline	0	20/23 (87)	Ref.	–	–
	>0	29/38 (76)	0.48	0.12–2.01	0.32
		pN0/Tot (%)			
VAF at baseline	0	22/23 (96)	Ref.	–	–
	>0	28/38 (74)	0.13	0.02–1.07	0.058

CI, confidence interval; OR, odds ratio; pCR, pathological complete response; pN0, pathologic N0; VAF, variant allele frequency.

Table 3. Univariable Cox proportional hazards regression models to evaluate the association between VAF at baseline with death and recurrence.

Variable	Level	Univariable analysis		
		HR	95% CI	*p* Value
Event: death				
VAF at baseline	0	Ref.		
	>0	1.18	0.35–4.06	0.79
Event: recurrence				
VAF at baseline	0	Ref.		
	>0	2.30	0.63–8.36	0.219

CI, confidence interval; HR, hazard ratio; VAF, variant allele frequency.

biopsy in a population of patients with LARC treated with CRT in a referral high-volume research center.

In the study population, one in five patients obtained a pCR, while downstaging was reported in 80% of the cases. We observed a numerically higher pCR and pN0 in patients with a baseline negative compared with positive ctDNA, even though not statistically significant.

The data are in line with previous findings.[10,14,15,17] In a large study by Tie *et al.*,[10] multiple plasma samples from 159 patients treated with CRT followed by total mesorectal excision were analyzed. No significant difference in terms of pCR was reported at different ctDNA timepoints including after neoadjuvant treatment. Moreover, the conversion of positive baseline ctDNA to negative did not have an impact on tumor response. Similar data were reported in a small population by

Table 4. Univariable Cox proportional hazards regression models assessing the relationship between change in VAF following neoadjuvant chemoradiotherapy with death and recurrence.

Variable	Level	Univariable analysis		
		HR	95% CI	*p* Value
Event: death				
VAF at baseline and after neoadjuvant	Stable	Ref.		
	Decrease	2.08	0.43–10.0	0.36
	Increase	3.26	0.45–23.5	0.24
Event: recurrence				
VAF at baseline and after neoadjuvant	Stable	Ref.		
	Decrease	2.88	0.62–13.4	0.18
	Increase	1.58	0.14–17.7	0.71

CI, confidence interval; HR, hazard ratio; VAF, variant allele frequency.

McDuff *et al.*[14] Despite a numerically increased pCR rate in negative pre-operative ctDNA patients (22% *versus* 11%), no statistical differences were observed. However, the absence of detectable ctDNA was correlated with higher rates of surgical outcomes such as margin-negative and node-negative resection (88% *versus* 44%, $p = 0.028$).

In our study, only two patients received surgery with R1 margins. Moreover, the presence of positive lymph nodes was observed only in 11 cases (18%). However, we observed a trend toward increased pN0 for patients with negative baseline ctDNA. The different populations included in the studies, the quality of the surgery, and the limited number of patients could explain these differences. The correlation of liquid biopsy with tumor response was evaluated as a preplanned *post hoc* biomarker analysis of the GEMCAD 1402 study investigating the combination of neoadjuvant treatment with FOLFOX+/− aflibercept followed by CRT in LARC.[15] In patients receiving a TNT approach, no significant relationship between pre-operative ctDNA status and pCR, ypT, or ypN status could be found.

On the contrary, Zhou and colleagues provided the first evidence about the association of pre-operative positive ctDNA with reduced pCR in a cohort of 104 patients LARC treated with concomitant capecitabine, oxaliplatin, and radiotherapy.[16] However, considering the limited number of patients with positive pre-surgical ctDNA (10.5%), these findings should be considered with caution.

The impact of a positive baseline ctDNA and negativization following surgery and adjuvant therapy represents a strong predictive biomarker of recurrence in localized colon cancer.[7,11,12] Results of the afore-mentioned studies confirmed the role of liquid biopsy to detect MRD after neoadjuvant treatment and surgery in LARC with a benefit in terms of DFS and OS in patients with postoperative negative ctDNA.[7,13–17]

In line with previous findings, patients with baseline positive ctDNA showed a twofold increased risk of recurrence. However, we did not observe a significant impact on OS; nevertheless, this might be explained by the reduced number of patients and the low rate of events that occurred in the cohort.

In this study, we were not able to catch significant differences in EFS or OS according to postoperative ctDNA status. Several factors could have contributed to these results. Together with few events during the follow-up period, in our study, only a limited subset of patients had available blood samples after surgery which prevents solid

interpretations. In addition, 36 out of 61 patients (59%) received adjuvant chemotherapy (27 patients with fluoropyrimidines monotherapy and 9 with platinum-based doublet) which could have potentially cleared some positive postoperative ctDNA and have impacted survival. At the ASCO GI 2024, preliminary results from the phase II AGITG DYNAMIC-Rectal trial were presented.[19] The trial tried to assess the role of ctDNA-based decision compared with standard of care, of adjuvant treatment following CRT, and surgery in patients with LARC. In case of a positive ctDNA test at weeks 4 and/or 7 after surgery, patients received and adjuvant treatment received 4 months of oxaliplatin-based or fluoropyrimidine chemotherapy. Patients with a negative ctDNA result received no chemotherapy if the node was negative or the clinician's choice if the node was positive. Only 46% of patients in the ctDNA-guided arm received adjuvant chemotherapy compared to 77% of patients in the standard management arm. Despite an early interruption of the study due to COVID-19 and the use of TNT, the 3-year recurrence-free survival rates for ctDNA-guided management and standard management were 76% and 82%, respectively. The risk of recurrence was more than twofold higher (38% *versus* 17%) in the ctDNA-positive group. Further studies on TNT strategies with the integration of liquid biopsy in the decision tree (intensification/deintensification of the multimodal therapies) are urgently required.

Our study has several limitations that should be taken into account. First, despite using a highly sensitive digital droplet PCR, we were able to evaluate only mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes that were found in both tumor tissue and ctDNA. Of course, a tumor-informed approach that takes into account other mutations and a different assay (Next generation sequencing rather than PCR) could increase the sensibility of the test. Moreover, we used tubes with anticoagulants available in daily practice, Streck or PaxGene tubes would have been a valuable addition to the results, reducing the number of false-negative cases. All these factors could partially explain the lower rate of ctDNA detection at baseline compared with previous findings (61% *versus* 74–83%).[10,12–17] Second, the use of consolidation chemotherapy in high-risk tumors could have an influence on ctDNA clearance before surgery. It could have been of interest to introduce a timepoint after CRT and before to consolidation of capecitabine. Taken together, these variables might have an impact on the correlation between post-neoadjuvant ctDNA status and clinical outcomes.

Third, a significant quote of blood samples at time points after surgery was not available for lack of compliance. Therefore, data derived from postoperative timepoints should be considered more as hypothesis generating for further studies than as conclusive results. Fourth, as previously reported, the highly selected population and the low rate of events could have limited the role of liquid biopsy in predicting tumor recurrence.

Conclusion

Although the use of liquid biopsy might represent an appealing tool in refining the treatment of colorectal cancer in different settings, the role in the management of LARC remains controversial. It is unclear if ctDNA clearance after neoadjuvant chemotherapy or CRT might be a surrogate of tumor response. In fact, emerging data support the idea that ctDNA analysis cannot differentiate between minimal *versus* no residual local disease. Probably, translating results derived from studies conducted in localized colon cancer, a liquid biopsy might be used to detect MRD after surgery, recognizing patients at risk of systemic spread who could benefit from adjuvant therapy. Moreover, non-operative management for patients that obtain a clinical CR might be the 'elephant in the room' for the treatment landscape of LARC. In this setting, liquid biopsy might have a space in identifying the subset of patients that could avoid demolitive surgery, without an increased risk of recurrence. Further translational analysis derived from larger randomized trials and dedicated studies is needed to clarify the role of liquid biopsy in the optimization of multimodal treatment in LARC.

Declarations

Ethics approval and consent to participate

The study has been approved by the ethic committee of the European Institute of Oncology (RE 1362/1). Written informed consent was required for study participation.

Consent for publication

Not applicable.

Author contributions

Lorenzo Gervaso: Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing.

Davide Ciardiello: Data curation; Investigation; Writing – original draft; Writing – review & editing.

Giuliana Gregato: Data curation; Investigation; Methodology.

Lorenzo Guidi: Data curation; Investigation.

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Marianna Alessandra Gerardi: Data curation; Investigation.

Luca Bottiglieri: Data curation; Investigation.

Darina Tamayo: Data curation; Investigation.

Emilio Bertani: Data curation; Investigation.

Uberto Fumagalli Romario: Data curation; Investigation.

Vincenzo Bagnardi: Data curation; Formal analysis.

Giuseppe Curigliano: Supervision.

Francesco Bertolini: Data curation; Investigation; Methodology.

Nicola Fazio: Data curation; Investigation; Supervision.

Maria Giulia Zampino: Data curation; Investigation; Supervision; Writing – review & editing.

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

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Supplemental material

Supplemental material for this article is available online.

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