Clinical Impact of Presurgery Circulating Tumor DNA after Total Neoadjuvant Treatment in Locally Advanced Rectal Cancer: A Biomarker Study from the GEMCAD 1402 Trial

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ABSTRACT

Purpose: Total neoadjuvant treatment (TNT) is a valid strategy for patients with high-risk locally advanced rectal cancer (LARC). Biomarkers of response to TNT are an unmet clinical need. We aimed to determine the value of circulating tumor DNA (ctDNA) to predict tumor response, recurrence, and survival in patients with LARC treated with TNT.

Experimental Design: The GEMCAD 1402 was a phase II randomized, multicentric clinical trial that randomized 180 patients with LARC to modified schedule of fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) +/− aflibercept, followed by chemoradiation and surgery. Plasma samples were collected at baseline and after TNT within 48 hours before surgery (presurgery). An ultrasensitive assay that integrates genomic and epigenomic cancer signatures was used to assess ctDNA status. ctDNA results were correlated with variables of local tumor response in the surgery sample, local/systemic recurrence, and survival.

Results: A total of 144 paired plasma samples from 72 patients were included. ctDNA was detectable in 83% of patients at baseline and in 15% following TNT (presurgery). No association was found between ctDNA status and pathologic response. Detectable presurgery ctDNA was significantly associated with systemic recurrence, shorter disease-free survival (HR, 4; $P = 0.033$), and shorter overall survival (HR, 23; $P < 0.0001$).

Conclusions: In patients with LARC treated with TNT, presurgery ctDNA detected minimal metastatic disease identifying patients at high risk of distant recurrence and death. This study sets the basis for prospective clinical trials that use liquid biopsy to personalize the therapeutic approach following TNT.

Introduction

Preoperative administration of systemic chemotherapy, either before or after chemoradiation (CRT) or short-course radiation, also known as total neoadjuvant therapy (TNT), is a standard strategy to treat high-risk locally advanced rectal cancer (LARC). Compared with the conventional preoperative CRT followed by surgery and postoperative adjuvant chemotherapy, the TNT strategy demonstrated better toxicity profile and compliance with the systemic treatment without compromising local efficacy (1). More recently, two phase III trials have shown that TNT is associated with higher pathologic complete response (pCR) rates and longer disease-free survival (DFS) or disease-related treatment failure (2, 3). Distant metastases, which potentially arise from systemic minimal residual disease (MRD) after surgery, undetectable by clinical examination, continue to be the main cause of treatment failure (1–3). While the value of pCR as a surrogate marker for survival is controversial, the neoadjuvant rectal (NAR) score (4–6) has recently been validated as a surrogate marker for DFS (6, 7). However, the NAR score is calculated after performing the rectal surgery because it integrates pathologic results from the tumor surgical specimen. Hence, presurgical identification of patients

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Clin Cancer Res 2021;27:2890–8

doi: 10.1158/1078-0432.CCR-20-4769

Translational Relevance

Total neoadjuvant therapy (TNT) is a valid strategy for patients with locally advanced rectal cancer (LARC) that may be followed by a watch-and-wait approach in selected patients. However, 30% of patients will still develop metastasis and die. In this prospective biomarker study, we assessed the role of circulating tumor DNA (ctDNA) before rectal surgery to predict response to TNT, relapse, and survival in patients with LARC. Using an ultrasensitive ctDNA assay that integrates genomic and epigenomic signatures, we found that presurgery ctDNA significantly identifies subjects at high risk of distant recurrence and death. On the contrary, presurgery ctDNA does not accurately predict local tumor response after TNT. Our data support the role of ctDNA as a biomarker of systemic minimal metastatic disease (MMD) rather than local tumor control, setting the basis for the design of prospective clinical trials to personalize the therapeutic approach in patients with LARC treated with TNT.

with minimal metastatic disease (MMD) who are potentially likely to recur at distant sites remains a challenge.

Several studies suggest that in patients who achieve a clinical complete response after administration of TNT, a close surveillance strategy [so called, nonoperative management (NOM) or watch-and-wait approach] is an acceptable alternative to rectal surgery, with the benefits of a proctectomy sparing approach (8). Biomarkers to select patients who are good candidates for a NOM strategy are not yet identified.

Circulating tumor DNA (ctDNA) analysis in patients with advanced solid tumors is a diagnostic alternative to traditional tissue molecular testing, with proven clinical utility (9–12). Emerging data support the use of ctDNA in patients with early-stage disease, treated with curative intent, to assess for MRD (12). Studies have shown that the presence of ctDNA is strongly associated with recurrence after curative treatment in localized tumors (13–15).

Our group completed a phase II randomized trial with the aim of examining the effect of an induction chemotherapy treatment with or without aflibercept plus a modified schedule of fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6), followed by standard CRT and total mesorectal excision (TME) surgery in patients with high-risk LARC treated with TNT in this trial. Early clinical results have been recently reported (15). Here, we report on a preplanned biomarker study of ctDNA in patients that participated in the substudy. The primary aim was to evaluate the association of baseline and post-TNT (presurgery) ctDNA status with clinical outcome markers, including pCR, NAR score, DFS, site of recurrence, and overall survival (OS).

Materials and Methods

Study design and participants

GEMCAD 1402 was an investigator-initiated phase II randomized trial, which recruited patients with high-risk LARC from 20 Spanish hospitals (16). The trial protocol was approved by the respective ethics committees of all participating institutions, and written informed consent was obtained from all patients prior to participation in the study. The study was conducted in accordance with the Declaration of Helsinki and followed the Consolidated Standards of Reporting Trials.

The eligibility criteria included patients ages 18–75 years with histologically confirmed rectal adenocarcinoma, with an inferior margin distal border below the peritoneal reflection. High-risk LARC was considered on the basis of high-resolution MRI clinical (c) staging. Tumors included were: (i) cT3 low-lying tumor at or below the elevators; (ii) cT3 tumors in the middle-third position extending 5 mm or more into the perirectal fat, or the presence of extramural venous invasion; (iii) cT3 tumors both at the distal- and middle-third positions with lymph node extending to within 1 mm of or beyond the mesorectal fascia; and (iv) any cT4 or cN2 tumors. Baseline thoracic and abdominal chemotherapy scan confirmed the absence of distant metastasis.

Patients were randomly assigned in a 2:1 ratio to arm A (induction chemotherapy with aflibercept plus mFOLFOX6) or arm B (induction chemotherapy with mFOLFOX6 alone). Both schemes were administered for six cycles, followed by 5 weeks of CRT with capecitabine. TME surgery was performed at 6–8 weeks after CRT completion in both treatment arms. The primary endpoint of the main study was the pCR rate defined as the absence of viable tumor cells in the primary tumor and lymph nodes (ypT0ypN0). Secondary endpoints included 3-year DFS and OS. Study protocol is available in Supplementary Data S1.

Serum carcinoembryonic antigen (CEA) level was collected at baseline and before surgery and measured by the local diagnostic laboratory at each participating site, with CEA concentrations of less than 5 ng/mL considered within reference range. Following completion of therapy, surveillance was performed according to standard of care, which included 3-monthly clinical review and CEA level and annual imaging for 3 years.

Biomarker substudy design and blood sample collection

The primary aim was to evaluate the association of ctDNA status with clinical outcome markers, including pCR, NAR score, DFS, site of recurrence, and OS. An independent written informed consent was obtained from all patients prior to participation in the biomarker substudy.

As per study design, blood samples were obtained from all patients prior to initiation of induction chemotherapy with mFOLFOX6 +/− aflibercept (baseline), and after completing all TNTs within 48 hours before surgery (presurgery). At each collection timepoint, 9–10 mL of whole blood was drawn into EDTA whole-blood collection tubes and centrifuged at 3,200 rpm for 15 minutes within the 3 hours after extraction. Plasma was aliquoted into 2 mL cryotubes for storage at −80°C. Samples were processed and aliquoted in each participant center.

Between January 2015 and March 2017, 180 patients were enrolled in the GEMCAD 1402 clinical trial and randomized to arm A (induction aflibercept plus mFOLFOX6 followed by CRT; $n = 115$) or arm B (induction mFOLFOX6 followed by CRT; $n = 65$). Patients with paired baseline and presurgery plasma samples and enough plasma quantity in both timepoints were selected for the biomarker substudy.

ctDNA analysis

The ctDNA assay selected for this study was a single-sample next-generation sequencing–based *in vitro* diagnostic assay validated for qualitative detection of cancer-derived cell-free DNA (cfDNA) in patients with colorectal cancer following resection and completion of standard-of-care therapy (MRD). The assay was performed in a single College of American Pathologists (CAP)-accredited and Clinical Laboratory Improvement Amendments–certified laboratory (LUNAR-1/Guardant Reveal Guardant Health). This ctDNA test integrated

assessment of somatic alterations with an epigenomic cancer signature to return a result of either ctDNA detected or ctDNA not detected without *a priori* knowledge of tumor mutational status.

Briefly, whole blood was collected in EDTA or Streck blood collection tubes and ctDNA was extracted from isolated plasma as described previously (11). ctDNA fragments were then partitioned on the basis of DNA methylation level, barcoded, pooled, and sequenced using a 500 kb panel targeting both somatic and epigenomic regions. Sequencing data files were analyzed using a proprietary bioinformatics pipeline software to detect the presence of ctDNA based on genomic variation and epigenomic signals, and to exclude common sources of interference, such as clonal hematopoiesis of indeterminate potential (CHIP), using a proprietary bioinformatics caller (17). This integrated approach alleviates the need for tumor tissue sequencing and creation of bespoke panels and the need for white blood cell sequencing for assessment of CHIP.

Minimum plasma volume for this ctDNA assay was 4 mL with the goal of at least 10 ng of extracted cfDNA. Given the plasma volume in this cohort was below the minimum plasma volume, a pilot feasibility analysis was completed on paired samples from three subjects to ensure sufficient sample quality prior to full-cohort analysis. This pilot analysis assessed cfDNA yield and diversity, sample contamination, and quality of sequencing reads with the aim of determining sample quality prior to exhausting study samples for full analysis. All ctDNA analyses were performed blind to the treatment regimen and clinical outcomes.

Statistical analysis

DFS was defined as the time from randomization until recurrence, second primary tumor, or death, whichever occurred first, independently of whether patient underwent surgery or not. OS was defined as the time from randomization to death from any cause. The NAR score was developed as a composite short-term endpoint for clinical trials involving neoadjuvant therapy for rectal cancer and was calculated on the basis of clinical (c)T stage (1–4), pathologic (p)T stage (0–4), and pN stage (0–2; Supplementary Data S2). The NAR formula gives a relative weight of five for pN and three for downstaging of T and serves as a pseudocontinuous variable with 24 possible discrete scores from 0 to 100, with higher scores representing a poorer prognosis. On the basis of tertiles, NAR score was validated using the NSABP R-04 clinical trial patient dataset, categorizing the observed scores as low, intermediate, or high (4).

To compare the overall study cohort with the biomarker substudy cohort and identify any statistically significant differences, patient baseline characteristics and clinical outcomes were assessed using χ^2 and Fisher exact test for categorical variables, Spearman and Kendall tau correlations for ordinal variables, and *t* tests and Mann–Whitney (rank sum) test for continuous variables. Differences in survival times were estimated using the Kaplan–Meier method and the log-rank test. HRs were estimated by Cox proportional hazards models. ctDNA results from both study arms were analyzed together.

Data analysis was performed using R software version 3.6.3 or higher. The threshold for statistical significance was established at $P < 0.05$.

Results

Biomarker substudy flowchart is summarized in **Fig. 1**. Paired plasma samples at baseline and after completing TNT (presurgery) were collected from 86 patients (47%) from both treatment arms. After processing, enough plasma at both timepoints was obtained from 72 patients (144 samples) that were included in the biomarker substudy. After ctDNA extraction and quality control (QC) analysis, 62 patients (97 samples) with good quality ctDNA at least at one timepoint were sequenced and included in the analysis.

Clinicopathologic and treatment arm characteristics according to ctDNA status are shown in **Table 1**. Median age was 60 years (range, 33–75) and 64.5% were male. Forty-four tumors (72.1%) were classified as cT3 and 43 (69.4%) as cN2. No significant differences in clinicopathologic features and treatment were found between the subset of patients included versus not included in the ctDNA analysis (Supplementary Table S1).

ctDNA analysis

Six paired baseline and presurgery samples from 3 patients were analyzed in the pilot feasibility assay prior to full-cohort analysis. Four of these (67%) samples passed QC analysis (total cfDNA yield between 40.3 and 120.25 ng). The two samples (33%) that failed QC analysis had less than 10 ng of isolated cfDNA. Following successful completion of the pilot assay, cfDNA analysis of the paired samples from the remaining 69 subjects was completed. Two of 138 samples failed cfDNA extraction (1%). Similar to QC results from the pilot assay, 43 samples (31%) failed QC analysis. The median volume of extracted cfDNA was 7.3 ng in the samples that failed QC analysis as compared with 21.4 ng in the 98 samples that passed QC.

For samples that passed QC, the median baseline cfDNA was 16.64 ng (range, 3.8–110 ng) and median presurgery cfDNA was 24.9 ng (range, 5.7–120.3 ng). No differences between cfDNA total yields were found between treatment arms. Overall, ctDNA was detected in 43 of 52 (83%) of baseline samples and in seven of 45 (15%) of presurgery samples. A total of 23 subjects with paired baseline and presurgery samples showed ctDNA clearance following neoadjuvant treatment (66%), in 4 patients (11%) ctDNA remained positive at both timepoints, and in 7 patients ctDNA remained negative (20%) at both timepoints. In one subject, ctDNA emergence was detected. No differences in ctDNA dynamics were found between the two trial arms. Therefore, we herein report the results of the joint analysis, without distinction by treatment arm.

ctDNA detection and pathologic response to TNT

After completing TNT, 60 patients (97%) went through curative surgery and were included in the analysis. Pathologic analysis of surgical samples revealed pCR in 8 (13.3%) patients, ypT3–4 in 32 (53.3%) patients, and persistent node-positive disease (ypN1–2) in 17 (28.3%) subjects. Divided into tertiles, 12 (19.4%) patients had a low NAR score, 35 (56.5%) patients had a medium NAR score, and 15 (24.2%) patients had a high NAR score.

In the substudy cohort, pCR was neither associated with DFS ($P = 0.24$) nor OS ($P = 0.49$). However, high NAR score was significantly associated with worse DFS ($P = 0.0018$) and worse OS ($P = 0.024$) compared with low or medium NAR score (Supplementary Fig. S1). No statistically significant differences in prognosis were found between patients with low and medium NAR score.

There was no association between baseline ctDNA detection and pCR ($P = 0.134$). Among 45 patients with evaluable presurgery ctDNA, 5 patients achieved a pCR, of which 4 patients were ctDNA negative and 1 patient was ctDNA positive ($P = 0.66$). Presurgery ctDNA detection was not associated with pathologic ypT or ypN status in the rectal surgical specimen ($P = 0.8969$ and $P = 0.586$, respectively; **Table 1**). Neither baseline nor presurgery ctDNA status was associated with NAR score ($P = 0.6$ and $P = 0.9$,

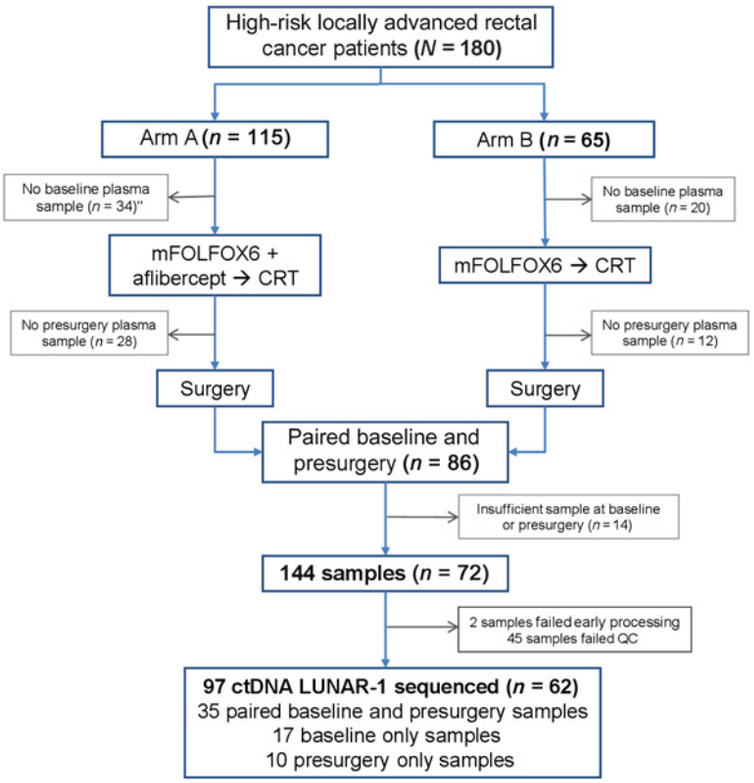

Figure 1.
Study flowchart. Number of patients included in each of the analysis endpoints and reasons for exclusion are depicted. CRT, chemoradiation.

respectively; **Table 1**). Altogether, these data suggest that ctDNA detection is not a predictive biomarker of treatment response on the primary tumor as measured by pCR or NAR.

ctDNA status and risk of recurrence or death

As of December 2019, the median follow-up was 38 months (range, 2.3–51.5 months). During this period of time, 12 of 62 patients (19%) experienced disease recurrence, all of them involving distant sites. Risk of recurrence was not statistically different in the cohort of patients without ctDNA result (Supplementary Table S1).

Baseline ctDNA detection was not associated with survival outcome, neither DFS ($P = 0.59$) nor OS ($P = 0.38$; **Fig. 2**). Moreover, patients with ctDNA clearance following TNT had a similar prognosis to patients with persistent undetectable ctDNA before and after TNT (**Fig. 3A** and **B**).

Patients with presurgery-positive ctDNA had an increased risk of recurrence compared with patients with negative ctDNA [HR, 4.029; 95% confidence interval (CI), 1.004–16.16; $P = 0.033$] and a marked reduced survival (HR, 23; 95% CI, 2.4–212; $P < 0.0001$; **Fig. 2**). Kaplan–Meier estimates for DFS at 3 years were 66% and 84% for patients with presurgery-positive and -negative ctDNA, respectively. At 4 years, 84% of patients with undetectable presurgery ctDNA remained recurrence free compared with 33% of patients with detectable presurgery ctDNA. Similarly, the probability of OS rate was 97% both at 3 and 4 years for patients with undetectable presurgery ctDNA compared with 67% and 33%, respectively, in the detectable presurgery ctDNA subset of patients.

Altogether, presurgery ctDNA was shown to be a promising prognostic marker of recurrence and survival, independently of baseline ctDNA detection and ctDNA dynamics.

ctDNA and site of recurrence

Among 9 patients with disease recurrence and presurgery ctDNA available, 7 patients were limited to a single organ (2 patients in the liver, 3 patients with lung metastasis, and 2 patients with peritoneal involvement) and 2 patients developed multiple metastasis (both including the liver). Presurgery ctDNA was detectable in 3 of 4 patients (75%) who developed liver metastasis during the follow-up compared with 4 of 41 patients (9.8%) who never developed recurrence to the liver ($P = 0.009$; after Bonferroni multiplicity correction, $P = 0.054$). Sensitivity and specificity for presurgery ctDNA and liver recurrence were 0.75 (95% CI, 0.19–0.99) and 0.9 (95% CI, 0.77–0.97). Presurgery ctDNA was undetectable in all patients that recurred to the lung only or peritoneum only (**Fig. 3C**).

ctDNA, CEA, and clinical outcome

Paired ctDNA and CEA was available in 49 of 52 (94%) patients at baseline and 33 of 45 (73%) patients presurgery. Median baseline and presurgery CEA was 3.72 (0.50–115.5) and 1.39 (0.30–7.60), respectively, in patients included in the biomarker substudy.

At baseline, 15 of 49 (31%) patients had concurrent ctDNA positivity and elevated CEA, 26 (53%) had detectable ctDNA, but negative CEA, 4 patients (8%) had only elevated CEA, and the remaining 4 patients were negative for both biomarkers. No association was found between baseline ctDNA and CEA status and clinical outcome (Supplementary Fig. S2A and S2B).

At presurgery, 5 of 33 patients (15%) remained with an elevated CEA, 4 of them with positive ctDNA and 1 with undetectable ctDNA. Patients with both ctDNA positive and CEA presurgery elevated or CEA elevated had worse DFS compared with patients who were negative for both biomarkers ($P = 0.044$). Patients with ctDNA

Table 1. Baseline and treatment characteristics of patients.

		Baseline ctDNA ($n = 52$)			Presurgery ctDNA ($n = 45$)		
	All patients ($N = 62$)	**Negative ($n = 9$)**	**Positive ($n = 43$)**	***P***	**Negative ($n = 38$)**	**Positive ($n = 7$)**	***P***
Age, median (range)	62 (33–75)	66 (45–72)	59 (33–75)	0.143	62.5 (33–77)	66 (44–75)	0.772
Gender, male, *n* (%)	40 (64.5%)	7 (77.8%)	28 (65.1%)	0.462	22 (57.9%)	5 (71.4%)	0.502
Treatment, *n* (%)							
mFOLFOX6 + aflibercept	36 (58.1%)	8 (88.9%)	21 (48.8%)	0.028	23 (60.5%)	3 (42.9%)	0.384
mFOLFOX6	26 (41.9%)	1 (11.1%)	22 (51.2%)		15 (39.5%)	4 (57.1%)	
cT stage, *n* (%)							
T2	1 (1.6%)	0 (0.0%)	1 (2.4%)	0.085	1 (2.6%)	0 (0.0%)	0.1026
T3	44 (72.1%)	9 (100%)	28 (66.7%)		24 (63.2%)	7 (100.0%)	
T4	16 (26.2%)	0 (0.0%)	13 (31.0%)		13 (34.2%)	0 (0.0%)	
Missing	1	0	1		0	0	
cN stage, *n* (%)							
1	19 (30.6%)	5 (55.6%)	12 (27.9%)	0.11	13 (34.2%)	1 (14.3%)	0.3008
2	43 (69.4%)	4 (44.4%)	31 (72.1%)		25 (65.8%)	6 (85.7%)	
Tumor location, *n* (%)							
Middle	44 (71%)	7 (77.8%)	32 (74.4%)	1	28 (73.7%)	6 (85.7%)	1
Distal	18 (29%)	2 (22.2%)	11 (25.6%)		10 (26.3%)	1 (14.3%)	
CEA basal, median (range)	3.7 (0.5–115.5)	5.37 (1.9–18.9)	3.3 (0.5–115.5)	0.903	5.51 (0.5–115.5)	3.52 (2–5.6)	0.202
ypT stage, *n* (%)							
ypT0	8 (13.3%)	2 (22.2%)	2 (4.8%)	0.19	4 (10.5%)	1 (16.6%)	0.897
ypTis	2 (3.3%)	0 (0.0%)	2 (4.8%)		2 (5.3%)	0 (0.0%)	
ypT1	3 (5%)	0 (0.0%)	1 (2.3%)		2 (5.3%)	0 (0.0%)	
ypT2	15 (25%)	3 (33.3%)	10 (23.8%)		10 (26.3%)	2 (33.3%)	
ypT3	30 (50%)	4 (44.4%)	25 (59.5%)		19 (50.0%)	3 (50%)	
ypT4	2 (3.3%)	0 (0.0%)	2 (4.8%)		1 (2.6%)	0 (0.0%)	
Missing	2	0	1		0	1	
ypN stage, *n* (%)							
0	43 (71.6%)	6 (66.7%)	29 (69%)	0.928	26 (68.4%)	4 (66.6%)	0.586
1	12 (20%)	3 (33.3%)	8 (19%)		11 (28.9%)	0 (0.0%)	
2	5 (8.3%)	0 (0.0%)	5 (12%)		1 (2.6%)	2 (33.3%)	
Missing	2	0	1		0	1	
Pathologic response, *n* (%)							
pCR	8 (13.3%)	2 (22.2%)	2 (4.8%)	0.077	4 (10.5%)	1 (16.7%)	0.660
Residual disease	52 (86.7%)	7 (77.8%)	40 (95.2%)		34 (89.5%)	5 (83.3%)	
Missing	2	0	1		0	1	
NAR, median (range)	8.4 (0–50.4)	8.43 (0.94–30.07)	14.98 (0.94–50.36)	0.556	8.43 (0–50.36)	11.71 (0.94–50.36)	0.181
NAR, *n* (%)							
Low	12 (19.4%)	2 (22.2%)	5 (11.6%)	0.598	17 (44.7%)	3 (42.8%)	0.900
Medium	35 (56.5%)	4 (44.4%)	26 (60.5%)		19 (50%)	3 (42.8%)	
High	15 (24.2%)	3 (33.3%)	12 (27.9%)		2 (5.3%)	1 (14.3%)	
CEA presurgery, median (range)	1.4 (0.3–7.6)	1.41 (1.1–3.68)	1.38 (0.3–7.6)	0.903	1.38 (0.5–6.93)	1.53 (1.23–4)	0.646

positive and CEA presurgery elevated had a significant worse OS compared with patients with both biomarkers negative or CEA positive, but ctDNA undetectable ($P = 0.014$; Supplementary Fig. S2C and S2D).

Discussion

GEMCAD 1402 was a phase II randomized, multicentric clinical trial which met its primary endpoint proving that the addition of aflibercept to mFOLFOX6 induction chemotherapy improves pCR rate compared with mFOLFOX6 alone (13.8% and 22.6%, respectively; $P = 0.15$; ref. 16) in patients with high-risk LARC. However, with a follow-up of 38 months, no benefit in DFS has been observed (18).

The current preplanned biomarker substudy from the GEMCAD 1402 trial shows that ctDNA before surgery is a promising predictive and prognostic biomarker that significantly detects MMD and predicts distant recurrence and survival in patients receiving a TNT approach. Patients with presurgery ctDNA detection had a worse DFS compared with ctDNA undetectable patients (HR, 4.029; $P = 0.033$). Even more dramatic was the impact of ctDNA on OS. Patients with detectable presurgery ctDNA had a statistically significant increase in the risk of death (HR, 23; $P < 0.0001$), with an estimated 4-year OS of 33% compared with 97% for patients with undetectable presurgery ctDNA. Similarly, previous studies in localized colon (13–15, 19) and two recent studies in patients with LARC treated with standard preoperative CRT (20, 21) have shown the value of ctDNA to predict distant recurrence. To the best of our knowledge, this study is the first to demonstrate the value of ctDNA to predict not only recurrence, but also OS in LARC. This may be explained, in part, by the fact that patients had received full-dose systemic chemotherapy to potentially treat micrometastatic disease as part of a TNT strategy. Another important differential characteristic of this study is the technical approach for detecting ctDNA. While most previous studies of MRD in LARC had used droplet digital PCR to interrogate plasma-specific

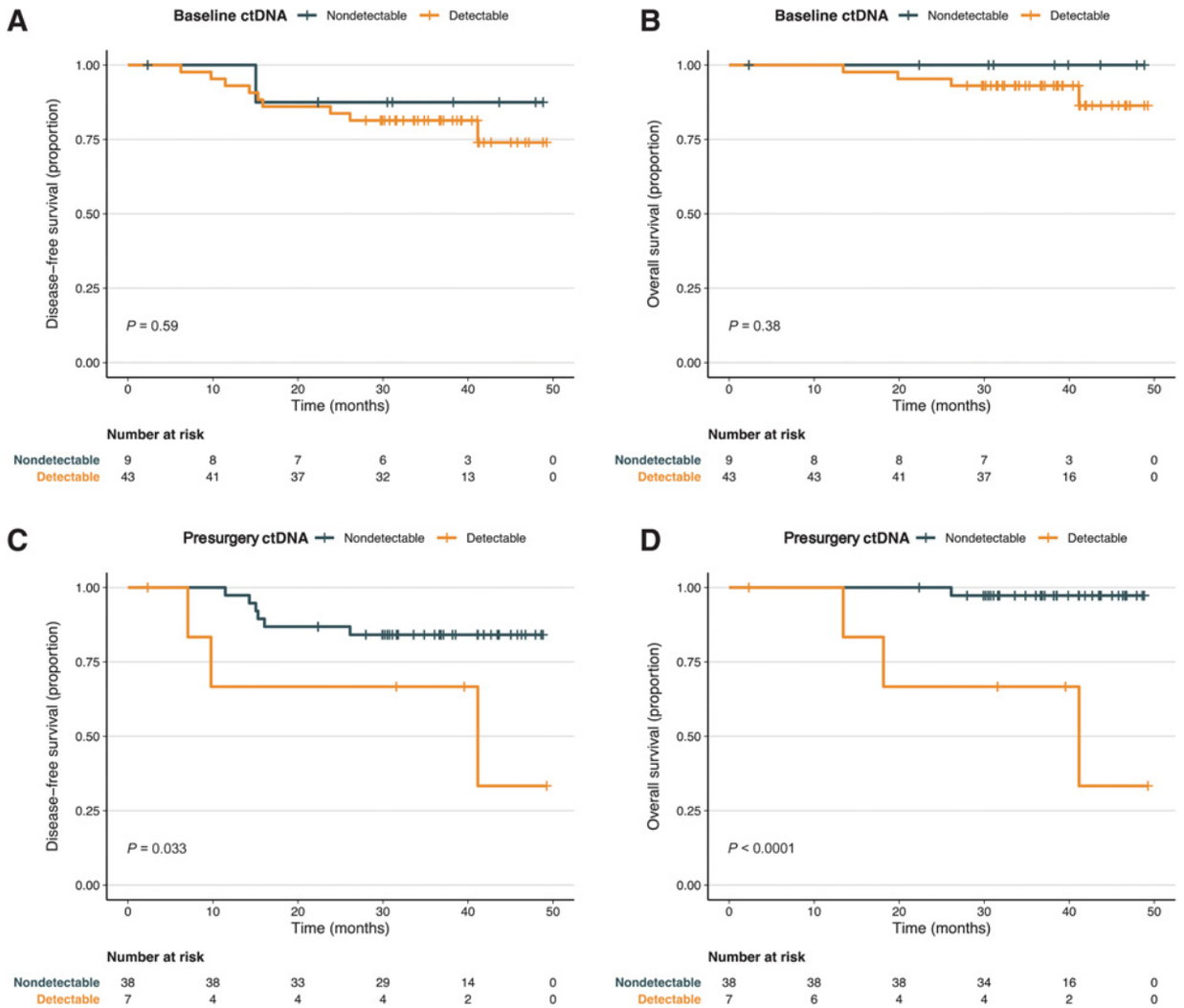

Figure 2.

Kaplan–Meier estimates of DFS and OS according to baseline and presurgery ctDNA status. **A,** DFS in patients with baseline ctDNA detectable versus nondetectable. **B,** OS in patients with baseline ctDNA detectable versus nondetectable. **C,** DFS in patients with presurgery ctDNA detectable versus nondetectable. **D,** OS in patients with presurgery ctDNA detectable versus nondetectable.

mutations detected previously in tumor tissue, this study used a next-generation sequencing approach that integrated both genomic and epigenomic signatures resulting in a more sensitive and more accurate detection of MMD before surgery.

Although presurgery blood samples were obtained within 48 hours before surgery, no association between ctDNA detection and pCR rates was found. These results are in line with other studies in LARC (18, 20, 21), supporting the idea that ctDNA analysis cannot differentiate between minimal versus no residual local disease. Moreover, we did not find a relationship with the NAR score. The prognostic role of NAR score by using the pathologic lymph node staging and the change between clinical and pathologic tumor stage were validated in the CAO/ARO/AIO-04 clinical trial (7). The lack of correlation between ctDNA and NAR score in our study suggests that ctDNA does not mirror changes in primary tumor staging. Our study supports the concept that ctDNA specifically reflects the presence of systemic disease rather than local minimal disease.

The concept of MRD refers to the persistence of disease after a potential curative surgery. We have proposed the term MMD to refer to the detection of ctDNA following total neoadjuvant systemic therapy in patients that are likely to recur at distant sites.

In the past years, there has been a dramatic improvement in the development of ctDNA technology. Currently, the efficacy of liquid biopsy is mainly limited by biology rather than a lack of technical sensitivity. In this study, although small numbers are presented, we can hypothesize that ctDNA may predict distant recurrence involving the liver more than metastasis to the peritoneum only or the lung only. This difference in ctDNA sensitivity related to the localization of the disease was also shown in previous works from our group and others in the metastatic setting (9, 22, 23). Another possibility is that this is related to tumor size, tumor growth rate, or cell turnover, as recently described using a mathematical theoretical model of ctDNA shedding in stage I–III lung cancer (24). A biological-based discovery to understand the multiple processes

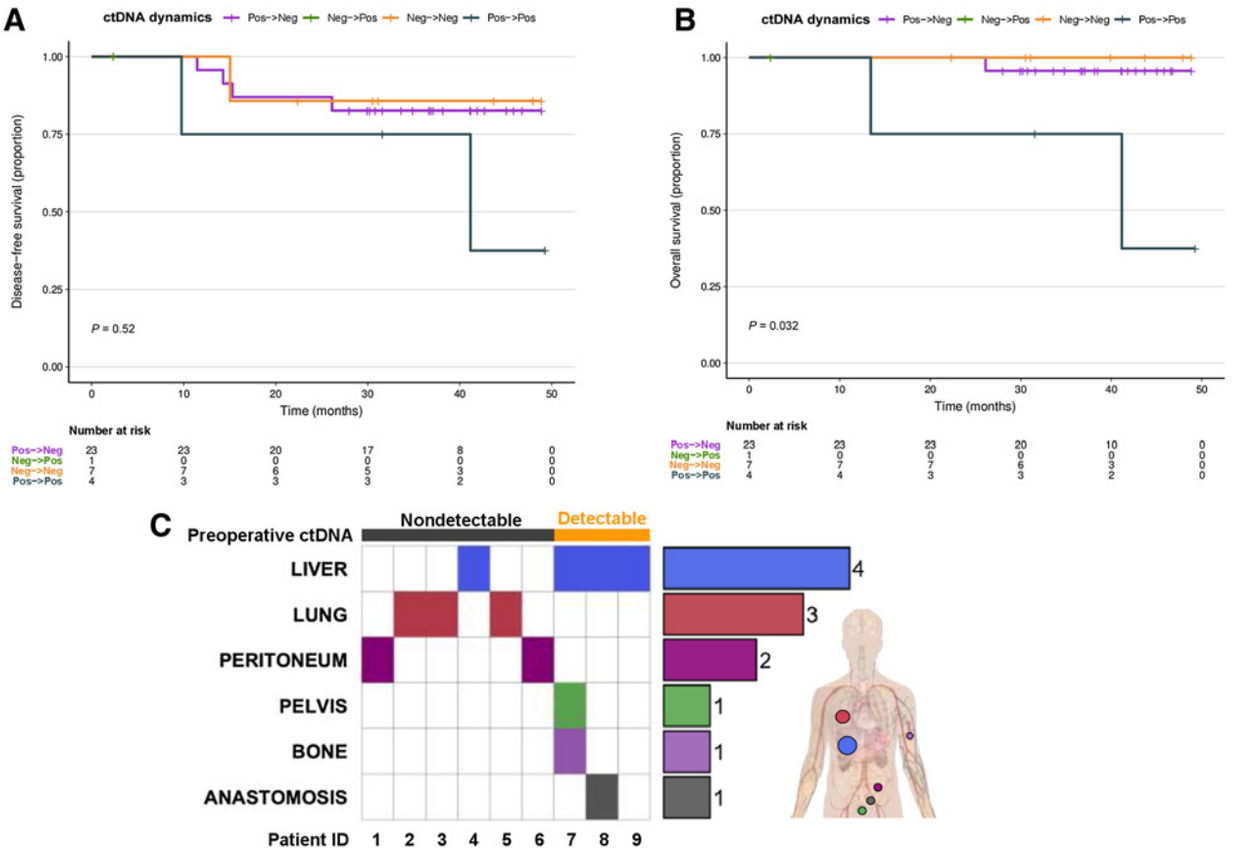

Figure 3.

ctDNA tumor dynamics and recurrence pattern. **A,** DFS according to ctDNA changes at baseline and presurgery. **B,** OS according to ctDNA changes at baseline and presurgery. Only patients with paired baseline and presurgery ctDNA are represented in both Kaplan–Meier curves. In purple, patients with baseline ctDNA positive (pos) and presurgery ctDNA negative (neg; clearance); in green, patients with baseline ctDNA negative and presurgery ctDNA positive (emergence); in orange, patients with baseline ctDNA negative and presurgery ctDNA negative (remained negative); and in gray, patients with baseline ctDNA positive and presurgery ctDNA positive (remained positive). **C,** Presurgery ctDNA detection and recurrence pattern. Nine patients with tumor recurrence during the follow-up are shown. For each patient, the location of metastasis and presurgery ctDNA results are shown. Presurgery ctDNA from patient 7 detected epigenomic changes (methylation). Presurgery ctDNA from patient 8 was positive for a somatic mutation (APC R232X). Presurgery ctDNA from patient 9 detected epigenomic changes and a somatic mutation (APC p.Pro1439fs).

involved in cfDNA metabolism will be critical for further development of liquid biopsy.

This study is mainly limited by the small subset of analyzed patients from an initially large cohort. This was due to the limitation of some participating centers to collect and process the blood samples and the very small quantity of plasma collected because the study was designed to extract only 4.5 mL of blood at each timepoint. While QC analysis allowed for inclusion of only samples with sufficient DNA quality for analysis, lower sample volume and cfDNA input may reduce the sensitivity of this assay to detect ctDNA. One strength is the fact that all samples were collected within a prospective clinical trial which guarantees good sample quality from a homogeneous group of patients, and reliable and robust results. The small subset of patients available for analysis also precluded us from doing robust multivariable analysis that would potentially establish the independent role of ctDNA positivity and other known prognostic variables, such as ypN2 or pCR.

In conclusion, this study suggests that in patients with high-risk LARC treated with a TNT approach, presurgery liquid biopsy detects systemic MMD. Hence, it is a significant predictive biomarker of systemic relapse (mainly to the liver) and ultimately a prognostic biomarker of death. Accordingly, future studies should evaluate whether presurgery ctDNA-positive patients should be managed as patients with metastatic rectal tumors (i.e., switch to chemotherapy, spare rectal surgery, and intensify treatment). This proof-of-concept study sets the basis for randomized prospective trials to investigate whether ctDNA-based therapeutic strategies improve outcomes in LARC treated with a TNT approach.

Authors' Disclosures

J. Vidal reports grants from SEOM (Sociedad Española de Oncología Médica) and TTD (Grupo de Tratamiento de los Tumores Digestivos) during the conduct of the study, and personal fees from Hoffman La-Roche, Merck-Serono, Sanofi, Guardant Health, and Bristol Myers Squibb outside the submitted work. B. Bellosillo reports personal fees from Biocartis, Thermo Fisher Scientific, Qiagen, Merck-Serono, BMS, and Pfizer, and grants and personal fees from Novartis, AstraZeneca, and Roche outside the submitted work. C. Pericay reports personal fees and nonfinancial support from Merck, and personal fees from Roche, Amgen, and Sanofi outside the submitted work. R. Garcia-Carbonero reports grants from Sanofi during the conduct of the study, and personal fees from AAA, Advanz Pharma, Bayer, HMP, Ipsen, Merck, Midatech Pharma, MSD, Novartis, PharmaMar, Pfizer, Pierre Fabre, Roche, Sanofi,

and Servier outside the submitted work. L. Layos reports personal fees from Sanofi, Ipsen, Merck, Amgen, and Roche outside the submitted work. V. Alonso reports personal fees from Servier, Amgen, Sanofi, Merck Serono, Ipsen, Advanced Accelerator Applications, and Roche outside the submitted work. J. Capdevila reports grants and personal fees from Bayer, Eisai, Ipsen, and Adacap; personal fees from Lilly, Exelixis, Novartis, Pfizer, Hutchinson MediaPharma, and Merck; and grants from AstraZeneca and Roche outside the submitted work. R. Vera reports personal fees from Roche, MSD, Sanofi, Lilly, Amgen, and Merck outside the submitted work. E. Cillán reports personal fees and nonfinancial support from Amgen, Merck-Serono, and Hoffman La-Roche, and nonfinancial support from Sanofi outside the submitted work. J. Maurel reports grants from Amgen, Merck, and Incyte; nonfinancial support from NanoString; personal fees from Advance Medical, Cancer Expert Now, Fundacion Clinica Universitaria, Sirtex, Pierre-Fabre, Servier, AstraZeneca, Roche, Sanofi, and Shire; and nonfinancial support from Biocartis outside the submitted work. I. Faull reports personal fees from Guardant Health Inc outside the submitted work. C. Fernández-Martos reports personal fees from Sanofi and grants from Amgen outside the submitted work. C. Montagut reports personal fees from Sanofi and grants and personal fees from Merck Serono, Roche, and Biocartis outside the submitted work. No disclosures were reported by the other authors.

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Acknowledgments

The authors thank the patients, their families, and the sites participating in the study. Data management support was provided by PIVOTAL S.L.U. This study was supported by Instituto de Salud Carlos III – FEDER grant PI18/00031 and Fundacion CRIS (Excellence 19-30) grant (to C. Montagut). J. Vidal was supported by SEOM and TTD grants. D. Casadevall was supported by ISCIII (Juan Rodés Research Contract JR18/00003).

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Received December 9, 2020; revised February 6, 2021; accepted March 10, 2021; published first March 16, 2021.

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