European Journal of Surgical Oncology xxx (xxxx) xxx

Contents lists available at ScienceDirect

European Journal of Surgical Oncology

journal homepage: www.ejso.com

Circulating tumor DNA detection after neoadjuvant treatment and surgery predicts recurrence in patients with early-stage and locally advanced rectal cancer

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ARTICLE INFO

Article history:
Received 3 November 2022
Received in revised form
30 December 2022
Accepted 24 January 2023
Available online xxx

Keywords:
Rectal cancer
Circulating tumor DNA
Neoadjuvant treatment
Liquid biopsies
Next-generation sequencing

ABSTRACT

Introduction: Patients with early-stage and locally advanced rectal cancer are often treated with neoadjuvant therapy followed by surgery or watch and wait. This study evaluated the role of circulating tumor DNA (ctDNA) to measure disease after neoadjuvant treatment and surgery to optimize treatment choices.

Materials and methods: Patients with rectal cancer treated with both chemotherapy and radiotherapy were included and diagnostic biopsies were analyzed for tumor-specific mutations. Presence of ctDNA was measured in plasma by tracing the tumor-informed mutations using a next-generation sequencing panel. The association between ctDNA detection and clinicopathological characteristics and progression-free survival was measured.

Results: Before treatment ctDNA was detected in 69% (35/51) of patients. After neoadjuvant therapy ctDNA was detected in only 15% (5/34) of patients. In none of the patients with a complete clinical response who were selected for a watch and wait strategy (0/10) or patients with ypN0 disease (0/8) ctDNA was detected, whereas it was detected in 31% (5/16) of patients with ypN + disease. After surgery ctDNA was detected in 16% (3/19) of patients, of which all (3/3) developed recurrent disease compared to only 13% (2/16) in patients with undetected ctDNA after surgery. In an exploratory survival analysis, both ctDNA detection after neoadjuvant therapy and after surgery was associated with worse progression-free survival ($p = 0.01$ and $p = 0.007$, respectively, Cox-regression).

Conclusion: These data show that in patients with early-stage and locally advanced rectal cancer tumor-informed ctDNA detection in plasma using ultradeep sequencing may have clinical value to complement response prediction after neoadjuvant therapy and surgery.

1. Introduction

Colorectal cancer is the third most commonly occurring cancer worldwide and almost 40% of these patients present with a tumor in the rectum [1]. The curative treatment strategy for most patients with early-stage and locally advanced rectal cancer consists of neoadjuvant radiotherapy with or without chemotherapy followed by surgery [2]. In the Netherlands, the conventional neoadjuvant

Abbreviations: CEA, carcinoembryonic antigen; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; CI, confidence interval; HR, hazard ratio; NGS, next-generation sequencing; VAF, variant allele frequency.

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https://doi.org/10.1016/j.ejso.2023.01.026

chemoradiotherapy was compared to total neoadjuvant therapy, consisting of short-course radiation followed by chemotherapy in the RAPIDO study [3]. This intensified neoadjuvant treatment strategy increased pathological complete response rates and is now often used as an alternative treatment in rectal cancer patients [3]. The observation of a substantial number of patients with a pathological complete response has led to emergence of an active surveillance strategy as an alternative to surgery with the benefit of organ preservation [4,5]. This so-called watch and wait policy is now frequently applied when complete response is suspected based on endoscopy and magnetic resonance imaging [6]. Despite improvements in local response, development of distant metastases after rectal cancer surgery continues to be the main cause of treatment failure [2,3]. Reliable biomarkers of treatment efficacy are required to optimize treatment management of patients with rectal cancer by improving both identification of good candidates for watch and wait policies as well as prediction of patients at risk of metastasis that potentially require intensification of systemic treatment.

The use of circulating tumor DNA (ctDNA) measurements for noninvasive and molecular assessment of colorectal tumors looks promising. Several studies reported on ctDNA analysis in patients with non-metastatic colorectal cancer for postoperative assessment of residual disease [7–10]. Additionally, studies specifically focusing on rectal cancer showed additive value of ctDNA levels to monitor response in the neoadjuvant setting of non-metastatic patients [11–13]. Furthermore, studies also showed the potential of postoperative ctDNA measurements to predict risk of recurrence in patients with non-metastatic rectal cancer [14–16]. More evidence could further establish the value of ctDNA analysis to improve personalized treatment management in these patients. Therefore, in this study, we evaluated the role of ctDNA measurements in patients with both early-stage and locally advanced rectal cancer using a tumor-informed ultradeep sequencing approach to identify patients with a good clinical response after neoadjuvant treatment as well as to predict patients at risk for metastasis.

2. Materials and Methods

2.1. Patient and sample collection

In this prospective observational study patients with rectal cancer who underwent neoadjuvant therapy in the Radboud university medical center, Institute for Radiation Oncology Arnhem, or Institute Verbeeten between November 2017 and July 2021 were consecutively enrolled. Both patients with early-stage rectal cancer (cT1-3N0) and locally advanced rectal cancer (cT1-3N+ and cT4Nx) were included. Patients with early-stage rectal cancer underwent radiotherapy of 25 fractions of 2 Gy daily, five times a week, with concomitant fluorouracil (5FU) chemotherapy taken orally. Patients with locally advanced rectal cancer underwent this treatment or the experimental arm of the RAPIDO study which consists of short-course radiotherapy (five fractions of 5 Gy) followed by chemotherapy consisting of capecitabine and oxaliplatin (CAPOX). There were no specific criteria for patients treated with standard chemoradiotherapy or short-course radiotherapy followed by chemotherapy and treatment in our region is based on shared decision making. Longitudinal plasma samples were obtained before treatment (T0), 6–8 weeks after neoadjuvant therapy (T1), and 1–2 weeks after surgery (T2). The ctDNA analysis was performed after collection of all plasma samples and clinicians were not aware of the ctDNA results during the treatment trajectory. Clinical data of all patients, such as cTNM and pretreatment carcinoembryonic antigen (CEA) levels, were retrieved. For estimation of tumor size before treatment the gross tumor volume (GTV, in cm^3) was used that is calculated by drawing the primary tumor on each relevant slice of the planning computed tomography scan prior to radiotherapy [17]. The clinical response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) [18]. Follow-up data were collected during three to six monthly visits at the outpatient clinic, according to the Dutch Colorectal Cancer guidelines. Survival data were requested from the Netherlands Cancer Registry (NCR). The study was ethically approved by the Internal Review Board of the Radboudumc (CMO 2017–3498). The study meets the criteria of the code of proper use of human samples of the Netherlands and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent before enrollment.

2.2. Tissue and plasma analysis

For all patients, diagnostic tissue biopsies were obtained and histologically evaluated by a gastrointestinal pathologist (RSvdP). Additionally, data was collected from evaluations of resection specimens for surgical margins, tumor diameter, pathological response and ypTNM status. Pathological response was classified according to tumor regression grade [19]. For molecular analysis tumor DNA was isolated by microdissection from the formalin-fixed paraffin-embedded diagnostic biopsies using the Chelex-100 (Bio-Rad, Hercules, California) method as previously described [20]. DNA concentrations were measured using the Qubit Broad Range kit (Thermo Fisher, Waltham, Massachusetts).

Blood samples were collected in special cell-free DNA (cfDNA) collection tubes (Roche, Basel, Switzerland) and processed within four days using two centrifugation steps: first at 1,600 g for 10 min to isolate plasma and subsequently at 16,000 g for 10 min to remove cellular debris. Plasma was stored at −80 °C until further processing. Isolation of cfDNA from 6 to 10 ml plasma was performed with QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany). cfDNA concentrations were measured using the Qubit High Sensitivity kit (Thermo Fisher).

For next-generation sequencing (NGS) analysis tumor tissue-derived DNA was mechanically sheared with a focused-ultrasonicator instrument (Covaris, Woburn, Massachusetts) and 150 ng of this sheared tissue DNA was used as input. For cfDNA, input ranged from 18 to 56 ng with a mean of 46 ng. Library preparation was performed with the Twist Library Preparation Kit (Twist Biosciences, San Francisco, California) in combination with xGEN consensus sequencing adaptors (IDT, Coralville, Iowa) with dual index and unique molecular identifiers. A hybridization capture was performed on the prepared libraries with a customized probe set (Twist Biosciences) covering 117 kb (Supplemental Table S1). This panel also includes 56 mononucleotide repeat markers to measure microsatellite instability in the tissue samples. Paired-end sequencing was performed on a NovaSeq 6000 instrument (Illumina, San Diego, California) using 2 × 150 cycles.

2.3. NGS data analysis

NGS data analysis was performed as described earlier [21]. cfDNA samples were sequenced to a total mean depth of 48,312× and after deduplication and singleton filtering the mean depth was 3,694×. For tissue-derived DNA this was 27,849× and 2,582×, respectively. Variant calling was performed on unique reads using Genomic Analysis ToolKit (GATK) Mutect2 (version 4.1.5.0, Broad Institute, Cambridge, Massachusetts). Exclusion of germline variants was done using population databases and comparison of variant allele frequencies (VAFs) with estimated tumor cell percentage. Only variants detected with Mutect2 in tissue were retrieved from the variants called with Mutect2 in plasma. The tumor-specific variants were also manually checked in

corresponding plasma samples in Integrative Genomics Viewer (IGV) (version 2.4, University of California, San Diego, California and Broad Institute, Cambridge, Massachusetts). Plasma from 22 healthy donors was used to create a panel of normals and this was used for variant calling with Mutect2. Subsequently, the 22 healthy plasma samples were used for exclusion of platform- and panel-specific artifacts and estimation of variant-specific background noise. To call somatic variants with a specificity of 99% a minimum number of mutant reads and a value for the variant-specific background was determined using artificial human control template DNA standards (SeraCare, Milford, Massachusetts and Horizon Discovery, Cambridge, United Kingdom). Based on this evaluation, the variant-specific background was calculated for every variant by multiplying the mean variant-specific background noise by fifteen and only variants with at least four unique mutant reads and a VAF higher than the variant-specific background were designated as true plasma variants. Plasma samples with at least one tumor-specific variant detected were called positive for ctDNA. The number of mutant molecules per ml plasma was calculated with the mean mutant VAF, volume of plasma used for isolation and the total number of unique deduplicated cfDNA molecules.

2.4. Statistical analysis

Differences in tumor characteristics and clinical and pathological measurements were compared using the Fisher exact test for categorical variables and the Mann-Whitney (rank sum) test or Kruskal-Wallis test for continuous variables. Correlation was assessed using Spearman rank correlation coefficient. For survival estimates we employed the Kaplan-Meier method. Differences in survival were compared with the Cox Proportional Hazards regression model to estimate hazard ratios (HR) with confidence intervals (CI). Statistical tests were performed in IBM SPSS Statistics (version 25) and figures were generated using R software (version 4.1.2) and GraphPad Prism (version 5.03). All p-values were based on two-sided testing and p-values <0.05 were considered significant.

3. Results

3.1. Patient characteristics

In this prospective observational study 58 patients with early-stage or locally advanced rectal cancer were enrolled. Diagnostic tissue biopsies for somatic mutation analysis were available for 51 patients (Supplemental Fig. S1). A total of 171 somatic mutations were detected in the 51 tumor samples with at least one tumor-specific mutation in 100% of tissues (Supplemental Table S2, Fig. 1). Most mutations were found in *APC*, *TP53*, and *KRAS*. None of the patients presented with a microsatellite unstable tumor.

The 51 patients with tumor analysis were included for ctDNA evaluation. A total of 104 plasma samples were analyzed. In these patients the median age was 66 years and 73% of the patients were male (Table 1). The majority of patients presented with cT3 disease (77%) and cN+ disease (90%). All patients received neoadjuvant therapy. Most patients (n = 37) were treated with standard chemoradiotherapy and fourteen patients underwent short course radiotherapy followed by chemotherapy. After neoadjuvant treatment 32 patients underwent surgery. In 19 patients, of which 2 had early-stage and 17 patients had locally advanced disease, a watch and wait policy followed. For 18/19 (95%) of patients this was applied based on a complete clinical response.

3.2. ctDNA analysis prior to treatment

At least one tumor-specific mutation could be identified in the plasma sample collected prior to treatment (T0) in 69% (35/51) of patients (Supplemental Table S2). Detection of ctDNA at this timepoint was higher in patients with a more advanced cN stage (86% in cN2 vs 40% in cN0, p = 0.01, Fisher exact) and patients with detected ctDNA had a significantly higher tumor volume (median 49 cm^3 vs 25 cm^3, p = 0.003, Mann-Whitney) (Table 1, Fig. 2A). The absolute levels of ctDNA (mean mutant molecules per ml plasma) were associated with tumor volume (ρ = 0.515, p = 0.0001, Spearman rank) and were significantly higher in patients with cN2 compared to cN0 (p = 0.005, Kruskal-Wallis) (Fig. 2B and C). CEA levels showed no association with ctDNA levels and tumor volume (p = 0.102 and p = 0.105, respectively, Spearman rank) (Supplemental Fig. S2). If ctDNA was not detected at T0, it consistently remained undetected at T1 and T2.

3.3. ctDNA analysis during treatment

In 34 of the total group of 51 patients a plasma sample 6–8 weeks after neoadjuvant therapy (T1) was collected. In ten of these 34 patients a clinically complete response was identified during restaging and a watch and wait strategy was started. The other 24 patients had residual disease at restaging and received surgery. At this timepoint ctDNA was detected in only 15% (5/34) of patients. More specifically, in all patients with a complete clinical response (0/10) or ypN- disease (0/8) ctDNA was not detected, whereas ctDNA was detected in 31% (5/16) of patients with ypN+ disease (Fig. 3).

In patients with detected ctDNA at T0, ctDNA was detected at T1 in 23% (5/22) of patients. ctDNA was detected in 39% (5/13) of patients with ypN+ disease. ctDNA was not detected in patients with a complete clinical response (0/4) or ypN- disease (0/5).

A postoperative plasma sample (T2) was available for only nineteen patients. In 16% (3/19) of these patients ctDNA was still detected after surgery. In patients with detected ctDNA at T0 ctDNA was detected at T2 in 21% (3/14). All these three patients with detected ctDNA after surgery developed distant metastases, while this was the case for only 13% (2/16) of patients with undetected ctDNA after surgery (Supplemental Fig. S3, Supplemental Fig. S4). ctDNA detection at both timepoints (T1 and T2) was not associated with neoadjuvant treatment regimen (p = 0.617 and p = 0.517, respectively, Fisher exact).

3.4. Association of ctDNA detection with progression-free survival

For the 36 patients with ctDNA analysis after neoadjuvant therapy (T1) with or without surgery median follow-up was 13 months (range 1–50 months) (Supplemental Fig. S4). In an exploratory survival analysis, patients with detected ctDNA after neoadjuvant therapy showed an increased risk of disease progression (HR 6.5; 95% CI 1.4–30.3, p = 0.02, Cox-regression) compared to patients with undetected ctDNA after neoadjuvant therapy (Fig. 4A). Also, patients with detected ctDNA after surgery had a higher risk of disease progression (HR 10.9; 95% CI 1.1–106.7, p = 0.04, Cox-regression) compared to patients with undetected ctDNA after surgery (Fig. 4B). Both associations were independent of ctDNA status at T0, which was not associated with progression-free survival (HR 0.9; 95% CI 0.3–2.5, p = 0.76, Cox-regression).

4. Discussion

In this study we evaluated the role of ctDNA measurements in patients with both early-stage and locally advanced rectal cancer after neoadjuvant treatment and surgery or watch and wait using ultradeep sequencing. After neoadjuvant treatment ctDNA could not be detected in patients with a clinically complete response who

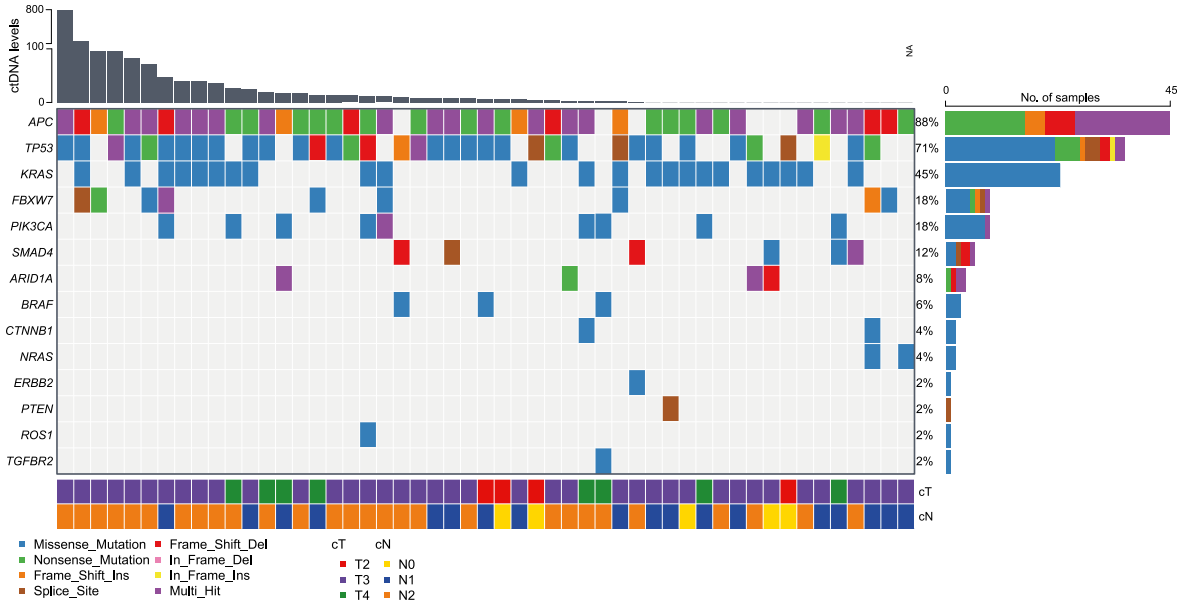

Fig. 1. Overview of all somatic mutations found in tumor tissue of patients (n = 51). Different patients are shown horizontally and the tissue alterations vertically with different colors. Clinical features are shown at the bottom. The top bar displays the ctDNA levels (mutant molecules/ml plasma) of the tumor-informed mutations detected in the paired plasma sample prior to treatment.

Table 1
Clinicopathological characteristics of patients with rectal cancer (n = 51). The total number of patients with plasma analysis and the ctDNA detection at T0 is shown with a percentage. p-values were calculated using Fisher exact test and Mann-Whitney test. Fluorouracil (5FU), capecitabine and oxaliplatin (CAPOX), low anterior resection (LAR), abdominoperineal resection (APR), transanal total mesorectal excision (taTME).

Clinicopathological characteristics			All patients (n = 51)	ctDNA before treatment (T0) (n = 51)		
				Negative	Positive	p-value
Age, median (range)			66 (48–84)	66 (52–80)	66 (48–84)	0.94
Gender, n (%)	Male		37 (73)	11 (30)	26 (70)	0.74
	Female		14 (28)	5 (36)	9 (64)	
Disease	Early (cT1-3N0)		5 (10)	3 (60)	2 (40)	0.31
	Locally advanced (cT1-3N+, cT4Nx)		46 (90)	13 (28)	33 (72)	
Location tumor	Upper rectum		5 (10)	0 (0)	5 (100)	0.19
	Mid rectum		13 (26)	3 (23)	10 (77)	
	Low rectum		33 (65)	13 (39)	20 (61)	
cT stage, n (%)	cT2		4 (8)	1 (25)	3 (75)	1.00
	cT3		39 (77)	13 (33)	26 (67)	
	cT4		8 (16)	2 (25)	6 (75)	
cN stage, n (%)	cN0		5 (10)	3 (60)	2 (40)	0.01
	cN1		18 (35)	9 (50)	9 (50)	
	cN2		28 (55)	4 (14)	24 (86)	
Gross tumor volume (cm^3), median (range)			42 (7–542)	25 (7–88)	49 (12–542)	0.003
CEA levels, median (range)			5 (0.6–929	4 (0.6–51)	5 (1–929)	0.334
Chemoradiotherapy, n (%)	25 × 2 Gy and 5FU		37 (73)	11 (30)	26 (70)	0.74
	5 × 5 Gy followed by CAPOX		14 (28)	5 (36)	9 (64)	
Clinical response (RECIST)	Complete response		18 (35)	8 (44)	10 (56)	0.45
	Partial response		29 (57)	8 (28)	21 (72)	
	Stable disease		3 (6)	0 (0)	3 (100)	
	Progressive disease		1 (2)	0 (0)	1 (100)	
Treatment traject, n (%)	Watch and wait		19 (37)	8 (42)	11 (58)	0.23
	Surgery		32 (63)	8 (25)	24 (75)	
	Type of surgery	LAR	15 (47)	4 (27)	11 (73)	0.81
		APR	15 (47)	4 (27)	11 (73)	
		Total exenteration	1 (3)	0 (0)	1 (100)	
		taTME	1 (3)	0 (0)	1 (100)	
	ypT stage, n (%)	ypT0	1 (3)	0 (0)	1 (100)	0.45
		ypT1	4 (13)	2 (50)	2 (50)	
		ypT2	12 (38)	4 (33)	8 (67)	
		ypT3	14 (44)	2 (14)	12 (86)	
		ypT4	1 (3)	0 (0)	1 (100)	
	ypN stage, n (%)	ypN0	11 (34)	4 (36)	7 (64)	0.42
		ypN1	18 (56)	4 (22)	14 (78)	
		ypN2	3 (9)	0 (0)	3 (100)	

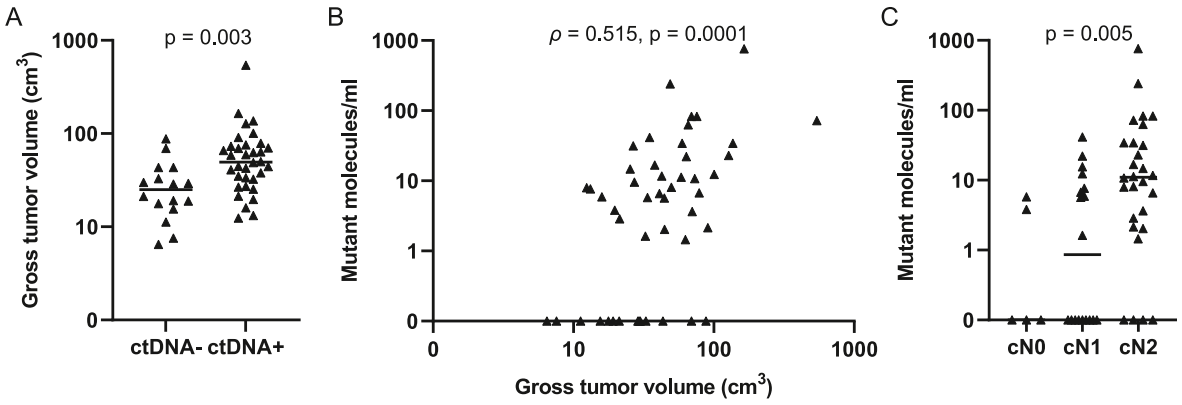

Fig. 2. ctDNA prior to treatment in association with clinical characteristics (n = 51). **A)** Comparison of ctDNA detection with tumor volume. Comparison of levels of ctDNA (mutant molecules per ml plasma) with **B)** tumor volume and **C)** cN stage. Correlation was assessed using Spearman rank correlation coefficient and p-values are calculated using Mann-Whitney test and Kruskal-Wallis test.

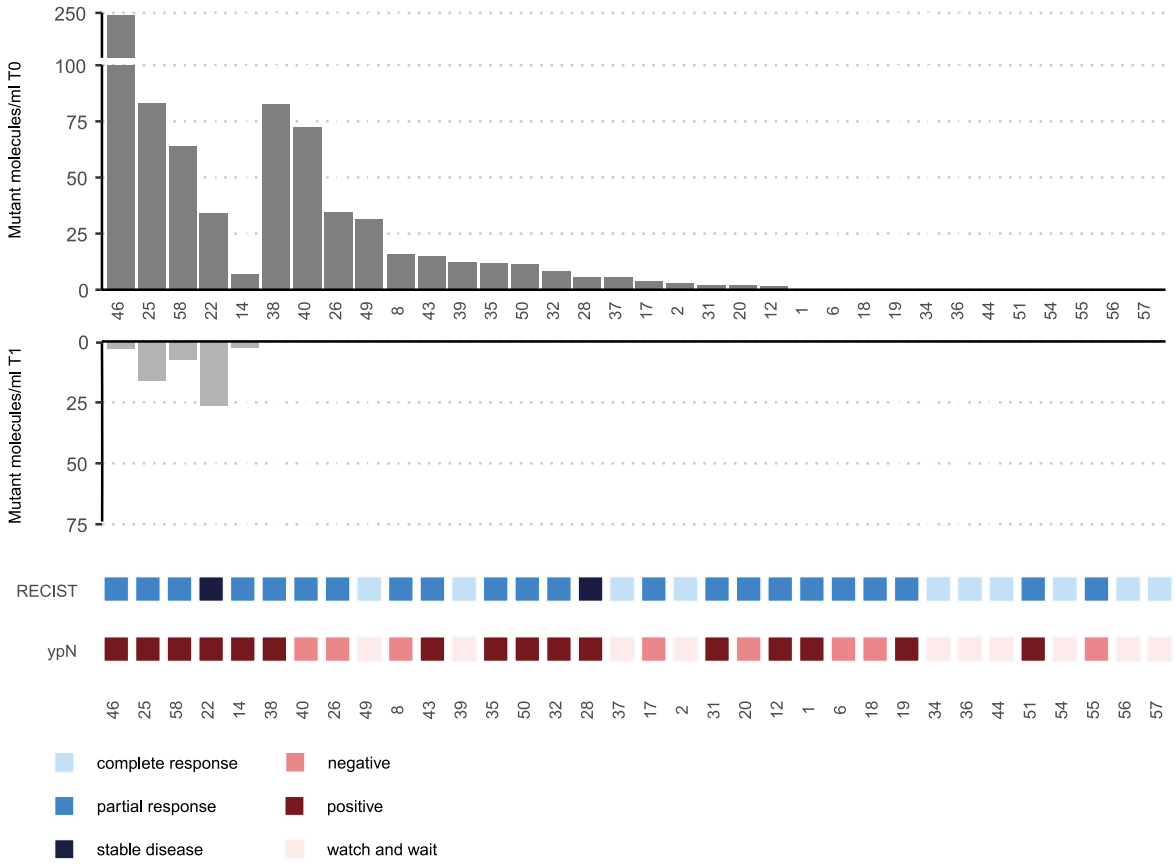

Fig. 3. ctDNA levels before (T0) and after neoadjuvant therapy (T1) combined with clinical response evaluation and postoperative lymph node status (n = 34). Patients are sorted according to ctDNA levels at T0 and presence of ctDNA at T1. No postoperative lymph node status was determined for patients with a clinically complete response as these patients did not receive surgery but watch and wait policy.

were selected for a watch and wait strategy and in patients without involved lymph nodes (ypN0) in the resection specimen. Furthermore, both ctDNA detection after neoadjuvant treatment as well as ctDNA detection after surgery were associated with an impaired progression-free survival.

To further personalize treatment of rectal cancer patients the clinical validity of ctDNA measurements to monitor response to neoadjuvant therapy and predict recurrence is explored. Previous ctDNA analysis studies using droplet digital PCR of hotspot mutations demonstrated that presence of ctDNA after neoadjuvant treatment and after surgery can be informative on outcome in patients with locally advanced rectal cancer harboring a hotspot mutation [13,16]. An advantage of the present study is the use of highly-sensitive ultradeep sequencing for ctDNA assessment with a tumor-informed approach. Sequencing enables simultaneous analysis of a variety of mutations in multiple genes, making it suitable to apply ctDNA evaluations for a larger proportion or even all tumors and increasing the chance of detecting very low levels of

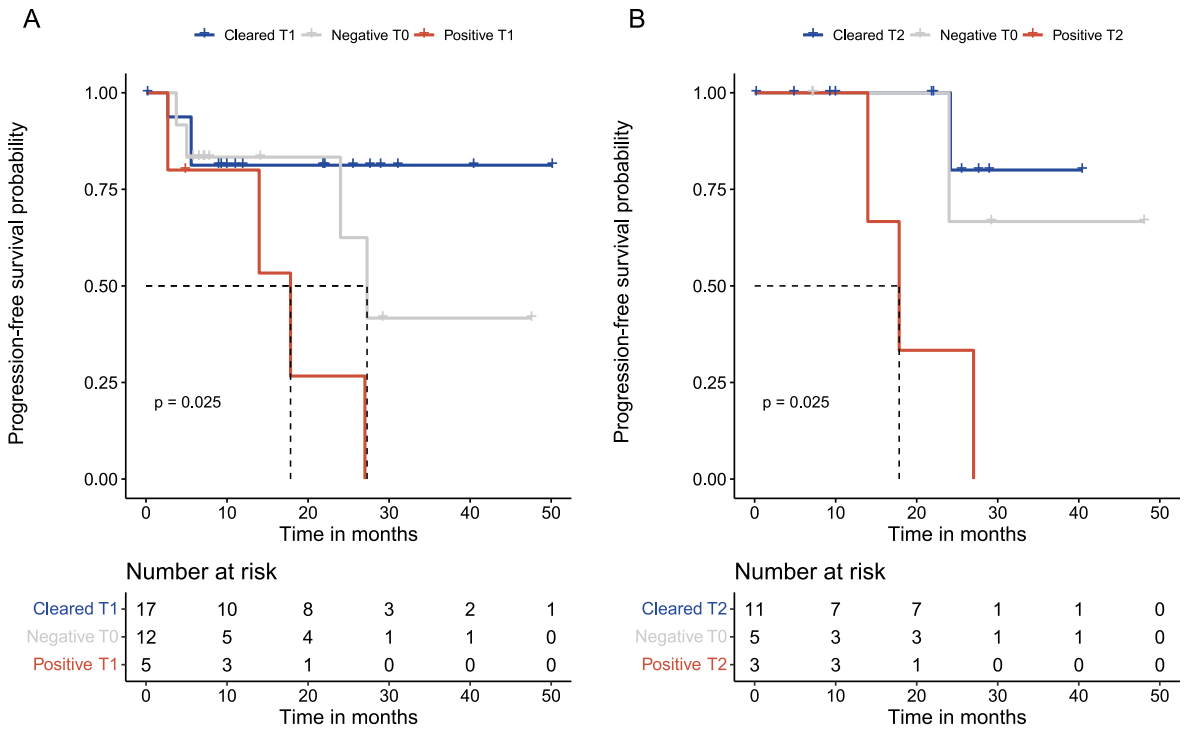

Fig. 4. Kaplan-Meier analysis of progression-free survival dividing patients according to **A)** ctDNA dynamics after neoadjuvant therapy (n = 34) and **B)** ctDNA dynamics after surgery (n = 19). p-values were calculated using Log-rank test.

ctDNA when multiple targets can be assessed per patient [22,23]. In addition, the tumor-informed approach decreases the probability of detecting a false-positive result ensuring higher specificity [24].

In the present study including both early-stage and locally advanced rectal cancer patients, detection rates of ctDNA prior to treatment, after neoadjuvant therapy and after surgery were 69%, 15% and 16%, respectively. The high proportion of patients with detected ctDNA before treatment is consistent with findings from previous studies where pretreatment detection rates in locally advanced rectal cancer patients ranged from 74 to 75% [12,13]. ctDNA detection and levels prior to treatment showed an association with clinically suspected lymph node metastases which was also described previously [12,13]. Additionally, ctDNA detection and ctDNA levels prior to treatment were also found to be associated with tumor volume. This confirms the suitability of ctDNA measurements to estimate tumor load, which is required for monitoring during treatment. Interestingly, both ctDNA measurements and tumor volume were not associated with CEA levels, which is currently an important biomarker to monitor rectal cancer treatments.

While previous studies focused on locally advanced rectal cancer patients receiving neoadjuvant treatment and surgery [12,25], our study also included early-stage rectal cancer patients and patients who entered a watch and wait strategy. Early-stage rectal cancer patients are treated more often in organ preservation trials such as the STAR-TREC [26,27]. Since the number of patients who are considered for a watch and wait trajectory after neoadjuvant treatment is rapidly growing [28], it is of interest to evaluate whether ctDNA measurements may support decisions to omit surgery. After neoadjuvant treatment no ctDNA could be detected both in patients with clinically complete response who did not undergo surgery and in patients with residual tumor without involved lymph nodes (ypN0), whereas in patients with involved lymph nodes in the resection specimen ctDNA was detected in 31%. Two other studies in similar cohorts also described increased ctDNA detection rates in patients with residual pathological disease [12,16]. Despite this, in most patients with involved lymph nodes no ctDNA was detected after neoadjuvant treatment, which implies that it remains challenging to discriminate between no or minimal residual disease. As such, negative ctDNA measurements should be used with caution, while detection of ctDNA after neoadjuvant treatment may be used as a strong argument for surgical treatment [29]. This could be useful when there is doubt about a complete response, such as identification of suspicious lymph nodes in a patient with clinical complete response of the primary tumor (ycT0N+). Therefore, ctDNA measurements can have a complementary role to current diagnostics to support the decision for surgery or watch and wait.

Both ctDNA detection after neoadjuvant treatment and ctDNA detection after surgery were associated with worse progression-free survival. This confirms findings from other studies that presence of ctDNA after neoadjuvant treatment and after surgery might identify patients at high risk of recurrence [11,14]. Furthermore, the association of ctDNA detection prior to surgery with development of progression after surgery suggests that ctDNA at that timepoint is informative on existence of micrometastatic disease [13,14]. The presence of ctDNA after surgery may identify patients with high risk of recurrence that could potentially benefit from adjuvant treatment. Currently, adjuvant treatment is not standard of care in rectal cancer patients in the Netherlands and subsequently none of the patients in this study received this treatment [30]. This opens up possibilities for ctDNA measurements to aid in the stratification for adjuvant treatment, which was recently shown useful in stage II colon cancer [31].

A limitation of our study includes the small and heterogeneous cohort and the small number of longitudinal samples collected during treatment precluding multivariate analyses. The different timepoints were logistically challenging and were hampered

during the COVID-19 pandemic. Moreover, the variable follow-up time due to the long inclusion period led to a short follow-up time for some patients and therefore limited the conclusions that could be drawn from the survival data.

In conclusion, the role of ctDNA measurements using ultradeep sequencing in patients with early-stage and locally advanced rectal cancer was explored, demonstrating the potential to complement current clinical assessments of response to neoadjuvant treatment and the risk of metastasis after surgery. These data need validation in larger prospective studies to further clarify how ctDNA measurements can adapt treatment in patients with both early-stage and locally advanced rectal cancer.

Data availability statement

The data generated in this study are available within the article and its supplementary data files or upon request from the corresponding author.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or the writing of the report.

CRediT authorship contribution statement

Lisa S.M. Hofste: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Final approval of manuscript. **Maartje J. Geerlings:** Methodology, Formal analysis, Investigation, Resources, Writing – review & editing, Final approval of manuscript. **Daniel von Rhein:** Methodology, Formal analysis, Writing – review & editing, Final approval of manuscript. **Heidi Rütten:** Investigation, Resources, Writing – review & editing, Final approval of manuscript. **A. Helen Westenberg:** Investigation, Resources, Writing – review & editing, Final approval of manuscript. **Marjan M. Weiss:** Methodology, Formal analysis, Writing – review & editing, Final approval of manuscript. **Christian Gilissen:** Methodology, Formal analysis, Writing – review & editing, Final approval of manuscript. **Tom Hofste:** Methodology, Formal analysis, Writing – review & editing, Final approval of manuscript. **Rachel S. van der Post:** Conceptualization, Investigation, Resources, Writing – review & editing, Final approval of manuscript. **Bastiaan R. Klarenbeek:** Conceptualization, Investigation, Resources, Writing – review & editing, Final approval of manuscript. **Johannes H.W. de Wilt:** Conceptualization, Investigation, Resources, Writing - original draft, Final approval of manuscript. **Marjolijn J.L. Ligtenberg:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Final approval of manuscript.

Declaration of competing interest

Prof. Marjolijn Ligtenberg received consulting fees from AstraZeneca, Bristol-Myers Squibb, GlaxoSmithKline, Illumina, Janssen Pharmaceuticals, Lilly, Merck Sharp & Dohme and Roche. All these relations were not related to this study and were paid to the institution. Prof. Johannes de Wilt received research funding from Dutch Cancer Society, ZonMw and Metronic. These relations were not related to this study and were paid to the institution. All other authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by funding from Bergh in het Zadel Foundation and Sacha Swarttouw-Hijmans Foundation. The authors thank the Radboudumc Technology Center Genomics and the Netherlands Cancer Registry.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejso.2023.01.026.

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