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Original Research Article

Circulating cell-free DNA as predictor of pathological complete response in locally advanced rectal cancer patients undergoing preoperative chemoradiotherapy

Christina Glismand Truelsen [a,b,*], Camilla Skovhus Kronborg [b], Brita Singers Sørensen [b], Louise Bach Callesen [a], Karen-Lise Garm Spindler [a,c]

[a] Department of Experimental Clinical Oncology, Arhus University Hospital, Denmark
[b] Danish Centre for Particle Therapy, Aarhus University Hospital, Denmark
[c] Department of Oncology, Aarhus University Hospital, Denmark

A B S T R A C T

Background: The watch and wait (W&W) strategy is proposed for patients with locally advanced rectal cancer (LARC) achieving clinical complete response (cCR) after neoadjuvant radiotherapy. cCR is only in partial concordance with pathological complete response (pCR) due to persisting viable tumour cells. The aim was to investigate circulating-free-deoxyribonucleic-acid (cfDNA) as a biomarker for prediction of pCR.

Materials and methods: Patients treated with neoadjuvant radiotherapy for LARC, were included in a prospective biomarker study in Aarhus, Denmark from 2017 to 2020. Plasma cfDNA levels were analysed by a direct fluorescent assay (DFA). Surgical specimens were reviewed by pathologists to categorize response to cytotoxic therapy.

Results: In total, 76 patients were included with plasma available at baseline (n = 70), mid therapy (n = 50), and end of therapy (n = 54). Higher cfDNA levels were observed in LARC patients compared with healthy subjects ($p < 0.01$). By ROC analysis (AUC: 0.87 (95% CI, 0.81–0.92)) the optimal cut-off was 0.71 ng/μL for differentiation between healthy subjects and LARC patients. Thirteen patients obtained pCR with a median cfDNA level of 0.57 ng/μL at end of therapy. Patients with cfDNA levels at end of therapy below the cut-off ($p < 0.02$) and 'cfDNA responders' with descending levels greater than the 75th percentile during therapy had a significantly higher chance of pCR ($p < 0.01$).

Conclusion: This hypothesis generating study indicates that low cfDNA levels at end of treatment or cfDNA responders might be associated with pCR. Quantification of cfDNA by the rapid and feasible DFA analysis could potentially facilitate personalized follow-up as a complementary tool to identify candidates for a W&W strategy.

Introduction

Colorectal cancer (CRC) continues to be a global health challenge as CRC ranks third in cancer incidences and second in mortality worldwide. Rectal cancer constitutes around one third of colorectal cancers and incidences surpassed 700.000 cases in 2020 [1].

Throughout decades surgical and radiotherapeutic methods have been developed and refined. Heald *et al.* [2] revolutionized the surgery of rectal cancer minimizing residual tumour and risk of local recurrence. The risk was further reduced with the introduction of preoperative (chemo)radiotherapy. The implementation of modern Intensity-Modulated Radiotherapy (IMRT) and Volumetric-Modulated Arc Therapy (VMAT) have reduced the dose delivery to healthy tissue without compromising tumour coverage.

The gold standard for locally advanced rectal cancer (LARC) is neoadjuvant (chemo)radiotherapy followed by surgery. This approach is associated with a risk of both surgical and radiotherapeutic morbidity including wound infection, anastomotic leak, and postoperative death [3]. Further, late morbidity with intestinal (especially permanent colostomy), urinary and sexual dysfunction can have a negative impact on

Abbreviations: cfDNA, circulating cell free deoxyribonucleic acid; LARC, locally advanced rectal cancer; DFA, direct fluorescent assay; CRT, chemoradiotherapy; RT, radiotherapy; ng/μL, nanogram per microliter.; VMAT, volumetric modulated arc therapy; pCR, pathological complete response; cCR, clinical complete response; qPCR, quantitative polymerase chain reaction; ddPCR, digital droplet polymerase chain reaction; NGS, next generation sequencing; W&W, watch and wait; IMRT, intensity modulated radiotherapy.

* Corresponding author at: Danish Centre for Particle Therapy and Department of Experimental Clinical Oncology, Aarhus University Hospital, Palle Juul-Jensens Boulevard 99, 8200 Aarhus N, Denmark.

E-mail addresses: chtrue@rm.dk, christinatruelsen@oncology.au.dk (C.G. Truelsen), camkro@rm.dk (C.S. Kronborg), loucal@oncology.au.dk (L.B. Callesen), k.g.spindler@rm.dk (K.-L.G. Spindler).

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quality of life.

A strategy with watchful waiting (W&W) aiming at organ preservation has emerged for patients with clinical complete response (cCR) after neoadjuvant (chemo)radiotherapy. A review of 3157 patients, estimated the overall pathological complete response (pCR) rate to 13.5 % [4] and higher response rates have been associated with high-dose radiotherapy [5]. The W&W strategy was presented by Habr-Gama [6], and recently the International Watch and Wait Database described outcomes of this approach with a 2-year cumulative local regrowth incidence of 25.2 % [7].

The challenge of a W&W strategy is to accurately determine cCR as previous findings have proposed magnetic resonance imaging (MRI) to be more useful in ruling out cCR than ruling it in [8]. Therefore, a complementary tool to MRI for prediction of pCR could be useful to both neoadjuvant and definitive chemoradiotherapy (CRT) as well as select patients for dose-escalation.

Liquid biopsies hold great predictive and prognostic value. Decades ago, Mandel *et al.* recognized circulating nucleic acids in the bloodstream [9]. Circulating cell-free DNA (cfDNA) is a combination of normal DNA fragments and cancer-specific DNA segments with tumour-specific alterations (circulating tumour DNA; ctDNA). cfDNA is elevated in a range of physiological and pathological conditions due to spontaneous and active release, necrosis, and apoptosis. cfDNA can be quantified with a range of time- and cost consuming methods incl. quantitative- and digital droplet polymerase chain reaction (ddPCR) and next generation sequencing (NGS). A rapid and cost-effective direct fluorescent assay (DFA) for cfDNA quantification, has been developed in-house. The method has proven feasible in patients with LARC and squamous cell carcinoma of the anus treated with CRT [9–11]. cfDNA appears to hold great predictive and prognostic value in LARC as few published studies found increased levels to be associated with risk of recurrence and decreased levels to be associated with CRT responders [10,12–15].

In this study, the aim was to assess whether cfDNA can discriminate between healthy control subjects and patients with LARC. Further to clarify the value of cfDNA in prediction of pCR.

Material and methods

Patients

Patients with LARC were included in a prospective Danish study. Inclusion criteria were histopathological rectal adenocarcinoma, localized $<$15 cm from the anal verge by rigid rectoscopy as well as indication for radiotherapy defined as T4 (except resectable high tumours), T3 tumours (or pathological lymph nodes) within 2–5 mm of the mesorectal fascia or growth through muscularis propria $\geq$5 mm. Exclusion criteria were prior pelvic radiotherapy or prior pelvic malignancy within 5 years before entering the study. In a subgroup of 76 patients, included from February 2017 to February 2020, blood samples were drawn for translational analysis.

Treatment

Patients underwent routine diagnostic evaluation and tumour staging including endoscopy, pelvic MRI, a computed tomography (CT) scan of the thorax, abdomen, and pelvis as well as assessment by a multidisciplinary team. Preoperative treatment was delivered as fixed field IMRT or VMAT consisting of short-course (25 Gy/5F/5w) or long-course (50.4 Gy/28F/5w) radiotherapy with or without concomitant capecitabine 850 mg/m^2 twice a day (BID). Standard elective clinical target volumes were mesorectal, pre-sacral and internal iliac regions. Surgery after national standardized guidelines was performed 6–8 weeks after neoadjuvant treatment. Pathologist reviewed the surgical specimens for pathological staging as stated by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC). The tumour regression grade (TRG) system of Mandard was used to categorize regressive changes after cytotoxic therapy ranging from TRG I (complete regression) to TRG V (absence of regressive changes).

Plasma samples and cfDNA measurement

Blood samples (30 ml) were collected at baseline, mid therapy and at end of therapy. Plasma samples were collected in EDTA tubes, centrifuged after 30 min. at 1200g for 10 min. at 21 °C, and stored at −80 °C. DFA, first described by Douvdevani *et al.* [16] and modified by Boysen *et al.* [9], were used for quantification of cfDNA directly in plasma. 40 μL of plasma was added to 160 μL of phosphate buffered saline (PBS). The PBS contained dimethyl sulfoxide (DMSO), (1:8) and SYBR® Gold Nucleic Acid Gel Stain (1:8,000), (*Invitrogen*). Samples were analysed in a black 96-well plate (Pio-Plex Pro Flat Bottom Plated, *Bio-Rad*). Fluorescence was detected with the 96-well fluorometer (Infinite F200 PRO, *Tecan*) at an emission wavelength of 535 nm and at an excitation wavelength of 485 nm. Human Control Genomic DNA (*Life Technologies*) was used for preparation of DNA standard curve, diluted 1:5 in PBS containing 10 % Bovine Plasma Albumin (*Sigma® Life Science*). Plasma concentration of cfDNA was calculated from the standard curve. Each sample was analysed four times and the final concentration was calculated as the mean value. From the individual values, outliers with a standard deviation exceeding 10 % were assessed according to Dixons q test, with a critical Q value at an alpha level of 5 % with one outlier removed. Samples were analysed blinded to clinical parameters and -endpoints.

Statistical analysis

Categorical and continuous variables were presented with descriptive statistics as number and percentages, and median values and range, respectively. The association between cfDNA, patient characteristics and pathological stage were evaluated by Mann-Whitney *U* test, Pearson's chi-squared test or Fischer's exact test when appropriate. The Wilcoxon signed rank test was used to compare baseline cfDNA to Mid- and End of therapy cfDNA. Receiver Operating Characteristic (ROC) was performed estimating the Area Under the Curve (AUC). The Youden index was applied for cut-off analysis. Survival analysis was estimated by Kaplan-Meier and differences among groups by log-rank test. Hazard ratios (HR) were estimated with a cox proportional hazard regression model. Disease-free survival (DFS) was calculated as time from inclusion in the study to recurrence (local or distant), death or censored at last observation, whichever came first. Overall survival (OS) was calculated as time from inclusion to death of any cause or censored at last follow-up. P-values $<$ 0.05 were considered statistically significant and reported p-values were two sided. NCSS software version c20.1 was used to perform statistical analyses.

Ethics

Signed informed consent was obtained from all included patients. The study was approved by The Danish National Committee on Health Research Ethics (no. 1-10-72-63-16) and reported to the Regional Data Protection Agency.

Reporting of this study is in accordance with REMARK guidelines [17].

Results

Patient characteristics and baseline circulating cell-free DNA

In total, 76 patients were included. Median age at diagnosis was 68 years (range: 24–94) with a slight overrepresentation of men (53.9 %). The majority was ECOG performance status (PS) 0 (73.7 %), and a large proportion was overweight (61.8 %). T3/T4 tumours dominated (93.4 %), the remaining was lymph node positive T2 tumours. Lymph node

positive disease was present in 73.7 % of cases. However, 10 patients with metastatic disease were included with a curative intent. Of those, nine had liver metastasis and underwent either surgery or radiofrequency ablation and one had a malignant inguinal lymph node removed by surgery. Dictated by national guidelines and clinical assessment 64 (84.2 %) received long-course CRT, only two without concomitant capecitabine and 12 (15.8 %) received short-course radiotherapy, two of those followed by neoadjuvant chemotherapy.

Baseline patient characteristics are presented in Table 1. Blood samples for translational analysis were available at baseline (n = 70), mid therapy (n = 50) and at end of therapy (n = 54). Mean and median baseline cfDNA levels were 0.91 ng/μL (95 % CI 0.85–0.96) and 0.87 ng/μL (95 % CI 0.79–0.96), respectively. The highest level of cfDNA was measured at baseline with a statistically significant reduction during (chemo)radiotherapy, thus a decrease was detected from baseline to mid therapy ($p < 0.001$) and from baseline to end of therapy ($p < 0.001$), presented in Fig. 1A. Median baseline levels of cfDNA were compared to baseline characteristics. No significant difference in baseline median cfDNA levels were observed for age, gender, BMI, tumour mutation status, tumour size or -localization, however a significant difference in median baseline cfDNA level was observed for patients in PS 0 (0.81 ng/μL) compared with PS 1 (0.92 ng/μL) ($p = 0.03$), see Fig. 1B. There was a trend towards higher median cfDNA levels with increasing T stage, however not statistically significant, demonstrated in Fig. 1C. Lastly, there was no significant difference in baseline cfDNA levels of patients achieving pCR vs patients not obtaining pCR ($p = 0.9$).

Table 1
Baseline patient characteristics of median cfDNA levels.

Characteristics	Number (%, n = 76)	Baseline median cfDNA, ng/μL, (95 % CI)	P value*
Age at primary diagnosis (years)			
Median: 68			
Range: 25–94			
<68			
>68	38 (50.0)	0.83 (0.75–0.96)	
	38 (50.0)	0.91 (0.81–1.01)	0.2
Sex			
Male	41 (53.9)	0.90 (0.79–1.03)	
Female	35 (46.1)	0.86 (0.79–0.97)	0.7
Performance status			
0	56 (73.7)	0.81 (0.76 – 0.95)	
1	20 (26.3)	0.92 (0.90–1.20)	**0.03**
BMI			
18.5–24.9 (normal)	29 (38.2)	0.82 (0.75–1.01)	
> 25 (overweight)	47 (61.8)	0.91 (0.79–1.01)	0.5
cT-stage			
cT2	5 (6.6)	0.82	
cT3	57 (75.0)	0.83 (0.79–0.92)	0.9 cT2 vs. cT3
cT4	14 (18.4)	1.01 (0.79–1.17)	0.08 cT3 vs. cT4
cN-stage			
cN0	19 (25.0)	0.80 (0.73 – 0.96)	0.8 cN0 vs. cN1
cN1	23 (30.3)	0.81 (0.71 – 1.03)	0.1 cN0 vs. cN2
cN2	33 (43.4)	0.91 (0.86 – 1.05)	0.1 cN1 vs. cN2
cM-stage			
cM0	66 (86.8)	0.87 (0.79 – 0.95)	
cM1	10 (13.2)	0.93 (0.71 – 1.03)	0.7 cM0 vs. cM1
AJCC stage			
II	15 (19.7)	0.80 (0.65 – 0.96)	
III	51 (67.1)	0.90 (0.79 –0.98)	0.3 II vs. III
IV	10 (13.2)	0.93 (0.71 – 1.03)	0.3 II vs. IV
Radiotherapy			
Short-course	12 (15.8)	0.91 (0.71–1.04)	
Long-course	64 (84.2)	0.86 (0.79–0.96)	0.8
Tumour size			
Median: 4.7 cm			
Range: 2–10 cm			
<4.7	37 (48.7)	0.85 (0.79–0.98)	
>4.7	38 (50.0)	0.90 (0.79–1.03)	0.6 < 4.7 vs. > 4.7
Not evaluated	1 (1.3)		
Tumour Localization			
Low: 0–5 cm	30 (39.5)	0.90 (0.79 – 1.01)	0.3 Low vs. Mid
Mid: 5–10 cm	37 (48.7)	0.83 (0.75 – 0.95)	0.2 Mid vs. Upper
Upper: 10–15 cm	9 (11.8)	1.01 (0.52 – 1.25)	0.4 Low vs. Upper
Tumour mutation status			
Wildtype	16 (21.1)	0.79 (0.75 – 1.03)	
Mutation**	38 (50.0)	0.97 (0.79 – 1.11)	0.3 Wildtype vs. Mutated
Not evaluated	22 (28.9)		

*Mann Whitney *U* test. **KRAS, NRAS or BRAF. Percentages deviates from exact 100 due to rounding.

Cut-off values, reduction of cfDNA levels during therapy, and pCR

Baseline cfDNA levels in the studied cohort were compared to cfDNA levels of a cohort of healthy individuals. In the cohort of 94 healthy individuals mean and median cfDNA levels were 0.54 ng/μL (95 % CI 0.49–0.59) and 0.52 ng/μL (95 % CI 0.48–0.57), respectively, thus statistically significant higher levels were observed in the cohort of rectal cancer patients ($p < 0.001$). A ROC analysis was applied, as depicted in Fig. 2, with an AUC of 0.87 (95 % CI 0.81–0.92, $p < 0.001$). The Youden index [18] for optimal cut-off value estimated a cut-off of 0.71 ng/μL with a sensitivity of 83 % and a specificity of 82 %, for distinction between healthy subjects and patients with LARC.

Intended radical surgical procedures were performed with subsequent pathological assessment. Three patients did not proceed to surgery after preoperative treatment. One had metastatic disease at preoperative evaluation and two did not go through with operation due to patient wish and went directly to follow-up.

Thirteen patients (17.1 %) obtained pCR (ypT0) with a median cfDNA level of 0.57 ng/μL (95 % CI 0.38–0.7) at end of therapy. A reduction in median cfDNA levels was associated with tumour regression grade, statistically significant from TRG I (no residual cancer) to TRG IV (residual cancer outgrowing fibrosis) ($p = 0.01$), of note, only a small number of patients were available for analysis, see Table 2. Using the cut-off value from the ROC curve, patients with a cfDNA level below 0.71 ng/μL at end of therapy had a statistically significant higher probability of pCR ($p = 0.015$).

Dynamic changes throughout treatment were analysed. The percentage increase or decrease in cfDNA level from baseline to the lowest obtained value at mid- or end of therapy was estimated. A significant association with pCR was found when cfDNA levels were reduced in percent by more than the 75th percentile ($p = 0.001$). Patients with a percentage reduction in cfDNA level greater than the 75th percentile throughout treatment are termed cfDNA responders, for illustration see Fig. 5.

Patients obtaining pCR had significantly longer DFS ($p < 0.005$) and a trend towards longer OS ($p = 0.17$), Supplementary Fig. 1. When applying a ROC curve analysis for cfDNA levels at end of therapy to pCR vs. non-pCR a cut-off value of 0.70 ng/μL (sensitivity: 0.88, specificity: 0.59), (AUC: 0.77 (95 % CI 0.55–0.88), $p < 0.01$) was estimated, depicted in Fig. 3, which is in accordance with the cut-off value for distinction between healthy subjects and rectal cancer patients in this study.

Prognostic value of cfDNA levels

After a median follow-up of 32 months, 20 (30.3 %) patients with initial M0 disease developed recurrence, of those 3 were local recurrences. Eight of the 10 primary metastatic patients had a failed

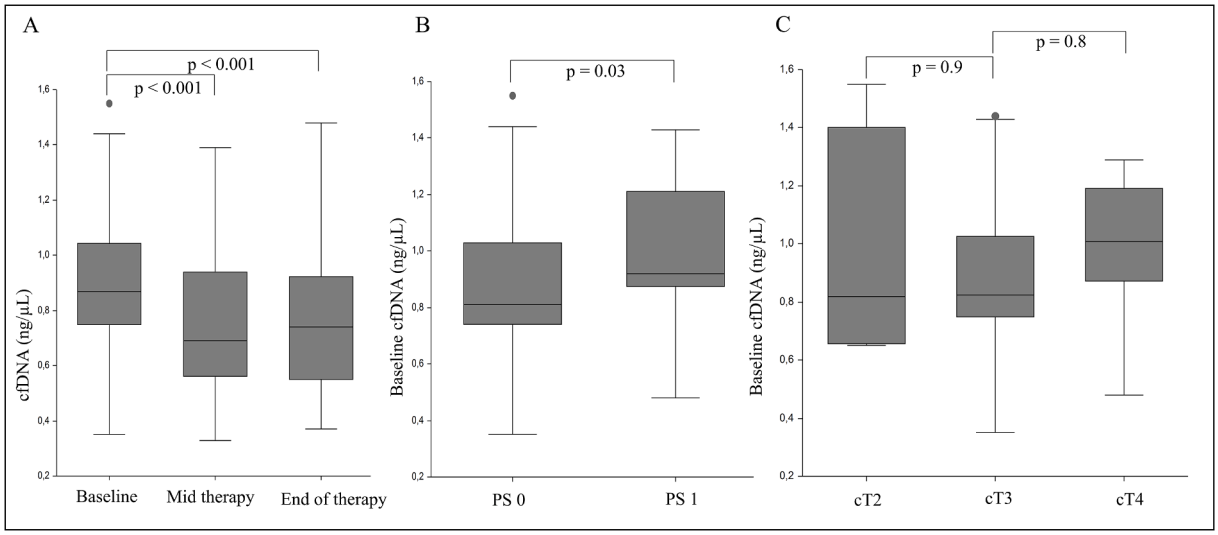

Fig. 1. Boxplots depicting medians, quartiles and whiskers indicating variability outside the upper and lower quartiles. A: cfDNA levels throughout treatment at baseline, mid therapy and at end of therapy. B: Baseline plasma cfDNA levels in relation to performance status. C: Baseline plasma cfDNA levels in relation to cT-stage.

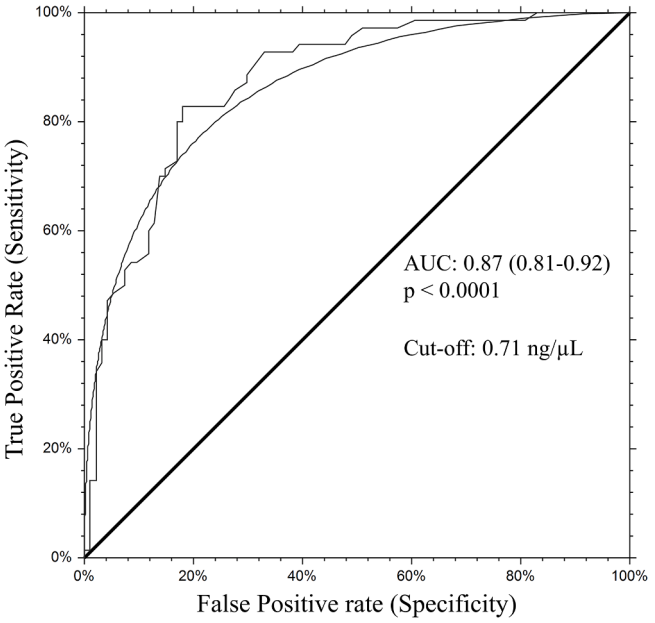

Fig. 2. Receiver Operating Characteristic of baseline plasma cfDNA levels between the healthy cohort and the studied cohort of LARC patients, depicted as both empirical and binormal curves. The Empirical Area Under the Curve was 0.87 (0.81–0.92) with a p-value < 0.001. The cut-off was estimated to 0.71 ng/µL with a sensitivity of 83 % and a specificity of 82 %.

curative intent. In this study, high baseline levels of cfDNA above the 75th percentile were not associated with either recurrence, shorter DFS or - OS. However, 'cfDNA responders' with descending levels greater than the 75th percentile during therapy had significantly longer DFS ($p < 0.05$). Kaplan-Meier plots for DFS and OS related to cfDNA responders are shown in Fig. 4.

Discussion

Liquid biopsies for a personalized tailored treatment have gained ground in recent years, but the true clinical utility needs to be clarified. ctDNA is limited to patients with detectable genetic and epigenetic alterations, whereas cfDNA quantification can be applied in most cancer cases despite geno- or phenotype. Limitations of using cfDNA is that quantification is not entirely cancer specific, as it is influenced by e.g., comorbidity and it cannot be adjusted for lymphocyte contamination. However, DFA quantification is a rapid and cost-effective analysis, which can be introduced into daily clinical practice with simplicity due to the laboratory feasibility.

Previous studies have evaluated cfDNA as a predictive and prognostic marker in patients with LARC. Zitt *et al.* demonstrated, cfDNA quantified by qPCR, as a surrogate marker of response, suggesting cfDNA as a tool for treatment monitoring [13]. Agostini - and Sun *et al.* supported these findings with a cfDNA integrity index being significantly lower in CRT responders [14,12]. Guo *et al.* provided information with promoter profiling of cfDNA enabling prediction of pCR [19]. Boysen *et al.* emphasized the importance of correct staging by a nodal involvement and pathological AJCC stage dependent variation in cfDNA [15].

Shou *et al.* reported on 123 patients using the same DFA method applied in this study. High baseline cfDNA were associated with higher risk of local or distant recurrence, shorter time to recurrence and negatively associated with DFS. Thus, suggesting the use of cfDNA as a prognostic marker for treatment strategy [20]. In this study, we were not able to verify these findings, however we will await further follow-up.

This prospective study aimed to confirm the hypothesis of cfDNA being able to distinguish LARC patients from healthy control subjects. Previously this has been documented in two studies as cfDNA levels (Alu 115, 247 and β globin gene) and ratio of 400-/100-bp DNA were higher in rectal cancer patients than healthy individuals [14,12]. The present study found significantly higher cfDNA levels by DFA quantification in rectal cancer patients. A ROC curve with an AUC of 0.87 (95 % CI 0.81–0.92) estimated by cut point analysis the ability to discriminate with a sensitivity of 83 % and a sensitivity of 82 %.

Accurate classification between histopathological responders and non-responders would be groundbreaking for patients with vital impact on treatment decision-making, especially with the introduction of W&W strategies. pCR after CRT allows for organ-sparing strategies either as watchful waiting or minimal surgery. In the PAN-EX study, radiological tumour regression after CRT on MRI scans (mrTRG) and pathological TRG was compared with an overall agreement of 71 % with mrTRG able to correctly identify pCR in 74 % of cases [21]. On the other hand, in a review by Glynne-Jones *et al.* cCR was only found to be associated with pCR in 30 % of cases [22]. Therefore, there is an unmet need for biomarkers complementary to imaging to predict pCR. This study was

Table 2
Median cfDNA level at end of therapy in relation to pathological TNM stage and TRG.

Characteristics	Number (%, n = 76)	Available blood test at end of therapy (%, n = 54)	Median cfDNA, ng/µL, (95 % CI)	P value*
ypT-stage				
ypT0	13 (17.1)	8 (14.8)	0.57 (0.38–0.7)	
ypT1	5 (6.6)	5 (9.3)	0.79 (NA)	0.2 ypT0 vs. ypT1
ypT2	11 (14.5)	8 (14.8)	0.75 (0.54–0.93)	0.06 ypT0 vs. ypT2
ypT3	39 (51.3)	28 (51.9)	0.76 (0.63–0.92)	**0.03** ypT0 vs. ypT3
ypT4	6 (7.9)	4 (7.4)	0.90 (NA)	**0.04** ypT0 vs. ypT4
NE	2 (2.6)			
ypN-stage				
ypN0	44 (57.9)	31 (57.4)	0.71 (0.60–0.89)	0.5 ypN0 vs. ypN1
ypN1	22 (28.9)	16 (29.6)	0.68 (0.54–0.79)	0.3 ypN0 vs. ypN2
ypN2	8 (10.5)	6 (11.1)	0.85 (0.66–1.04)	0.07 ypN1 vs. ypN2
NE	2 (2.6)			
ypV-stage				
ypV0	49 (64.5)	33 (61.1)	0.71 (0.59–0.89)	0.6 ypV0 vs. ypV1
ypV1	9 (11.8)	8 (14.8)	0.83 (0.49–1.01)	0.9 ypV0 vs. ypV2
ypV2	16 (21.1)	12 (22.2)	0.75 (0.62–0.84)	0.6 ypV1 vs. ypV2
NE	2 (2.6)			
TRG				
TRG-I	13 (17.1)	8 (14.8)	0.57 (0.38–0.70)	
TRG-II	16 (21.1)	11 (20.4)	0.79 (0.54–1.16)	0.07 TRG I vs. II
TRG-III	24 (31.6)	19 (35.2)	0.74 (0.54–0.92)	0.1 TRG I vs. III
TRG-IV	12 (15.8)	9 (16.7)	0.76 (0.64–1.03)	**0.01** TRG I vs. IV
TRG-V	9 (11.3)	6 (11.1)	0.82 (0.63–0.94)	**0.02** TRG I vs. V
NE	2 (2.6)			

NE: Non-evaluable. Percentages deviates from exact 100 due to rounding.

limited as clinical response to CRT according to MRI was not systematically graded by radiologist at pre-operative evaluation, therefore we were not able to include this in our analysis. This needs to be incorporated and addressed in future radiological and translational study designs.

In the present study, there was a trend but no significant correlation between cfDNA levels and T/N-stage arising the question of whether cfDNA should be applied as a simplified pseudo-marker for tumour burden. Nygaard *et al* [23] assessed the correlation between cfDNA and volumetric parameters of positron emission tomography (PET)-CT and found no correlation suggesting that cfDNA reflects a complex biological picture beyond tumour burden.

In this study, a significant reduction in cfDNA was observed from baseline to mid- and end of therapy but with a slight increase from mid- to end of therapy. This increment might reflect toxicity, as cfDNA levels at end of therapy has been associated with higher scores of toxicity in a study on anal cancer [11]. Therefore, we applied the lowest achieved value obtained at either mid- or end of therapy in our analysis to

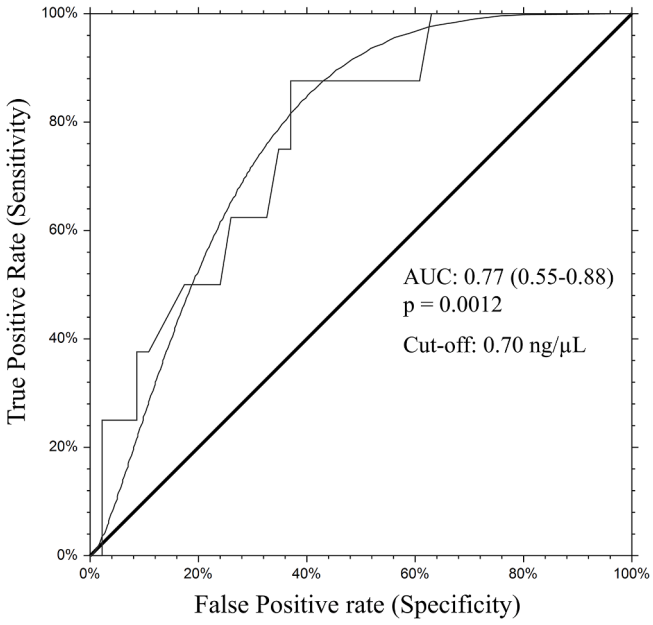

Fig. 3. Receiver Operating Characteristic of cfDNA levels at end of therapy amongst patients achieving pathological complete response vs. non-pathological complete response depicted as empirical and binormal curves. The Empirical Area Under the Curve was 0.77 (0.55–0.88), $p < 0.01$. Using the Youden index a cut-off value of 0.70 ng/µL was estimated giving a sensitivity of 0.88 and a specificity of 0.59.

circumvent toxicity related increase in cfDNA at end of therapy. Our results showed that cfDNA responders and low cfDNA levels at end of therapy under the cut-off value from the ROC curve were associated with pCR. However, major limitations of this study represent the small patient number resulting in small subgroup analysis. Therefore, conclusions should be drawn with caution. Further, this exploratory study applied descending levels of cfDNA greater than the 75th percentile. This cut-off and its significant association with DFS have not been independently validated in external cohorts and data should be interpreted with caution but may guide the direction for future translational studies. Results are regarded as hypothesis-generating suggesting that the use of cfDNA quantification by a rapid, feasible DFA laboratory method could potentially be a complementary tool for patient selection for a W&W strategy.

The International W&W multicentre registry study identified 1009 patients with cCR after neoadjuvant treatment. The rate of local regrowth was ≈25 %, with distant metastasis occurring in 17.8 %. In patients undergoing surgery after neoadjuvant CRT the risk of local recurrence is reduced to ≈5 %, with a 5-year distant metastasis rate of ≈30 % [24]. There is no clear-cut knowledge on how survival and curative treatment chance may be compromised, when local regrowth is diagnosed after a W&W strategy. In locally recurrent rectal cancer previously resected, radical curative surgery is only obtained in 20–30 %. Thus, the benefits of surpassing surgery must be weighed against risk in shared decision making and patient preferences. The true analytical validity and clinical utility of cfDNA still needs to be further clarified in studies with larger sample size and comprehensive study designs.

Conclusion

This hypothesis-generating study suggests that low levels of cfDNA at end of therapy and cfDNA responders' might be an indicator of pCR. We propose cfDNA as a biomarker and complementary tool to imaging to identify candidates for a W&W strategy in hopes of an individualized tailored follow-up for future rectal cancer patients. The true clinical utility needs to be validated in larger cohorts with comprehensive study

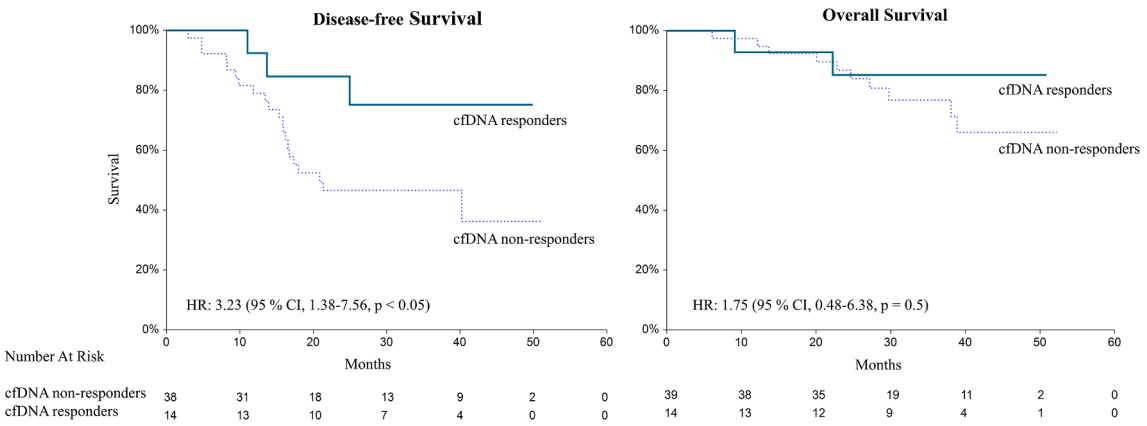

Fig. 4. Kaplan Meier survival curves of disease-free survival and overall survival according to cfDNA responders and cfDNA non-responders. The solid (green) line represents cfDNA responders with descending levels of cfDNA greater than the 75th percentile during therapy and the dashed (blue) line represents cfDNA non-responders with a change in cfDNA levels below the 75th percentile.

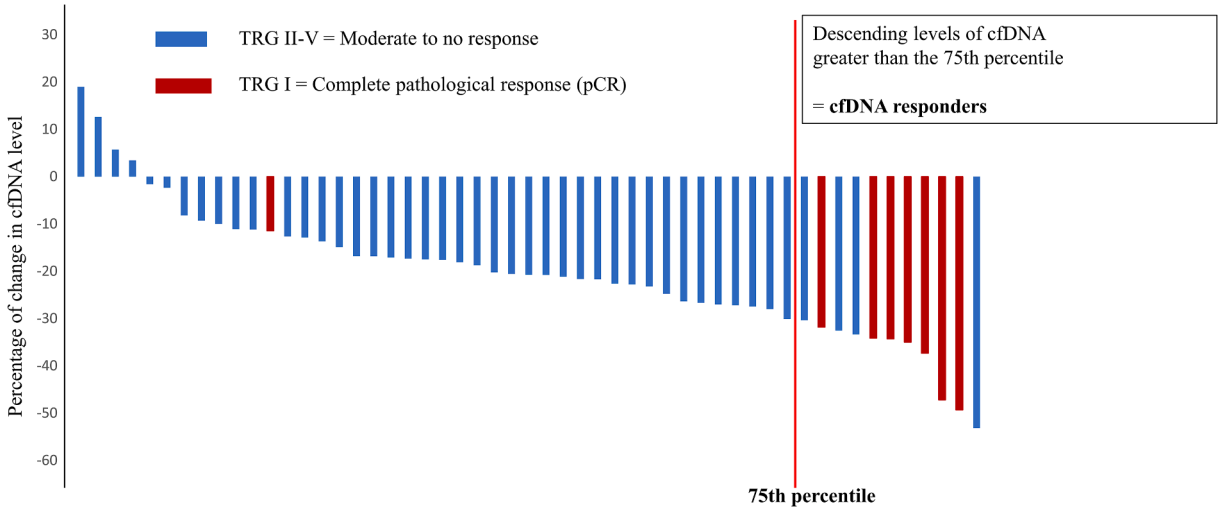

Fig. 5. Waterfall plot of percentage change in cfDNA levels from baseline to the lowest obtained value at mid- or end of therapy and its association with pCR. Patients with a percentage reduction in cfDNA level greater than the 75th percentile were significantly associated with pCR (p = 0.001), these are termed cfDNA responders.

designs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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