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The Importance of Feasibility Assessment in the Design of ctDNA Guided Trials – Results From the OPTIPAL II Study

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Abstract

Before conducting randomized clinical trials in a new research field, feasibility must be tested. We evaluated feasibility of mutational testing on cell-free DNA for selecting primary systemic therapy for patients with metastatic colorectal cancer and for response evaluation during therapy. The setup was tested on 48 patients, proved feasible, and can be applied in randomized clinical trials evaluating clinical utility of circulating tumor DNA.

Introduction: Both quantitative and molecular changes in ctDNA can hold important information when treating metastatic colorectal cancer (mCRC), but its clinical utility is yet to be established. Before conducting a large-scale randomized trial, it is essential to test feasibility. This study investigates whether ctDNA is feasible for detecting patients who will benefit from treatment with epidermal growth factor receptor inhibitors and the prognostic value of circulating tumor DNA (ctDNA) response. **Materials and methods:** Patients with mCRC, who were considered for systemic palliative treatment and were eligible for ctDNA analysis. Mutational testing on cell-free DNA (cfDNA) was done by ddPCR. ctDNA response from baseline to the third treatment cycle was evaluated in patients with detectable ctDNA at baseline. ctDNA maximum response was defined as undetectable ctDNA at the third treatment cycle, ctDNA partial response as any decrease in the ctDNA level, and ctDNA progression as any increase in the ctDNA level. **Results:** Forty-nine patients were included. The time to test results for mutational testing on cfDNA was significantly shorter than on tumor tissue ($p < .001$). Progression-free survival were 11.2 months (reference group), 7.5 months (HR = 10.7, $p = .02$), and 4.6 months (HR = 11.4, $p = .02$) in patients with ctDNA maximum response, partial response, and progression, respectively. Overall survival was 31.2 months (reference group), 15.2 months (HR = 4.1, $p = .03$), and 9.0 months (HR = 2.6, $p = .03$) in patients with ctDNA maximum response, partial response, and progression, respectively. **Conclusion:** Pretreatment mutational testing on cfDNA in daily clinic is feasible and can be applied in randomized clinical trials evaluating the clinical utility of ctDNA. Early dynamics in ctDNA during systemic treatment hold prognostic value.

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Introduction

The majority of patients with metastatic colorectal cancer (mCRC) suffer from incurable disease, and chemotherapy is recommended to reduce symptoms and prolong survival. Flouropyrimidine-based chemotherapy regimens with antiangiogenic agents or monoclonal antibodies directed against the epidermal growth factor receptor (EGFRi) are approved worldwide for first-line treatment.[1-3] However, patients harboring *KRAS, BRAF*, or *NRAS* mutations in tumor tissue cannot be offered treatment with EGFRi since these mutations are resistance-conferring.[4-7] The accurate prescription of anti-EGFR agents is of high clinical importance. Recently, *BRAF-targeted* therapies have been added to the treatment options in *BRAF*-mutated disease, and new drugs targeting subtypes of *KRAS* mutations are undergoing evaluation in clinical trials.

Pretreatment mutational testing is therefore mandatory. According to the standard of care, mutational testing is performed on

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previously stored tumor tissue from surgery or diagnostic biopsy, where the tumor tissue may have been removed several years prior.

Tissue-based genotyping can be time-consuming, leading to patients beginning therapy before biomarker results are available, precluding their use in therapy selection.[8] In case of insufficient tumor tissue for mutational testing, rebiopsy is required, further delaying mutational testing and adding a risk of complications such as hemorrhage and infection.

Molecular spatial and temporal heterogeneity are well-known phenomenas in mCRC; hence, mutational testing on archival tumor tissue may not be representative.[9] During treatment with EGFRi, tumor subclones with resistance-conferring aberrations may be selected to grow under the treatment pressure. Moreover, de novo mutations may drive resistance to EGFRi. Repetitive tumor tissue biopsies to detect changes in mutational status during treatment are neither feasible nor ethical. Hence, treatment resistance is only detected when it leads to radiological progression or gives rise to physical deterioration.

In the past decade, there has been an increasing interest in circulating tumor DNA (ctDNA) for optimizing the treatment of patients with mCRC. Retrospective studies indicate that mutational testing on cell-free DNA (cfDNA) from plasma can predict who will benefit from treatment with EGFRi, and that quantitative levels of ctDNA seem to be important for outcome.[10–13] Furthermore, early changes in ctDNA during systemic treatment hold a prognostic value in patients with incurable mCRC.[14]

A pretreatment plasma sample for mutational testing on cfDNA may give a faster and more timely picture of molecular biology and therefore be a better selection criterion for treatment in mCRC. Furthermore, the procedure is minimally invasive and can be repeated consecutively throughout the treatment course, capturing dynamics in ctDNA level and genomic changes over time.

However, the true clinical utility of ctDNA when treating patients with mCRC is yet to be established. Hence, before implementing ctDNA into daily clinic, it is of utmost importance to establish the clinical utility in randomized clinical trials (RCT), and it is highly relevant to test the feasibility before conducting large-scale RCT. Feasibility parameters such as inclusion rate and detection rate of ctDNA positive samples are essential to optimize sample size calculations. Furthermore, it is crucial to test the technical setup, including the quality and turnaround time of ctDNA analysis and the clinical feasibility, ie, disease control and survival rates in treatment strategy based on ctDNA.

The primary aim of the present prospective study was to investigate if mutational testing on cfDNA in plasma is feasible for selecting the most optimal systemic treatment for patients with mCRC. Secondary, to evaluate the prognostic value of early changes in ctDNA level and analyze developments in mutational status in cfDNA in plasma during therapy.

Materials and Methods

The study was approved by The Central Denmark Region Committees on Health Research Ethics (1-10-72-111-17), and it was prospectively registered with ClinicalTrials.gov (NCT03750175). Written and orally informed consent according to the Helsinki II Declaration was obtained from all patients. The study is reported in accordance with the REMARK guideline.[15]

Study Design

The OPTIPAL II study was a prospective biomarker study for patients receiving standard systemic treatment for mCRC. The study prospectively enrolled patients with mCRC prior to the start of systemic palliative treatment. The study was explorative and designed to include approximately 50 patients for analysis. The examined variable was "circulating free DNA." The term "circulating free DNA" covers both measurements of total cfDNA and measurements of the proportion of cfDNA originating from tumor cells (ie, ctDNA). In the present study, the ctDNA results are presented. The cfDNA results have previously been published.[16]

Patients

Patients with mCRC, who were eligible for standard combination therapy with palliative intent (noncurable), were included at the Department of Oncology, Aarhus University Hospital (Aarhus, Denmark). The key inclusion criteria were: Histopathologically verified mCRC, indication for systemic palliative treatment with EGFRi, fit for treatment with EGFRi, and age $\geq$ 18 years. The key exclusion criteria were World Health Organization (WHO) performance status $>$ 2, significant other cancer disease within 5 years of inclusion, and conditions precluding sampling during therapy and treatment breaks. Off note, the indication for systemic palliative treatment was evaluated prior to mutational testing, and after inclusion in the OPTIPAL II study, patients were offered EGFRi treatment based on mutation status in ctDNA.

Treatment

RAS/RAF status in plasma was determined by ddPCR. In the case of *RAS/RAF* wild-type treatment consisted of chemotherapy and EGFRi (cetuximab or panitumumab). Patients with a *RAS/RAF* mutation were treated with chemotherapy without concomitant EGFRi.

Response Evaluation

Treatment response was determined based on RECIST version 1.1.[17] CT Scans of the chest, abdomen, and pelvis were performed at baseline, every 8 to 9 weeks during treatment, and hereafter every third month during treatment breaks until progression, death, or end of follow-up, whichever came first.

Blood Sampling

Blood samples were collected prospectively at baseline (pretreatment), prior to the third treatment cycle, and at every response evaluation. The last sample was drawn at the documented time of progression. A total of 30 mL of whole blood was drawn at each time point. Plasma samples were obtained in EDTA tubes. Plasma was isolated by double centrifugation at 1,600 *g* for 10 minutes and 10,000 *g* for 10 minutes. The centrifugations were done at room temperature within 2 hours and stored at -80°C until further analysis. At baseline, a blood sample was collected in a Streck tube and shipped for mutational analysis in a central laboratory (Zealand University Hospital, Denmark). Plasma was isolated as described above.

ddPCR

An in vitro generated spike-in DNA fragment (191 base pair [bp]) was added to 4 mL plasma and DNA purified on a Chemagic 360 robot (PerkinElmer, Waltham, MA) using a 1304 cfDNA purification kit (PerkinElmer).[18] To control cfDNA amount and sample fragmentation, an in-house multiplex ddPCR reaction was performed, amplifying 65 bp and 250 bp fragments of the *EMC7* gene using a QX200 AutoDG ddPCR system (Bio-Rad, Berkeley, CA). As a reference for cfDNA measurement, the *EMC7* gene on chromosome 15 was chosen. The EMC7 gene has not been reported to be mutated in cancer, it is located close to the centromere and, therefore, unlikely to be involved in chromosomal gains or losses. Furthermore, when searching the human genome no *EMC7* pseudo-genes have been reported or found.[19] Potential contamination of the purified DNA with white blood cells DNA was evaluated using an immunoglobulin gene-specific assay (PBC), and loss of DNA during purification and handling was assessed by measuring the amount of spike-in fragment as previously reported by multiplex ddPCR.[18] For mutational screening, an input volume of 5 µL of cfDNA in a single-well setup was used. For the detection of specific mutations, 5 µL of cfDNA was used in duplicates. ctDNA was analyzed by 8 multiplex ddPCR assays at baseline covering mutations in the *KRAS* (codons 12, 13, 61, 117, and 146), *NRAS* (codons 12, 13, and 61), and *BRAF* (codon 600), and have a limit of detection of at least 0.1%. In the case of a positive finding, another multiplex assay was performed to find the specific mutation. If a mutation was found at baseline, the following plasma samples were analyzed by the relevant multiplex assay with a typical limit of detection of 0.01%.

Failure parameters for analysis of baseline blood samples were defined as

- Quality of samples; PBC > 0.5%, CPP1 major loss corresponding to <20% remaining.
- Transportation >3 working days.
- Analysis >3 working days.
- Total results delivered > 7 working days.

The test turnaround time was calculated from the sample acquisition date to the results report date. ctDNA fraction was calculated as mutated alleles divided by the sum of mutated and wild-type alleles.

MassARRAY

Paired plasma samples from baseline and end of the study were additionally analyzed retrospectively by a mass spectrometric-based multiplexed platform (MassARRAY Agena Bioscience) to evaluate whether a MassARRAY gene panel would enable the detection of a higher fraction of different mutations than mutation analyses by ddPCR, and to investigate the evolution in mutational status. MassARRAY genotyping utilizes PCR reactions, DNA extension, and mass spectrometry in a 96-well format. The UltraSEEK MassARRAY Colon Panel (Agena Bioscience) was used. It is a high-throughput assay with the capacity to screen for more than 100 somatic mutations in the 5 key oncogenes *KRAS* (codons 12, 13, 59, 61, 117, and 146), *NRAS* (codons 12, 13, 59, 61, 117, and 146), *BRAF* (codons 469, 594, and 600), *EGFR* (extracellular domain mutations in exon 12), and *PIK3CA* (codons 542, 545, and 1047) with a limit of detection of 0.1%. All mutations targeted by the ddPCR were included in the MassARRAY gene panel. The technology is based on matrix-assisted laser desorption/ionization time-of-flight spectrometry (MALDI-TOF). According to the manufacturer, an input of 10 to 15 ng of cfDNA in a volume of 35 µL was used.

Tumor Tissue

Patients were referred from several different hospitals to our department. Mutational testing on tumor tissue was performed at the referring hospital according to each hospital's standard of care. Information was retrospectively collected from pathology reports. The methods and coverage are listed in Supplemental Table 1. All samples were analyzed blinded to clinical parameters.

Definition of Groups

ctDNA response was evaluated in patients with detectable ctDNA at baseline. Patients were divided into 3 subgroups based on ctDNA response as change from baseline to before the third treatment cycle. ctDNA maximum response was defined as undetectable ctDNA at the third treatment cycle, ctDNA partial response as any decrease in the ctDNA level from baseline to third treatment cycle, and ctDNA progression as any increase in the ctDNA level from baseline to third treatment cycle (Figure 1). A blood sample was categorized as drawn at progression if the sample was drawn no more than 30 days before or after radiological progression, according to RECIST version 1.1.

Statistical Analyses

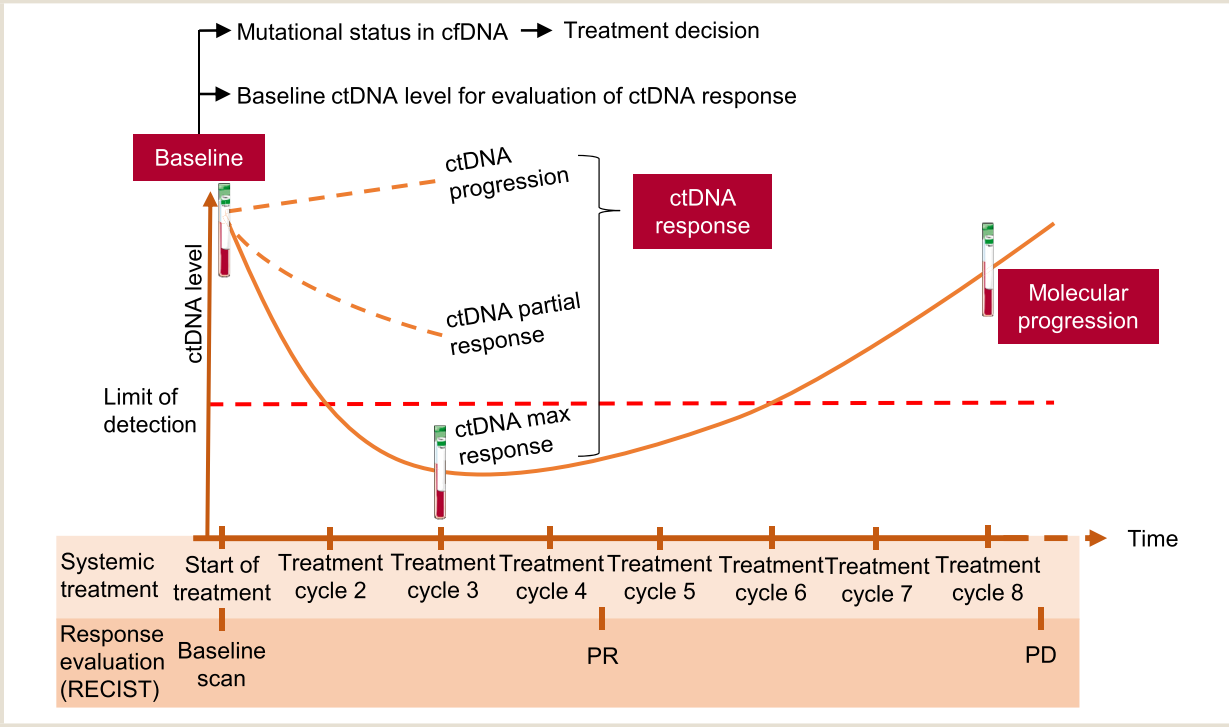
Categorized variables were expressed as counts and proportions, and continuous variables as median and/or mean values and ranges. Wilcoxon's signed-rank test was applied for the comparison of nonparametric paired samples. Progression-free survival (PFS) was measured from the date of inclusion to progression according to RECIST version 1.1 or death, whichever came first, and censored at the last documented follow-up if the patient was free of progression. Overall survival (OS) was calculated from the date of inclusion to the date of death from any cause. Patients still alive were censored at the last known date alive. PFS and OS were analyzed using the Kaplan–Meier method and compared via log-rank test. Median follow-up was estimated using the reverse Kaplan–Meier method. All reported *P*-values were 2-sided. $P < .05$ were considered to indicate statistical significance. Effect sizes were indicated by 95% confidence intervals (95% CI). Statistical analyses were performed using STATA/IC17.0 (StataCorp LLC).

Results

Feasibility of Clinical Workflow

Between 2018 and 2020, a total of 49 patients were included, and blood samples were collected consecutively from 2018 to 2022. Due to equipment failure, 1 baseline sample was not analyzed, and the patient was excluded. A total of 151 blood samples were analyzed by ddPCR, including 48, 31, 31, and 41 samples drawn at baseline, before the third treatment cycle, at the first radiological response evaluation, and at the end of the study, respectively. Of the end-of-study samples, 29 were drawn at progression. All samples showed

Figure 1 Schematic overview of the OPTIPAL II study. PR, partial response; PD, progressive disease; cfDNA, circulating free DNA; ctDNA, circulating tumor DNA. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.



acceptable low levels of PBC (mean 0,05%, median 0%, range 0%-0.4%).

The average transportation time for baseline samples was 1.1 working days (range 1-5 working days), and the average duration of analysis was 1.6 working days (range 1-3 working days), adding up to an average turnaround time of 2.7 working days (range 2-6 working days). In comparison, analysis of stored tumor tissue lasted on average 5.2 working days (range 1-11 working days; $p < .001$). One sample failed the feasibility parameters due to transportation to the laboratory of more than 3 working days; however, the sample was analyzed, and the prolonged transportation time did not affect the results' reliability and the overall time to result was only 6 working days.

Feasibility of Mutational Testing at Baseline

When comparing mutational testing on tumor tissue and cfDNA in a baseline plasma sample by ddPCR, we report a concordance of 70% (95% CI 55%-83%, n = 33/47; Table 1a). One patient did not have tumor tissue available for mutational testing and was excluded from the comparison. Discordant cases were mutated in tumor tissue but wild-type in plasma. In 11 discordant cases, the report from the standard of care mutational analysis on tumor tissue did not specify the exact mutation detected. Due to that, it could not be determined whether the detected mutation in tumor tissue was included in the ddPCR panel. In the remaining 3 discordant cases, the mutations detected in tumor tissue were included in the ddPCR panel.

The concordance between mutational testing on tumor tissue and baseline plasma samples by MassArray was 80% (95% CI 65%-90%, n = 35/44; Table 1b). One patient did not have tumor tissue available for mutational testing, and in 3 patients, the plasma volumes in the baseline samples were insufficient for MassARRAY analyses. Hence, 4 patients were excluded from the comparison. Discordant cases were mutated in tumor tissue but wild-type in plasma. In 8 discordant cases, the report from the standard of care mutational analysis on tumor tissue did not specify the exact mutation detected. Due to that, it could not be determined whether the detected mutation in tumor tissue was included in the MassARRAY panel. In the remaining discordant case, the mutation detected in tumor tissue was included in the MassARRAY panel.

Concordance between mutational testing by ddPCR and MassARRAY on cfDNA in baseline plasma samples was 87% (95% CI 73%-95%, n = 39/45; Table 1c). In 3 patients, the plasma volumes in the baseline plasma samples were insufficient for MassARRAY analyses, and these patients were excluded from the comparison. All but 1 mutation detected by ddPCR was detected by MassARRAY. Of note, the mutation not detected by MassARRAY was in the MassARRAY panel. In 4 patients, who were wild-type by ddPCR, MassARRAY detected a mutation, which in 4 of the cases was in the ddPCR panel and also present in the tumor tissue. In the last case, the MassARRAY response covered 2 mutations, of which one was in the ddPCR panel and present in the tumor tissue, and the other was neither. Results from mutational testing are seen in Table 2.

Table 1 Comparison of Mutational Testing on Tumor Tissue and Plasma by ddPCR and MassARRAY

a

Plasma, ddPCR \ Tissue	Wild-type	Mutation	Total
Wild-type	8 (17%)	14 (30%)	22
Mutation	0 (0%)	25 (53%)	25
Total	8	39	47[a]

b

Plasma, MassARRAY \ Tissue	Wild-type	Mutation	Total
Wild-type	6 (14%)	9 (20%)	15
Mutation	0 (0%)	29 (66%)	29
Total	6	38	44[b]

c

Plasma, MassARRAY \ Plasma, ddPCR	Wild-type	Mutation	Total
Wild-type	15 (33%)	1 (2%)	16
Mutation	5 (11%)	24 (53%)	29
Total	20	25	45[c]

[a]One patient did not have tumor tissue available for mutational testing.
[b]One patient did not have tumor tissue available for mutational testing and in 3 patients, the plasma volume in the baseline sample was insufficient for MassARRAY analyses.
[c]In 3 patients the plasma volume in the baseline sample was insufficient for MassARRAY analyses.
Percentages may not total 100 due to rounding.

Table 2 Mutational Testing on Tumor Tissue and cfDNA by ddPCR and MassARRAY

***RAS/BRAF* status**	**Tissue**		**Plasma**			
			ddPCR		***MassARRAY***	
KRAS mutated	29	(60%)	18	(38%)	22	(46%)
NRAS mutated	4	(8%)	3	(6%)	3	(6%)
BRAF mutated	6	(13%)	4	(8%)	4	(8%)
All wild-type	8	(17%)	23	(48%)	16	(33%)
NA	1	(2%)	0	(0%)	3	(6%)

Patient Characteristics and Outcome

Data cutoff for updated survival analyses was performed on October 10, 2022. All baseline patient characteristics collected are summarized in Table 3. The median age was 66 years (range 37-81 years). Of the 48 patients, 28 were male. The performance status was 0 to 1 in all patients except 3, with a performance status of 2. A total of 27 patients had their primary tumor located on the left side of the colon or rectum. The majority of patients had not previously been treated with palliative chemotherapy (45/48), whereas 2 patients were included prior to second-line treatment and 1 before third-line treatment.

In the study, 37 patients were treated with FOLFIRI (a combination of folinic acid, fluorouracil, and irinotecan) with the addition of EGFRi in 19 patients. Due to contraindications to fluorouracil, 4 patients were treated with irinotecan in combination with EGFRi. One patient was treated with CAPOX (a combination of capecitabine and oxaliplatin). Six patients did not receive study treatment due to deterioration before initiation of treatment. None of the patients were treated with vascular endothelial growth factor inhibitors in first-line.

Regarding the best response from study treatment, partial response was achieved in 11 patients and stable disease in 26. No patients achieved a complete response. Early disease progression occurred in 11 patients, including 5 patients with progressive disease at first radiological response evaluation and 6 patients where radiological response evaluation was not possible due to symptomatic deterioration.

At the end of follow-up, disease progression had occurred in 47 patients, and 37 patients had died. No patients were lost to follow-up. Median follow-up time was 46 months (28-n.r. months, n = 48), median PFS was 8 months (95% CI 6-8 months, n = 48), and median OS was 21 months (95% CI 13-28 months, n = 48).

ctDNA Response and Outcome

A total of 25 patients had a mutation detected in the plasma sample drawn at baseline with a median ctDNA level of 918 mutated alleles per mL plasma (range 30-548,000 mutated alleles per ml plasma, n = 25) and a median ctDNA fraction of 8.5% (range 0.1%-88.5%, n = 25). Of the 25 patients with mutations in cfDNA from the baseline plasma sample, 14 patients had a blood sample drawn before the third treatment cycle and were included in the exploratory analysis of ctDNA response and outcome (Figure 2). Off note, all patients included in the ctDNA response analyses were treated with FOLFIRI without EGFRi.

In the 14 patients available for ctDNA response analyses, the ctDNA-level at baseline and before the third treatment cycle were 412 mutated alleles per mL plasma (range 30-27,822 mutated alleles per ml plasma, n = 14) and 63 mutated alleles per mL plasma (range 0-166,000 mutated alleles per ml plasma, n = 14), respectively. This corresponded to a median ctDNA fraction of 8.5% (range 0.1%-50.2%, n = 14) at baseline and 0.9% (range 0%-51.8%, n = 14) before the third treatment cycle.

We evaluated the association between changes in ctDNA level and outcome. The median PFS were 11.2 months in patients with ctDNA maximum response (reference group; Figure 3a), 7.5 months in patients with ctDNA partial response (HR = 10.7 95%

Table 3 Patient Characteristics

Number of Patients	48 (100)
Age, median (range)	66 (37-81)
Sex	
Male	28 (58)
Female	20 (42)
Performance status (PS)	
0-1	43 (90)
2	3 (6)
Missing	2 (4)
Location primary tumor	
Colon	32 (67)
Rectum	16 (33)
Sidedness primary tumor	
Right	21 (44)
Left	27 (56)
Resection status primary tumor	
Resected	31 (65)
Not resected	17 (35)
Number of lines of previous anticancer therapies	
0	45 (94)
≥1	3 (6)
Time of metastases	
Synchronous	28 (58)
Metachronous	20 (42)
Number of metastatic sites	
1 site	20 (42)
>1 site	28 (58)
Liver metastasis	
Yes	26 (54)
No	22 (46)
Lung metastasis	
Yes	20 (42)
No	28 (58)
Tissue mutation status	
RAS/BRAF wild-type	8 (17)
RAS/BRAF mutation	39 (81)
Missing	1 (2)
LDH	
<UNL	24 (50)
>UNL	19 (40)
Missing	5 (10)
Total cfDNA level, median alleles per mL plasma (IQR)	7,048 (3,966-22,766)

Values are expressed as n(%) unless otherwise specified.
IQR, interquartile range; WHO, World Health Organization; PS, performance status; LDH, lactate dehydrogenase; ULN, upper limit of normal (<205 U/L).

Figure 2 Flow chart of mutational testing on cfDNA for evaluation of ctDNA response. cfDNA, circulating free DNA; ctDNA, circulating tumor DNA.

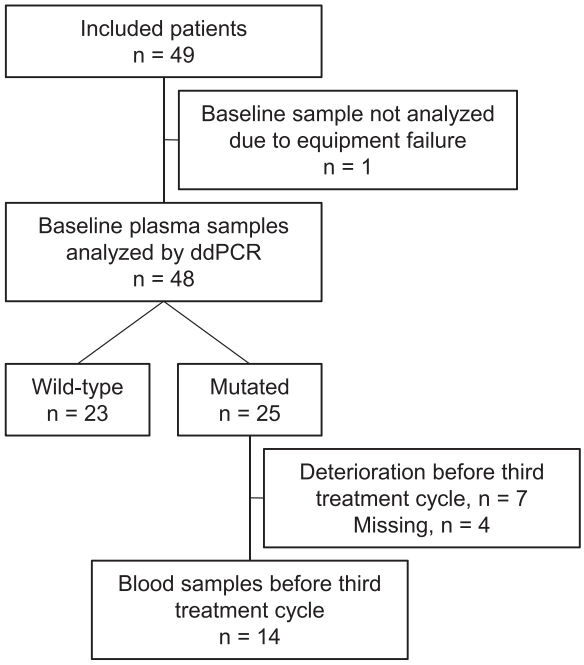
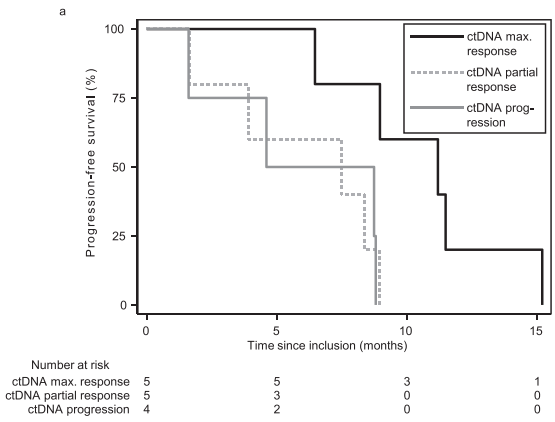
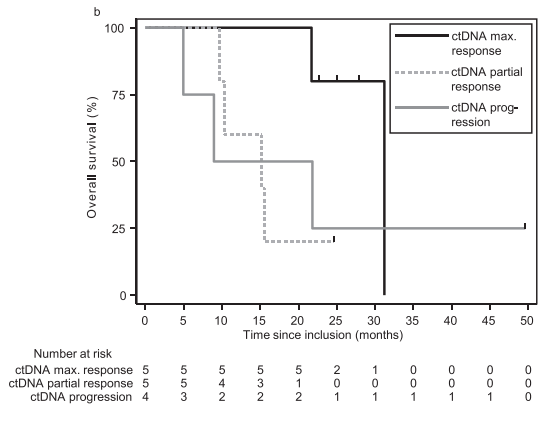


Figure 3 Kaplan-Meier estimates of progression-free survival (a) and overall survival (a) according to ctDNA response (based on early changes in ctDNA level). Comparison by log-rank test. Max., maximum; ctDNA, circulating tumor DNA; PFS, progression-free survival; OS, Overall survival; CI, confidence interval.



	ctDNA response		
	Maximum	*Partial*	*Progression*
No. of events	5	5	4
Median PFS (months)	11.2	7.5	4.6
(95% CI)	(6.5-n.r.)	(1.6-n.r)	(1.6-n.r.)
Hazard Ratio (95% CI)	ref.	10.7 (1.2-94.4)	11.4 (1.2-111.4)
	-	p=0.02	p=0.02



	ctDNA response		
	Maximum	*Partial*	*Progression*
No. of events	2	4	3
Median OS (months)	31.2	15.2	9.0
(95% CI)	(21.7-n.r.)	(9.6-n.r.)	(5.0-n.r.)
Hazard Ratio (95% CI)	ref.	4.1 (0.7-24.3)	2.6 (0.5-17.1)
	-	p=0.03	p=0.3

CI 1.2-94.4, p = .02; Figure 3a), and 4.6 months in patients with ctDNA progression (HR = 11.4 95% CI 1.2-111.4, p= .02; Figure 3a). The median OS were 31.2 months in patients with ctDNA maximum response (reference group; Figure 3b), 15.2 months in patients with ctDNA partial response (HR = 4.1 95% CI 0.7-24.3, p= .03; Figure 3b), and 9.0 months in patients with ctDNA progression (HR = 2.6 95% CI 0.5-17.1, p= .3; Figure 3b). Comparable results were obtained when evaluating the association between changes in ctDNA fraction and PFS and OS. (Supplemental Figure 1).

Molecular Progression

When comparing mutational testing on cfDNA by MassArray in baseline samples and relevant end-of-study samples, a new mutation (ie, *EGFR S492R*) was only detected in 1 patient (n = 1/36). Interestingly, this patient was not treated with EGFRi.

Discussion

The overall aim of the present study was to test feasibility parameters to prepare for randomized clinical trials investigating the utility of ctDNA. The clinical workflow for prospective analyses of baseline plasma samples was acceptable with good quality of analyses and a short average turnaround time of less than 3 working days, which is significantly shorter than mutational testing on tumor tissue. Previous studies have also reported a significantly shorter turnaround time for mutational testing on cfDNA compared to tumor tissue.[8,20]

Concordance between mutational testing on tumor tissue and cfDNA by ddPCR was 70%, which is lower than previously reported.[8,21-23] *RAS/RAF* mutations are early mutations in colorectal cancer (CRC) development,[24] and it is expected that they would be present throughout the disease, but with time and different systemic treatments, it can vary how dominant each cancer cell clones are.[25,26] Discordance between mutational status in tumor tissue and plasma could be explained by spatial and temporal heterogeneity and varying dominance of different cancer cell clones. Another explanation could be differences in the shedding of DNA from tumor cells to the bloodstream depending on the localization of metastases. For example, liver metastases are known to give rise to high shedding of DNA into the bloodstream with a high probability of a ctDNA level above the limit of detection.[23,27] However, the quantitative measure of *RAS/RAF* mutations in plasma appears to have a more significant impact on sensitivity to EGFRi compared to the binary outcome of mutational testing on archival tumor tissue.[10-13] Hence, a concordance of less than 100% is not necessarily a shortcoming of the test but could be a clinically relevant expression of the actual biology of the disease.

Mutational testing on cfDNA by MassARRAY covered more mutations than ddPCR, and future studies could investigate MassARRAY for selecting patients with mCRC who will benefit from EGFRi. Off note, mutational testing by MassARRAY does not provide a quantitative measurement of ctDNA, and hence this method has limitations in evaluating the prognostic value of quantitative baseline ctDNA level or ctDNA responses. Despite a quantitative measurement of ctDNA, mutational testing on cfDNA in plasma by ddPCR also has a shortcoming since it depends on the presence of a detectable, representative mutation.

Hypermethylation of the NPY promotor region (meth-NPY) has been suggested as a promising biomarker in CRC.[28-30] Meth-NPY is a universal biomarker in mCRC and, in opposite to mutational testing, is not dependent on the presence of specific mutations or influenced by varying dominance of tumor clones. Early dynamics in meth-NPY have been associated with clinical outcomes in patients with mCRC.[31,32]

Despite deficiencies, an objective response rate is frequently used as a surrogate end-point for OS when evaluating the effect of systemic treatment.[33,34] It has been suggested that early ctDNA response might serve as a better marker for OS.[32]

Our group has conducted a systematic review and meta-analysis evaluating ctDNA and its clinical utility in predicting treatment response or survival in patients with mCRC.[14] We report growing evidence that early ctDNA response during systemic treatment in patients with mCRC holds prognostic value, but the clinical utility is yet to be tested. Unfortunately, the reporting in the majority of studies is not standardized. An example is a response, which was defined differently in the various studies, and the definition was often unclear. For example, in some studies, the response was defined as a decrease without further description and consideration of measurement uncertainty. The deficient reporting hampers the preparation for prospective randomized clinical trials based on these studies, underlining the importance of standardized reporting (ie, REMARK guidelines[15]).

The results from our study should be interpreted with caution due to the low number of participants and events but support the theory of early ctDNA response as a potential strong valid surrogate end-point for OS.

In the present study, the emergence of a new mutation during systemic palliative treatment was a rare event. When comparing mutational testing by MassARRAY on cfDNA in a baseline sample and an end-of-study sample, only 1 patient had a mutation detected at the end of the study, which was not detected at baseline. The mutation detected was an *EGFR* ectodomain mutation (S492R), known to be resistance-conferring to cetuximab.[35] However, in this study, the mutation was detected in a patient treated with FOLFIRI without EGFRi. The rarity of acquired mutations after first-line treatment is in line with previous findings.[36]

This study has some limitations. We included a limited sample size of patients for the current purpose, and fulfilled the feasibility investigations. However, with this small sample size the utility of ctDNA-response criteria for therapy evaluation cannot be adequately assessed, but the signal from our dataset has generated a hypothesis to be tested in larger scale settings. The RAS coverage in the OPTIPAL II study was carefully selected, taking into account the known prevalence of mutations at the time and the feasibility of a ddPCR multiplex assay. However, the more rare subtypes in KRAS codon 59, NRAS codons 59, 117, and 146 were not included 1 to 3. Consequently, there are limitations in the assessment of acquired mutations by the ddPCR, and the MassARRAY assay was explored.

Conclusion

Pretreatment mutational testing on cfDNA in daily clinic is feasible and can be applied in randomized clinical trials evaluating the clinical utility of ctDNA. Early dynamics in ctDNA during systemic

treatment hold prognostic value and should be evaluated against radiological response evaluation in future larger-scale prospective trials. An earlier and more precise pseudo marker of treatment response will potentially limit ineffective treatment, avoid unnecessary treatment-related toxicity, enable an earlier change in treatment, and is likely to be more cost-effective compared to the current standard of practice.

Clinical Practice Points

- Both quantitative and molecular changes in ctDNA can hold important information in the treatment of metastatic colorectal cancer (mCRC), but its clinical utility is yet to be established.
- Before conducting a randomized clinical trial (RCT) in a new research field, it is of utmost importance to test the feasibility to assess study setup and sample size.
- In patients with mCRC, pretreatment mutational testing on cfDNA in daily clinic is feasible and can be applied in randomized clinical trials evaluating the clinical utility of ctDNA.
- Early dynamics in ctDNA during systemic treatment for mCRC hold prognostic value.
- In future larger-scale prospective studies, the early dynamics in ctDNA should be evaluated against radiological response evaluation.

Ethics Approval

The study was approved by Regional Committees on Health Research Ethics for Central Denmark Region (June 12, 2017; no. 1-10-72-111-17) and Danish Data Protection Agency (May 2, 2017; no. 1-16-02-153-17).

Informed Consent

Both oral and written informed consent was obtained from all participants for inclusion in the study, also specifically for the collection of blood samples prior to, during and after therapy (as described in the methods section) and for the use of the samples in scientific research.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' Contributions

KS designed the outline of the study. LC, KS, AB, CA, and NP collaborated in the acquisition of data. KS and LC analyzed and interpreted the data. NP, CA and AB participated in analyzing and interpreting of the data. LC wrote the manuscript. KS, AB, CA and NP revised the manuscript critically for important intellectual content. KS, NP, CA and LC confirm the authenticity of all raw data. All authors have read and agreed to the published version of the manuscript.

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Disclosure

The authors declare no competing interests.

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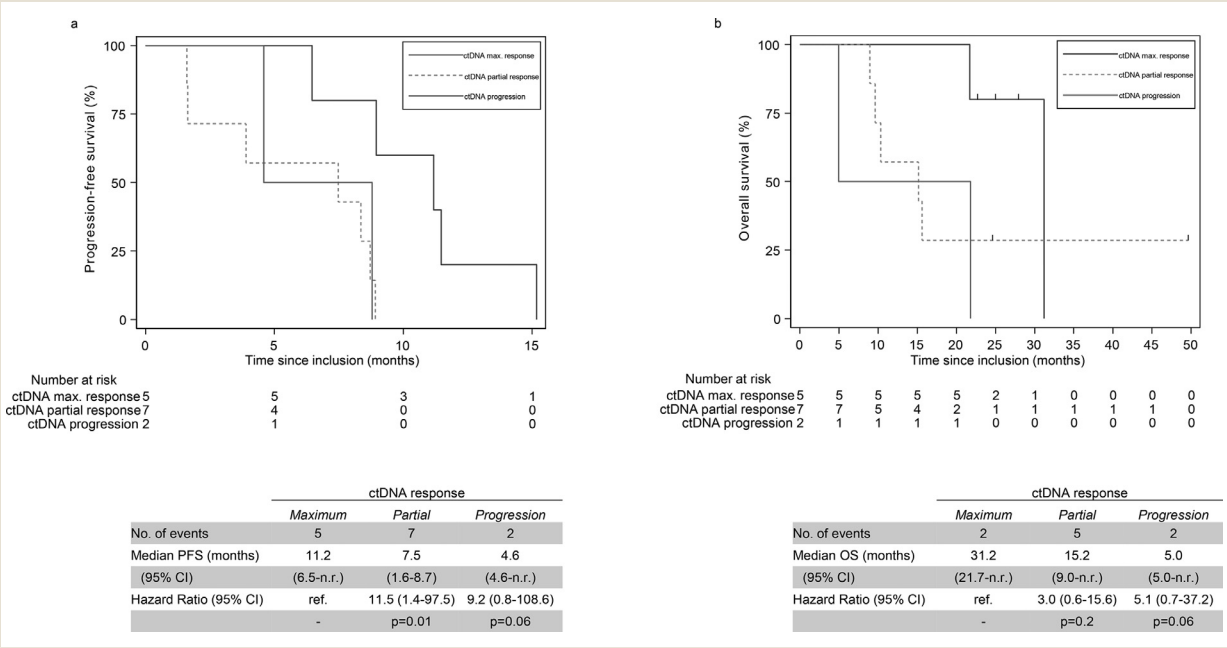
Supplementary materials

Supplemental Table 1 Mutational Testing on Tumor Tissue

N	Method	Coverage
28	PCR, idylla	KRAS codons 12, 13, 59, 61, 117, and 146. NRAS codons 12, 13, 59, 61, 117, and 146. BRAF V600D, E, K, R
17	NGS	KRAS codon 12,13,59,61,117 og 146. NRAS codon 12,13,61 og 146. BRAF codon V600
1	PCR, other	KRAS codons 12, 13, 58, 59, 61, 117, and 146. NRAS codons 12, 13, 58, 59, 61, 117, and 146. BRAF.
1	PCR, other	KRAS codons 12, 13, 58, 59, 117, and 146. NRAS codons 12, 13, 58, 59, 117, and 146. BRAF.
1	PCR, Stratagene	KRAS codons 12, 13, 59, 61, 117, and 146. NRAS codons 12, 13, 59, 61, 117, and 146. BRAF V600D, E, K, R
1	-	No tumor tissue available

Abbreviation: N, number of patients; PCR, polymerase chain reaction; NGS, next-generation sequence

Supplementary Figure 1 Kaplan-Meier estimates of progression-free survival (A) and overall survival (B) according to ctDNA response (based on early changes in ctDNA fraction). Comparison by log-rank test. Max., maximum; ctDNA, circulating tumor DNA; PFS, progression-free survival; OS, Overall survival; CI, confidence interval.



	ctDNA response		
	Maximum	*Partial*	*Progression*
No. of events	5	7	2
Median PFS (months)	11.2	7.5	4.6
(95% CI)	(6.5-n.r.)	(1.6-8.7)	(4.6-n.r.)
Hazard Ratio (95% CI)	ref.	11.5 (1.4-97.5)	9.2 (0.8-108.6)
	-	p=0.01	p=0.06

	ctDNA response		
	Maximum	*Partial*	*Progression*
No. of events	2	5	2
Median OS (months)	31.2	15.2	5.0
(95% CI)	(21.7-n.r.)	(9.0-n.r.)	(5.0-n.r.)
Hazard Ratio (95% CI)	ref.	3.0 (0.6-15.6)	5.1 (0.7-37.2)
	-	p=0.2	p=0.06