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Abstract

Purpose

Analysis of methylation markers in liquid biopsies is a promising technique for the follow-up of metastatic colorectal cancer (mCRC) patients, since they can be used in all patients, regardless of their mutational status. Therefore, we studied the value of *NPY* methylation analysis in circulating tumor DNA (ctDNA) for accurate response monitoring in mCRC patients in the PANIB trial.

Experimental design

The PANIB trial was a randomized phase two trial designed to compare FOLFOX plus panitumumab and FOLFOX plus bevacizumab in patients with *RAS* wild-type unresectable mCRC. The results of sequential liquid biopsies were correlated with results of imaging.

Results

Forty patients were included from six Belgian hospitals. Analysis of the liquid biopsies revealed that higher baseline levels of methylated ctDNA was associated with a significantly shorter overall survival (HR, 1.015; 95% CI 1.005 -1.025 and p=0.002). Furthermore, thirty-seven patients provided at least two liquid biopsies. Thirty-one of them showed a decrease in the methylation ratio after the start of therapy, which corresponded with stable disease or response on imaging at the first evaluation.

When comparing the panitumumab and bevacizumab arm, significantly higher objective response and early tumor shrinkage rates were observed in the panitumumab arm (p=0.048 and p=0.015, respectively). However, due to a small study population, the trial was underpowered to detect a significant difference in survival.

Conclusions

The results of this study confirm that baseline methylated ctDNA is a prognostic marker and indicate that *NPY* methylation is a promising marker for response monitoring in patients with mCRC.

Translational relevance

Previous studies have indicated that quantification of circulating tumor DNA (ctDNA) based on NPY methylation could provide more accurate response monitoring in metastatic colorectal cancer patients (mCRC), but its practical value remains unclear. The results of the sequential liquid biopsies of 40 patients with mCRC in this trial indicate that a decrease in methylated ctDNA after two cycles of therapy (4 weeks) corresponds to stable disease or partial response on imaging (at 8 weeks). In contrast, an increase in methylated ctDNA during follow-up indicates progressive disease. In one patient, the increase in methylated ctDNA preceded radiologic progression, indicating that methylated ctDNA can also be used for the prediction of progressive disease. This universal biomarker for mCRC might allow for earlier response evaluation, more accurate response monitoring, and prediction of progressive disease. However, larger studies with a more frequent collection of liquid biopsies are necessary to confirm this finding.

Introduction

Colorectal cancer (CRC) is the third most common cancer and a major cause of cancer-related death. In 2020, there were approximately 1.9 million new cases of CRC and an estimated 935,000 deaths from CRC, representing approximately 10% of all cancer cases and deaths (1). Of newly diagnosed patients, 15–25% have metastatic disease at diagnosis, and up to 50% of all patients eventually develop metastatic disease (2,3).

Advances in systemic therapies, including monoclonal antibodies against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), have significantly improved the survival of CRC patients. Currently, anti-VEGF or anti-EGFR combined with cytotoxic therapy is the standard first-line therapy for patients with *RAS* wild-type (WT) metastatic colorectal cancer (mCRC). However, results from several prospective clinical trials comparing anti-EGFR and anti-VEGF in combination with chemotherapy in *RAS* WT mCRC have been inconsistent (4). Therefore, at time of this study, it remained unclear which combination was the optimal first-line treatment in patients with *RAS* WT mCRC. In addition, since there is still high variability in therapeutic responses among patients, determining the optimal personalized treatment plan remains challenging.

Conventional monitoring of therapy response is based on imaging (RECIST 1.1)(5) and measurements of carcinoembryonic antigen (CEA). However, radiological assessments are usually limited in frequency (owing to radiation exposure and costs), have a poor detection limit, are not suited for detecting small metastases, and cannot describe the intrinsic characteristics of each tumor. Therefore, the development of new biomarkers would be of great value in enabling early treatment response evaluation and more accurate response monitoring. Early detection of disease progression based on these new biomarkers could allow for earlier changes in therapy, thus avoiding unnecessary side effects, enhancing efficacy, and minimizing costs.

Quantification of circulating tumor DNA (ctDNA) provides real-time information on tumor burden and has been shown to be associated with treatment responses in mCRC (6-9). Recently, it has become apparent that the methylome holds great potential for biomarker discovery, not only in pan-cancer settings (10,11), but also in colorectal cancer (12). Furthermore, previous studies have shown that quantifying ctDNA through the methylation analysis of *NPY* correlates with total tumor burden and can therefore be used for the follow-up of mCRC patients (7-9). As progressive disease might be detected earlier using liquid biopsies than radiographic evaluation, the use of liquid biopsies might be a promising tool to guide treatment.

The initial aim of this study was to compare the efficacy and tolerability of anti-EGFR therapy (panitumumab) versus anti-VEGF (bevacizumab) in combination with oxaliplatin–5FU

(FOLFOX) as first-line treatment for patients with *RAS* WT unresectable mCRC. The second aim of this study was to explore whether quantification of ctDNA based on *NPY* methylation analysis can lead to better, individualized response evaluation and real-time follow-up using non-invasive liquid biopsies in mCRC patients.

Materials and Methods

Study population

The PANIB trial (20139173 study) was a randomized, multicenter phase II trial comparing FOLFOX plus panitumumab with FOLFOX plus bevacizumab in patients with previously untreated *RAS* wild-type metastatic unresectable colorectal cancer. In this trial, patients aged ≥18 years, with an Eastern Cooperative Oncology Group performance status score of ≤2, and histologically or cytologically confirmed metastatic adenocarcinoma of the colorectum were included from six centers in Belgium.

Since the primary aim of this study was to compare the efficacy of panitumumab and bevacizumab in combination with FOLFOX, the sample size for this trial was calculated to detect a difference in progression-free survival (PFS). To demonstrate a hazard ratio of 0.66, assuming a median PFS of 10 months in the bevacizumab group, 83 patients per arm were planned to be recruited, with a minimum follow-up of 12 months. To account for a 5% dropout rate in both treatment arms, 175 patients had to be included. With this sample size, 80% power would be reached to demonstrate statistical significance at a one-sided alpha level of 0.10. However, owing to slow patient accrual, the trial was closed after the inclusion of 40 patients.

Wild-type *KRAS* and *NRAS* tumor status was confirmed by the pathology laboratory of the Antwerp University Hospital using formalin-fixed paraffin-embedded tumor tissue from the primary tumor or metastasis. The full eligibility criteria can be found in the supplemental materials and methods. Using a computer-generated minimization sequence, the patients were randomized to one of the two treatment arms in a one-to-one ratio. Tumor response evaluation was performed every 8 weeks by a blinded radiologist and based on RECIST 1.1 criteria. Patients received the assigned treatments until progressive disease (PD), consent withdrawal, or unacceptable toxicity. Safety assessments were performed at baseline, during each treatment cycle, and 30 days after the last drug administration. Adverse events (AEs) were graded using the Common Terminology Criteria for Adverse Events (version 4.0).

The study protocol was approved by the independent ethics committee at the participating study centers, and all patients provided signed informed consent before any study-related procedures were performed (Ethical Committee of Antwerp University Hospital protocol number 14/24/256 and EUDRACT 2014-000543-33). The trial was conducted in accordance with the Declaration of Helsinki.

Blood samples

Blood samples were collected at three different time points during treatment. The first liquid biopsy sample was collected at enrolment before the start of treatment (T1). A second liquid

biopsy was collected between the second and third cycles (T2; approximately 1 month after enrolment), and the last liquid biopsy was obtained at the end of the study (progressive disease, unacceptable toxicity, or change of therapy, T3). At enrolment, 30 ml blood (EDTA tubes) was collected, and 20 mL blood was collected at the following two time points. The tubes were centrifuged at 400g for 10 minutes, followed by another centrifugation step (10 minutes at 16,000g). Aliquots of plasma were stored at -80 °C until cell-free DNA (cfDNA) extraction was performed using a QIAsymphony DSP Circulating DNA Kit (Qiagen, Hilden, Germany). cfDNA was stored at -20°C, and cfDNA concentration was determined using the Qubit 2.0 fluorometer with the double strand DNA high sensitivity assay (Thermo Fisher Scientific, Eugene, OR, USA). The downstream ctDNA analyses were not pre-planned but performed with pre-specified hypotheses.

Droplet digital PCR analysis

The full droplet digital PCR (ddPCR) protocol was performed as described previously (6-9,13). First, up to 45µL of cfDNA was bisulfite-converted in a 50µL reaction using the Zymo EZ DNA methylation kit (Zymo Research, Research, Freiburg, Germany) before elution into 10.5µL. For each bisulfite conversion of new samples, two control samples were incorporated to ensure adequate bisulfite conversion and DNA recovery, namely, universal methylated DNA (Zymo Research) and human genomic DNA. These control samples were also included as control samples in the further ddPCR analysis.

The *ALB/NPY* duplex ddPCR was performed using 9µl of bisulfite converted cfDNA per well in a 20µl reaction with Bio-Rad ddPCR supermix for probes (No dUTP). A more detailed description of the ddPCR assay is provided in the supplemental materials and methods section.

Droplets were generated using the QX200 Automated Droplet Generator (Bio-Rad, CA, USA), and the PCR step was run on a Veriti thermal cycler (Applied Biosystems, Waltham, MA, USA). A QX200 Droplet Digital Reader (Bio-Rad) was used to read the samples. Data analysis was performed using QuantaSoft version 1.0 (Bio-Rad).

The limit of blank (LOB) and limit of detection (LOD) were calculated, as previously described (14-17). The LOB is defined as the frequency of positive droplets measured in negative control samples (n=26, human genomic DNA of healthy volunteers) and is a finite number of false positive droplet events detected per analysis. The LOB and LOD values for the *NPY* methylation assay were 1.60 and 5.43, respectively (Supplemental Table S1). The methylation ratio was calculated for each positive sample. This ratio determines the fraction of ctDNA (containing tumor-specific methylation of the *NPY* gene promoter) in the total amount of cell-free DNA. This was calculated as the ratio of the normalized number of

droplets containing *NPY* methylated sequences minus the LOB value over the number of droplets containing albumin sequences.

Next generation sequencing

Liquid biopsies of patients with progressive disease during anti-EGFR therapy were studied to identify potential mechanisms for the acquired resistance. The cfDNA of liquid biopsies collected after detection of progressive disease was sequenced using the Oncomine™ Colon cfDNA Assay (Thermo Fisher Scientific). This cfDNA panel covers 14 genes with >240 hot spots (SNVs and short indels), including *AKT1, BRAF, CTNNB1, EGFR, ERBB2, FBXW7, GNAS, KRAS, MAP2K1, NRAS, PIK3CA, SMAD4, TP53,* and *APC*. This assay uses tag sequencing technology (unique molecular identifiers) and allows for the detection of rare variants with up to 0.1% allelic frequency.

Library preparation, molecular barcoding, and sequencing were performed according to the manufacturer's instructions, using a maximum of 50ng of cfDNA as input. The concentration of each Oncomine cfDNA library was determined using the Ion Library TaqMan Quantitation Kit. Sequencing was performed on an ION S5 system using Ion 540 chips (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis was performed using Ion S5 Torrent Server software and Ion Report software with hg19 as the human reference genome.

The NGS data for library performance and variant calling quality are presented in Supplemental Table S2. The manufacturer recommends a median read coverage (MedReadCov) of >25,000 and median molecular coverage (MedMolCov) of >2500 to detect a variant with a minor alle frequency of 0.1%.

Classification of the somatic variants was carried out based on the standardized approach of the Belgian ComPerMed Expert Panel(18).

Shallow whole-genome sequencing of cell-free DNA

12.5 ng of cfDNA was used as input for shallow whole-genome sequencing (sWGS), aiming for a coverage of x0.2-0.4-fold. Library preparation was performed using the TruSeq Nano DNA High Throughput Library Prep Kit (Illumina, San Diego, CA, USA) on an automated Hamilton STAR liquid handling system (Hamilton, Germany GmbH, Robotics, Gräfeling, Germany) with dual indexing, and sequencing was performed on the NextSeq500/550 platform (Illumina). The fraction of tumor-derived DNA in cell-free DNA was estimated using the R package ichorCNA(19).

Statistical analysis

Analyses were performed using SPSS version 27 (IBM Corp., Armonk, NY, USA) and R version 3.6. Patient characteristics were compared between the treatment arms using the independent samples t-test for continuous variables and the chi-square or Fisher's exact test for categorical variables. Because of the small sample size, the Monte Carlo chi-square test was used for non-dichotomous categorical variables. Survival and duration of response were modeled using Kaplan-Meier curves and described with medians and 95% confidence

interval (CI), comparing groups using the log-rank test. The association between survival and baseline characteristics was assessed using Cox proportional hazard models, and effect sizes were reported using the hazard ratio (HR) and its 95% CI. All Cox proportional hazards analyses were adjusted for sex and age. Objective response and early tumor shrinkage rates were compared using Pearson's chi-square test. Resection rates were compared using Fisher's exact test. The time to response was studied using the Mann-Whitney U test. For the analyses of liquid biopsies, methylation ratios were compared using the nonparametric Wilcoxon signed-rank test. Statistical significance was set at $P < 0.05$.

The figures were designed using GraphPad Prism version 9 (GraphPad Software Inc., La Jolla, CA, USA). The REMARK (REporting recommendations for tumor MARKer prognostic studies) checklist was used to ensure transparent reporting (Supplemental Table S3) (20).

Data availability

The sequencing data generated in this study are publicly available in European Nucleotide Archive (ENA) at EGAS00001006820. The ddPCR data generated in this study are available upon reasonable request from the corresponding author.

Results

Patient population

Between 17/2/2015 and 07/03/2019, 40 mCRC patients from six institutes were included. Seventy percent of patients was male and the median age was 68 years. The primary tumor was located in the left or rectal colon in 73.7% of patients and in the right colon in 23.7% of patients. One patient had a left-sided and right-sided tumor (2.6%). Baseline patient characteristics are provided in Table 1.

Using a computer-generated minimization sequence, 20 patients were allocated to receive FOLFOX combined with panitumumab and 20 were assigned to receive FOLFOX with bevacizumab. The demographic and baseline characteristics of the two treatment arms were similar, except for ECOG PS (p=.034, Supplemental table S4). Supplemental figure S1 shows a CONSORT diagram of the study.

Comparison of anti-EGFR therapy to anti-VEGF therapy

At the time of data cutoff, all subjects had discontinued first-line treatment. The reasons for end of study were, for panitumumab vs bevacizumab respectively, disease progression (25% vs 50%), toxicity (20% vs 5%), withdrawal of consent (10% vs 0%), metastasectomy (15% vs 5%), and change to alternative treatment (30% vs 40%).

Cox proportional hazard analysis showed no significant difference in PFS (HR 0.789; 95% CI 0.24-2.63; p=.700) or overall survival (OS) (HR 0.673; 95% CI 0.28-1.60; p=.371) between the treatment arms. However, the objective response and early tumor shrinkage rates were significantly higher in the panitumumab arm (16/19 and 15/17) than in the bevacizumab arm (11/20 and 9/18, p=0.048 and p=0.015, respectively). In addition, there was a trend towards a higher resection rate, shorter time to response and a longer duration of response in the panitumumab arm, but these differences did not reach statistical significance. Furthermore, there was a trend towards increased toxicity in the panitumumab arm, with significantly more patients experiencing grade 3 or 4 adverse events (p=0.004). (All results presented in supplemental figure S2 and S3, supplemental table S5 and S6 and the supplemental data file)

Methylation analysis – liquid biopsy

At study closure, 96 liquid biopsy samples were available (40 at enrolment (T1), 36 before the third cycle (T2), and 20 at the end of the study (T3)). At the end of the study, 11 of 20 patients that provided a liquid biopsy developed progressive disease, 1 patient experienced intolerable toxicity, 3 underwent curative surgery and 5 patients changed to other therapies.

Methylated circulating tumor DNA at baseline

At baseline, 35 of 40 patients (87.5%) had detectable levels of methylated circulating tumor DNA. There were no statistically significant associations between presence of methylated ctDNA at baseline and patient characteristics. However, there was a trend towards a higher SLDTL in the group with detectable methylated ctDNA (Supplemental table S7).

The median *NPY* methylation ratio at baseline was 5.58% (range 0.00%-285.82%) (Supplemental figure S4). The only variable significantly associated with the baseline methylation ratio was sex, with females having higher *NPY* methylation levels at baseline (p=.001, Table 1).

Baseline methylation ratio was not significantly associated with PFS but showed a trend towards shorter PFS with higher methylation ratios (HR, 1.007; 95% CI 0.998-1.017, p=0.143).

However, the baseline methylation ratio (studied as a continuous variable) was significantly associated with overall survival (HR, 1.015; 95% CI 1.005 -1.025 and p=0.002). In this model, sex was also significantly associated with OS (female sex was associated with better survival, HR = 0.192, p=0.024). Figure 1 shows Kaplan-Meier curves for PFS and OS according to the baseline methylation ratio.

Early response evaluation based on methylated circulating tumor DNA

To study the value of methylated ctDNA analysis for early response evaluation, the *NPY* methylation ratios of the liquid biopsy at T1 (baseline) and T2 (after two cycles) were compared. Thirty-seven of 40 patients provided at liquid biopsy at T1 and T2 (Table 2). Thirty-one of them (83.8%) had detectable methylated ctDNA at baseline and showed a decrease in the *NPY* methylation ratio after the start of therapy. Five patients had no detectable *NPY* methylation in ctDNA at baseline, and in all of these patients, methylated ctDNA remained undetectable after two cycles of therapy. For these two groups of patients, a decrease in the methylation ratio or no detectable methylated ctDNA at T2 corresponded with response on imaging (stable disease (SD), partial response (PR), or complete response (CR)) in all patients where imaging data and liquid biopsy samples were both available (n=34) at the first evaluation. In contrast, one patient had a positive sample at baseline but showed an increase in the methylation ratio at T2. In this patient, progressive disease was detected during the first evaluation. Overall, the median *NPY* methylation ratio decreased from 5.58% at baseline to 0.05% after two cycles of therapy. This was a statistically significant decline in the methylation ratio (p=<.0001).

Normalized methylated ctDNA levels

The initial effect of therapy on methylated ctDNA was also evaluated by comparing the baseline and follow-up samples of all patients (who provided two samples with the baseline sample being positive). In Supplemental figure S5, this change in methylated ctDNA levels after the start of treatment is described by normalizing the *NPY* methylation ratio to 100% at baseline. The median normalized fraction of methylated ctDNA was 100% at baseline, 0.29% after two cycles of therapy, and 3.13% at the end of the study (all reasons for the end of the study combined). In patients selected for curative surgery, the mean normalized fraction of methylated ctDNA was 0% at the end of the study and 183.07% in patients with progressive disease.

ctDNA response group

In correspondence to the publication by Garlan et al. (6) and Thomsen et al.(8), patients were divided into two groups based on the methylated ctDNA at baseline (T1) and after two cycles of therapy (T2); low-level methylated ctDNA (LctDNA) and high-level methylated ctDNA (HctDNA). The LctDNA group consisted of patients with a *NPY* methylation ratio of zero at baseline as well as those decreasing to a level with zero included in the 95% CI after two cycles of therapy. This LctDNA group represents the patients with a deep, early molecular response to treatment. The HctDNA group included all other patients with a decreased level of ctDNA at T2, but who did not reach 0.

In this study, twenty-eight patients belonged to the LctDNA group and eight to the HctDNA group. (One patient with an increase of ctDNA from T1 to T2 was excluded from this analysis.) The objective response rate was 74.1% in the LctDNA group and 87.5% in the HctDNA group (Supplemental table S8). There was a trend towards longer PFS in the LctDNA group compared to the HctDNA group (median PFS 17.26 months vs 9.34 months), however this association was borderline significant (HR .276 with 95% CI .075-1.014, p=.052). Likewise, there was a trend towards a longer OS in the LctDNA group, but this association did not reach statistical significance, with a median OS of 30.99 months in the LctDNA and 23.10 months in the HctDNA group (HR .352 with 95% CI 0.115-1.072, p=.066, corrected for age and sex). Figure 2 provides the Kaplan Meier curves according to ctDNA response.

Slope of ctDNA decrease

To study the clinical value of an early and sharp decrease of the *NPY* methylation ratio after start of therapy, we studied the slope of ctDNA decrease from T1 to T2. The slope was calculated as ((T2-T1)/T1)*100 (6). When studying the slope of ctDNA decrease as a continuous variable, no statistically significant association was observed with OS or PFS. However, when the slope of ctDNA decrease was studied as a categorical variable (and dividing the patients in two groups: below and above the median slope), a statistically significant association with PFS was observed (logrank: p=.025).

Follow-up of individual patients

Supplemental figure S6 provides graphs and additional clinical information for all the patients included in this study. In figure 3, an overview is provided of the results of liquid biopsies and CT imaging of all patients with a liquid biopsy at PD.

As demonstrated in the graphs, in patients who responded to therapy, a decrease in the methylation ratio was observed after initiation of therapy, and the methylation ratio remained low or undetectable during response. In patients who underwent curative metastasectomy, the methylation ratio remained low. In contrast, patients with progressive disease show an increase in *NPY* methylation ratio.

Furthermore, patients who underwent curative metastasectomy had a lower methylation ratio at baseline (mean 9.80% with SD 19.45%) and after two therapy cycles (mean 1.09% with SD 2.17%) than all other patients (baseline mean 24.45% with SD 49.56%; T2, mean 1.01% with SD 2.26%). However, this difference was not statistically significant.

In contrast, patients who reached the end of the study due to progressive disease had a higher methylation ratio at baseline (mean 40.81% with SD 72.73%) and after two cycles of therapy (mean 1.21% with SD 2.56%) than all other patients (baseline, mean 12.29% with SD 16.04% and T2, mean 0.90% with SD 2.03%). Furthermore, there was a statistically significant association between a higher methylation ratio at T2 and developing PD at the end of the study in this patient population. Out of 7 patients that provided a liquid biopsy at progressive disease, in 5 patients the progressive disease was detectable as a rise in methylation ratio (range: 0.05% - 157.32%) compared to the lowest methylation ratio at time point 2. Of the two patients that did not show an increase in the methylation ratio, one patient did not provide a liquid biopsy after the start of therapy (T2) and therefore had no methylation ratio to compare the liquid biopsy at progressive disease to. The second patient had no detectable methylated ctDNA at baseline or during the follow-up.

Detection of resistance mechanisms against anti-EGFR – liquid biopsy

Five patients developed progressive disease during anti-EGFR therapy. From three patients, sufficient cfDNA was acquired from liquid biopsies that were collected after the detection of progressive disease to study acquired resistance mechanisms against anti-EGFR using the Oncomine™ Colon cfDNA Assay. In these three patients, no mutations were detected in *KRAS* (exon 2,3 and 4) or *NRAS* (exons 2 and 3) (data on coverage is provided in Supplemental Table S2).

Two variants were detected in Patient 1: an *FBXW7* R479Q mutation with a variant allele frequency (VAF) of 12.57% and a *TP53* variant (C135Y) with a VAF of 17.95% (Table 3).

This corresponds to a ctDNA level (methylation ratio) of 14.17%, indicating that these mutations are present in the tumor.

SMAD4, *TP53* and *BRAF* mutations were detected in patient 13. The *BRAF* mutation was a V600E mutation with a VAF of 41.38%. The *NPY* methylation ratio was 42.06% in the same liquid biopsy sample. However, before starting therapy, a formalin-fixed paraffin-embedded (FFPE) tumor sample was tested for *BRAF* mutations but did not reveal the V600E mutation. To determine whether this mutation was acquired during therapy or was missed in the baseline FFPE sample, we tested liquid biopsies of T1, T2, and T3 using the BioRad ddPCR *BRAF* V600 Screening Kit. *BRAF* V600E mutation was detected in all liquid biopsy samples, including the baseline sample (Supplemental Table S9).

In the last patient, 24, only a *TP53* variant with low VAF (0.07%) was detected. However, this patient showed an interesting pattern of *NPY* methylation ratios, with values above 100%. We hypothesized that this could be due to copy number alterations (CNA) of *NPY* (gene of interest) or *ALB* (reference gene). For this reason, low-pass WGS was performed on cfDNA of the liquid biopsies (since no tumor material was available). This showed a CNA profile (Supplemental figure S7) with a hemizygous deletion of chromosome 4 and high-level amplification of part of chromosome 7 (five copies). The reference gene, *ALB*, is located on chromosome 4 and the target gene of the *NPY* methylation assay is located on chromosome 7p15. This resulted in a 5 times higher number of copies of *NPY* compared to *ALB*. The *NPY* methylation ratio measured at baseline was 285.82%, which corresponded to a ratio of 57.16% of ctDNA in all cell-free DNA when considering the CNA. Interestingly, this corresponded to a tumor fraction of 58.46%, as measured by the ichorCNA tool.

Discussion

The first aim of this study was to compare the efficacy and tolerability of panitumumab versus bevacizumab in combination with FOLFOX as first-line treatment for patients with *RAS* WT mCRC. As a result of the limited sample size, no conclusions can be drawn from the results of the current trial. However, our results show a trend in accordance with the latest trials on this subject (Supplemental Table S10). For example, in the PARADIGM trial panitumumab plus mFOLFOX6 was compared to bevacizumab plus mFOLFOX6 as first-line treatment in patients with *RAS* wild-type mCRC. In this phase 3 study in 802 patients, a significant better OS was observed in the panitumumab group compared to the bevacizumab group (HR 0.84; 95% CI 0.72-0.98; p=.030). Even though PFS was comparable in the two groups, a higher response rate and curative resection rate were observed in the panitumumab arm (21). Likewise, in the STRATEGIC-1 multi-line therapy trial, two treatment strategies were compared (FOLFIRI-cetuximab followed by mFOLFOX6-bevacizumab vs OPTIMOX-bevacizumab followed by FOLIRI-bevacizumab and anti-EGFR therapy with or without irinotecan). The treatment strategy starting with anti-EGFR therapy led to significantly higher response rates in first-line and a trend towards a longer OS (RR, p=.003; OS, HR 1.26 ; 95% CI 0.94-1.7, p=0.121)(22). The current trial, however, showed no significant difference in OS between treatment with panitumumab or bevacizumab, as can be expected based on the limited sample size. However, regardless of the small sample size, a significantly higher objective response rate and early tumor shrinkage rate was observed in the panitumumab arm. Furthermore, the panitumumab arm showed a trend toward a longer PFS, longer duration of response, shorter time to response, and higher resection rate, which corresponds to the previously cited trials.

The results of these trials all strengthen the proposed treatment strategy of the current ESMO guidelines in which anti-EGFR therapy is recommended as first-line therapy in *RAS* WT patients in which cytoreduction is the treatment goal (4).

Since the start of this trial, extensive research has been conducted on the predictive value of primary tumor location (21,23-32). Since anti-EGFR therapy is less effective in patients with right-sided tumors, it is especially recommended in patients with left-sided tumors to start with anti-EGFR therapy in first-line. Owing to the small study population, it was not possible to study the effect of primary tumor location in this trial.

The second aim of this study was to explore whether quantification of ctDNA based on *NPY* methylation analysis can lead to better individualized treatment monitoring and real-time follow-up using minimally invasive liquid biopsies. In this study, 87.5% of patients had detectable ctDNA levels at baseline. This is similar to that reported in other studies of patients with mCRC (76.8% (6), 87.5%(7)), confirming that the percentage of non-shedding samples is relatively low in these patients (33). It is of interest to study why five patients had no detectable *NPY* methylation in the liquid biopsy at baseline. This could be because the tumor was negative for *NPY* methylation or the concentration of *NPY* methylated ctDNA was too low to be detected.

Corresponding to previous research, in this patient population, a higher baseline *NPY* methylation ratio was significantly associated with shorter overall survival (6,9,34). This is in line with other studies showing that baseline cfDNA or ctDNA concentrations are prognostic factors (35,36). Even though this was a relatively small study population, it can be noted that a decrease in the methylation ratio or undetectable methylated ctDNA after two cycles of therapy corresponded to stable disease or response on the first radiologic evaluation. Only one patient showed an increase in the methylation ratio after two cycles and interestingly, this patient already showed progressive disease on the first evaluation (after 4 cycles of therapy). Even though no conclusions can be drawn from one patient, it is striking that an increase in *NPY* methylation ratio, which is known to reflect tumor burden, might be detected before progressive disease is noticed on imaging. This earlier detection of progressive disease in ctDNA has also been observed in previous studies on metastatic colorectal cancer (lead time ranging from 0.5–10 months) (9,37,38) and in other cancer types (39-41).

The fact that PFS was longer in the LctDNA arm compared to the HctDNA arm (borderline significance) confirms, as suggested by Garlan et al.(6), the importance of not only a decrease in ctDNA level alone but also a decrease below a negligible ctDNA threshold (0.1 ng/ml in the study of Garlan et al, with 0% in the 95% CI in this study). Likewise, Garlan et al. did not observe a significant difference in OS in the two groups. This could be due to the fact that OS is determined by many more other factors (2^{nd} line therapy, comorbidities, …) than PFS. PFS might have a more direct association with the initial decrease in ctDNA level. However, Thomsen et al. did find a significant better PFS and OS in the LctDNA group. (6,8)

Patients selected for curative metastasectomy had a lower methylation ratio at baseline or T2 than all other patients. If this finding could be replicated in a larger cohort of patients, this could indicate that a low *NPY* methylation ratio could be used to select patients who are candidates for metastasectomy. Furthermore, it is interesting to note that the methylation ratio at the end of the study (before surgery) was 0.00% in the three patients that were selected for metastasectomy. However, the relationship with disease-free survival and metastasectomy could not be studied in this trial.

When progressive disease on liquid biopsy was defined as any increase in the methylation ratio from T2 to T3, the sensitivity for detection of progressive disease was 83.3% (5/6) with a specificity of 100% (6/6) in this study. Patients who reached the end of the study due to progressive disease had a higher methylation ratio at baseline and after two cycles of therapy than all other patients. This might indicate that a smaller molecular response after two cycles of therapy can select patients at greater risk for early progressive disease, and that these patients should be followed more closely.

In addition, ctDNA was sequenced to identify the acquired resistance mechanisms in patients who developed resistance against anti-EGFR. In patient 13, a *BRAF* V600E mutation was detected, which was not detected in the FFPE tumor sample at baseline. As *BRAF* V600E mutations are rarely detected as an acquired resistance mechanism (42,43), this may be a false negative result on the FFPE sample due to insufficient tumor cell percentage or lower sensitivity of the used test compared to liquid biopsy.

In patients 1 and 13, mutations were detected with a VAF that was comparable to the *NPY* methylation ratio. This indicates that mutated and methylated ctDNA are correlated, which corresponds to results of previous studies (7,9). However, methylated ctDNA has an advantage over mutated ctDNA since it can be used in all mCRC patients, irrespective of mutational status.

An important strength of this study is the uniform collection and processing of the liquid biopsies. However, it is important to emphasize that the small study population and low number of liquid biopsies limit our interpretation.

To confirm that quantification of ctDNA based on *NPY* methylation can be used for response monitoring and that progressive disease can be detected earlier with liquid biopsies than with conventional CT imaging, clinical trials with serial collection of liquid biopsies at more frequent time points are needed. Furthermore, the relationship between methylated ctDNA and CEA is of interest and should be studied further. To answer these questions, the FOLICOLOR lead-in trial is currently being conducted at our center (ClinicalTrials.gov Identifier: NCT04735900). This is a prospective study wherein biweekly liquid biopsies are collected from patients with *RAS* and *BRAF* WT mCRC. This study will allow us to study the changes in ctDNA levels based on *NPY* methylation during treatment in more detail.

Conclusions

In this study of 40 *RAS* WT mCRC patients, significantly higher objective response and early tumor shrinkage rates were observed in patients treated with FOLFOX and panitumumab compared to patients treated with FOLFOX and bevacizumab (p=0.048 and p=0.015, respectively). However, due to the small study population, the trial was underpowered to detect a significant difference in survival or to draw further conclusions.

The results of liquid biopsy analyses in this study confirm that baseline methylated ctDNA is a prognostic marker. In addition, our results indicate that analysis of methylated ctDNA of sequentially collected liquid biopsies can also be used for the follow-up of patients with mCRC. We believe that this marker, which can be used in all patients with mCRC, might allow accurate response monitoring and early detection of progressive disease. However, larger studies with a more frequent collection of liquid biopsies are necessary to confirm this hypothesis.

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683 # Tables

684 **Table 1.** Baseline patient characteristics and median baseline ctDNA level

	N (%)	Median baseline ctDNA (IQR)	*p*-value
All patients	40 (100%)	5.575 (29.13)	
Age group			
<65	13 (32.5%)	1.740 (20.06)	.291
≥65	27 (67.5%)	15.790 (32.08)	
Sex			
Female	12 (30%)	35.685 (44.00)	**.001**
Male	28 (70%)	1.435 (16.95)	
ECOG PS			
0	23 (59.0%)	2.630 (19.37)	.324
1	15 (38.5%)	21.730 (40.80)	
2	1 (2.6%)	6.500 (one patient)	
Primary tumor location			
Right	9 (24.3%)	19.440 (33.69)	
Left + rectal	28 (75.7%)	1.750 (21.70)	.196
Primary tumor location			
Right	9 (24.3%)	19.440 (33.69)	
Left	20 (54.1%)	9.610 (27.82)	.311
Rectal	8 (21.6%)	1.435 (5.33)	
Synchronicity of metastases	27 (67.5%)	15.790 (32.34)	
Synchronous	13 (32.5%)	4.650 (19.24)	.851
Metachronous			
Number of metastatic sites			
1	23 (62.2%)	6.500 (29.63)	.839
≥2	14 (37.8%)	10.045 (34.58)	
Liver involvement			
N**o**	7 (18.9%)	6.500 (32.34)	0.698
Y**es**	30 (81.1%)	9.325 (31.87)	
SLDTL			
Below median	18 (50%)	3.205 (21.73)	0.159
Above median	18 (50%)	18.890 (40.49)	
Treatment arm			

Bevacizumab	20 (50%)	5.575 (34.59)	.978
Panitumumab	20 (50%)	8.775 (29.10)	

685 N, Number; IQR, Interquartile range; ECOG PS, Eastern Cooperative Oncology Group
686 Performance Status; SLDTL, Sum of largest diameter of target lesions according to RECIST
687 criteria. *p*-values in bold are below 0.05 and are considered statistically significant.
688

Table 2. Evolution of methylated ctDNA

Methylated ctDNA at T1 and T2	Number of patients (n= 36)	
	No	**%**
Positive at T1 and decreased at T2	30	83.3
Negative at T1 and negative at T2	5	13.9
Positive at T1 and increased at T2	1	2.8

T, timepoint.

691 **Table 3.** Overview of mutations identified in patients that developed resistance against anti-EGFR

Patient	Methylation ratio (*NPY*/*ALB*)	MedReadCov	MedMolCov	Gene	Variant	Class	VAF	MolCov	Theoretical LOD
1	14%	52.837	2.249	*TP53*	c.404G>A p.C135Y	Suspected deleterious	18%	841	0.26-0.40%
				FBXW7	c.1436G>A p.R479Q	Suspected deleterious	13%	3197	
13	42%	10.987	231*	*SMAD4*	c.989A>T p.E330V	Unknown	55%	170	2.5-12.6%
				BRAF	c.1799T>A p.V600E	Deleterious	41%	12	
				TP53	c.824G>A p.C275Y	Suspected deleterious	67%	8	
24	169%	30.346	4315	*TP53*	c.530C>G p.P177R	Suspected deleterious	0.07%	4	0.14-0.16%

692

693 * Reduced sensitivity; MedReadCov, median read coverage; MedMolCov, median molecular coverage; VAF, variant allele frequency; MolCov,
694 molecular coverage; LOD, limit of detection.

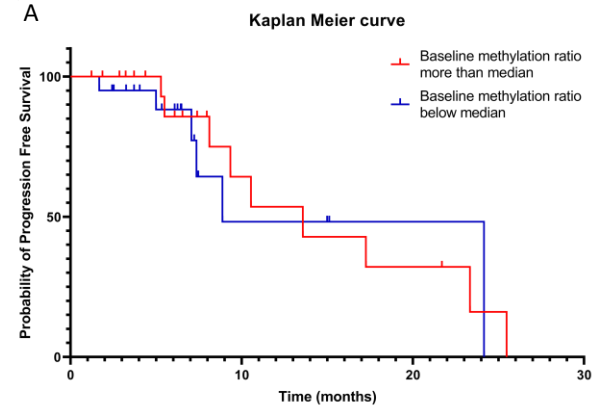
Figure legends

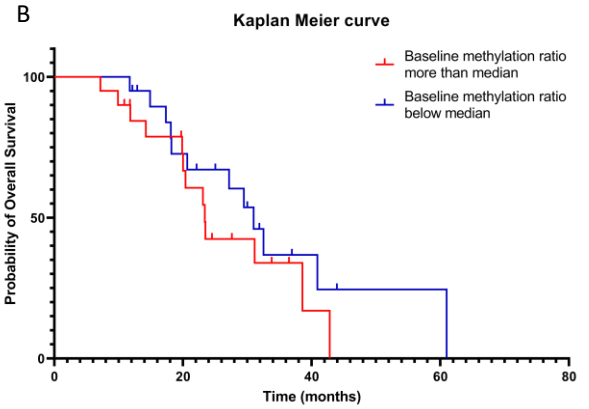
Figure 1. Kaplan Meier curve for (a) PFS and (b) OS according to baseline methylation ratio above or below the median. Bmet, baseline methylation ratio.

Figure 2. Kaplan Meier curve for (a) PFS and (b) OS according to methylated ctDNA response.

Figure 3. Changes in levels of ctDNA based on *NPY* methylation (dots) and results of CT imaging (squares, sum of largest diameter of target lesions; triangles, response evaluation according to RECIST criteria) are presented in function of time after treatment start (days): (**a-g**) all patients with a liquid biopsy at progressive disease. SD, stable disease; PR, partial response; PD; progressive disease.

Figure 1

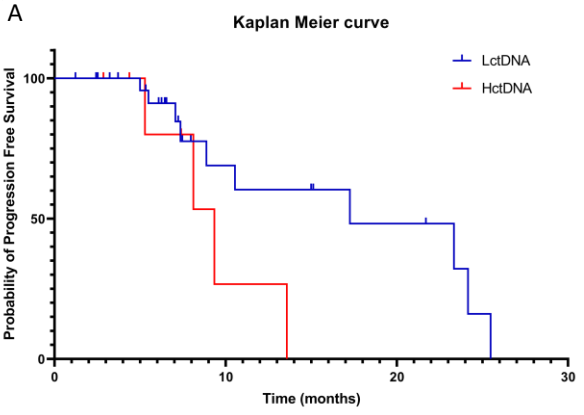


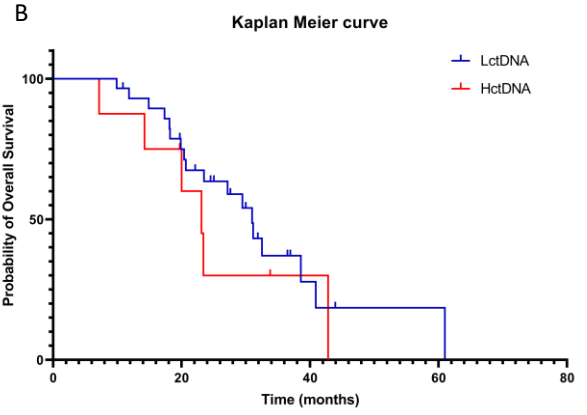


	Bmet < median	Bmet > median
Reached PD	6/20 (30%)	9/20 (45%)
Median PFS (months)	8.88 (95% CI 0.00-18.20)	13.58 (95% CI 7.43-19.73)
Log Rank test: *P* = 0.795		

	Bmet < median	Bmet > median
Deceased	12/20 (60%)	13/20 (65%)
Median OS (months)	30.99 (95% CI 25.34-36.64)	23.40 (95% CI 19.36-27.44)
Log Rank test: *P* = 0.305		

Figure 2

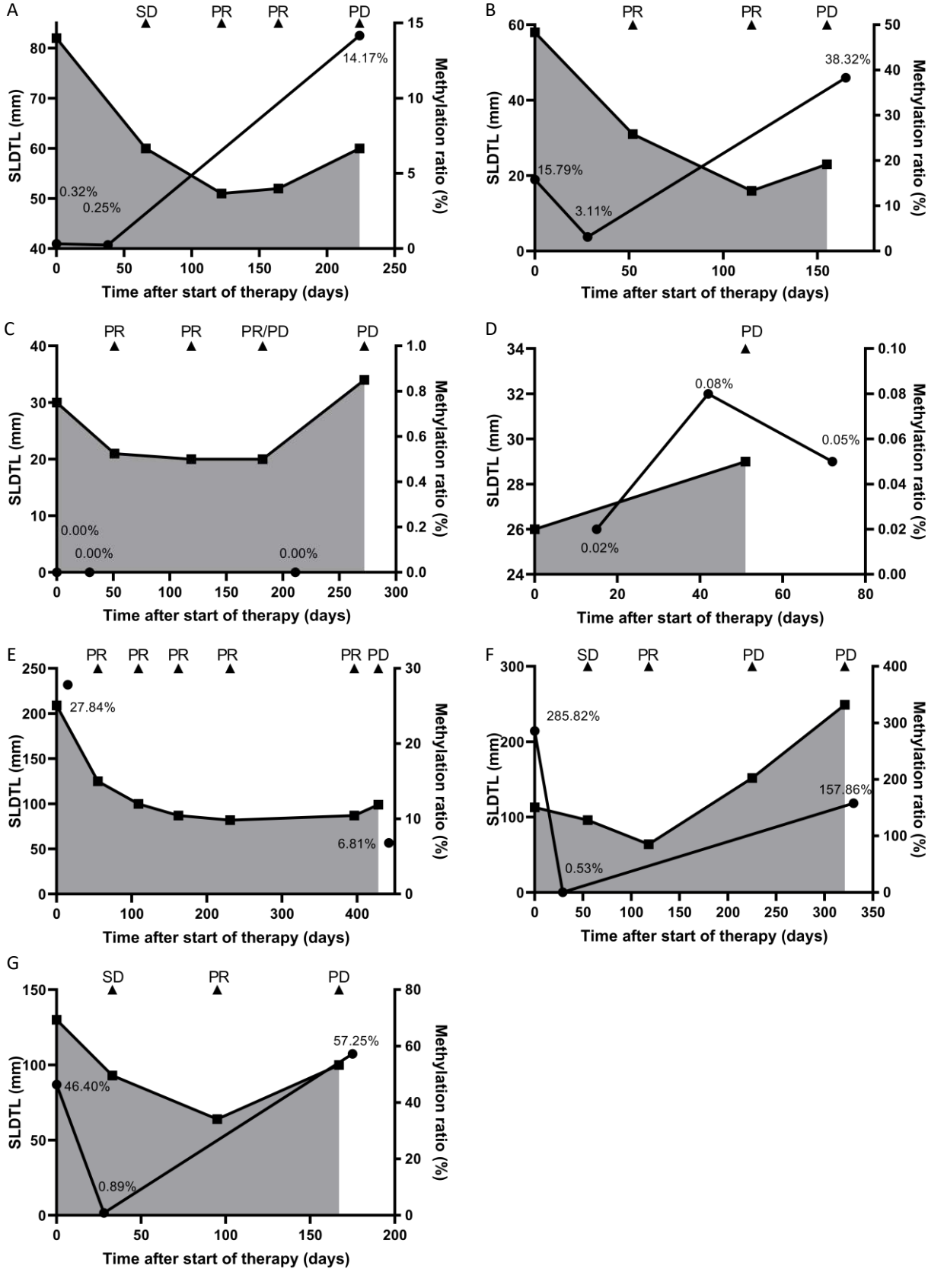




	LctDNA	HctDNA
Reached PD	10/28 (35.7%)	4/8 (50%)
Median PFS (months)	17.26 (95% CI 3.73-30.79)	9.34 (95% CI 5.98-12.7)
Log Rank test: *P* = 0.081		

	LctDNA	HctDNA
Deceased	18/28 (64.3%)	6/8 (75%)
Median OS (months)	30.99 (95% CI 25.81-36.17)	23.10 (95% CI 15.50-30.70)
Log Rank test: *P* = 0.417		

Figure 3


A
SD
PR
PR
PD
SLDTL (mm)
Methylation ratio (%)
14.17%
0.32%
0.25%
Time after start of therapy (days)
B
PR
PR
PD
38.32%
15.79%
3.11%
C
PR
PR
PR/PD
PD
0.00%
0.00%
0.00%
D
PD
0.08%
0.05%
0.02%
E
PR
PR
PR
PR
PR
PD
27.84%
6.81%
F
SD
PR
PD
PD
285.82%
0.53%
157.86%
G
SD
PR
PD
57.25%
46.40%
0.89%