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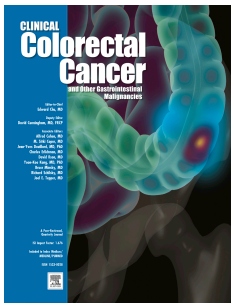

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Conflict of interest

Nele Boeckx, Ken Op de Beeck, Matthias Beyens, Vanessa Deschoolmeester, Christophe Hermans, Peggy De Clercq, Sonia Garrigou, Corinne Normand, Els Monsaert, Konstantinos Papadimitriou, Patrick Pauwels and Guy Van Camp declare no potential conflicts of interest. Valerie Taly declares consulting for Raindance Technologies and Boehringer Ingelheim; Pierre Laurent-Puig for AstraZeneca, Amgen, Boehringer Ingelheim, Sanofi, Roche, Lilly and BMS. Marc Peeters received honoraria (lectures) from Merck Serono and an educational grant from Roche.

Microabstract

Improvements in follow-up methods of mCRC patients during treatment are warranted to ensure response to therapy. The potential of ctDNA for follow-up was investigated using methylation and mutation analyses in different blood samples collected during treatment. Our results corresponded to the results of radiographic evaluation and therefore the analysis of ctDNA is valuable to complement the current follow-up methods.

Abstract

Background: Targeted therapies, although contributing to survival improvement in metastatic colorectal cancer (mCRC), are expensive and may cause side effects. Therefore, confirming that patients are responding to these therapies is extremely important. Currently, follow-up is performed using radiographic evaluation, which has its limitations. Liquid biopsies, reflecting real-time tumor characteristics, hold great potential in monitoring tumor disease.

Methods: Blood samples were collected at different time points during treatment of 24 mCRC patients. Mutation and *NPY* methylation picoliter droplet-based digital PCR (ddPCR) assays were performed on circulating DNA to investigate whether these assays can be used for disease monitoring.

Results: The results of the mutation and methylation assays were correlated with each other and corresponded with the results of radiographic evaluation. There was a steep decrease in circulating tumor DNA (ctDNA) levels immediately after treatment start. Furthermore, ctDNA levels were increased in progressive samples and undetectable in patients undergoing curative surgery.

Conclusion: This prospective study shows that tumor-specific mutation and *NPY* methylation ddPCR assays performed on circulating DNA can be used for the follow-up of mCRC patients during treatment and could complement the current follow-up methods. The analysis of *NPY* methylation is promising as it has the additional advantage that no prior knowledge of tumor mutations is needed.

Keywords: liquid biopsies; metastatic colorectal cancer; follow-up marker; digital droplet PCR; methylation and mutation assays

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, accounting for 694 000 deaths in 2012.[1] When detected in early stages, primary tumors can be surgically and effectively removed. This highlights the importance of screening and early diagnosis. However, there are still a lot of patients diagnosed in later stages with metastasis and 30 to 50% patients diagnosed in early stage will develop metastatic CRC (mCRC).[2] Although novel therapeutics including anti-epithelial growth factor receptor (anti-EGFR) and anti-vascular endothelial growth factor (anti-VEGF) therapy have led to survival improvement in mCRC, primary and acquired resistance to these therapies are frequently observed. Therefore, it is important to ensure that patients who are receiving these expensive targeted therapies and who are dealing with the associated toxicities and side effects, benefit from the administered treatment.[3] In order to achieve this goal, follow-up of mCRC patients during treatment needs to be improved.

Currently, the image-based Response Evaluation Criteria in Solid Tumors (RECIST) are used for the initial determination of the tumor load and the evaluation of response during treatment. However, the use of these criteria has multiple limitations, such as poor inter- and intra-observer reproducibility and limited categorization. Furthermore, radiographic evaluation is expensive, time-consuming, may lead to accumulation of ionizing radiation and is only performed every six to eight weeks.[4] In complement to radiographic evaluation, carcinoembryonic antigen (CEA) is routinely analyzed as a tumor marker for CRC. However, this marker has its limitations too. In fact, CEA monitoring has limited sensitivity and specificity and its variation observed during disease monitoring does not always reflect tumor response or progression. In addition, CEA levels are also elevated in different other malignancies and benign conditions.[5]

Liquid biopsies allow non-invasive and repetitive analysis of tumor DNA, which holds great potential to improve current disease monitoring. During the last decade, a lot of research has been

performed on circulating tumor DNA (ctDNA). This ctDNA can be detected in the bloodstream of cancer patients and originates from apoptosis, necrosis and active secretion of tumor cells. Thanks to the development of highly sensitive technologies such as optimized next generation sequencing (NGS) and microfluidic droplet-based digital PCR (ddPCR), it became possible to study ctDNA which is present in a large background of circulating DNA derived from the turn-over of normal cells.[6] Multiple studies already reported the possibility to detect tumor mutations in the blood of cancer patients.[7-11]

In contrast to genetic mutations, which are very heterogeneous, DNA hypermethylation often targets specific loci in the regulatory sequences of genes.[12] Epigenetic changes in the promotor region of neuropeptide Y (*NPY*) have been described in CRC.[13] In addition, Garrigou et al. (2016) showed that *NPY* hypermethylation was higher in CRC tissue than in normal tissue, irrespective of tumor stage. Furthermore, they found that methylated DNA was detectable in plasma of patients with early stage disease, which might suggest that hypermethylation of these genes is an early event in tumorigenesis and therefore might be promising to monitor patients during treatment.[14] Here, we describe the results of mutation and methylation assays performed on different blood samples collected during first-line treatment of mCRC patients. The aim of this prospective study was to investigate whether mutation and methylation analysis on circulating DNA can be used to monitor tumor disease during mCRC treatment.

Materials and Methods

Study population

For this study, 24 mCRC patients receiving a combination of chemotherapy and targeted (anti-EGFR or anti-VEGF) therapy as first-line treatment were prospectively recruited in three different Belgian hospitals (Antwerp University Hospital (UZA), Maria Middelares Ghent and O.L.V. Van

Lourdes Hospital Waregem). Blood samples were collected at different time points during treatment: T1) before the start of treatment (baseline), T2) before the second cycle of treatment, T3) at the moment of the first radiographic evaluation and T4) at the moment of disease progression or curative surgery. Additional monthly blood samples were collected between T3 and T4 for two patients (#20, #21). FFPE tissue of the primary tumor and metastasis (if available) was requested at the pathology department of the recruiting hospitals. The study was approved by the central ethical committee of the UZA (protocol number 14/5/47), an informed consent was obtained from every patient.

Blood samples

At each time point, one plasma EDTA-tube (replaced by one Cell Preparation Tube at T1) and one serum tube (both 10 mL) were collected and centrifuged at 1500g for 10 minutes, followed by the isolation of plasma, buffy coat and serum. Aliquots were stored at -80°C before isolation of circulating DNA from plasma and serum and isolation of DNA from buffy coat. This was performed using the QIAamp® Circulating Nucleic Acid Kit and DNeasy Blood & Tissue Kit (Qiagen), respectively. Following isolation, DNA was stored at -20°C. DNA concentrations were quantified using the Qubit 2.0 fluorometer with the dsDNA High Sensitivity Assay (Thermo Scientific).

DNA isolation of FFPE samples

Ten sections of 5µm were cut from each FFPE tissue block. One slide was stained with hematoxylin and eosin and checked by the pathologist to exclude tissue blocks without tumor cells. In addition, tumor regions were indicated for macrodissection and the percentage of tumor cells in these regions was estimated by the pathologist. Thereby, contamination of the tumor samples with stromal cells could be considered in later variant analysis. Samples were excluded

when the percentage of tumor cells was below 15%. Following macrodissection, the nine remaining slides were deparaffinized and DNA was isolated using the QIAamp® DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions.

Haloplex enrichment and next generation sequencing

FFPE and buffy coat samples were enriched using custom targeted Haloplex Enrichment, followed by Illumina NGS. A custom Haloplex enrichment panel (Agilent Technologies) was developed to sequence all exons of 66 genes, selected based on their involvement in CRC or their presence in targeted pathways (EGFR and VEGF pathway) (Supplemental Table 1). Quality and degradation of DNA isolated from FFPE samples was checked using the Fragment Analyzer (Advanced Analytical Technologies, Inc.) in order to determine the amount of input DNA for Haloplex enrichment. For samples with high DNA quality an input concentration of 250ng was used, as described in the Haloplex protocol. The input concentration was increased to 500ng and 1000ng for samples with intermediate quality and highly degraded samples with poor quality, respectively. Next, DNA of all tumor samples and corresponding normal samples (buffy coat) were prepared for targeted resequencing using a custom Haloplex Design enrichment, optimized for FFPE samples, following manufacturer's instructions. The enriched samples were hybridized, amplified and sequenced using the Illumina HiSeq 1500 (Illumina).

Read alignment, variant calling and Haloplex amplicon filtering

Raw sequencing reads were analyzed using an in-house developed Perl-based workflow. First, quality of the raw data was assessed using FastQC software (version 1.0). Cutadapt (version 1.2.1) and an in-house developed quality trimmer were used to trim adaptors and low quality bases, respectively. Next, paired-end reads were aligned to the human reference genome (hg19, NCBI Build 37) using Burrows-Wheeler Aligner (BWA mem, version 0.7.4). Picard (version 1.88) was

used to mark and remove duplicates. Alignments with mapping quality smaller than 17 or nucleotides with base quality smaller than 17 were ignored. The maximal read amount per BAM-file was set on 30 000 reads avoiding excessive memory usage. Then, matched tumor-normal somatic variant calling was performed on the tumor-normal aligned data with VarScan2 (2.3.9). Within the same patient, the data of the tumor sample was compared to the data of the normal sample. During variant calling, corrections for the percentage of tumor cells estimated by the pathologist, were set (Supplemental Table 2). Taking into account the percentage of tumor cells and the presence of the alternative nucleotide in tumor and normal samples, variants were categorized into different somatic state classes: germline (present in both tumor and normal), somatic (exclusively tumor), loss-of-heterozygosity (heterozygous in normal and loss of its distribution in tumor) and unknown (different alternative bases in both normal and tumor).

In order to decrease the amount of false positive variants, the variant calling files were filtered using an in-house developed amplicon filtering strategy, adjusted to the Haloplex protocol. The filtering was performed using pyAmpli, an amplicon-based variant filter pipeline for targeted enriched resequencing data, described by Beyens et al. (2017).[15] Resulting variants were checked using Integrative Genomics Viewer in order to remove false positive variants.

A first validation of these results was performed by comparing the clinically important mutations identified in *KRAS* and *BRAF* to the results of the mutation analyses performed at the pathology department, required before starting anti-EGFR treatment. Thereafter, selected variants with an allelic balance larger than 20% (sensitivity Sanger sequencing) detected in patients of whom still enough DNA was available were validated in both tumor and normal paired tissue with Sanger sequencing on the 3130xl Genetic Analyzer platform (Applied Biosystems Inc.) and analyzed using CLC DNA Workbench v5 software (CLC Bio).

Droplet-based digital PCR

Mutation and methylation assays

Among the identified mutations using NGS, there were seven mutations for which validated ddPCR mutation assays were available. Fifteen patients harbored one of these seven mutations. First, the mutation assays were performed on the plasma and serum samples of these 15 patients (Figure 1). As the validation of other new tumor-specific mutation assays is time-consuming and expensive, it was decided to use methylation assays instead of mutation assays for the other nine patients. To compare the mutation and methylation results, the 15 patients for whom validated mutation assays were available, were also subjected to methylation analysis. Therefore, the *NPY* hypermethylation assay was performed on the plasma and serum samples of all 24 patients. See Supplemental Methods for a detailed description of the preparation of the ddPCR assay reactions.

Bisulfite conversion

Prior to the ddPCR methylation assays, bisulphite conversion of circulating and FFPE-isolated DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research) as described in.[14] For each bisulphite treatment of new samples, two control samples were incorporated ensuring proper realization of the modification treatment (ie. Universal hypermethylated DNA (Zymo Research) and normal human genomic DNA (Promega)).

Calculation limit of blanc and limit of detection

The LOB has been calculated as described previously.[16-18] For the methylation assay, the LOB is defined by the amount of positive *NPY* droplets measured in control DNA samples that do not contain hypermethylated *NPY* (n=17). As Garrigou et al. (2016) reported that the rate of false positive *NPY* droplets was independent on the total amount of DNA, the LOB was determined as a finite number of false mutant droplets detected per analysis, and not as a mutant allele

205 percentage.[14] The LOB for the NPY methylation analysis of 5 million droplets is 3.29. The LOD was
206 7.93, calculated as described in[16], based on the LOB. An overview of the LOB and LOD values for
207 the methylation and mutation assays is presented in Supplemental Table 3.

208 ***Data analysis***

209 The sensitivity of ddPCR procedures is only limited by the number of molecules that can be
210 amplified. The sample analysis was performed following the procedure described earlier.[18] Samples
211 were considered positive when the number of observed droplets was higher than LOB value. The
212 fraction of methylated or mutated DNA was only calculated when the normalized number of
213 observed droplets was higher than LOB. First, the LOB value was subtracted from the normalized
214 number of observed positive droplets. Then, the fraction of methylated *NPY* or mutated DNA was
215 calculated. The fractions are highlighted in pink in Supplemental Table 4, 5 and 6 when the
216 normalized number of observed droplets is lower than LOD value. For each positive sample, the
217 fraction of hypermethylated DNA, which determines the fraction of ctDNA, was calculated as the
218 ratio of the normalized number of droplets containing *NPY* methylated sequences minus LOB
219 value, over the number of droplets containing albumin sequences (reference for total amount of
220 circulating DNA). The fraction of mutated DNA, which determines the fraction of ctDNA, was
221 calculated as the ratio of the normalized number of droplets containing mutant sequences minus
222 LOB value, over the total number of droplets containing wild-type and mutant sequences.

223 ## Statistical analyses

224 Correlation tests were performed using the non-parametric Spearman test. The difference
225 between the amount of circulating DNA and ctDNA in serum and plasma was assessed using the
226 Wilcoxon Signed-Rank test. All tests have been assessed using IBM SPSS Statistics version 24.

Results

Patient population

Twenty-four mCRC patients treated in first line with a combination of chemotherapy and targeted therapy were prospectively recruited for this study of which 16, 5 and 3 patients were treated with bevacizumab, cetuximab and panitumumab, respectively. At the moment of final blood collection (Time point (T) 4), 14 patients showed progressive disease while seven patients underwent curative surgery. One patient (#18) was lost to follow-up and two other patients were still responding to treatment (#19, #21) when the analysis was started. For these patients, we lack the last blood sample. Patient characteristics are summarized in Table 1.

Results next generation sequencing

Analysis of the NGS data resulted in the identification of zero to five somatic mutations per patient. The majority of these mutations was detected in *TP53*, *APC*, *KRAS* and *BRAF*. Fourteen patients (58.3%) had a mutation in *TP53* and 11 patients (45.8%) had at least one mutation in *APC*. Only four patients (16.7%) had no mutation in *APC* or *TP53*. Three of these patients had a mutation in *KRAS*. No mutation was detected in patient 4. In total, 22 different mutations were found in *APC* (12 mutations) and *TP53* (10 mutations); only three of them were detected in multiple patients (*TP53* R123W (n=3), *TP53* R43H (n=3), *APC* E1295X (n=2)). Nine patients (37.5%) showed a mutation in *KRAS*; recurrent G12V and G12D mutations were detected in three and two patients, respectively. The G12A, G13D, Q61L and Q61H mutations were each identified in one patient. The *BRAF* V600E mutation was found in four patients. An overview of all identified mutations and the clinically and Sanger validated mutations is presented in Supplemental Table 7.

Mutation assays

Previously validated mutation assays for ddPCR were available for 15 patients (Figure 1). First, the mutation assays were performed on the formalin-fixed paraffin-embedded (FFPE) samples of the corresponding patients to confirm the presence of the mutations, determined by NGS, in tumor tissue. Using ddPCR, all mutations were confirmed in the FFPE samples (Supplemental Table 4). Next, baseline plasma and serum samples of these patients were assessed using the corresponding ddPCR mutation assays. In 93% of patients (14/15), the mutations were clearly detected in baseline plasma samples. The sample of patient 6 contained a very low amount of DNA (357 amplifiable genomic equivalents of which 2 contained tumor DNA, this is below limit of blanc (LOB)), which did not allow us to draw definitive conclusions. All data are shown in Supplemental Table 5. Thereafter, the plasma samples collected at T2, T3 and T4 were analyzed. The changes in the ratio of ctDNA in plasma, determined based on the mutation assays, are presented for every patient in Figure 2.

Regarding those patients that underwent surgery at T4 (n=4) (Figure 2A-D), a steep decrease in the ratio of ctDNA, reaching zero at T2, was seen immediately after the first cycle of chemotherapy in three patients. During treatment, the level of ctDNA remained undetectable. In patient 6, the mutation could not be detected in any of the blood samples, even though it was detected in the primary tumor.

In most patients showing progressive disease at T4 (n=8) (Figure 2E-L), a steep decrease in the ratio of ctDNA was seen immediately after treatment start. At T3, the ctDNA level was further decreased or remained low, while an increase was observed at T4. Only in patient 24 the level of ctDNA was already increased at T3; this patient had an aggressive disease and died within 80 days after starting treatment. Patient 9 had an obstruction of the small intestine for which adhesiolysis was necessary a few days after his first radiographic evaluation. Manipulation of the tumor due to

this procedure has potentially damaged tumor cells and might explain the increase in ctDNA level, which was observed three days later. In patient 12, levels of ctDNA, measured one month after the second radiographic evaluation showing partial response, were increased at the end of the treatment break. Two months later, progression was observed by imaging, followed by a change in treatment. The last blood sample was only collected two months after detection of progressive disease due to logistical reasons. This might explain the low level of ctDNA at the final blood collection. Importantly, the dashed lines in the figure are only connections between two consecutive time points and no indication for the level of ctDNA. Therefore, the point at which the levels of ctDNA start increasing cannot be determined based on these data.

Only three samples were available for patient 18 (lost to follow-up) and 19 (still under treatment) (Figure 2M-N). Unfortunately, exact dates on which blood was collected for patient 22 were not available. Therefore, it was impossible to make a graph for this patient. However, as the order of the samples was known, these results were incorporated in the correlation assays below. The data of all patients are shown in Supplemental Table 5.

Methylation assays

In addition to the ddPCR mutation analysis, which were only available and validated for seven mutations detected in 15 patients, *NPY* hypermethylation was assessed for all patients (n=24). Again, FFPE tissue was first analyzed to confirm the presence of *NPY* hypermethylation in tumor tissue. It was detected in all primary tumor and metastatic tissue samples (Supplemental Table 4). Next, the methylation assay was performed on all plasma and baseline serum samples. *NPY* hypermethylation in baseline plasma samples was detected in 21 of 24 patients (87.5%). In the baseline samples of four of these patients, the number of positive droplets was higher than the LOB but did not exceed the limit of detection (LOD). The samples of the other three patients had values below LOB since the amount of DNA was very low (151 to 365 amplifiable genomic

296 equivalents of which 0 to 3 contained tumor DNA). All data can be found in Supplemental Table
297 5.
298 The methylation results are shown in Figures 2 and 3. Similar results were found using
299 methylation and mutation assays for samples analyzed with both assays (Figure 2). Only one
300 discordance is present for patient 11 at T3 (Figure 2H). For the methylation assay, the amount of
301 DNA was too low (only 365 genomic equivalents of which 1 contained tumor DNA) to detect
302 ctDNA. Unfortunately, there was no DNA left to redo the analysis.
303 Among the patients for which only methylation assays were performed, three patients underwent
304 surgery (Figure 3A-C). In patient 16, a steep decrease in ctDNA level was seen immediately after
305 treatment start which remained low during treatment while patient 20 had very low ctDNA levels
306 in all blood samples. Patient 23 experienced an adverse reaction including heart problems and
307 acute renal problems after his first treatment cycle. After recovery, the patient underwent
308 metastasectomy.
309 Five patients developed progressive disease at T4 (Figure 3D-H). Patient 4 had undetectable levels
310 of ctDNA before progression, which became detectable at T4. Patient 3, 14 and 15 showed
311 decreasing ctDNA levels during treatment that increased at progression in patient 3 and 15.
312 Although increased, the value at T4 in patient 14 was rather low which might be explained by
313 restarting treatment after a treatment break of eight months, three weeks before blood collection.
314 Patient 8 had very low ctDNA levels, which slightly increased after the first radiographic
315 evaluation.
316 Patient 21 was still under treatment when this analysis was started. This patient showed a steep
317 decrease in ctDNA level upon treatment start, which remained very low during monthly follow-
318 up. All data are presented in Supplemental Table 5.

Comparison mutation and methylation assays

In general, the ctDNA levels calculated by mutation and methylation assays corresponded to the results of radiographic evaluation. Even though the ctDNA levels were not strictly identical for both assays, the same trend was observed for all patients (Figure 2). Non-parametric Spearman tests assessing the correlation between mutation and methylation results for the different time points and for the differences between two consecutive time points, confirm the correlation between both assays (ρ>0.8 for each test, Supplemental Table 8). Furthermore, a strong correlation between mutation and methylation ddPCR assays was found for all plasma samples (ρ=0.944).

Plasma versus serum

In addition to the plasma samples, all assays were also performed on baseline serum samples (Supplemental Table 6). Although the concentration of total circulating DNA measured following DNA isolation from serum and plasma samples collected at T1, was higher in serum (P<0.001), the fraction of ctDNA was higher in plasma for both mutation and methylation assays (P<0.001) (Figure 4).

Discussion

In this study, we report the results of ddPCR mutation and methylation assays performed on circulating DNA isolated from blood samples collected at different time points during treatment of mCRC patients. Validated ddPCR mutation assays were available for mutations detected in 15 patients. These mutations were confirmed in all primary tumor and metastatic samples. Furthermore, the mutations were detected in baseline plasma samples in 93% of patients. Highly concordant results (>80%) of mutation analyses performed on blood and tissue samples have been

reported in literature.[19-23] Our findings confirm a high concordance between tissue and plasma biopsies. Discordances can be explained by very low levels of circulating DNA, often associated with low tumor burden.[20] Although not assessed in our study, different research groups also reported the presence of mutations in plasma samples, which were undetectable in tumor tissue.[19-21, 23] These findings strengthen the idea that liquid biopsies are able to reflect tumor heterogeneity of both primary tumor and metastasis and might be of additional value when analyzing tumor tissue.

Disadvantages of the mutation assay approach are the need for prior knowledge of the mutations present in the tumor and the limited availability of validated assays. For patients not harboring recurrent mutations for which validated assays are available, patient-specific mutation assays should be developed which is an expensive and time-consuming process. In order to overcome these limitations, *NPY* hypermethylation was assessed in the samples of all our patients, since it was recently shown to be present in CRC tissue but absent in normal tissue.[14] We were able to detect *NPY* hypermethylation in 87.5% of baseline plasma samples. Three patients had very low concentrations of circulating DNA, which made it impossible to draw conclusions.

Follow-up of cancer patients using mutated ctDNA (mutctDNA),[8, 14, 22, 24] methylated ctDNA (metctDNA)[12, 14, 25, 26] and total circulating DNA[19] has been reported in multiple studies. However, the number of patients assessed is often limited and the time points for blood collection differ between studies. Most studies investigated the value of mutated or methylated ctDNA measured shortly after treatment start to predict response to treatment or survival outcomes.[8, 22, 25, 26] Garrigou et al. (2016) monitored mutctDNA and metctDNA levels in a limited cohort of three mCRC patients during three years of treatment. Increases in both metctDNA and mutctDNA levels were detected before evidence of disease progression.[14] In addition, it was recently shown that early changes in ctDNA concentration, measured using ddPCR methylation (*NPY*, *WIF1*) and

mutation assays, is a marker of therapeutic efficacy in mCRC patients.[25] Recently, Barault et al. (2017) presented a five-gene methylation panel, containing *EYA4, GRIA4, ITGA4, MAP3K14-AS1* and *MSC*, for the analysis of liquid biopsies to monitor advanced CRC. They performed a longitudinal assessment in 45 mCRC patients, of which 10, 6 and 29 were treated with conventional chemotherapy, targeted therapy and Temozolomide as part of a clinical trial, respectively. Based on their results, which are comparable to ours, they hypothesized that repetitive cfDNA assessments in combination with radiological evaluation will improve monitoring of mCRC patients.[27]

Our study investigated the value of methylated and mutated ctDNA for long-term follow-up in multiple mCRC patients. A strong correlation between the level of mutctDNA and metctDNA in plasma samples was detected confirming the results of Garrigou et al. (2016).[14] Although the results of the methylation and mutation assays were not numerically identical, the same trend was observed for both assays and the results were in line with radiographic evaluation. The ratio of ctDNA for both assays was strongly decreased 14 days after the first cycle of chemotherapy, which might indicate response to treatment. Furthermore, increased ratios were generally seen upon progression and undetectable ratios were usually seen when surgery was performed. These results indicate that both mutation and methylation assays of repeatedly collected blood samples can be used for patient follow-up to monitor tumor disease. Unfortunately, no samples were available between time point 3 and 4. Therefore, no conclusions can be made for these time frames. We want to emphasize the importance of the *NPY* hypermethylation assay as this assay can be used for the follow-up of almost every mCRC patient, while mutation assays can only be used for the follow-up of tumors harboring specific mutations. One small disadvantage of methylation assays is the need for bisulphite conversion, which can destroy DNA and therefore requires higher DNA input.

In addition, we compared serum and plasma samples, collected at the same time point. Although the fraction of cfDNA was higher in serum samples compared to plasma samples, the amount of ctDNA was higher in the plasma samples. Different studies suggested that cfDNA in serum is contaminated by genomic DNA that originates from lysis of white blood cells during ex vivo clotting,[28-30] which might explain the higher concentration of circulating DNA.

In conclusion, our data show that ddPCR assays can be used to detect mutations and *NPY* hypermethylation in circulating DNA of mCRC patients. Results from both analyses are correlated and correspond with imaging results. Hence, we believe that mutation and methylation assays can be used to monitor tumor disease during treatment by analyzing repeatedly collected blood samples and are a promising tool to complement radiographic evaluation. The finding that *NPY* hypermethylation can be assessed to monitor treatment is encouraging as methylation assays avoid the prior knowledge of mutations. Larger studies analyzing *NPY* hypermethylation in blood samples collected during mCRC treatment at multiple additional time points are warranted to check whether response and nonresponse to treatment can be detected earlier in blood compared to radiographic evaluation, which we do expect. This could lead to earlier therapy switches, resulting in shorter exposure to ineffective therapies, minimizing unnecessary side effects and costs. Therefore, we believe that ctDNA assessment can be used to complement standard imaging-based evaluation.

Clinical Practice Points

Current follow-up methods during mCRC treatment consist of radiographic evaluation and the analysis of carcinoembryonic antigen. These methods have their limitations. However, it is extremely important to ensure that patients who are receiving expensive targeted therapies who are dealing with the associated toxicities and side effects, are still responding to treatment. Liquid biopsies hold great potential to improve current disease monitoring as they allow non-invasive and repetitive analysis of tumor DNA. In this study, we evaluated the potential of liquid biopsies using droplet-based digital PCR. Tumor-specific mutation and *NPY* methylation analyses were performed on circulating DNA isolated from blood samples collected at different time points during treatment. Our results show that the results of the mutation and methylation assays are associated with each other and with the results of radiographic evaluation. Steep decreases in ctDNA levels were observed immediately after the start of treatment. These levels remained low in patients undergoing curative surgery and were increased again in progressive patients. Other studies already reported that liquid biopsies are able to predict progression before it is detected via radiographic evaluation. However, further research on serially collected blood samples is important. In conclusion, both mutation and *NPY* methylation assays could be of major clinical value and could complement the current follow-up methods. Importantly, *NPY* methylation can be used in almost every mCRC patient which avoids the need for prior knowledge of mutations present in the tumor and has the potential to become a follow-up marker for CRC.[31]

Abbreviations

ALB	albumin
anti-EGFR	anti-epithelial growth factor receptor
anti-VEGF	anti-vascular endothelial growth factor
CEA	carcinoembryonic antigen
CRC	colorectal cancer
ctDNA	circulating tumor DNA
ddPCR	droplet-based digital PCR
FFPE	formalin-fixed paraffin-embedded
LOB	limit of blanc
LOD	limit of detection
mCRC	metastatic colorectal cancer
metctDNA	methylated ctDNA
mutctDNA	mutated ctDNA
NGS	next generation sequencing
NPY	neuropeptide Y
RECIST	response evaluation criteria in solid tumors
T	time point
UZA	Antwerp university hospital

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542 **Table 1** Patient characteristics

543

Patient	Location primary tumor	Location metastasis	Age (years)*	Targeted therapy	Outcome	Sex
1	colon ascendens	liver	62	bevacizumab	surgery	F
2	rectum	liver & lung	64	bevacizumab	surgery	M
3	sigmoid	liver	79	cetuximab	progression	F
4	sigmoid	lung	65	cetuximab	progression	M
5	sigmoid	liver & peritoneum	77	cetuximab	progression	M
6	sigmoid	liver & lung	65	bevacizumab	surgery	M
7	cecum	liver & peritoneum	79	panitumumab	progression	F
8	rectum	lung	71	bevacizumab	progression	M
9	rectum	liver	60	bevacizumab	progression	M
10	rectosigmoid	liver	58	bevacizumab	surgery	F
11	rectum	liver	55	bevacizumab	progression	F
12	sigmoid	liver	63	panitumumab	progression	F
13	colon descendens	liver	71	bevacizumab	progression	M
14	rectum	liver	77	cetuximab	progression	M
15	rectum	pleura	82	bevacizumab	progression	M
16	sigmoid	liver	74	bevacizumab	surgery	M
17	rectum	liver	77	bevacizumab	progression	M
18	rectum	liver	66	bevacizumab	lost to follow-up	M
19	splenic flexure	liver	80	bevacizumab	still under treatment	M
20	rectum	lung	57	cetuximab	surgery	F
21	rectum	liver & lung	38	bevacizumab	still under treatment	F
22	colon ascendens	liver	77	bevacizumab	progression	M
23	rectum	liver	64	cetuximab	surgery	M
24	cecum	liver & peritoneum	45	bevacizumab	progression	F

544

545 F, female; M, male; *, Age at diagnosis (years).

546

Figure legends

Figure 1. Flowchart study.

ddPCR, droplet-based digital PCR; FFPE, formalin-fixed, paraffin-embedded; mCRC, metastatic colorectal cancer.

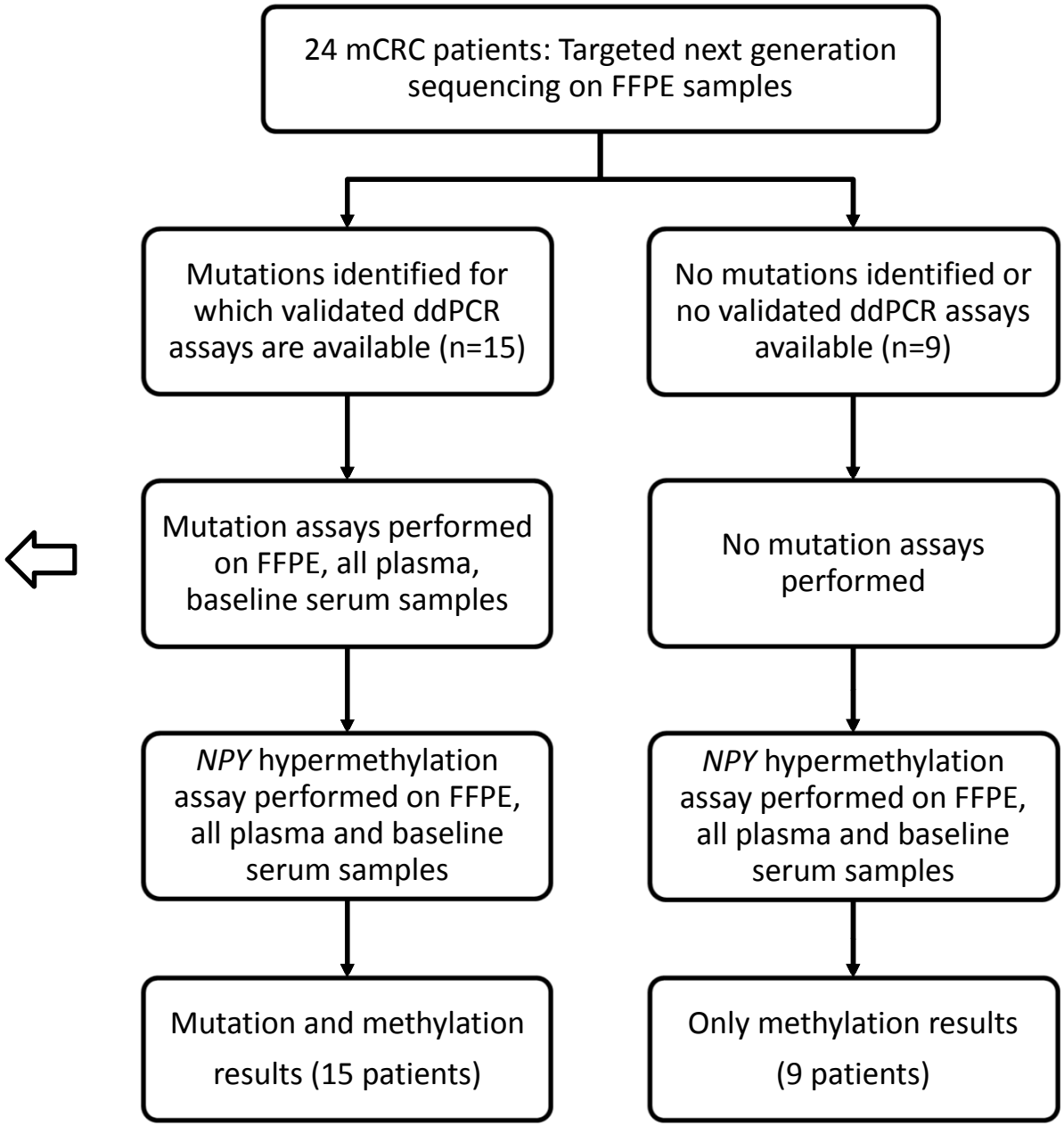
Figure 2. Follow-up mCRC patients during treatment using both mutation and methylation ddPCR assays. The changes in the levels of ctDNA (relative ratio on the amount of total circulating DNA) are presented in function of the time after treatment start (days). A-D) Patients undergoing surgery at last time point; E-L) Progressive patients; M) Lost-to-follow-up; N) Still under treatment.

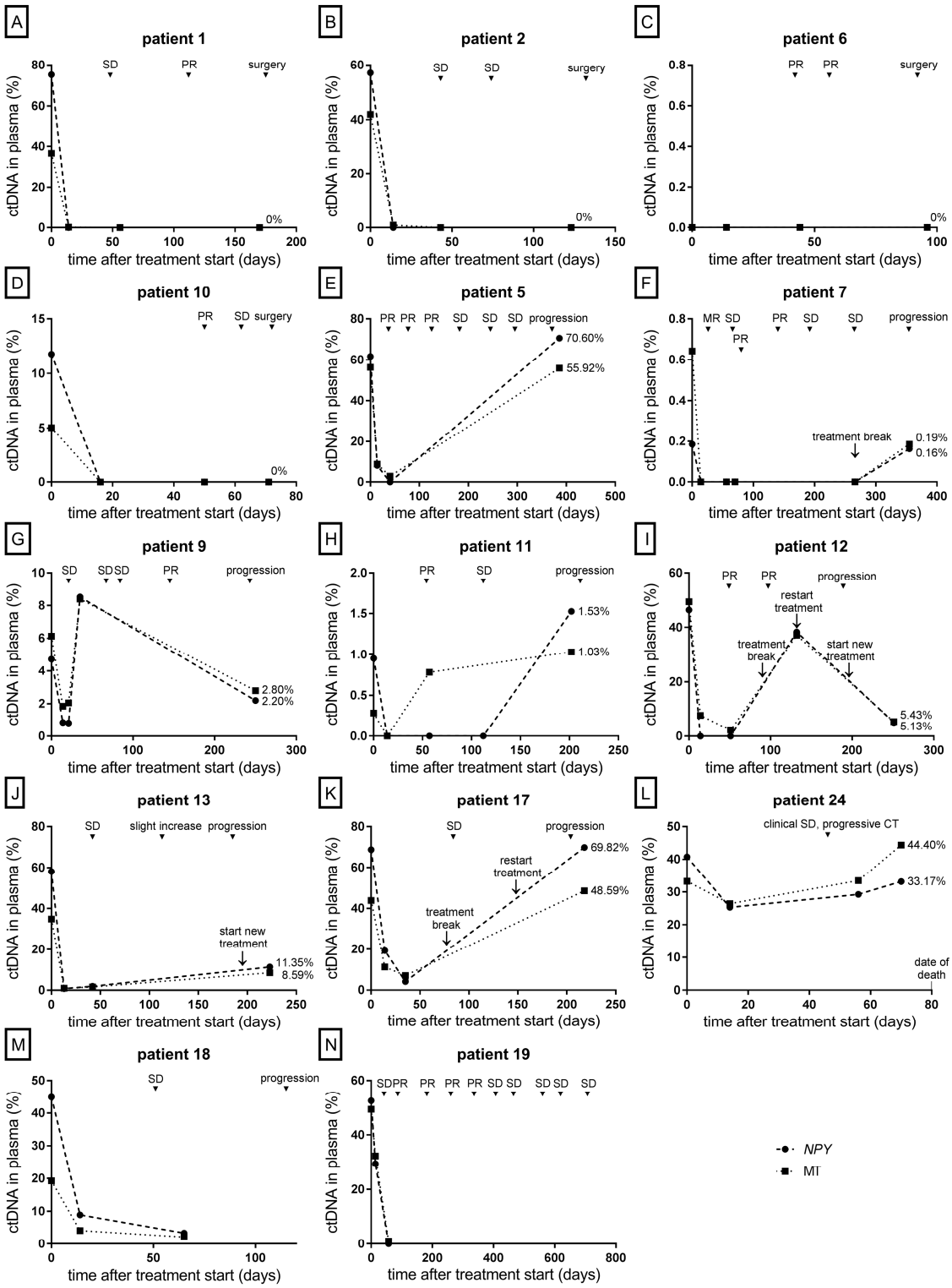
MR, mixed response; MT, mutated; PR, partial response; SD, stable disease

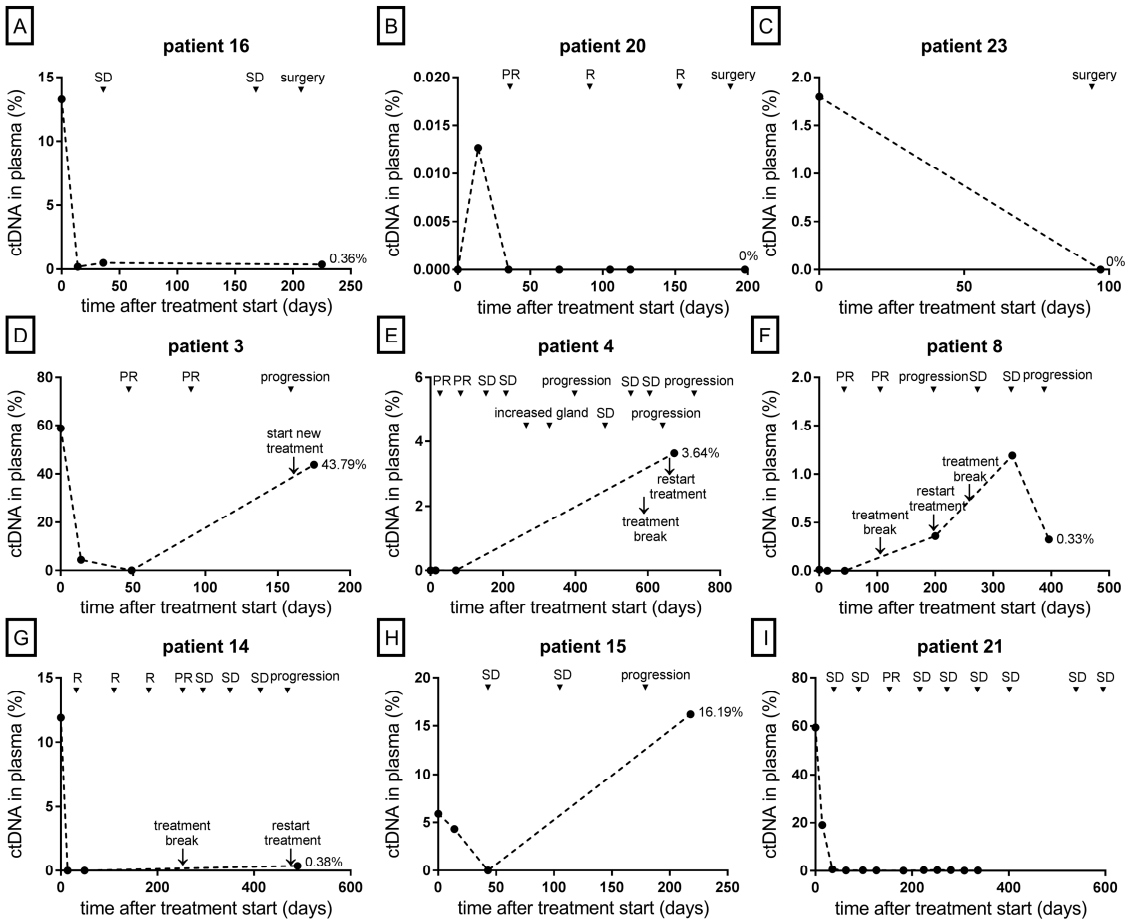
Figure 3 Follow-up of mCRC patients during treatment using *NPY* methylation ddPCR assays. The changes in the levels of ctDNA (relative ratio on the amount of total circulating DNA) are presented in function of the time after treatment start (days). A-C) Patients undergoing surgery at last time point; D-H) Progressive patients at last time point; I) Patient still under treatment. PR: partial response; R: response; SD: stable disease.

Figure 4. Comparison between plasma and serum collected at time point 1, showing A) the ratio of ctDNA calculated using methylation assays for every patient, B) the ratio of ctDNA calculated using mutation assays, C) the concentration of total circulating DNA (ng/µL) in plasma and serum measured after circulating DNA isolation using Qiagen circulating nucleic acid kit for every patient.

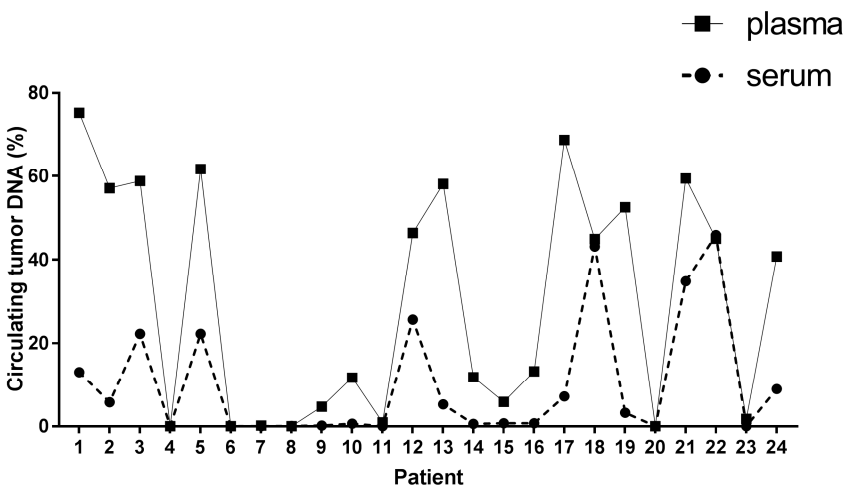
Patient	Assay
1	***KRAS* G12D**
2	***KRAS* G12V**
5	***TP53* R175H**
6	***KRAS* G13D**
7	***BRAF* V600E**
9	***KRAS* G12V**
10	***BRAF* V600E**
11	***KRAS* G12D**
12	***TP53* R175H**
13	***TP53* R273H**
17	***KRAS* G12V**
18	***BRAF* V600E**
19	***TP53* R175H**
22	***KRAS* G12A**
24	***BRAF* V600E**



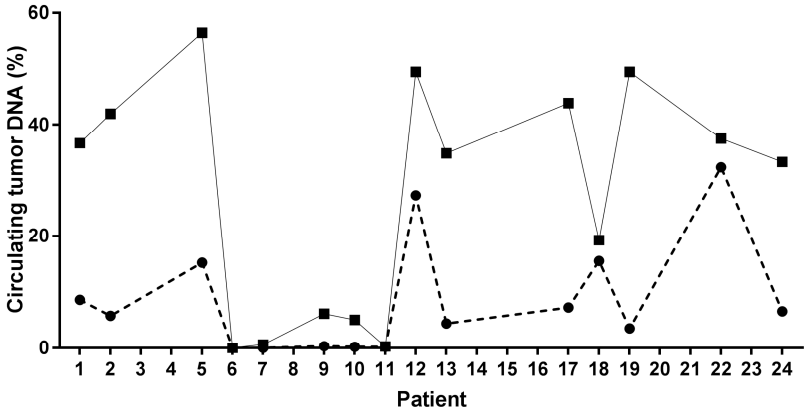

A
patient 1
SD
PR
surgery
0%
B
patient 2
SD
SD
surgery
0%
C
patient 6
PR
PR
surgery
0%
D
patient 10
PR
SD
surgery
0%
E
patient 5
PR PR PR SD SD SD progression
70.60%
55.92%
F
patient 7
MR SD
PR
PR
SD
SD
progression
treatment break
0.19%
0.16%
G
patient 9
SD
SD SD
PR
progression
2.80%
2.20%
H
patient 11
PR
SD
progression
1.53%
1.03%
I
patient 12
PR
PR
progression
restart treatment
treatment break
start new treatment
5.43%
5.13%
J
patient 13
SD
slight increase
progression
start new treatment
11.35%
8.59%
K
patient 17
SD
progression
restart treatment
treatment break
69.82%
48.59%
L
patient 24
clinical SD, progressive CT
44.40%
33.17%
date of death
M
patient 18
SD
progression
N
patient 19
SD PR PR PR PR SD SD SD SD SD
ctDNA in plasma (%)
time after treatment start (days)
NPY
MT



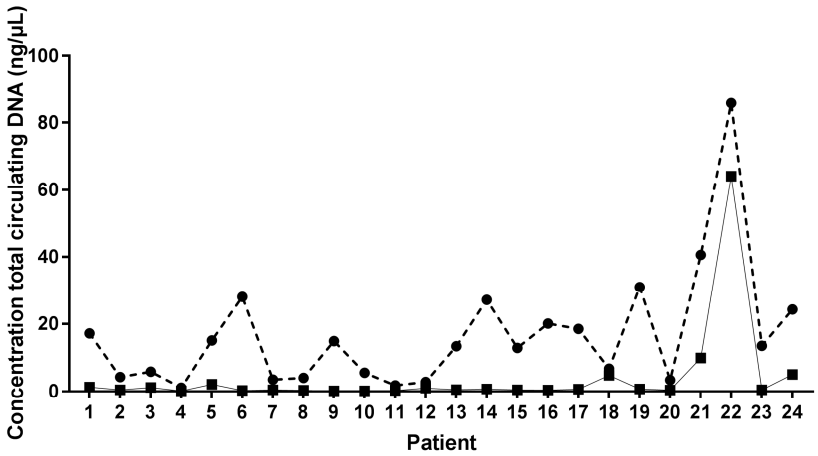
A


plasma
serum
Circulating tumor DNA (%)
0
20
40
60
80
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Patient

B


Circulating tumor DNA (%)
0
20
40
60
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Patient

C


Concentration total circulating DNA (ng/µL)
0
20
40
60
80
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Patient