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Personalized Medicine and Imaging

Clinical Cancer Research

Early Evaluation of Circulating Tumor DNA as Marker of Therapeutic Efficacy in Metastatic Colorectal Cancer Patients (PLACOL Study)

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Abstract

Purpose: Markers of chemotherapy efficacy in metastatic colorectal cancer (mCRC) are essential for optimization of treatment strategies. We evaluated the applicability of early changes in circulating tumor DNA (ctDNA) as a marker of therapeutic efficacy.

Experimental Design: This prospective study enrolled consecutive patients with mCRC receiving a first- or second-line chemotherapy. CtDNA was assessed in plasma collected before the first (C_0), second (C_1) and/or third (C_2) chemotherapy cycle, using picodroplet-digital PCR assays based either on detection of gene mutation (*KRAS, BRAF, TP53*) or hypermethylation (*WIF1, NPY*). CT scans were centrally assessed using RECIST v1.1 criteria. Multivariate analyses were adjusted on age, gender, ECOG performance status (PS), metastatic synchronicity, and treatment line.

Results: Eighty-two patients with mCRC treated in first- (82.9%) or second- (17.1%) line chemotherapy were included. Patients with a high (>10 ng/mL) versus low ($\leq$0.1 ng/mL) ctDNA concentration at C_0 had a shorter overall survival (OS; 6.8 vs. 33.4 months: adjusted HR, 5.64; 95% CI, 2.5–12.6; $P < 0.0001$). By analyzing the evolution of the ctDNA concentration between C_0 and C_2 or C_1 (C_{2or1}), we classified the patients in two groups (named "good" or "bad ctDNA responders"). In multivariate analysis, patients belonging to the group called "good ctDNA responder" ($n = 58$) versus "bad ctDNA responder" ($n = 15$) had a better objective response rate ($P < 0.001$), and a longer median progression-free survival (8.5 vs. 2.4 months: HR, 0.19; 95% CI, 0.09–0.40; $P < 0.0001$) and OS (27.1 vs. 11.2 months: HR, 0.25; 95% CI, 0.11–0.57; $P < 0.001$).

Conclusions: This study suggests that early change in ctDNA concentration is a marker of therapeutic efficacy in patients with mCRC. *Clin Cancer Res; 23(18); 5416–25. ©2017 AACR.*

Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in Europe and a leading cause of death both in Europe and worldwide (1). Increasing number of active agents and the associated biomarker-driven selection have improved outcomes of patients with metastatic CRC (mCRC; ref. 2). One crucial goal of patient treatment strategy is to improve survival while maintaining the quality of life and avoiding needless toxic effects of an ineffective treatment, especially for patients without curative intent. To reach this aim, the early evaluation of therapeutic efficacy is a key point in the patient management strategy.

The current gold standard for assessing tumor response and treatment efficacy is the radiographic imaging based on the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (3). However, there are some limitations to its use: the evaluation occurs at least after 2 months of treatment, it can be assessed only in patients with measurable lesions, it has poor interobserver reproducibility (4), and finally the introduction of targeted therapies modifies the correlation between efficacy and tumor shrinkage (5). Furthermore, serial radiographic imaging is expensive, time consuming, inconvenient, and contributes to an accumulation of ionizing radiation. Several attempts have been made to introduce biomarkers for response evaluation without strong success (6, 7). Measurement of carcinoembryonic antigen (CEA) level has reasonable sensitivity but its variation during the course of the disease does not always reflect tumor response or progression, being sometimes misleading (8, 9). Therefore, development of new biomarkers of tumor response is required (10).

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Translational Relevance

This study evaluated the relevancy of circulating tumor DNA (ctDNA) as a marker of treatment efficacy in metastatic colorectal cancer. The initial concentration of ctDNA appeared as a prognostic marker in patients with metastatic colorectal cancer. Furthermore, an early and deep decrease of ctDNA was shown as a strong predictive marker of therapeutic efficacy.

Circulating tumor DNA (ctDNA) has been suggested as a potential biomarker for tailoring treatment in various solid cancers (11–13). Although, in mCRC, some studies have demonstrated the prognostic value of ctDNA measurement (14, 15), as well as in deciphering the mechanism of treatment resistance to anti-EGFR therapy by identifying the occurrence of new RAS and EGFR mutations (16–18), the clinical utility of ctDNA measurement in mCRC has been insufficiently explored.

CtDNA generally represents a small fraction of the total circulating DNA (19, 20). In this context, picodroplet digital PCR has been demonstrated as a technology enabling accurate and sensitive quantification of ctDNA (21, 22). Most of the strategies for monitoring changes of ctDNA concentration during treatment are based on prior analysis of tumor tissue for the identification of somatic mutations (23). More recently, ctDNA monitoring was also demonstrated using epigenetic modifications such as hypermethylation of specific genes involved in colorectal carcinogenesis (24, 25). In particular, ctDNA detection based on hypermethylation of *WIF1* and *NPY* genes using picodroplet digital PCR has been recently validated as a surrogate marker of mutations and recognized as a potential universal biomarker in both localized and mCRC (24).

In this prospective exploratory study, using picodroplet digital PCR quantitative analysis of mutated or hypermethylated alleles, we investigated the pertinence of monitoring plasmatic ctDNA concentration as a prognostic marker and an early predictive marker of treatment efficacy in patients with mCRC receiving chemotherapy.

Patients and Methods

Patients

This prospective study was performed at the European Georges Pompidou hospital (Paris, France) and enrolled all consecutive patients with histologically proven mCRC receiving a first- or second-line chemotherapy with or without targeted therapy between October 2012 and July 2015 (Placol Cohort). Radiotherapy or previous malignancy other than colorectal cancer in the last 5 years was considered as exclusion criteria, as well as the medical, sociological, psychologic, or legal conditions that would compromise the patient ability to sign informed consent. This study (NCT01983098) received ethical approval from the "Ile-de-France ethics committee" (ID CRB: 2013-A00680-45) and all patients provided written informed consent.

Analyzed blood samples were prospectively collected from patients before the first cycle (at baseline, C_0), and then before the second (at 2 weeks, C_1) and/or third cycle (at 4 weeks, C_2) of chemotherapy. Analysis of ctDNA at baseline was based either on detection of: (i) a mutation previously identified in tumor tissue or, (ii) hypermethylation of *WIF1* or *NPY* genes when no digital PCR assay was available for the identified mutation(s) or when no mutation was detected in tumor tissue (Fig. 1). In case of several detectable mutations by our assays, we prioritized the analysis of *KRAS* mutation.

CEA serum levels and CT scans of the chest, abdomen, and pelvis were performed at baseline and then every 8 weeks during treatment (or earlier for patients with suspected disease progression). CT scans were centrally reviewed by a single radiologist to document response to treatment according to the RECIST criteria, version 1.1. This clinical and radiologic evaluation was conducted blindly from ctDNA results.

Circulating DNA measurement

Determination of the mutational status on tumor tissues, isolation of circulating DNA, and analyses of circulating mutated or hypermethylated DNA using picodroplet digital PCR was done as previously reported (17, 24) and are detailed in the Supplementary Data file.

Statistical analysis

Sample size determination. We planned to assess the additional value of ctDNA on known prognostic factors for prediction of progression-free survival (PFS) and overall survival (OS). For patients with mCRC, there are five well-characterized risk factors: age, gender, ECOG PS, synchronicity of metastasis and treatment line. Thus, our model will contain at most six prognostic factors. In this case, in absence of other data, in particular of prespecified cutoff, it is not formally possible to determine the appropriate sample size. However, according to Ogundimu and colleagues (26), it is necessary to have a minimum of 10 events per variable of a model to develop a statistically stable prognostic model. We therefore needed to include at least 60 patients for this study evaluating the predictive impact of early changes in ctDNA on survival. Considering potential 20% of failures due to missing data, we needed to include at least 81 patients.

Survival and response analysis. Disease assessment was performed every 8 weeks until documented progression. Response was assessed according to RECIST criteria, version 1.1. Progression-free survival (PFS) was defined as the time elapsed from the first cycle of chemotherapy until the date of first progression or death (all causes), whichever came first. Surviving patients without disease progression were censored at the last follow-up date. Overall survival (OS) was defined as the time elapsed from the first cycle of chemotherapy until death (all causes). Surviving patients were censored at the last follow-up date. Survival curves were drawn with the Kaplan–Meier method and compared with the log-rank test.

The quantification of ctDNA at baseline (C_0) was evaluated as a prognostic factor. The change in ctDNA concentration was evaluated between C_0 and C_2 as an early predictive marker of treatment efficacy [better objective response rate (ORR), PFS and OS]. When a blood sample at C_2 was not available, the change in ctDNA concentration was evaluated between C_0 and C_1. This change is referred to as C_{2or1}. For nine patients, neither ctDNA concentration at C1 or C2 was assessed, and these patients were excluded from the analysis. Therefore, the different survival analyses were performed on 73 patients. All analyses were carried out with a bilateral alpha type 1 error of 5%. Data were described as frequencies (percentages) or median (interquartile range).

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A Cox regression model was used to estimate HRs with 95% confidence intervals. Multivariate analyses were adjusted for age, gender, ECOG performance status (PS), metastatic synchronicity and treatment line. None of these variables was missing. The PFS could not be assessed for one patient, and the analysis was done on 72 patients. When performing survival analyses to evaluate the impact of the change in ctDNA concentration during treatment, an adjustment to the cycle of plasma collection was applied (cycle C_1 or C_2). Analyses were performed using R Survival and Survisc packages. *P* values below 0.05 were considered to indicate a statistical significance. The Pearson χ^2 test with Yates' continuity correction if necessary was used to compare the distribution of qualitative variables and nonparametric Kruskal–Wallis test for comparing ctDNA concentration within different groups.

Patients from the validation cohort

To validate the main results of this study, we analyzed a subgroup of 35 new independent patients coming from a study prospectively conducted from November 2010 to August 2014 (Coca-Colon study, Charles Nicolle Hospital, Rouen, France). Patients were aged more than 18 years old, had a histologically proven stage IV colorectal adenocarcinoma with at least one measurable lesion and an ECOG PS $\leq$2. All patients had a pretreatment CEA >5 μg/L and/or CA19.9 >30 μg/L and were candidate for a chemotherapy based-regimen as first or subsequent lines with a free-interval period from the last cycle of at least 15 days. All common baseline characteristics and outcome were collected. This subgroup of patients was selected because plasma samples were collected before the first cycle and the third cycle of chemotherapy and they do not receive more than one cycle of chemotherapy before the inclusion. The evaluation of response to treatment was assessed by investigators at baseline and every 12 weeks according to RECIST criteria (version 1.1). The study was approved by the Ethics Committees and all patients provided a written informed consent (NCT01212510). On the basis of the tumor mutational status, the plasma samples were tested following the same procedures as the main study. The ctDNA was assessed by mutations assays in 19 cases (54.3%) and by methylation assays in 16 cases (45.7%).

Results

Characteristics of patients (Placol cohort)

A total of 82 patients with mCRC were included in this study (male, 59.8%; median age, 67.6 years; ECOG PS 0-1, 82.7%). These patients were included before first- (82.9%) or second- (17.1%) line of chemotherapy with cytotoxic drugs alone (62.2%) or in combination with a targeted therapy (37.8%). The primary tumor site was located in the proximal colon (36.6%), distal colon (40.2%), and rectum (23.2%). The other patient characteristics at the time of inclusion are summarized in Table 1.

Table 1. Patients' characteristics and ctDNA concentration at baseline

	n (%)	Median ctDNA (IQR)	*P*
All patients	82	0.38 (0.02–7.73)	
Age years (mean)	64.6 ± 12.0		
Median age [range]	67.6	(35.0–90.7)	
Age group			
<65	35 (42.7)	1.39 (0.02–8.78)	
65+	47 (57.3)	0.36 (0.02–5.96)	0.52
Gender			
Male	49 (59.8)	0.40 (0–9.13)	0.87
Female	33 (40.2)	0.36 (0.05–5.17)	
ECOG PS (n = 81)			
0–1	67 (82.7)	0.36 (0.01–4.8)	**0.02**
2–3	14 (17.2)	8.09 (0.34–57.6)	
Primary tumor site			
Proximal colon	30 (36.6)	0.41 (0.02–8.32)	
Distal colon	33 (40.2)	0.36 (0.01–9.13)	
Rectum	19 (23.2)	0.36 (0.01–4.07)	0.78
Synchronicity of metastases			
Metachrone	19 (23.2)	0.03 (0–0.41)	
Synchrone	63 (76.8)	1.65 (0.05–9.50)	**0.002**
Primary tumors in synchronous disease			
Nonresected	27 (42.8)	6.85 (0.34–25.3)	
Resected	36 (57.2)	0.35 (0.01–4.7)	**0.02**
Number of metastatic site(s)			
1	40 (48.8)	0.38 (0.02–6.84)	0.77
2	25 (30.5)	0.36 (0–8.71)	
≥ 3	17 (20.7)	0.85 (0.13–6.85)	
Line of chemotherapy at inclusion			
First-line	68 (82.9)	0.36 (0.02–6.78)	0.64
Second-line	14 (17.1)	1.12 (0.02–37.5)	
Protocol regimen			
Chemotherapy	51 (62.2)	1.65 (0.02–8.99)	0.15
Chemotherapy +targeted therapy	31 (37.8)	0.32 (0.012–1.65)	
CEA U/mL			
<10 × ULN	40 (52.6)	0.38 (0.02–5.43)	0.31
≥10 N	36 (47.4)	1.73 (0.06–9.18)	

NOTE: Numbers in boldface correspond to *P* values below 0.05 and are considered statistically significant.
Abbreviations: CEA, carcinoembryonic antigen; ULN, upper limit of normal.

ctDNA detection at baseline

Among the 82 patients tested, 63 patients (76.8%) had detectable ctDNA at baseline. No significant difference was observed in the frequency of positive ctDNA detection between samples analyzed by assays targeting either mutations or methylation. Among the 43 patients with *KRAS* ($n = 37$), *TP53* ($n = 3$), or *BRAF* mutations ($n = 3$) identified in tumor tissue, 36 patients have an identical mutation detectable at baseline in plasma (83.7%), while for the 39 remaining patients, plasmatic ctDNA analyzed by *WIF1* or *NPY* hypermethylation was positive in 27 cases (69.2%; $P = 0.19$; Fig. 1). The only variable (see Supplementary Table S1) significantly associated with the presence of detectable ctDNA at baseline was the absence of resection of primary tumor (93% vs. 69%).

Prognostic impact of ctDNA at baseline

The concentration of ctDNA at baseline was significantly higher for patients with synchronous versus metachronous metastatic disease ($P = 0.002$), as well as for patients with ECOG PS 2–3 versus ECOG PS 0-1 ($P = 0.02$). Adjustments for these two variables were performed in addition to age, gender, and treatment line for the following analyses. No significant difference in ctDNA concentration was associated with the other patient characteristics tested (Table 1).

We observed that the ctDNA concentration at baseline recorded as a continuous variable was significantly associated with shorter OS after adjustment (HR, 1.01; 95% CI, 1.0052–1.018; $P = 0.0003$). We divided the population into three groups according to the ctDNA concentration at baseline. No significant difference was observed between patients whom ctDNA concentration was below or equal to 0.1 ng/mL ($n = 31$) and those

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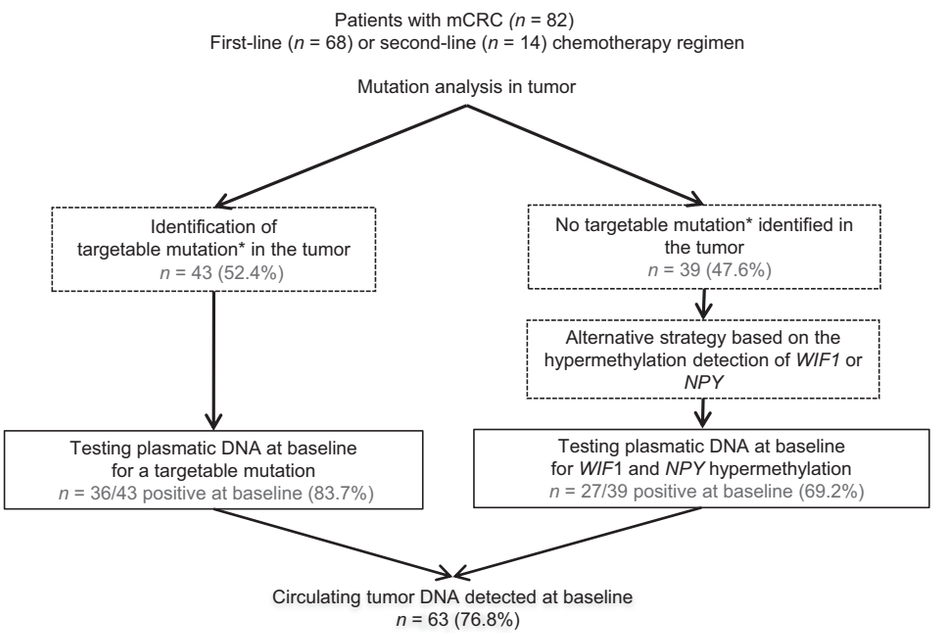

Figure 1.
Workflow of the exploratory prospective study. *Targetable mutation was defined as a mutation for which a digital PCR assay was developed: KRAS (p.Gly12Ala, p.Gly12Cys, p.Gly12Asp, p.Gly12Val, p.Gly12Ser, p.Gly12Arg, p.Gly13Asp, p.Gln61Lys, p.Ala146Thr), BRAF (p.Val600Glu), or TP53 (p.Arg175His, p.Arg248Gln).

with a ctDNA concentration between 0.1 and 10 ng/mL ($n = 36$; HR, 0.79; 95% CI, 0.35–1.8; $P = 0.58$). However, patients with ctDNA above 10 ng/mL ($n = 15$) had a significantly shorter OS in comparison with the reference group (ctDNA≤0.1 ng/mL; HR, 5.64; 95% CI, 2.5–12.6; $P < 0.0001$; Fig. 2; Supplementary Figs. S1 and S2). The median OS was 33.4, 36.8, and 6.8 months for patients with ctDNA concentration at baseline below 0.1 ng/mL, between 0.1 and 10 ng/mL and above 10 ng/mL, respectively. In multivariate analyses, a ctDNA concentration above 10 ng/mL was significantly associated with a short OS (HR, 3.36; 95% CI, 1.3–8.6; $P = 0.01$; Table 2).

Predictive impact of ctDNA concentration variations on treatment efficacy

The predictive value of ctDNA concentration variation between baseline (C_0) and the third cycle of chemotherapy (C_2) was evaluated. In 16 cases, the C_2 sample was missing, and we used the sample collected just before the second cycle (C_1). In nine cases, both samples were missing. Among the 73 evaluable patients, we compared ctDNA concentration between C_0 and C_{2or1} and observed a decrease of ctDNA concentration for 51 patients, an increase of ctDNA concentration for seven patients and, for the remaining 15 patients, no ctDNA was detected at both C_0 and C_{2or1}. Indeed, among the 73 patients who were evaluated for changes in ctDNA between C0 and C1 or C2, 15 patients had no detectable ctDNA at both pretreatment and ontreatment time points. On the basis of the C_{2or1} ctDNA concentration and the variation from C_0, we classified the patients into three groups. These groups correspond to patients with a ctDNA concentration (i) remaining or decreasing below 0.1 ng/mL at C_{2or1} ("$D_{<0.1\ ng/mL}$", $n = 40$, Fig. 3, A1), (ii) decreasing but remaining above 0.1 ng/mL at C_{2or1} ("$D_{\geq 0.1\ ng/mL}$", $n = 26$, Fig. 3, A2), and (iii) increasing at C_{2or1} ("$I_{\geq 0.1\ ng/mL}$", $n = 7$, Fig. 3, A3). The survival of the patients according to these groups was studied.

In univariate analysis, the $D_{<0.1\ ng/mL}$ patients group had a significant longer PFS and OS than the $D_{\geq 0,1\ ng/mL}$ patients group (PFS: HR, 2.22, 95% CI, 1.18–4.16; $P = 0.013$; OS: HR, 2.85; 95% CI, 1.38–5.89; $P = 0.004$), and significant longer PFS than the $I_{\geq 0.1\ ng/mL}$ group (PFS: HR, 3.71; 95% CI, 1.46–9.45; $P = 0.006$; OS: HR, 2.95, 95% CI, 0.84–10.34; $P = 0.09$; Fig. 3, B1 and B2). The median PFS and OS were respectively 8.6 and 36.8 months for $D_{<0.1\ ng/mL}$, 6 and 13 months for $D_{\geq 0,1\ ng/mL}$ and 2.8 and 14 months for $I_{\geq 0.1\ ng/mL}$ groups.

In multivariate analysis, patients who had a ctDNA concentration lower than 0.1 ng/mL at C_{2or1} ($D_{<0.1\ ng/mL}$ group) remained with a significant longer PFS than patients having a ctDNA concentration decreasing at C_{2or1} but remaining superior or equal to 0.1 ng/mL ($D_{\geq 0,1\ ng/mL}$ group; HR, 2.3; 95% CI, 1.2–4.7; $P = 0.02$), and than patients with an increasing concentration ($I_{\geq 0.1\ ng/mL}$ group; HR, 3.6; 95% CI, 1.3–10.0; $P = 0.01$; Table 3; Supplementary Table S2). No significant difference in OS was observed between these three groups (Table 3; Supplementary Table S2). Such results demonstrate the main influence on prognosis of reaching and passing below a negligible ctDNA threshold (0.1 ng/mL) rather than observing a decrease alone. Furthermore, when patients with no detectable ctDNA at both pretreatment and on-treatment time points were removed ($n = 15$) from the analysis, the ctDNA concentration remains significantly associated with clinical outcomes (Supplementary Fig. S3; Supplementary Table S3).

To test the clinical interest of an early and sharp decrease of the concentration of ctDNA, we evaluated in the $D_{\geq 0,1\ ng/mL}$ group the prognostic impact of the slope of ΔctDNA according to the following ratio $(|C_{2or1}\text{-}C_0)/C_0|)\times 100$, named SlopeΔctDNA. In an univariate Cox model, we observed a significant decrease in PFS HR by increasing unit of SlopeΔctDNA (HR, 0.96; 95% CI, 0.92–0.99; $P = 0.015$). The best cutoff of this continuous variable (SlopeΔctDNA) was estimated using the log-rank test statistic. The retained value was 80%. The PFS curve according to this cut-off value is given in Supplementary Fig. S4 (PFS: HR, 0.13; 95% CI, 0.03–0.54; $P = 0.005$; and OS: HR, 0.27; 95% CI, 0.08–0.92; $P = 0.04$). We also observed a significant enrichment in tumor

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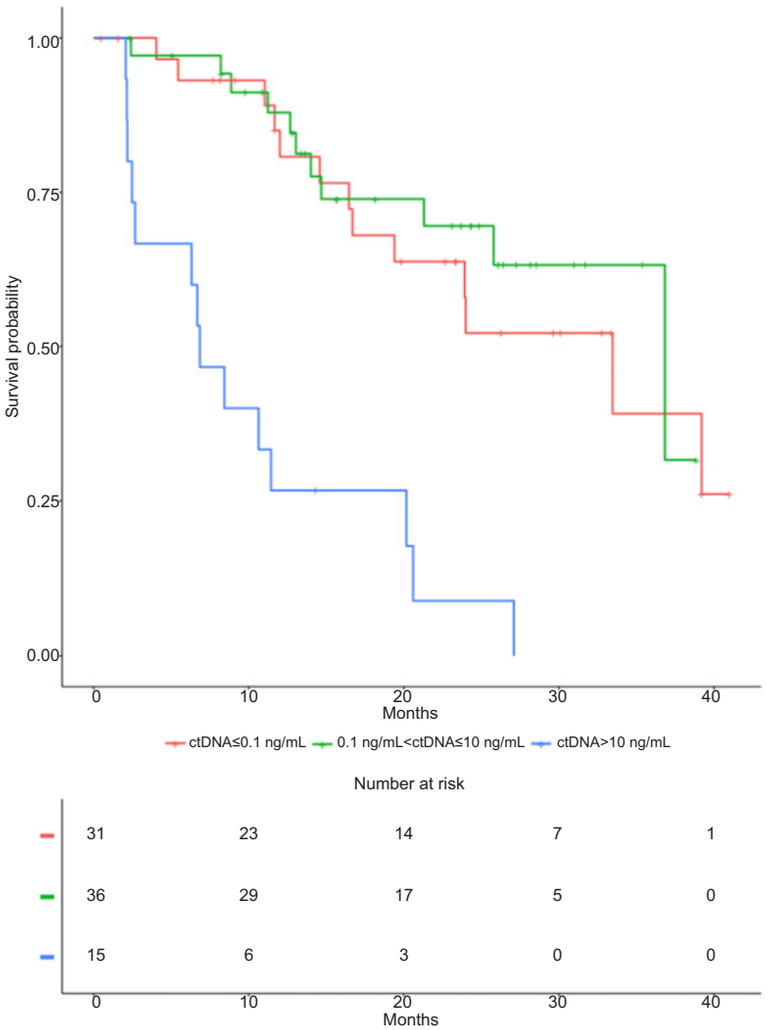

Figure 2.
Impact of baseline ctDNA concentration on overall survival (OS). Three groups of patients were identified according to ctDNA concentrations at baseline: below 0.1 ng/mL (red), between 0.1–10 ng/mL (green), and above 10 ng/mL (blue).

Table 2. Prognostic value of ctDNA concentration at baseline on overall survival adjusted on gender, age, ECOG PS, treatment line and metastatic synchronicity

Variable	HR[a] (95% CI)	*P*
ctDNA concentration (ng/mL)		
<0.1	1	
0.1–10	0.66 (0.27–1.61)	0.36
>10	3.36 (1.31–8.60)	**0.01**
Gender		
Female	1	
Male	1.04 (0.50–2.16)	0.91
Age (years)		
≥65	1	
<65	0.72 (0.33–1.59)	0.42
ECOG PS[b]		
0–1	1	
2–3	3.58 (1.56–8.23)	**0.003**
Treatment line		
1	1	
2	1.79 (0.75–4.27)	0.19
Synchonicity		
Metachronous	1	
Synchronous	2.70 (0.87–8.34)	0.08

NOTE: Numbers in boldface correspond to *P* values below 0.05 and are considered statistically significant.
[a]HR = 1 for reference variable in the Cox test.
[b]Eastern Cooperative Oncology Group Performance Status.

response rate in the group of patients with a SlopeΔctDNA≥80% as compared with the group of patients with a SlopeΔctDNA<80% (best ORR was 47.1% vs. 0%; $P = 0.03$; Fig. 4, B1).

Finally, we created a composite marker based on both ctDNA concentration at C_{2or1} and SlopeΔctDNA. The patients were divided in two groups according to this composite marker. Patients with a ctDNA concentration below 0.1 ng/mL at C_{2or1}, and those with a SlopeΔctDNA≥80% were grouped and named "good ctDNA responder" ($n = 58$). The other patients (SlopeΔctDNA<80% and ctDNA concentration above 0.1 ng/mL at C_{2or1}) were named "bad ctDNA responder" ($n = 15$). In comparison with "bad ctDNA responder" patients, patients belonging to the "good ctDNA responder" group has a longer median PFS (8.5 vs. 2.4 months: HR, 0.19; 95% CI, 0.09–0.40; $P < 0.0001$) and OS (27.1 vs. 11.2 months: HR, 0.25; 95% CI, 0.11–0.57; $P < 0.001$; Fig. 4, A1 and A2). These results remained significant after adjustment for age, gender, treatment line, ECOG PS, metastatic synchronicity, and the use of C_1 or C_2 for ctDNA measurement, either for PFS (HR, 0.21, 95% CI, 0.09–0.45; $P < 0.0001$) and OS (HR, 0.35; 95% CI, 0.14–0.86; $P = 0.02$). Furthermore, all patients who experienced an objective tumor response belonged to the good ctDNA responder group as defined by the composite marker (Fig. 4, B2). In contrast,

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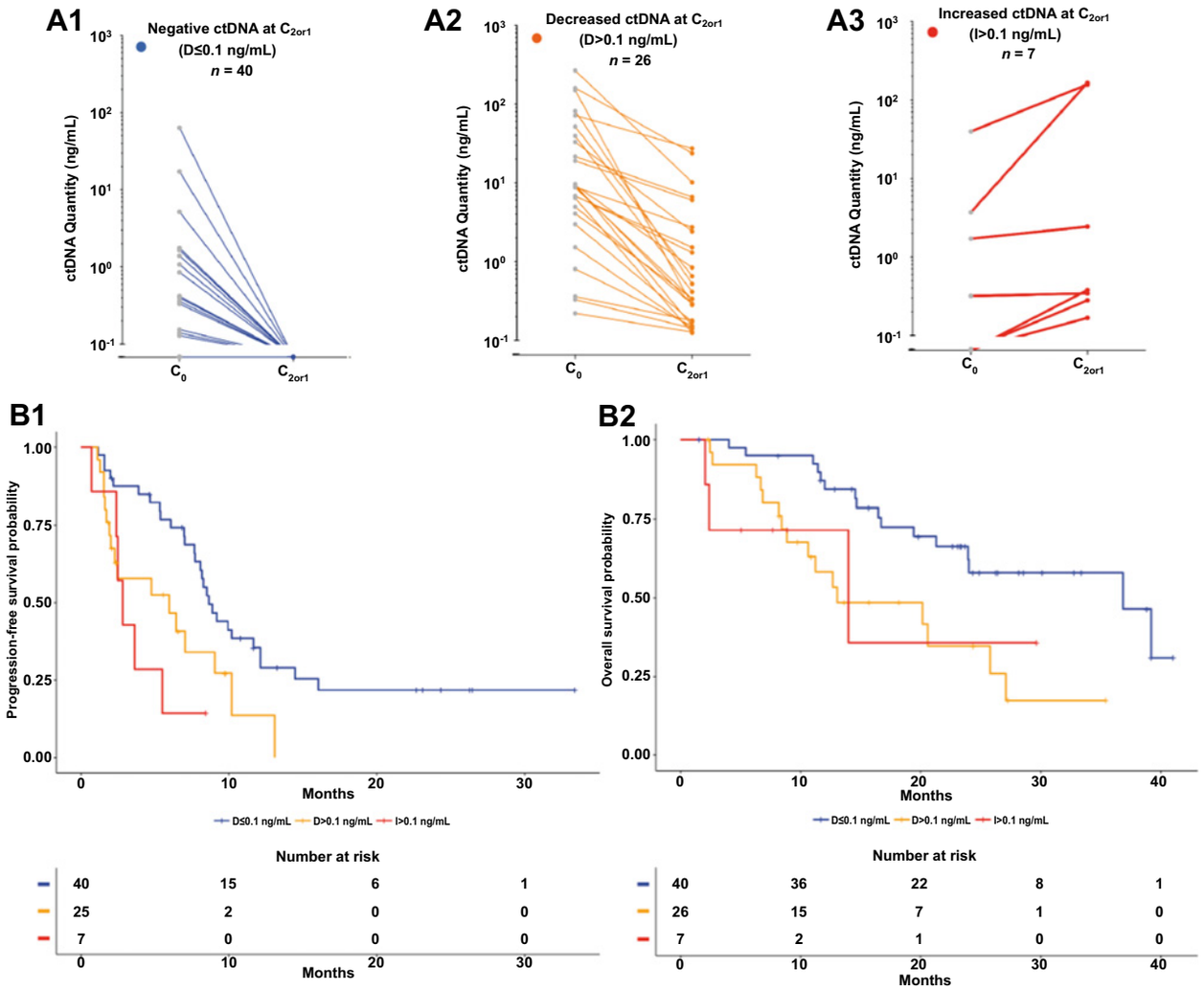

Figure 3.

ctDNA variations. **A1–A3,** Profiles of circulating tumor DNA variations observed in patients of the PLACOL study between C_0 (first cycle of chemotherapy) and C_{2or1} (second or third cycle of chemotherapy). **B1** and **B2**, Progression-free survival (PFS, **B1**) and overall survival (OS, **B2**) of patients with metastatic colon cancer according to variations of ctDNA concentration categorized in three groups: decreasing group with a ctDNA normalization at C2or1 (D $\leq$ 0.1 ng/mL, blue), decreasing group without a ctDNA normalization at C2or1 (D>0.1 ng/mL, yellow), and the increasing group with a higher ctDNA concentration at C2or1 than at C0 (I>0.1 ng/mL, red).

among the 16 patients with progressive disease at the first evaluation, 50% of them belonged to the bad ctDNA responder group ($P < 0.001$). The objective response rate was 41.3% versus 0%, in the good and bad ctDNA responder group, respectively ($P < 0.001$).

Validation cohort

To validate our results, 35 patients with mCRC from the Coca-Colon cohort were included in this study (male, 48.5%; median age, 64 years; ECOG-PS 0–1, 97.1%). These patients were included before first (62.9%) or second (37.1%) line of chemotherapy with cytotoxic drugs alone (45.7%) or in combination with a targeted therapy (54.3%). The primary tumor site was located in the proximal colon (22.8%), distal colon (42.8%), and rectum (34.4%).

This cohort was used to validate the impact of ctDNA at baseline and the predictive value of the composite marker as defined by our exploratory study. We confirmed the prognostic impact of ctDNA at baseline superior to 10 ng/mL. In multivariate analysis, patients with ctDNA above 10 ng/mL ($n = 7$) had a significantly shorter OS in comparison with the reference group (ctDNA $\leq$ 0.1 ng/mL; HR, 12.65; 95% CI, 2.2–72.5; $P < 0.005$). Accordingly to the exploratory study, we divided the patients of the validation cohort in two groups based on the composite marker into good ($n = 30$) and bad ctDNA responder ($n = 5$). In comparison with "bad ctDNA responder" patients, those belonging to the "good ctDNA responder" group have a longer median PFS (6.5 vs. 1.8 months: $HR_{adjusted} = 0.24$; 95% CI, 0.06–0.98; $P < 0.05$) and OS (14.7 vs. 6.9 months: $HR_{adjusted} = 0.16$; 95% CI, 0.03–0.74; $P < 0.02$; Fig. 4, C1 and C2). The HRs are

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Table 3. Impact on progression-free survival and overall survival of ctDNA variations observed between C_0 and C_{2or1} and adjustment on variables

	Progression-free survival		Overall survival	
Variable	**HR[a] (95% CI)**	***P***	**HR[a] (95% CI)**	***P***
ctDNA group				
D $\leq$ 0.1 ng/mL	1		1	
D $\geq$ 0.1 ng/mL	2.28 (1.12–4.66)	0.02	1.99 (0.90–4.38)	0.09
I $\geq$ 0.1 ng/mL	3.62 (1.30–10.04)	0.01	2.26 (0.59–8.63)	0.23
Gender				
Female	1		1	
Male	1.27 (0.70–2.31)	0.43	1.23 (0.58– 2.58)	0.59
Age (years)				
$\geq$65	1		1	
<65	0.90 (0.49–1.66)	0.73	0.77 (0.33– 1.78)	0.54
ECOG PS[b]				
0–1	1		1	
2–3	1.47 (0.69–3.17)	0.32	2.86 (1.24– 6.63)	**0.01**
Treatment line				
1	1		1	
2	1.50 (0.75– 3.00)	0.25	2.06 (0.82– 5.17)	0.12
Synchronicity				
Metachronous	1		1	
Synchronous	1.16 (0.54– 2.47)	0.70	2.94 (0.93– 9.32)	0.07
C_{2or1}[c]				
C_1	1		1	
C_2	0.91 (0.46– 1.78)	0.78	0.94 (0.37– 2.42)	0.91

NOTE: Numbers in boldface correspond to *P* values below 0.05 and are considered statistically significant.
[a]HR = 1 for reference variable in the Cox test.
[b]Eastern Cooperative Oncology Group Performance Status.
[c]Adjustment on time point of ctDNA measurement.

adjusted on age, gender, treatment line, ECOG PS, metastatic synchronicity.

Discussion

In previous published studies, total circulating DNA concentration has been reported to be higher in patients with colorectal cancer than in healthy people, but also in patients with advanced cancers as compared with patients with localized ones (11). In the case of cancer, a part of this circulating DNA is carrying specific tumor genetic alterations (27). The amount of tumor-derived DNA in the circulation was thus suggested as being a surrogate marker of tumor burden (15). This relationship is challenging to firmly demonstrate owing to the difficulty in making precise imaging measurements of the size of primary and metastatic tumor sites. Some associations between clinical parameters, potential surrogates of tumor burden, and the ctDNA concentration at baseline have been suggested (14, 15). In our study, median ctDNA concentration at baseline was significantly higher in patients whom ECOG PS is 2–3 in comparison with others (ECOG PS 0–1 patients) and also in the case of synchronicity of metastases as compared with metachronous ones.

In our series, ctDNA baseline concentrations, highlighted by the presence of tumor-specific genetic or epigenetic alterations, vary in a broad range from 0 to 300 ng/mL. We found that the ctDNA concentration at baseline is a prognostic marker of clinical outcome in patients with mCRC when considered as a continuous variable and adjusted for prognostic factors. Furthermore, patients with ctDNA concentration at baseline higher than 10 ng/mL had a shorter median PFS and OS than the others. These results are in agreement with previously reported studies (14, 28). The value of the ctDNA concentration threshold depends on patient population included in these different series; around 20 ng/mL for patients tested before second line of chemotherapy (28) and, around 35 ng/mL for patients having received all approved standard therapy (14).

Moreover, we showed that early changes in ctDNA concentration could be a surrogate maker of chemotherapy efficacy in colorectal tumors and may be a pertinent tool for treatment monitoring. We demonstrated that early changes of the ctDNA concentration could predict PFS of patients with mCRC starting a first- or second-line chemotherapy. The patients were grouped according to ctDNA concentration change during treatment. Three groups of patients were defined on the basis of a decrease or an increase in ctDNA concentration during treatment and on the residual ctDNA concentration for those having a decrease of ctDNA above or below a threshold of 0.1 ng/mL. The PFS was significantly different between these defined groups of patients. One group has an intermediate prognosis (i.e., patients with a decreasing ctDNA concentration but not reaching the "normalization threshold" of 0.1 ng/mL). In this group, we observed that a decrease above 80% in ctDNA concentration was associated with significant better ORR, longer PFS and OS, than those who have a decrease under 80%. On the basis of our results, a composite marker was defined combining the thresholds of 0.1 ng/mL and the cutoff of 80% for the slope of ctDNA concentration decrease before second or third chemotherapy cycle. According to this composite marker, patients' population was divided in bad or good ctDNA responder groups, and the latter group was associated with a better tumor response rate, and a longer PFS and OS in multivariate analysis. Recently, Tie and colleagues (15) have evaluated ctDNA as a marker of therapeutic efficacy in a prospective cohort of 52 patients with mCRC receiving first-line chemotherapy. A reduction of 90% of ctDNA concentration level between baseline and before the second cycle of chemotherapy was significantly associated with tumor response based on RECIST criteria. This reduction was also associated with a trend for a longer PFS. Therefore, the variation of ctDNA concentration under treatment appears to be a relevant early biomarker of treatment efficacy that warrants validation in a large prospective series.

Monitoring ctDNA concentration levels usually requires prior identification of somatic alterations in tumor. In this study, ctDNA concentration measurement was performed on the basis of digital PCR assays using specific probes targeting specific tumor mutations in 52.4% of the patients. For the remaining patients, an alternative strategy was used based on the detection of hypermethylation of two genes *WIF1* and *NPY*, as previously described (24, 29). In one of these previous work, a hypermethylation of *NPY* or *WIF1* genes was present in 100% of tumors tested (24). This high rate of positivity allowed the monitoring of patients with mCRC without knowing the mutational status of the tumor. In our series, the follow-up of ctDNA concentration was performed using hypermethylation assays in 47.6% of the patients. Among the patients of whom the ctDNA was assessed by hypermethylation, 69.2% were positive at baseline. There was no significant difference in rate of positivity between the group of patients for which the ctDNA was assessed by point mutations (83.7%) and the group whom ctDNA was assessed by hypermethylation (69.2%), suggesting the absence of detection bias induced by the assay used for ctDNA detection. Furthermore, the overall rate of detectable

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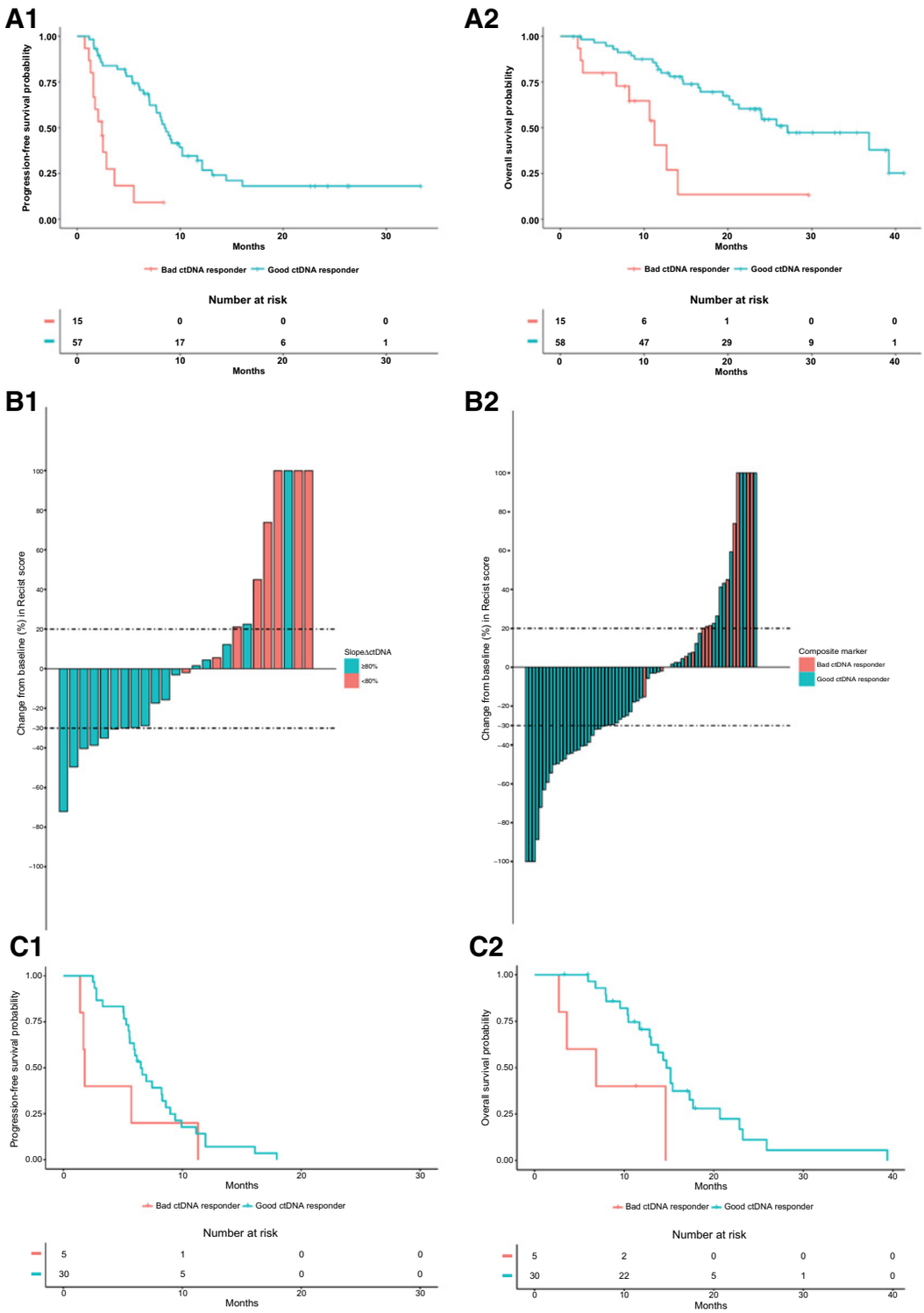

Figure 4.

Impact of the composite marker on progression-free survival (**A1**) and overall survival (**A2**) in the defined bad ctDNA responder and good ctDNA responder groups. **B1** and **B2,** Changes in RECIST scores (*y*-axis, %) in patients in the $D_{\geq 0.1\ ng/mL}$ group according to the decreasing slope of ctDNA concentration (orange: <80%; cyan: ≥80%; **B1**) or bad/good ctDNA responder groups according to the composite marker (orange: bad responder group; cyan: good responder group; B2). **C1** and **C2,** Impact of the composite marker on the progression-free survival (PFS, **C1**) and overall survival (OS, **C2**) in the defined bad ctDNA responder (orange) and good ctDNA responder (cyan) groups for the patients of the validation cohort.

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ctDNA at baseline of 76.8% is similar to that reported in others studies for patients with mCRC (11, 17, 22).

The relative small sample size of our exploratory study impairs the generalization of our conclusions; however, the fact that similar results were found in our validation cohort reinforces the potential clinical applicability of our findings. Nevertheless our data are insufficient to determine the optimal time for assessing the ctDNA concentration after initiation of treatment [i.e., after one (C_1) or two cycles (C_2) of chemotherapy] and larger cohorts of patients are needed to compare the impact on PFS or OS of determination of ctDNA concentration at C1 or C2. Furthermore, one limitation is the interpretation of small variations around the threshold of ctDNA detection. The clinical interpretation of such variations needs larger cohorts of patients to draw robust conclusions. Finally, not all patients with an mCRC have detectable ctDNA at baseline, this subgroup of patients seems to have a better prognosis and have been classified as "good ctDNA responder" in our series. It would be interesting to confirm this result and understand its underlying biological significance.

In conclusion, the ability to assess early and reliably the nonresponse to treatment with serial ctDNA concentration analysis may be of benefit for patients due to the possibility to change earlier to alternative therapy and to minimize the side-effects of an inefficient therapy, notably for patients with nonmeasurable disease. Our study showed that patients who do not experienced an "early normalization" (below 0.1 ng/mL) or "early decrease greater than 80%" of ctDNA concentration have a lower benefit from chemotherapy. Further prospective clinical trials including serial ctDNA analysis are ongoing to validate this promising early predictive marker of therapeutic efficacy for mCRC.

Disclosure of Potential Conflicts of Interest

P. Laurent-Puig is a consultant/advisory board member for Amgen, AstraZeneca, Boerhingher-ingelheim, Lilly, Merck-Serono, and Roche. G. Perkins is a consultant/advisory board member for Sanofi and Servier. J. Taieb is a consultant/advisory board member for Amgen, Baxalta, Celgene, Lilly, Merck, Roche, and Servier. V. Taly is a consultant/advisory board member for Boehringer Ingelheim and Raindance Technologies. A. Zaanan is a consultant/advisory board member for Amgen, Lilly, Merck Serono, Roche, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

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Clinical Cancer Research

Early Evaluation of Circulating Tumor DNA as Marker of Therapeutic Efficacy in Metastatic Colorectal Cancer Patients (PLACOL Study)

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