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TUMOR MARKERS AND SIGNATURES

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Clinicopathological factors associated with tumour-specific mutation detection in plasma of patients with *RAS*-mutated or *BRAF*-mutated metastatic colorectal cancer

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Abstract

Detection of tumour-specific circulating cell-free DNA in plasma (ctDNA) fails in a significant number of cases depending on the clinical context. The primary aim was to investigate clinicopathological factors associated with detection of ctDNA in patients with *RAS*-/*BRAF*-mutated metastatic colorectal cancer (mCRC) prior to first-line therapy. A secondary aim was to evaluate the prognostic impact of ctDNA compared to other biomarkers. Patients were included from the NORDIC-VII study ($N = 253$). ctDNA was sampled prior to treatment and analysed for hotspot tissue mutations

Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; cfDNA, total circulating cell-free DNA; CI, confidence interval; ctDNA, tumour-specific circulating cell-free DNA; ddPCR, droplet digital polymerase chain reaction; HR, hazard ratio; IL-6, interleukin 6; MAF, minor allele frequency; mCRC, metastatic colorectal cancer; OR, odds ratio; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; SIR, systemic inflammatory response; SLD, sum of the longest diameter of target lesions measured according to RECIST 1.0 at baseline; ULN, upper limit of normal; VIF, variance inflation factor; WBC, white blood cell.

Karen-Lise Garm Spindler and Elin H. Kure equally shared senior authorship.

(*KRAS*, *NRAS*, and *BRAF*) using droplet digital PCR. Multivariable regression models were constructed to predict the probability of mutation detection and survival. Increasing radiological size of target lesions by increments of 1 cm (odds ratio [OR] = 1.18; 95% confidence interval [CI] 1.09-1.27; $P < .001$), intact primary tumour (OR = 3.17; 95% CI 1.22-8.22; $P = .018$) and more than one metastatic site (OR = 3.08; 95% CI 1.32-7.19; $P = .009$) were associated with mutation detection in plasma. Metastatic involvement of the lung was associated with non-detection (OR = 0.26; 95% CI 0.12-0.58; $P = .001$). Preanalytical and analytical factors modulated detection. High allele frequencies of ctDNA indicated poor prognosis independently of CEA and CA19-9 (hazard ratio [HR] = 2.38; 95% CI 1.74-3.26; $P < .001$; $N = 206$). Clinicopathological characteristics should be carefully considered when evaluating ctDNA results from mCRC patients, especially when confronted with a plasma negative result. ctDNA may prove to be a clinically useful marker in the evaluation of mCRC treatment.

KEYWORDS
blood biomarkers, carbohydrate antigen 19-9, carcinoembryonic antigen, colorectal cancer, tumour-specific circulating cell-free DNA

1 | INTRODUCTION

Colorectal cancer is the third most common cancer worldwide.[1] Survival of patients with metastatic colorectal cancer (mCRC) has improved considerably during the past thirty years, partly due to improved and individualised surgical and oncological treatments. Somatic hotspot *RAS* and *BRAF* mutations are two examples of established predictive and prognostic markers. *RAS* mutations are associated with resistance to anti-EGFR therapy in first-line[2,3] and late-line[4] mCRC; *BRAF* mutations are associated with inferior prognosis[5] and may predict response to BRAF inhibition in combination with anti-EGFR therapy.[6]

Much research effort is currently focused on capturing tumour-specific somatic mutations in blood (ie, a liquid biopsy), rather than a tissue sample; providing predictive and prognostic information that can support treatment decisions, and ultimately improve quality of life and survival.[7] Although a representative tumour tissue sample still remains the gold standard for somatic mutation analysis in the clinic, utilising plasma-derived tumour-specific circulating cell-free DNA (ctDNA) seems to be a promising liquid biopsy approach in mCRC.[8,9] Potential advantages include the minimally invasive nature of sampling, the possibility to capture data at multiple time points and to identify clinically relevant clonal changes during the course of treatment.

However, there are still unresolved challenges related to this approach. Tumour-specific mutation detection in plasma depends on several preanalytical[10-12] and analytical factors,[13,14] including plasma preparation and the sensitivity of the method used. Detection is also dependent on cancer type and stage, and metastatic disease generally demonstrates superior detection rates compared to localised disease.[15,16] Despite this, detection fails in a significant number of mCRC patients (4%-23%) depending on the analytical and clinical context.[17-28] It has been observed that additional clinicopathological factors may correlate with mutation detection in this patient group,[21,22,27] but few studies have rigorously investigated this selectively for patients prior to first-line therapy.[25]

What's New?

In a "liquid biopsy", plasma samples that contain cell-free DNA from a tumor (ctDNA) are analyzed. While promising, this technique isn't always reliable. Are there factors that affect the ability to detect tumour-specific mutations in the plasma of cancer patients? In this study, the authors identified several such factors in patients with metastatic colorectal cancer (mCRC). They also found that ctDNA outperformed CEA and CA19-9 when used to predict prognosis. With some caveats, ctDNA may thus provide a clinically useful biomarker for evaluating mCRC treatment and prognosis.

The primary aim of our study was to investigate clinicopathological factors associated with tumour-specific mutation detection in plasma of patients with *RAS*-mutated or *BRAF*-mutated mCRC prior to first-line therapy. A secondary aim was to evaluate the prognostic impact of ctDNA compared to other tumour biomarkers.[29,30]

2 | MATERIALS AND METHODS

2.1 | Study designs

We used a post hoc design based on a prospective phase III trial, the NORDIC-VII study (NCT00145314).[31] In short, NORDIC-VII

investigated the effects of combining cetuximab with bolus 5-fluorouracil/folinic acid and oxaliplatin in first-line therapy of patients with mCRC. There were no statistically significant differences in outcome between the treatment arms[31,32]; in the present study, data were analysed across all arms. Patients harbouring hotspot tissue mutations in *RAS* or *BRAF* with available plasma before start of first-line chemotherapy were eligible for our study. Clinicopathological characteristics, preanalytical/analytical factors and biochemical tumour markers were included as recommended for phase 3 trials of systemic treatment in mCRC, with some modifications (Table S1).[33] Primary endpoint was the detection rate of predefined tumour-specific hotspot *RAS* and *BRAF* mutations in plasma. Clinical endpoints were progression-free survival (PFS), overall survival (OS) and overall response rate (ORR).

2.2 | Tumour tissue *RAS* and *BRAF* mutation status

Tumour tissue *RAS* and *BRAF* mutation status was previously determined by examining hotspot mutations in *KRAS* (codons 12, 13, 61, 117 and 146), *NRAS* (codons 12, 13 and 61) and *BRAF* (codon 600) (Table S2).[31,32]

2.3 | cfDNA purification

Blood was collected using K_2EDTA BD Vacutainer tubes at baseline prior to first-line therapy. The tubes were incubated in room temperature for 30 minutes before centrifugation for 10 minutes at 1400*g*. Plasma was pipetted into polypropylene cryogenic tubes and immediately stored at -70°C to -80°C in an upright position. DNA was purified from approximately 480 μL of EDTA-plasma using a chemagic CMG-1107 cfNA 1k Kit special H24 on a PerkinElmer chemagic 360 robot (PerkinElmer, Baesweiler, Germany), according to the manufacturer's instructions. DNA was eluted in 100 μL of the supplied elution buffer.

2.4 | Quantification of total cfDNA

Total cfDNA was quantified by droplet digital polymerase chain reaction (ddPCR) using an assay of the beta-2-microglobulin gene (*B2M*). The upper limit of normal (ULN) of total cfDNA was 6418 alleles/mL plasma.[30] A ddPCR assay for detecting immunoglobulin heavy chain rearrangements in B cells was performed in duplicates for all samples to identify genomic DNA contamination from leukocytes.[30,34]

2.5 | Detection and quantification of ctDNA

ctDNA was quantified by ddPCR using a combination of commercially available and previously published assays targeting selected mutations and wild type in hotspot loci of *KRAS*, *NRAS* and *BRAF* (Table S2). The level of blank and call cutoff was determined for mutation assay used in the study (Table S3). A sample was scored as positive if the number of positive events is greater than or equal to the call cut-off for the respective assay and the minor allele frequency (MAF) ≥0.1%. A sample was scored as negative if the number of positive events is less than the call cut-off for the respective assay or the MAF <0.1%.

Depending on the hotspot mutation in question, ctDNA (mutant) was defined as the absolute number of mutant allele copies per millilitre plasma as quantified by ddPCR. If the sample was scored as negative, ctDNA (mutant) was set to zero. Similarly, cfDNA (wild type) was defined as the absolute number of wild type allele copies per mL plasma as quantified by ddPCR. MAF in plasma was defined as $100 \times$ [ctDNA (mutant)]/[ctDNA (mutant) + cfDNA (wild type)]. Since the MAF reflects plasma status, the cfDNA (wild type) has contribution from both tumour cells and normal cells. More details on detection and quantification in Supporting Information Methods.

2.6 | Biochemical tumour markers

Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were analysed from serum, and levels were dichotomised as specified in Supporting Information Methods. CEA and CA19-9 results have previously been reported.[29]

2.7 | Radiological assessment of tumour burden

The computed tomography sum of the longest diameter of target lesions measured according to RECIST 1.0 at baseline (SLD) was used as a radiological proxy of tumour burden, which included up to 5 target lesions per organ and up to 10 target lesions in total.[30,31,36]

2.8 | Systemic inflammatory response

Serum interleukin 6 (IL-6) was used as a proxy of systemic inflammatory response. IL-6 was analysed in serum, and levels were dichotomised as specified in Supporting Information Methods. IL-6 results have previously been reported.[35]

2.9 | Statistical analyses

Values were summarised as median and range for continuous variables and proportions and percentages for categorical variables. Blood analyte levels were not normally distributed and hence log transformed where relevant. The frequencies of categorical variables were statistically compared using the Chi-square test. Levels in different groups were statistically compared using the Mann-Whitney *U* or Kruskal-Wallis test. Correlations were investigated using the Spearman's rho test.

A logistic regression model was constructed to predict the probability of mutation detection, using a stepwise analysis approach. Clinicopathological characteristics, preanalytical and analytical factors (proxy of maximum analytical sensitivity and the presence of

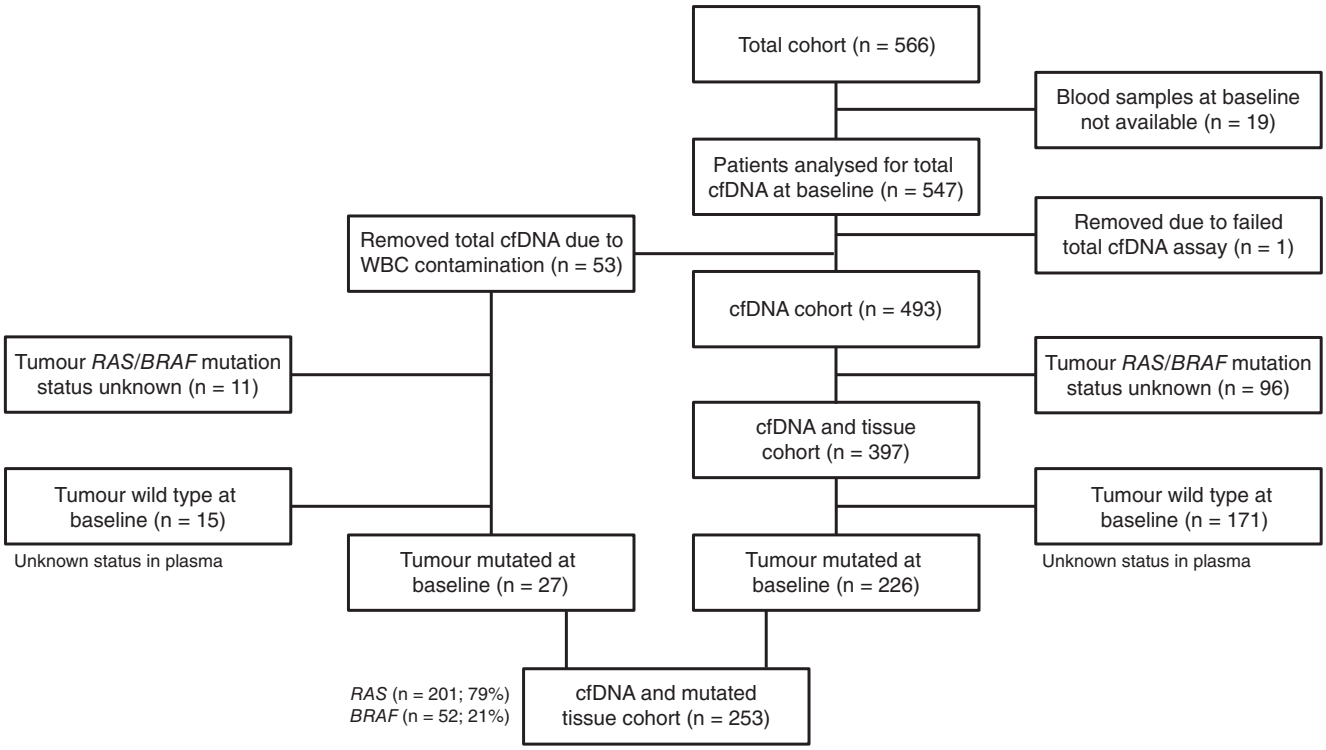

FIGURE 1 CONSORT diagram

leukocyte contamination) were included as independent variables (Table S1). Multicollinearity between independent variables was evaluated using variance inflation factors (VIF). Linearity of continuous independent variables with respect to the logit of the dependent variable was evaluated using the Box-Tidwell procedure.

The prognostic value of ctDNA MAF levels was initially assessed by unadjusted log-rank and univariable Cox regression tests, using data driven cutoffs of ctDNA detection and MAF close to the median (MAF 20%); and cutoffs from the literature (MAF 10% and 5.8%).[22,37] Subsequently a multivariable Cox regression model for OS was constructed similarly to the stepwise approach above. Clinicopathological characteristics and relevant biochemical tumour markers (CEA, CA19-9, total cfDNA and ctDNA MAF) were included as independent variables (Table S1). The assumption of proportional hazards for the final model was evaluated visually using log minus log plots and statistically using the scaled Schoenfeld residual procedure. Linearity of continuous variables was evaluated using Martingale residuals.

For all regressions, independent variables significant in univariable analyses ($P < .1$) were included in multivariable regression modelling. To identify the best predictive model, we used a backward elimination process. For each step in this stepwise procedure, we evaluated odds ratios (OR)/hazard ratios (HR), 95% confidence intervals (CI) and *P* values. Nonsignificant independent variables in multivariable analysis ($P > .05$) were omitted sequentially from the model until all remaining variables were statistically significant.

Statistical analyses were computed using SPSS version 25 (IBM Corp., Armonk, New York) and R version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria). A *P* value less than .05 was considered statistically significant, unless otherwise stated. Laboratory analyses were performed blinded to the clinical endpoints. Results were reported according to the Recommendations for Tumour Marker Prognostic Studies (REMARK) checklist.[38]

3 | RESULTS

3.1 | Mutation detection

There were 253 patients that harboured hotspot *KRAS*, *NRAS* or *BRAF* mutations in tumour tissue with plasma available prior to first-line therapy. This cohort did not differ significantly from the original study population in terms of clinicopathological characteristics and biochemical tumour marker levels, except that patients with *RAS*/*BRAF* wild type in tissue were excluded (Table S4). A CONSORT diagram summarises patient selection from the original intention to treat population of the NORDIC-VII study (Figure 1).

Total cfDNA levels were comparable to those of the cfDNA cohort previously reported.[30] The maximum test sensitivity ranged from .01% to 10% with a median of about 1.6% (Figure 2A). A total of 10.7% ($N = 27/253$) of all patients had plasma samples that were significantly contaminated with leukocyte DNA. A retrospective evaluation revealed that one study site was overrepresented with more than half of their samples being contaminated (Figure 2B; Supporting Information Results).

The overall mutation detection rate was 63% ($N = 159/253$). A considerably higher rate of 85% was observed in patients with total

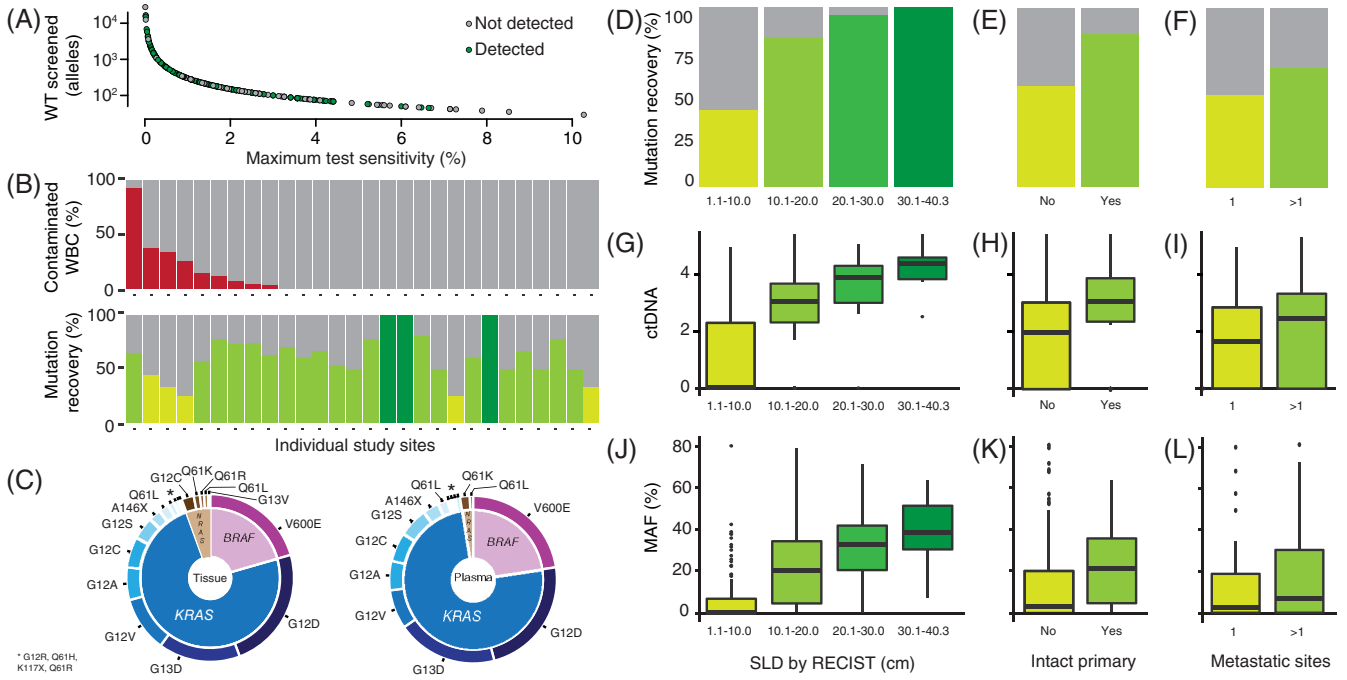

FIGURE 2 Relationship between maximum test sensitivity and the number of wilt type (WT) alleles screened by the test, where each dot represents a detected or nondetected patient sample in the study (A). Relative frequency of contaminated white blood cells (WBC) and mutation recovery per individual study site (B). Distribution of mutations detected in tissue ($N = 253$) and plasma ($N = 159$) (C). Detection rate of predefined tumour-specific hotspot *RAS* and *BRAF* mutations in plasma according to proxies of tumour burden (sum of the longest diameter of target lesions measured according to RECIST 1.0 at baseline [SLD]; intact primary tumour; number of metastatic sites) (all Chi-square $P < .05$; D-F). Absolute log-transformed ctDNA in plasma, with significantly higher levels with increasing SLD (Kruskal-Wallis $P < .001$; G), intact primary tumour (Mann-Whitney U $P < .001$; H) and > 1 metastatic site (Mann-Whitney U $P = .012$; I). Relative ctDNA minor allele frequencies (MAF) in plasma, with significantly higher levels with increasing SLD (Kruskal-Wallis $P < .001$; J), intact primary tumour (Mann-Whitney U $P < .001$; K) and > 1 metastatic site (Mann-Whitney U $P = .032$; L)

plasma cfDNA level above ULN and no significant leukocyte contamination ($N = 98/115$). There were no major differences in mutation detection with regard to gene involved. However, we noted numerically fewer *NRAS* mutations detected in plasma than tissue (Figure 2C). The median level of ctDNA (mutant) was 220 alleles/mL plasma (range 0-272 500 alleles/mL plasma) for patients in our study. When a mutation was recovered in plasma, both low ctDNA (MAF 0.2-19%; $N = 78$) and high ctDNA loads (MAF 20%-80%; $N = 81$) were observed. There was a statistically significant and strong correlation between ctDNA (mutant) and total cfDNA, and between ctDNA (mutant) and MAF (data not shown).

3.2 | Clinicopathological characteristics and mutation detection

Mutation detection was significantly more frequent in patients with high SLD, intact primary tumour and more than one metastatic site (Table 1; Figure 2D-F), all proxies of tumour burden. Similarly, the same groups were associated with significantly higher levels of ctDNA and MAF (Figure 2G-L). Additionally, metastatic involvement of the liver or lymph nodes, a systemic inflammatory response as measured by elevated IL-6 and poor performance status were statistically significantly associated with mutation detection in plasma. On the contrary, metastatic involvement of the lungs was statistically significantly associated with nondetection (Table 1).

Of note, 11 patients had lung metastases only. In terms of tumour burden proxies, these patients tended to have a lower SLD (median 3.7 cm; range 1.4-10.5 cm) compared to the rest of the cohort, none had intact primary tumour and all had only one metastatic site. None of the patients with lung metastases only had detectable ctDNA in plasma. Furthermore, 4 patients had peritoneal metastases only. In terms of tumour burden proxies, these patients tended to have lower SLD (median 9.1 cm; range 4.3-13.0 cm) compared to the rest of the cohort, half had intact primary tumour ($N = 2/4$) and all had only one metastatic site. None of the patients with peritoneal metastases only had detectable ctDNA in plasma.

3.3 | Model of clinicopathological and preanalytical/analytical factors associated with mutation detection

A logistic regression model was constructed to predict the probability of mutation detection based on clinicopathological characteristics.

TABLE 1 Clinicopathological characteristics, preanalytical/analytical factors and biochemical tumour markers in patients negative and positive for targeted mutation in plasma ($N = 253$)

	Negative for targeted mutation in plasma ($N = 94$)	Positive for targeted mutation in plasma ($N = 159$)	*P* value
Age, n (%)			
Below median	43 (34%)	84 (66%)	.276
Above median	51 (40%)	75 (60%)	
Gender, n (%)			
Male	49 (35%)	93 (65%)	.324
Female	45 (41%)	66 (59%)	
Performance status, n (%)			
WHO 0	71 (42%)	99 (58%)	.030
WHO 1-2	23 (28%)	60 (72%)	
Systemic inflammatory response (IL-6), n (%)[a]			
IL-6 normal (≤ 5.6 ng/L)	59 (51%)	57 (49%)	<.001
IL-6 elevated (> 5.6 ng/L)	24 (22%)	85 (78%)	
Primary tumour location, n (%)			
Colon	53 (34%)	103 (66%)	.184
Rectum	41 (42%)	56 (58%)	
Primary tumour mutation, n (%)			
RAS mutation	78 (39%)	123 (61%)	.285
BRAF mutation	16 (31%)	36 (69%)	
Metastatic involvement, n (%)			
Liver involvement	48 (27%)	133 (73%)	<.001
Liver only	12 (35%)	22 (65%)	.809
Lung involvement	54 (47%)	61 (53%)	.003
Lung only	11 (100%)	0 (0%)	<.001
Lymph node involvement	22 (27%)	61 (73%)	.014
Lymph node only	1 (9%)	10 (91%)	.049
Peritoneal involvement	16 (50%)	16 (50%)	.108
Resection of primary tumour, n (%)			
Resected	85 (44%)	108 (56%)	<.001
Not resected	9 (15%)	51 (85%)	
Metastatic sites, n (%)			
1 site	31 (48%)	33 (52%)	.031
>1 site	63 (33%)	126 (67%)	
Radiological size of target lesions, n (%)			
SLD (≤10 cm)	79 (57%)	59 (43%)	<.001
SLD (>10 cm)	15 (13%)	100 (87%)	
Number of wild type screened, n (%)			
Below median	67 (53%)	60 (47%)	<.001
Above median	27 (21%)	99 (79%)	
Significant WBC contamination, n (%)			
No contamination	80 (35%)	146 (65%)	.094
Contamination	14 (52%)	13 (48%)	
CEA, n (%)			
Normal (<5 μg/L)	38 (66%)	20 (35%)	<.001
Elevated (≥5 μg/L)	56 (29%)	139 (71%)	

TABLE 1 (Continued)

	Negative for targeted mutation in plasma ($N = 94$)	Positive for targeted mutation in plasma ($N = 159$)	*P* value
CA19-9, n (%)			
Normal (<35 kU/L)	64 (59%)	45 (41%)	<.001
Elevated (≥35 kU/L)	30 (21%)	114 (79%)	
Total cfDNA, n (%)[b]			
Normal (<6418 alleles/mL)	63 (57%)	48 (43%)	<.001
Elevated (≥6418 alleles/mL)	17 (15%)	98 (85%)	

Note: The groups are compared using Chi-square test.
Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; cfDNA, total circulating cell-free DNA; IL-6, interleukin 6; SLD, sum of the longest diameter of target lesions measured according to RECIST 1.0 at baseline; WBC, white blood cell; WHO, World Health Organisation.
[a]Analysed on $N = 225$ since 28 patients lacked IL-6 at baseline.
[b]Analysed on $N = 226$ in patients since 27 patients lacked total cfDNA at baseline.

TABLE 2 Logistic regression model to predict the probability of mutation detection in plasma based on known clinicopathological and preanalytical/analytical factors (maximum analytical sensitivity and the presence of leukocyte contamination)

	Univariable ($N = 253$)				Multivariable ($N = 225$)[a]			
	OR	Lower 95% CI	Upper 95% CI	*P* value	OR	Lower 95% CI	Upper 95% CI	*P* value
Demographic characteristics								
Age (<median/≥median)	0.75	0.45	1.26	.277				
Gender (male/female)	0.77	0.46	1.29	.325				
Host characteristics								
Performance status (WHO 0/WHO 1-2)	1.87	1.06	3.31	.031				
SIR IL-6 (≤5.6/>5.6 ng/L)[a]	3.67	2.05	6.56	<.001				
Tumour characteristics								
Primary tumour location (colon/rectum)	0.70	0.42	1.18	.185				
Primary tumour mutation (*RAS/BRAF*)	1.43	0.74	2.74	.287				
Liver involvement (no/yes)	4.90	2.74	8.78	<.001				
Lung involvement (no/yes)	0.46	0.27	0.78	.003	0.26	0.12	0.58	.001
Lymph node involvement (no/yes)	2.04	1.15	3.62	.015				
Peritoneal involvement (no/yes)	0.55	0.26	1.15	.111				
Tumour burden								
Resected primary tumour (yes/no)	4.46	2.08	9.57	<.001	3.17	1.22	8.22	.018
Number of metastatic sites (1 site/>1 site)	1.88	1.06	3.34	.032	3.08	1.32	7.19	.009
Radiological SLD (+1 cm)	1.25	1.17	1.33	<.001	1.18	1.09	1.27	<.001
Preanalytical/analytical factors								
Number of wild type screened (+1 decile)	1.30	1.18	1.44	<.001	1.25	1.08	1.46	.004
Significant WBC contamination (no/yes)	0.51	0.23	1.14	.099	0.18	0.05	0.69	.013

Abbreviations: CI, confidence interval; IL-6, interleukin 6; L, lower; OR, odds ratio; SIR, systemic inflammatory response; SLD, sum of the longest diameter of target lesions measured according to RECIST 1.0 at baseline; U, upper; WBC, white blood cell; WHO, World Health Organisation.
[a]Analysed on $N = 225$ since 28 patients lacked IL-6 at baseline.

The model also included preanalytical and analytical factors; that is, proxy of maximum analytical sensitivity (number of wild type alleles screened) and the presence of leukocyte contamination. VIF of the independent variables typically ranged between 1 and 2, hence multicollinearity was not considered a problem. Other model assumptions were evaluated and considered met.

The final multivariable regression model was statistically significant, $\chi^2(6) = 96.12$, $P < .001$. The model explained 48% of the

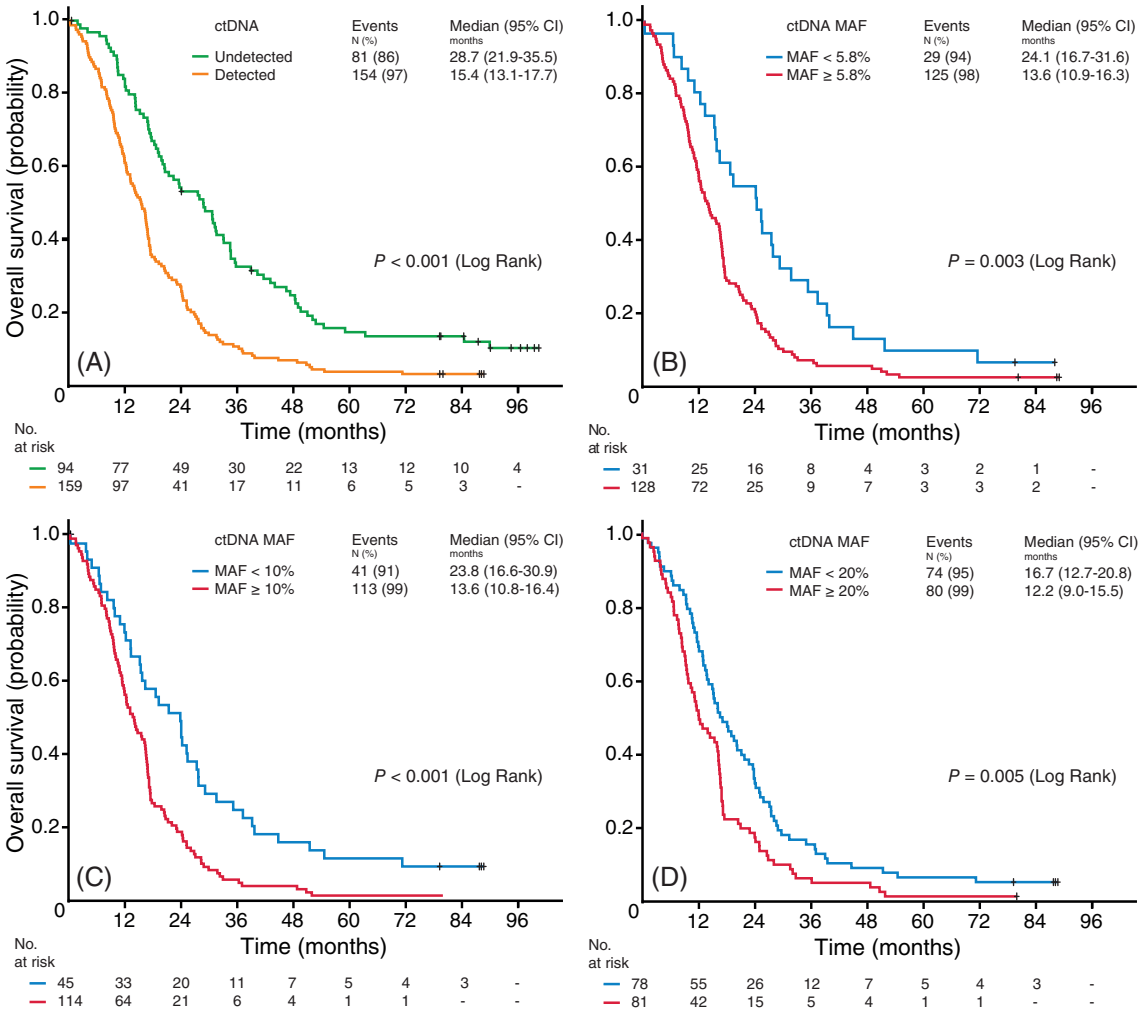

FIGURE 3 Overall survival (OS) of patients with ctDNA undetected vs detected (A), ctDNA MAF <5.8% vs MAF ≥5.8% (B), MAF <10% vs MAF ≥10% (C) and MAF <20% vs MAF ≥20% (D)

variance in mutation detection in plasma (Nagelkerke R^2), and correctly classified 78.2%. Of the initial 15 independent variables, 6 were found to be statistically significant in the final model (Table S5). Increasing radiological size of target lesions by increments of 1 cm (OR = 1.18; 95% CI 1.09-1.27; $P < .001$), intact primary tumour (OR = 3.17; 95% CI 1.22-8.22; $P = .018$) and more than one metastatic site (OR = 3.08; 95% CI 1.32-7.19; $P = .009$) were all associated with mutation detection in plasma. Metastatic site seemed to be of less importance with regard to detection, except for lung involvement which was associated with nondetection (OR = 0.26; 95% CI 0.12-0.58; $P = .001$). Furthermore, increasing number of wild type alleles screened was associated with detection and significant leukocyte contamination was associated with nondetection (Table 2).

The constructed multivariable model was subsequently applied to the subset of patients with detectable mutation in plasma in order to evaluate if the same factors could predict the probability of presenting with high MAF (ie, ≥20%, ≥10% or ≥5.8%). Increasing radiological size of target lesions was the only variable significantly and consistently associated with a high MAF across all three cutoffs (Table S6).

3.4 | Mutation detection, minor allele frequency levels and clinical outcome

Median OS was 15.4 months (95% CI 13.1-17.7 months) for patients with detectable ctDNA compared to 28.7 months (95% CI 21.9-35.5 months) for undetectable ctDNA (unadjusted HR = 2.11; 95% CI 1.61-2.79; $P < .001$; $N = 253$) (Figure 3A). Similarly, median PFS was 6.7 months (95% CI 6.2-7.1 months) for patients with detectable ctDNA compared to 8.5 months (95% CI 6.6-10.4 months) for undetectable ctDNA (unadjusted HR = 1.63; 95% CI 1.25-2.14; $P < .001$; $N = 253$) (Figure S1A).

High MAF was associated with a particularly poor prognosis in patients with detectable ctDNA, when applying cut-offs of 20%, 10% or 5.8%. Median OS was 13.6 months (95% CI 10.8-16.4 months) for MAF ≥10% compared to 23.8 months (95% CI 16.6-30.9 months) for MAF < 10% (unadjusted HR = 2.02; 95% CI 1.39-2.92; $P < .001$; $N = 159$). Comparable, albeit somewhat weaker associations with OS were obtained with MAF ≥20% (unadjusted HR = 1.58; 95% CI 1.14-2.17; $P = .005$; $N = 159$) and MAF ≥5.8%

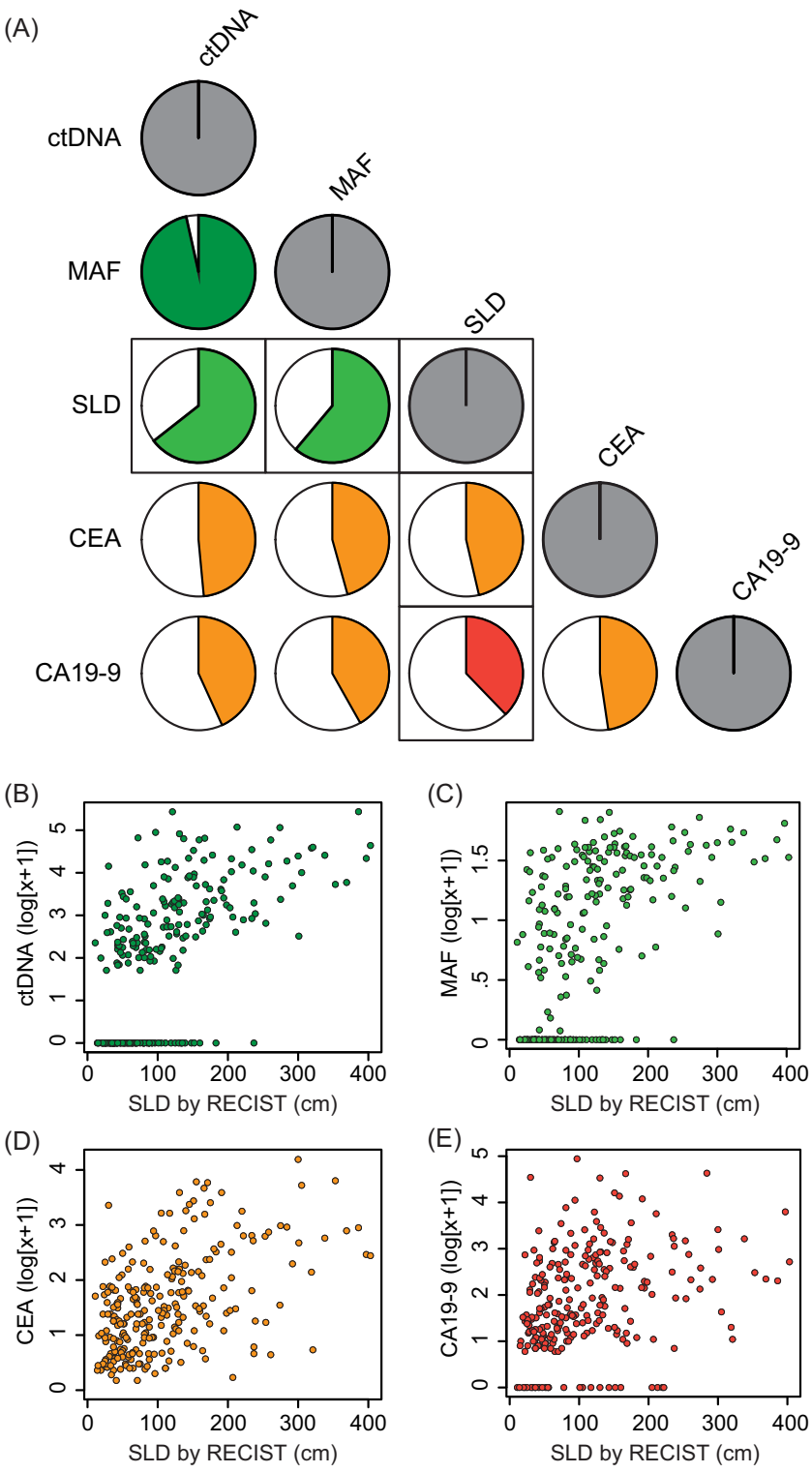

FIGURE 4 Log-transformed correlation matrix highlighting strengths of correlations between sum of the longest diameter of target lesions measured according to RECIST 1.0 at baseline (SLD) and blood levels of absolute ctDNA, relative ctDNA MAF, CEA and CA19-9; empty pie represents correlation coefficient 0; full colour pie represents correlation coefficient 1 (A). Scatter plots illustrating correlation between SLD and blood levels of absolute ctDNA, relative ctDNA MAF, CEA and CA19-9, where each dot represents a patient in the study (B-E)

(unadjusted HR = 1.86; 95% CI 1.23-2.80; $P = .003$; $N = 159$) (Figure 3B-D). Median PFS was 6.4 months (95% CI 6.0-6.9 months) for MAF ≥10% compared to 7.9 months (95% CI 6.9-9.0 months) for MAF <10% (unadjusted HR = 1.72; 95% CI 1.18-2.52; $P = .005$; $N = 159$). Comparable associations were obtained with MAF ≥5.8%, whereas MAF ≥20% did not show any statistically significant association with PFS (Figure S1B-D). There were no statistically significant associations between ctDNA detection or MAF at baseline and confirmed ORR.

When stratifying for patients with *RAS*-mutated or *BRAF*-mutated tumours, having detectable ctDNA and MAF ≥10% were associated with poor OS in both patient groups (Figure S2).

3.5 | Correlations between radiological and biochemical tumour markers

The computed tomography sum of the longest diameter of target lesions measured according to RECIST 1.0 at baseline (SLD) and biochemical tumour markers were correlated (Figure 4A). SLD demonstrated the strongest positive correlation to the absolute ctDNA levels (Spearman's rho 0.64; $P < .001$; $N = 253$) followed by the relative MAF level (Spearman's rho 0.61; $P < .001$), both measures of tumour-derived cfDNA in plasma. There were weaker associations between SLD and serum levels of CEA (Spearman's rho 0.46; $P < .001$), and SLD and serum levels of CA19-9 (Spearman's rho 0.38; $P < .001$) (Figure 4B-E).

3.6 | Model of clinicopathological factors and tumour markers associated with overall survival

A Cox regression model for OS was constructed based on clinicopathological characteristics. The model also included biochemical tumour markers; that is, CEA, CA19-9, total cfDNA and ctDNA MAF (cutoff 10%). We observed a weak statistical deviation from the proportional hazards assumption for primary tumour mutation ($P = .032$), but not for the overall model ($P = .176$). When stratifying the model for this variable, survival estimates of the remaining independent variables remained similar. Hence, this observation was not considered a serious violation of the proportionality assumption. Other model assumptions were evaluated and considered met.

Of the initial 17 independent variables, 4 were found to be statistically significant in the final model (Table S7). Poor performance status (HR = 1.80; 95% CI 1.32-2.46; $P < .001$), a systemic inflammatory response as measured by elevated IL-6 (HR = 1.71; 95% CI 1.27-2.30; $P < .001$), the presence of mutated *BRAF* in tumour tissue (HR = 2.66; 95% CI 1.84-3.83; $P < .001$) and ctDNA MAF ≥10% (HR = 2.38; 95% CI 1.74-3.26; $P < .001$) were all associated with poor OS. Of note, the other biochemical tumour markers CEA, CA19-9 and total cfDNA were not significantly associated with OS in the final model (Table S7).

4 | DISCUSSION

In our study, the absolute level of plasma ctDNA spans a wide range; the highest absolute level is more than 4000 times higher than the lowest level detected. The relative level of ctDNA as measured by MAF shows equally a broad range from 0.2% to 80%. We show compelling evidence that mutation detection and the levels of ctDNA are strongly associated with proxies of tumour burden, both in univariable and multivariable analyses.

Bachet et al. reported that having intact primary tumour and liver metastases prior to first-line therapy increased the probability of ctDNA detection,[25] in line with our results from univariable analysis. It has also been shown that liver involvement was associated with higher ctDNA levels.[21] However, these studies did not account for more detailed estimations of tumour burden. Grasselli et al. reviewed radiological imaging of patients where ctDNA in plasma was non-detected. They observed qualitatively that these patients were characterised by low tumour burden as estimated by the radiological size of tumour lesions.[22] We therefore accounted for tumour burden as assessed radiologically in our multivariable model, and report that the presence of liver metastases per se may be of lesser importance with regard to ctDNA detection than previously assumed.

Metastatic involvement of the lung seems to modulate the probability of recovering tumour-specific mutations in plasma. This association seems to be driven by patients with lung metastases only, a small subgroup ($N = 11$) where there was no mutation detection. This is in line with previous reports stating that lung metastases alone were associated with low ctDNA detection rates[26] and low MAFs.[21] Bando et al. found that lung metastases alone was associated with non-detection, especially in patients presenting with fewer than 10 lung lesions and a maximum lesion diameter less than 20 mm, in line with our findings.[27] Low tumour burden obviously matters, but additional mechanisms could make single-locus ctDNA detection more difficult in patients with lung metastases only (eg, low ctDNA shedding, tumour heterogeneity and/or clonal evolution).

It is well known that preanalytical and analytical factors modulate ctDNA detection.[10,11] We therefore accounted for potential contamination during leukocyte cell lysis, and the number of screened wild type alleles which is a proxy of the maximum assay sensitivity. This is recommended,[9] but not routinely reported in previously published studies. Given the real-world nature of our study we find that leukocyte contamination can occur despite using K_2EDTA tubes and downstream plasma processing close to current consensus guidelines.[9] A retrospective review of our data identified one study site being responsible for most of the contaminated plasma samples, suggesting human error in the preanalytical sample handling. Furthermore, we show that leukocyte contamination reduces the probability of ctDNA detection. Hence, contamination may have serious consequences if a false negative ctDNA result guides treatment in the metastatic setting, and perhaps even more detrimental consequences if used for de-escalating adjuvant therapy.

We further explored the prognostic impact of MAF. When a mutation was recovered, high MAFs were associated with a particularly poor survival. We investigated this trend using a data driven cutoff close to the median (20%) and cutoffs from the literature (10% and 5.8%).[22,37] The cutoff of 10% performed better in predicting survival in this patient group compared to a cutoff of 5.8% or 20%. Elez et al. derived a MAF cutoff of 5.8% from a development cohort and subsequently validated the association between MAF and survival in an independent cohort. These patients were included prior to first-line therapy, but unlike our population included only *RAS* mutated mCRC.[37] Another study reported a borderline significant association between MAF and survival when using a cutoff of 4.14%.[39] Grasselli et al. found a significant association between MAF and survival by applying a cutoff of 10%, although it should be noted that more than half of the patients included were not treatment naïve.[22]

Patients without detected mutation in plasma had superior PFS and OS. This group represents patients with relatively low tumour burden, and it is therefore plausible that ctDNA was not detected because of subsampling issues (low DNA shedding and low sample volume). Vidal et al. found a significant association between MAF and survival when using a cutoff of 1%,[21] but their study included a mixed patient population where not everyone was treatment naïve. We reached a median test sensitivity of 1.6% in our study, but given a higher plasma volume we could have reached better overall sensitivities (eg, 0.1%) allowing us to test the clinical significance of MAF cutoffs below 1% and perform separate cutoff analyses.

Several serum and plasma protein markers have been associated with CRC. CEA level in serum has traditionally been regarded as a tumour marker, in some cases reflecting disease burden and survival. CEA has been used in the follow-up of mCRC patients receiving systemic life-prolonging treatment or curative metastatectomy. However, the implementation has not been recommended in consensus management guidelines.[40,41] CA19-9 does not have an established role as tumour marker in mCRC, although it has been suggested to provide prognostic information in subgroups of mCRC patients.[29,42] Of note, both CEA and CA19-9 can be noninformative in a substantial number of mCRC patients (eg, poorly differentiated tumours,[43] and patients with Le [a-b-] genotype in the Lewis blood group system,[44] respectively). Only modest correlations between ctDNA and CEA levels in patients with mCRC are reported in the literature,[20,45] a finding which is supported by our study. In fact, ctDNA in plasma demonstrated a stronger correlation to SLD than the tumour markers CEA and CA19-9 in serum.

Our results suggest that MAF is a marker primarily of tumour burden in the context of *RAS*-mutated or *BRAF*-mutated mCRC prior to first-line therapy. There is no consensus as to where to apply a clinically relevant MAF cutoff, and it is likely that the cutoff will depend on previous exposure to systemic therapy.[46-48] In our study, a cutoff of 10% provided potentially useful prognostic information without being compromised by the test sensitivity. When we therefore evaluated the prognostic impact of a MAF cutoff of 10% in a multivariable model, integrating known clinicopathological and biochemical tumour markers associated with prognosis, ctDNA MAF was the only blood-based biomarker that remained significantly associated with OS, outperforming CEA, CA19-9 and total cfDNA. This suggests that ctDNA may be a clinically more useful marker than CEA and CA19-9 when evaluating patients prior to first-line therapy.

One of the main strengths of our study is that we were able to comprehensively model tumour-specific mutation detection in plasma using high quality clinicopathological data. Importantly, the effect of cytotoxic drugs that could modulate detection was minimal since sampling was performed prior to first-line therapy. However, we acknowledge several limitations. Firstly, the plasma sampling protocol of the NORDIC-VII study was not optimal regarding preanalytical conditions for ctDNA analyses (eg, sampling tubes, preservation fluids and centrifugation protocols), which could make our samples extra prone to leukocyte contamination. Secondly, there was low plasma volume available for ctDNA extraction, making the analyses prone to subsampling errors and limiting the maximum analytical sensitivity. We have tried to partially account for this in the multivariable model. Thirdly, we chose a tumour-guided, single-locus marker as a measure of ctDNA since both *RAS* and *BRAF* are considered early events of colorectal cancer carcinogenesis. However, there is a possibility that nondetection of ctDNA was partially caused by intratumour or intertumour heterogeneity, which could only be accounted for if we used a tumour-agnostic and/or multilocus analysis approach. Fourthly, we excluded patients with double wild type status in tissue and included only patients with *RAS*-mutant and *BRAF*-mutant mCRC, which limits generalisability to the latter patient groups.

Our study demonstrates that tumour-specific mutation detection in plasma of patients with *RAS*-mutated or *BRAF*-mutated mCRC is predominantly associated with tumour burden and modulated by metastatic site, the presence of leukocyte contamination due to preanalytical sample handling and analytical sensitivity. The results may have clinical implications when interpreting liquid biopsy results from patients with mCRC, supporting the idea that "one size does not fit all". Clinicopathological characteristics like proxies of tumour burden and metastatic involvement should be carefully considered when evaluating ctDNA results from a patient with mCRC, especially when confronted with a plasma negative result. Further research is needed to highlight strengths and limitations of liquid biopsies in the care pathways of mCRC patients, including a broader understanding of health economic impacts.

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CONFLICT OF INTEREST

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DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request within the framework of a data transfer agreement.

ETHICS STATEMENT

The NORDIC-VII study and its translational substudies were approved by the national ethics committees and governmental authorities in each country and conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent. The NORDIC-VII study is registered with ClinicalTrials.gov, number NCT00145314.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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