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Circulating tumour DNA at baseline for individualised prognostication in patients with chemotherapy-naïve metastatic colorectal cancer. An AGEO prospective study

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1 **Title:** Circulating tumor DNA at baseline for individualized prognostication in patients with
2 chemotherapy-naïve metastatic colorectal cancer. An AGEO prospective study.

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40

1 **Abstract**

2 **Purpose:** Baseline circulating tumor DNA (ctDNA) is a potential prognostic marker in
3 metastatic colorectal cancer (mCRC) patients. However, few studies have compared ctDNA
4 with the usual prognostic factors, and no ctDNA cut-off has been proposed for daily use in
5 clinical practice.

6 **Patients and methods:** Chemotherapy-naive patients with mCRC were prospectively
7 included. Plasma samples were collected at diagnosis and analyzed centrally by both NGS
8 and methylation digital PCR. Baseline patient and disease characteristics, treatment
9 regimens, and secondary surgeries were collected. The restricted cubic spline method was
10 used to define the optimal cut-off of ctDNA mutated allelic frequency (MAF). Prognostic
11 values were assessed on overall survival (OS) using Cox models.

12 **Results:** From July 2015 to December 2016, 412 patients were included. ctDNA was
13 undetectable in 83 patients (20%). ctDNA was an independent prognostic marker for OS
14 considering the whole study population. The optimal cut-off for ctDNA MAF was 20% with
15 median OS of 16.0 and 35.8 months for patients with MAF \geq 20% and $<$ 20%, respectively
16 (HR=0.40; 95% CI: 0.31-0.51; $P<0.0001$). The independent prognostic value of ctDNA MAF at
17 20% was confirmed in subgroups defined by *RAS/BRAF* status or resectability of metastases.
18 Combining ctDNA MAF and CEA levels allowed us to define three different prognostic groups
19 with median OS of 14.2, 21.1, and 46.4 months ($P<0.0001$).

20 **Conclusion:** ctDNA with a MAF cut-off of 20% improves prognostication of chemotherapy-
21 naïve mCRC patients and may be useful in the future for individualized therapeutic decisions
22 and as a stratification factor in clinical trials.

23

24 **Trial registration:** Clinicaltrials.gov, NCT02502656

- 1 **Keywords:** colorectal cancer, circulating tumor DNA, CEA, prognostic value, *RAS*, *BRAF*,
- 2 metastases
- 3

1 **Introduction**

2

3 During the last thirty years, the overall survival (OS) of patients with metastatic colorectal
4 cancer (mCRC) has progressively increased.[1,2] These improvements have been possible by
5 developing new polychemotherapy regimens, targeted therapies, and multidisciplinary
6 treatment approaches.[3]

7 Identifying and validating *RAS* and *BRAF* mutations as prognostic and predictive biomarkers
8 has profoundly impacted therapeutic strategies, and all guidelines now recommend upfront
9 testing for these markers.[3-5] The strong prognostic value of resection of metastases has
10 also led to a recommendation of systematic discussion of patient files in multidisciplinary
11 meetings and classification of metastases as resectable, potentially/borderline resectable
12 and unresectable.[3] In addition, many other prognostic factors have been described in the
13 literature, including the patient's general condition, baseline carcinoembryonic antigen (CEA)
14 level, number of metastatic sites and other parameters such as white blood cell count, LDH,
15 alkaline phosphatases, and blood albumin that may be integrated in prognostic scores.[3,6]
16 Circulating tumor DNA (ctDNA) is a promising emerging biomarker in solid tumors, including
17 mCRC. ctDNA is highly specific, but its sensitivity is influenced by technical issues and by
18 tumor characteristics, especially tumor sites and tumor burden [7,8]. Its strong prognostic
19 value has been reported in most cancers, but the assessment of its prognostic value
20 according to validated prognostic factors is lacking in mCRC, and to date, no cut-off has been
21 defined for its use in clinical practice.

22 The RASANC trial is a prospective multicenter study initially designed to assess the
23 concordance of *RAS* mutational status between paired plasma and tumor samples from
24 chemotherapy-naive patients with mCRC.[9,10] The positive results of this trial and those of

1 other teams have led to a modification of French and European guidelines with the
2 possibility of assessing *RAS/BRAF* status through ctDNA analysis in selected patients.[3,9-15]
3 In this article, we analyze the prognostic value of ctDNA in the RASANC trial population and
4 in different prognostic subgroups defined by *RAS/BRAF* status and resectability of
5 metastases.
6

1 **Patients and Methods**

2

3 *Study design and patients*

4 The RASANC study is a prospective translational study. Patients were eligible if they were 18
5 years or older and had pathologically confirmed chemotherapy-naive mCRC (adjuvant
6 chemotherapy completed \geq 6 months prior to enrollment was allowed).[9,10]

7 The Ile-de-France IV ethics committee approved the research protocol, and all the patients
8 gave their written informed consent. The trial conformed to the Declaration of Helsinki, the
9 Good Clinical Practice guidelines of the International Conference on Harmonization, and
10 relevant French and European laws and directives. The protocol was registered with
11 Clinicaltrials.gov (No: NCT02502656).

12

13 *Tumor tissue mutation analyses, Electronic Case Report Form, Plasma collection and ctDNA*
14 *assessment have been previously reported and are detailed in appendix A.1.*[9,10]

15 For ctDNA, the mutated allelic frequency (MAF) was defined as the highest frequency of
16 mutated genes found through NGS when at least one mutated gene was identified or as the
17 highest frequency of methylated biomarkers detected when no mutated gene was identified
18 through NGS.

19

20 *Statistical analyses*

21 The primary objective of the RASANC study was to assess the concordance of *RAS* mutations
22 in tumor tissue and plasma. The kappa coefficients were previously reported for *RAS*
23 mutations and *BRAF* mutations.[9,10]

1 The median (interquartile range) and frequency (percentage) were used to describe
2 continuous and categorical variables, respectively. Medians and proportions were compared
3 using the Wilcoxon-Mann-Whitney test and chi-square test, respectively, or with Fisher's
4 exact test if appropriate.

5 Median follow-up duration (95% CI) was estimated using the reverse Kaplan-Meier method.
6 OS was estimated using the Kaplan-Meier method described with median and rates at
7 specific time points with 95% CI and compared with log-rank test. Hazard ratios (HRs) with
8 95% CIs were estimated with Cox proportional hazard models.

9 The restricted cubic spline method was used to model the relationship between parameters
10 in their continuous form with OS to define the optimal cut-off. The association of baseline
11 parameters with OS was first assessed using univariate Cox analyses, and then parameters
12 with *P*-values of less than .05 were entered into a final multivariable Cox regression model,
13 after considering collinearity among variables with a correlation matrix.

14 The assumption of proportionality was checked by plotting log minus-log survival curves and
15 by cumulative martingale process plots.

16 The predictive value that a parameter added to a reference risk model was evaluated with
17 the use of the C-statistic. This analysis was repeated 1000 times with the use of bootstrap
18 samples to derive 95% confidence intervals for the difference in the C-statistic between
19 models.

20 All analyses used SAS software version 9.4 (SAS Institute, Cary NC) and R (version 4.0.5). *P*-
21 values below 0.05 were considered statistically significant, and all tests were two-sided.

22

1 **Results**

2

3 *Study population and ctDNA detection*

4 Between July 2015 and December 2016, 425 patients were enrolled in 14 centers. Thirteen
5 (3%) patients were excluded from analyses (not meet the inclusion criteria or no available
6 tumor tissue or plasma).

7 Among the 412 patients included (supplementary Figure A.1), at least one mutation of the
8 22 genes sequenced by NGS was detected in plasma from 299 (73%) patients. In the
9 remaining 113 (27%) patients, WIF1 and/or NPY methylation was detected in plasma from
10 30 patients (7%). Thus, ctDNA was detected at inclusion in 329/412 patients (80%) who are
11 defined as ctDNA-positive group. Patients in whom ctDNA was not detected through NGS
12 and methylation assay were defined as the ctDNA-negative group. Median MAF was of
13 22.0% (range from 0.5 to 95.0%) in patients with at least one mutated gene detected
14 through NGS and 3.3% (range from 0.1 to 66.7%) in those with methylation assay and no
15 mutated gene detected.

16 When looking at tissue molecular profiling and after excluding the 6 patients with *RAS* wild-
17 type tumor but *BRAF* status unknown, the rate of ctDNA detection at baseline according to
18 *RAS/BRAF* status was similar in the three subgroups: 76% (102/134) in patients with
19 *RAS/BRAF* wild-type tumor, 82% (198/242) in patients with *RAS* mutated tumor and 80%
20 (24/30) in patients with *BRAF* mutated tumor.

21 ctDNA detection was positively associated with synchronous metastases, metastatic sites >3,
22 the absence of primary tumor resection, liver metastases, low blood albumin, high alkaline
23 phosphatase, and LDH and CEA levels in all patient subgroups (tissue-based *RAS/BRAF* wild-
24 type, *RAS* mutated and *BRAF* mutated; supplementary Tables A.1-3).

1 Among the 329 patients of the ctDNA-positive group, *RAS* and *BRAF* V600E mutations were
2 detected in 187 (56.8%) and 25 (7.6%) of plasma samples (characteristics of the patients are
3 described in supplementary Table A.4).

4

5 *Treatment patterns*

6 372/412 (90%) patients received at least one line of chemotherapy and 234 (57%) two or
7 more lines: 366 (98%) patients received a fluoropyrimidine, 321 (86%) oxaliplatin, 235 (63%)
8 irinotecan, 226 (61%) bevacizumab, 43 (12%) aflibercept, 95 (26%) panitumumab or
9 cetuximab, 28 (8%) regorafenib, and 60 (16%) another treatment. Two hundred fifteen
10 patients (52%) had primary tumor resection before inclusion; during the follow-up, 84 (20%)
11 additional patients had a primary tumor resection and 123 (30%) at least one metastasis
12 resection.

13 Rates of primary tumor resection during follow-up and types of chemotherapy drugs
14 administered were not different between ctDNA-positive and -negative patients. Patients in
15 the ctDNA-positive group had significantly fewer metastasis resections, and the types of
16 resection were different between ctDNA-positive and ctDNA-negative groups as metastases
17 were not located similarly between these two groups (Table 1).

18

19 *ctDNA and overall survival*

20 After a median follow-up of 45.0 months (95% CI: 43.2-47.4), median OS was 26.0 months
21 (95% CI: 23.2-30.3) for the whole population. Follow-up data were not available for 8
22 patients (2%) (supplementary Figure A.1).

23 Median OS of patients in the ctDNA-positive group was significantly shorter than in the
24 ctDNA-negative group (23.2 vs 43.0 months; HR=0.54; 95% CI: 0.38-0.75; $P=0.0002$) (Figure

1 1a). Restricted cubic spline analysis identified a linear relationship between the MAF and the
2 risk of death. The optimal cut-off to define low- and high-risk populations was 20% (Figure
3 1b). Indeed, median OS was 16.0 and 35.8 months for patients with $MAF \geq 20\%$ and $< 20\%$,
4 respectively (HR=0.40; 95% CI: 0.31-0.51; $P<0.0001$) (Figure 1c).

5 A significant prognostic value of ctDNA detection (ctDNA-negative vs ctDNA-positive) or a
6 MAF greater than or equal to 20% was observed in the three subgroups defined by
7 metastasis resectability at baseline (data not shown), as in the three subgroups defined by
8 *RAS/BRAF* tumor status (supplementary Figure A.2).

9 A multivariate analysis was performed with significant variables in univariable analyses
10 (Table 3) and showed that MAF of ctDNA below 20% was independently associated with a
11 shorter OS (Table 2).

12

13 *Added prognostic value of combining CEA and ctDNA*

14 CEA was also an independent prognostic factor in our multivariable model with an optimal
15 threshold of 30 ($\approx \log_{10} 1.5$), as identified by the restricted cubic spline method, we
16 analyzed the respective prognostic values of both ctDNA and CEA alone, and in combination.

17 The concordance between the level of CEA and ctDNA MAF was generally poor, particularly
18 in the case of non-hepatic metastases (supplementary Figure A.3). The best risk model of
19 death was obtained when both biomarkers, CEA and ctDNA, were added to the other

20 prognostic factors (Figure 2a). Patients with a high level of both biomarkers (n=116, 34%,
21 reference) had the worst prognosis, with a median OS of 14.2 months (HR=1.75 ; 95% CI :

22 1.29-2.37), those (n=127, 37%) with a low level of both biomarkers had the best prognosis,
23 with a median OS of 48.1 months (HR=0.27 ; 95% CI: 0.20-0.38), and those with only a high
24 MAF of ctDNA (n=30, 9%) or only a high CEA level (n=68, 20%) had an intermediate prognosis

- 1 with a median OS of 21.8 months (reference) (Figure 2b; supplementary Table A.5 and
- 2 supplementary Figure A.4).
- 3

1 Discussion

2

3 In this article, we have assessed the prognostic value of ctDNA at baseline in a large
4 prospective cohort of patients with chemotherapy-naive mCRC. To detect ctDNA, we used a
5 two-step methodology: firstly, an NGS method adapted to plasma analysis and then, in the
6 absence of detection of a mutated gene, a multiplex digital PCR analysis of CpG island
7 methylation in two genes (WIF1 and NPY) to differentiate patients with ctDNA and no
8 identified mutations from those without ctDNA. The detection of ctDNA was significantly
9 associated with some clinical and laboratory features as previously reported.[9,10] A linear
10 relationship between ctDNA MAF and the risk of death was observed with an optimal cut-off
11 of 20%. The dismal prognosis of ctDNA-positive patients was observed in the whole
12 population, as in all subgroups. Combination of ctDNA MAF and CEA levels improved
13 prognostication as compared to the use of one marker alone.

14 A strong prognostic value of ctDNA has been reported in mCRC.[16] However, at diagnosis of
15 metastases, ctDNA is not detectable in around 20% of patients and the rate of detection is
16 correlated with the presence of liver metastases and the tumor burden.[9,10,16-19] In the
17 absence of liver metastases, a baseline longest diameter ≥ 20 mm or a number of lesions ≥ 10
18 or both were associated with ctDNA detection in patients with lung metastases or peritoneal
19 carcinomatosis.[19] Moreover, despite evidence of the prognostic value of ctDNA, no clear
20 cut-off of ctDNA MAF has been reported in the literature. Recent publications assessed the
21 median MAF as cut-off with various absolute MAF percentages (from 3.3% to 12.6%) in
22 limited cohorts of patients [17,18]. However, firstly, positivity versus negativity could be
23 suboptimal and, secondly, median MAF varies according to the different series and cannot
24 be used for an individual patient in routine practice. As we had here a large prospective

1 cohort with clinical annotations, we used a restricted cubic spline analysis to define an
2 optimal cut-off for the relationship between MAF and the risk of death, and found an
3 optimal cut-off of 20%. Using this cut-off, ctDNA was found to be an independent prognostic
4 marker in the whole population and in all subgroups. This cut-off of ctDNA MAF should now
5 be tested in external validation cohorts of mCRC patients to see whether it is confirmed or
6 can be improved.

7 CEA is an oncofetal antigen produced by some cancers (most often adenocarcinomas) and
8 shed from the cell surface and metabolized by the liver.[20] ctDNA release is believed to be
9 proportional to tumor growth, which is linked to the ratio of cell death and cell proliferation.
10 In mCRC, ctDNA detection is correlated with the presence of liver metastases and tumor
11 burden, at least in part as CEA. The comparative prognostic value of CEA and ctDNA tests is
12 rarely evaluated. Our results clearly demonstrated that these two biomarkers are not
13 correlated and that a combined analysis is superior to the analysis of just one. Because no
14 clear prognostic cut-off of CEA level is defined in mCRC, we used a restricted cubic spline
15 analysis to define the optimal cut-off in our cohort (30 ng/mL), as for the ctDNA MAF (20%).
16 With this methodology, the concordance rate between ctDNA and CEA levels was 71%
17 (n=243/341). This rate is relatively close to the overall concordance rate of 75% reported in a
18 cohort of 110 patients with mCRC and using other cut-offs (0.15% for MAF and 5 ng/mL for
19 CEA level).[21] In our study, among the patients with discordant ctDNA and CEA levels, 31%
20 (n=30/98) had only a high ctDNA level and 69% (n=68/98) only a high CEA level. These rates
21 are also close to those reported previously: 41% (n=11/27) and 59% (n=16/27),
22 respectively.[21] In comparison to patients with low ctDNA and high CEA levels, those with
23 high ctDNA and low CEA levels more frequently had an ECOG PS 2 (33.3% vs 21.7%) and liver
24 metastases (93.3% vs 58.6%). Conversely, patients with low ctDNA and high CEA levels more

1 frequently had peritoneal carcinomatosis (38.6% vs 20%), while other metastatic sites did
2 not seem to differ between the two groups. Both biomarkers appear to give complementary
3 information. Combination of the MAF ctDNA and CEA levels allowed us to define three
4 different prognostic groups of patients of about one-third each. Such analysis was previously
5 done in a small cohort of patients who had liver metastasis resection. In this study, the
6 combination of MAF ctDNA (cut-off 3.3%) and CEA level (cut-off 4.9 ng/mL) was also
7 associated with a strong prognostic value.[22] Thus, with the exception of the metastatic
8 sites and perhaps the performance status, it is currently not easy to explain why some
9 patients have only a high ctDNA level and others a high CEA level.

10 Despite a trend for worse prognosis associated to right colon cancer, the primary tumor
11 location did not have a significant prognostic value in our study in univariate analysis and
12 thus was not included in multivariate analysis. When the left colon and rectal cancer were
13 pooled and compared to right colon cancer, the difference was significant in univariate
14 analysis but not in multivariate analysis and did not modify the risk model of death (data not
15 shown). Our study population comprised patients with right and transverse colon cancer
16 (31%), left colon cancer (41%) or rectal cancer (28%). The relatively limited number of
17 patients included in each subgroup as the heterogeneous characteristics of patients at
18 inclusion (all patients whatever *RAS/BRAF* status, all types of metastases resectability) may
19 have limited the prognostic impact of the primary tumor location in our study.

20 The strengths of our study are the large homogeneous population of patients with
21 chemotherapy-naïve mCRC, its prospective multicenter conditions, and optimal conditions
22 to assess *RAS* and *BRAF* mutations and ctDNA. However, the design of this prospective study
23 and its primary objective did not allow assessment of dynamic follow-up of ctDNA, whereas
24 early dynamic changes in ctDNA levels according to treatment efficacy and development of

1 resistance mutations before clinical progression are promising in helping to guide
2 therapeutic strategy.[15,23-26] Only one plasma point was collected during the study and
3 monitoring of ctDNA analyses was not possible. So, the proposed cut-off is only valuable in
4 chemotherapy-naïve mCRC patients and cannot be generalized to others clinical situations
5 (localized CRC, mCRC under chemotherapy, refractory mCRC, others tumor types). Another
6 limitation could be that we used a non-tumor-informed methodology of ctDNA which may
7 be less sensitive.

8

9 **Conclusion**

10 In the large prospective RASANC cohort, ctDNA MAF at baseline had a significant prognostic
11 value at the optimal cut-off of 20%. CEA gave additional prognostic information and the
12 combination of both biomarkers classified patients in three subgroups of significantly
13 different prognosis. ctDNA with a MAF cut-off of 20% at baseline improves prognostication
14 of chemotherapy-naive mCRC patients and may be useful in the future for individualized
15 therapeutic decisions and as a stratification factor in clinical trials.

16

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22

23 **Authors' Contributions**

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- 4 etc.): all authors.
- 5 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational
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- 8 Writing, review, and/or revision of the manuscript: all authors.
- 9 Administrative, technical, or material support (i.e., reporting or organizing data, constructing
- 10 databases): J.B. Bachet, A. Meurisse, J. Taieb, H. Blons, D. Vernerey, P. Laurent-Puig.
- 11 Study supervision: J. B. Bachet, J. Taieb, D. Vernerey, P. Laurent-Puig.

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1 **Figure legends**

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3 Figure 1: Overall survival according to ctDNA at baseline

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5 Figure 1a: Overall survival according to ctDNA detection at baseline (ctDNA-negative group
6 vs ctDNA-positive group).

7 Figure 1b: Restricted cubic spline method to define the relation between the MAF of ctDNA
8 and overall survival.

9 Figure 1c: Overall survival according to the defined threshold of the MAF of ctDNA (< vs ≥
10 20%).

11

12 Figure 2: Prognostic analyses combining CEA and ctDNA MAF levels.

13

14 Figure 2a: Risk model of death with or without the addition of CEA and/or ctDNA.

15 The variables included in the first risk model were: Age (<70 vs ≥70); Köhne score;
16 Resectability of metastases; *RAS/BRAF* status.

17 Figure 2b: Overall survival of the three subgroups of patients defined by the combination of
18 CEA level (< or ≥ 30 ng/mL) and the MAF of ctDNA (< or ≥ 20%) at baseline.

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Table 1: Baseline characteristics, surgical resections and chemotherapy received during follow-up according to ctDNA detection at baseline

	All patients <i>n</i> =412	ctDNA-positive <i>n</i> =329	ctDNA-negative <i>n</i> =83	<i>P</i> -value
Baseline clinical and pathological characteristics				
Sex - <i>n</i> (%)				
Male	247 (60%)	202 (61%)	45 (54%)	0.233
Female	165 (40%)	127 (39%)	38 (46%)	
Age at diagnosis of metastases - years				
Median (range)	67 (20-100)	67 (20-100)	68 (29-88)	0.638
IQR	59-74.5	58-74	60-75	
ECOG PS at diagnosis of metastases - <i>n</i> (%)				
Unknown	17 (2%)	12 (4%)	5 (6%)	0.106
0	126 (31%)	93 (28%)	33 (40%)	
1	180 (44%)	148 (45%)	32 (39%)	
≥2	89 (22%)	76 (23%)	13 (16%)	
Primary tumor location - <i>n</i> (%)				
Right and transverse colon	126 (31%)	100 (30%)	26 (31%)	0.811
Left and sigmoid colon	171 (41%)	139 (42%)	32 (39%)	
Rectum	115 (28%)	90 (28%)	25 (30%)	
Primary tumor resection before inclusion - <i>n</i> (%)				
No	197 (48%)	177 (54%)	20 (24%)	<0.0001
Yes	215 (52%)	152 (46%)	63 (76%)	
Differentiation - <i>n</i> (%)				
Unknown	75 (18%)	57 (17%)	18 (22%)	0.295
Well differentiated	121 (30%)	98 (30%)	23 (28%)	
Moderately differentiated	198 (48%)	157 (48%)	41 (49%)	
Undifferentiated	18 (4%)	17 (5%)	1 (1%)	
<i>RAS-BRAF</i> status from tumor samples				
<i>RAS/BRAF</i> wild-type	134 (33%)	102 (31%)	32 (39%)	0.602
<i>RAS</i> mutated	242 (59%)	198 (60%)	44 (53%)	
<i>BRAF</i> mutated	30 (7%)	24 (7%)	6 (7%)	
<i>BRAF</i> unknown	6 (1%)	5 (2%)	1 (1%)	
Metastases - <i>n</i> (%)				
Synchronous	310 (75%)	269 (82%)	41 (49%)	<0.0001
Metachronous	102 (25%)	60 (18%)	42 (51%)	
Resectability of metastases - <i>n</i> (%)				

Unknown	9 (2%)	9 (3%)	0	
Resectable	51 (12%)	38 (11%)	13 (16%)	
Potentially resectable	121 (30%)	92 (28%)	29 (35%)	
Not resectable	231 (56%)	190 (58%)	41 (49%)	0.255
Number of metastatic sites - <i>n</i> (%)				
Unknown	9 (%)	9 (3%)	0	
Median (range)	1 (1-7)	1 (1-7)	1 (1-4)	0.341
IQR	1-2	1-2	1-2	
Location of metastases - <i>n</i> (%)				
Liver	293 (71%)	269 (82%)	24 (29%)	<0.0001
Lung	130 (32%)	99 (30%)	31 (37%)	0.203
Peritoneal carcinomatosis	104 (25%)	72 (22%)	32 (39%)	0.002
Adenopathy subdiaphragmatic	40 (10%)	32 (10%)	8 (10%)	0.981
Adenopathy supradiaphragmatic	21 (5%)	14 (4%)	7 (8%)	0.158
Bone	18 (4%)	15 (5%)	3 (4%)	1
Brain	5 (1%)	4 (1%)	1 (1%)	1
Other	34 (8%)	21 (6%)	13 (16%)	0.006
Baseline laboratory characteristics				
Leukocytes (mm ³) - <i>n</i> (%)				
Unknown	23 (6%)	16 (5%)	7 (8%)	
Median (range)	7990 (771-22910)	8200 (771-22910)	7065 (2880-22600)	0.011
IQR	6140-10200	6200-10500	5750-9410	
Albumin (g/L) - <i>n</i> (%)				
Unknown	97 (24%)	72 (22%)	25 (30%)	
Median (range)	37.6 (16-52)	36.0 (16-50)	40.5 (24.9-52)	<0.0001
IQR	32-42	31.9-41	37.6-43	
CEA (ng/mL) - <i>n</i> (%)				
Unknown	48 (12%)	34 (10%)	14 (17%)	
Median (range)	35.3 (0.7-23980)	64.3 (0.7-23980)	7.5 (0.8-360.5)	<0.0001
IQR	7-192.3	10.7-277.8	3.6-14	
LDH (xULN) - <i>n</i> (%)				
Unknown	192 (47%)	144 (44%)	48 (58%)	
Median (range)	1 (0.1-32.7)	1.04 (0.09-32.66)	0.80 (0.48-1.80)	0.0001
IQR	0.8-1.7	0.81-1.83	0.65-0.96	
ALP (xULN) - <i>n</i> (%)				
Unknown	53 (13%)	38 (12%)	15 (18%)	
Median (range)	0.9 (0.2-22)	1.02 (0.20-22.03)	0.66 (0.34-2.13)	<0.0001
IQR	0.6-1.7	0.63-1.97	0.53-0.81	

Köhne score				
Missing	30 (7%)	20	10	
Low risk	179 (43%)	138 (44.7%)	41 (56.2%)	
Intermediate risk	133 (32%)	108 (34.9%)	25 (34.2%)	
High risk	70 (17%)	63 (20.4%)	7 (9.6%)	0.0661
Treatments received during follow-up				
Primary tumor resection during follow-up				
Unknown	30	26	4	
Primary tumor not resected at baseline	n=205	n=183	n=22	
During follow-up	82 (40.0%)	72 (39.3%)	10 (45.5%)	
No	123 (60.0%)	111 (60.7%)	12 (54.5%)	0.5804
Metastasis resection				
Unknown	34	29	5	
No	255 (67.5%)	212 (70.7%)	43 (55.1%)	
Yes	123 (32.5%)	88 (29.3%)	35 (44.9%)	0.0091
Type of metastasis resection*	n=123	n=88	n=35	
Unknown	0	0	0	
Liver	87 (70.7%)	75 (85.2%)	12 (34.3%)	<0.0001
Lung	22 (17.9%)	10 (11.4%)	12 (34.3%)	0.0028
Peritoneum / CHIP	21 (17.1%)	10 (11.4%)	11 (31.4%)	0.0076
Other	23 (18.7%)	12 (13.6%)	11 (31.4%)	0.0224
Chemotherapy				
No	40 (9.7%)	33 (10.0%)	7 (8.4%)	
Yes	372 (90.3%)	296 (90.0%)	76 (91.6%)	0.6606
Chemotherapy number of line				
Mean (std)	2.4 (1.9)	2.5 (2.1)	2.3 (1.8)	
Median (min-max)	2.0 (0-10)	2.0 (0-10)	2.0 (0-10)	0.5733
Q1-Q3	1.0-3.0	1.0-4.0	1.0-3.0	

Abbreviations: IQR, interquartile range; ECOG PS, Eastern Cooperative Oncology Group performance status; *BRAF*: *BRAF* V600E; ULN, upper limit of normal; CEA, carcinoembryonic antigen; LDH, lactate dehydrogenase; ALP, alkaline phosphatases.

ctDNA-positive: Patients with conclusive plasma result were defined as patients with at least one mutation identified by the Ampliseq technique (*RAS* or other) or at least one methylated biomarker identified in the plasma sample (patients without identified mutations).

* some patients had more than one metastasis resection

Table 2: Univariate and multivariate Cox analysis for overall survival

Parameters		Univariate analysis				Multivariate analysis		
		No. of patients	No. of events	HR	95% CI	P-value	95% CI	p-value
Gender	Male	242	166	1				
	Female	162	107	0.940	0.737-1.200	0.6214		
Age	<70	243	153				1	
	≥70	161	120	1.516	1.193-1.926	0.0007	1.307	0.991-1.725 0.0580
ECOG PS	Missing	14						
	0-1	304	188	1				
	≥2	86	75	2.977	2.266-3.910	<0.0001		
Primary tumor location	Right and transverse colon	123	90	1				
	Left and sigmoid colon	167	108	0.761	0.575-1.007			
	Rectum	114	75	0.789	0.580-1.072	0.1303		
Primary tumor resection	No	221	199	1				
	Yes	183	112	0.605	0.475-0.771	<0.0001		
Grade of tumor differentiation	Missing	73						

Parameters		No. of patients	No. of events	Univariate analysis			Multivariate analysis		
				HR	95% CI	P-value	95% CI	p-value	
	Well	120	74	1					
	Moderate	193	131	2.518	1.460-4.343				
	Poor	18	16	1.214	0.912-1.615	0.0039			
Metastases	Synchronous	303	216	1					
	Metachronous	101	57	0.559	0.417-0.749	<0.0001			
Resectability of metastases	Missing	2							
	Resectable	50	25	1			1		
	Potentially resectable	121	72	1.527	0.968-2.408		1.511	0.917-2.490	
	Not resectable	231	174	3.098	2.029-4.732	<0.0001	2.049	1.265-3.318	0.0085
No. of metastatic sites	Missing	9							
	<3	360	238	1					
	≥3	35	27	1.250	1.041-1.500	0.0167			
Köhne score	Missing	28							
	Low risk	178	104	1			1		
	Intermediate risk	132	90	1.461	1.100-1.940		1.286	0.943	
	High risk	69	61	3.380	2.450-4.662	<0.0001	2.182	1.754	<0.0001

Parameters		No. of patients	No. of events	Univariate analysis			Multivariate analysis		
				HR	95% CI	P-value	95% CI	p-value	
Mutation	Missing	6							
	Double wild-type	133	76	1			1		
	RAS	235	170	1.713	1.306-2.248		1.437	1.047-1.972	
	BRAF	30	25	2.777	1.761-4.378	<0.0001	2.235	1.377-3.959	0.0037
Leuko/Lympho ratio	Missing	28							
	<5	189	114	1					
	≥5	187	144	1.874	1.463-2.401	<0.0001			
PAL ratio	Missing	46							
	<3	318	209	1					
	≥3	40	36	3.462	2.413-4.966	<0.0001			
CEA (ng/mL)	Missing	48							
	<30	170	94	1			1		
	≥30	186	152	2.360	1.820-3.061	<0.0001	1.464	1.082-1.983	0.0136
ctDNA	Missing	7							
	<20% or ctDNA-negative	236	137	1			1		
	≥20%	161	134	2.546	1.998-3.244	<0.0001	1.920	1.416-2.605	<0.0001

Figure 1: Overall survival according to ctDNA at baseline

Figure 1a: Kaplan-Meier estimation for overall survival according to ctDNA detection at baseline (ctDNA-negative group vs ctDNA-positive group)

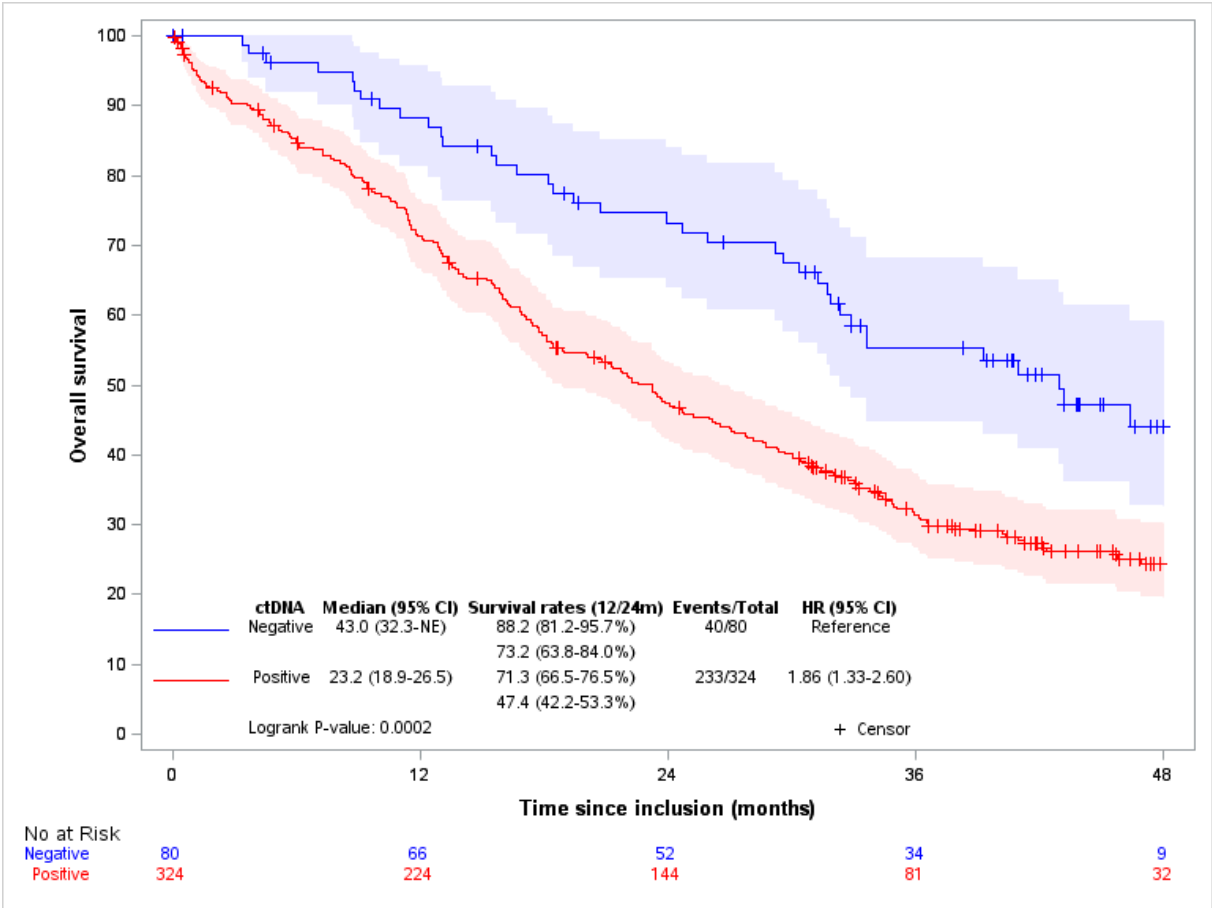


Figure 1b: Restricted cubic spline method to define the relation between the MAF of ctDNA and overall survival

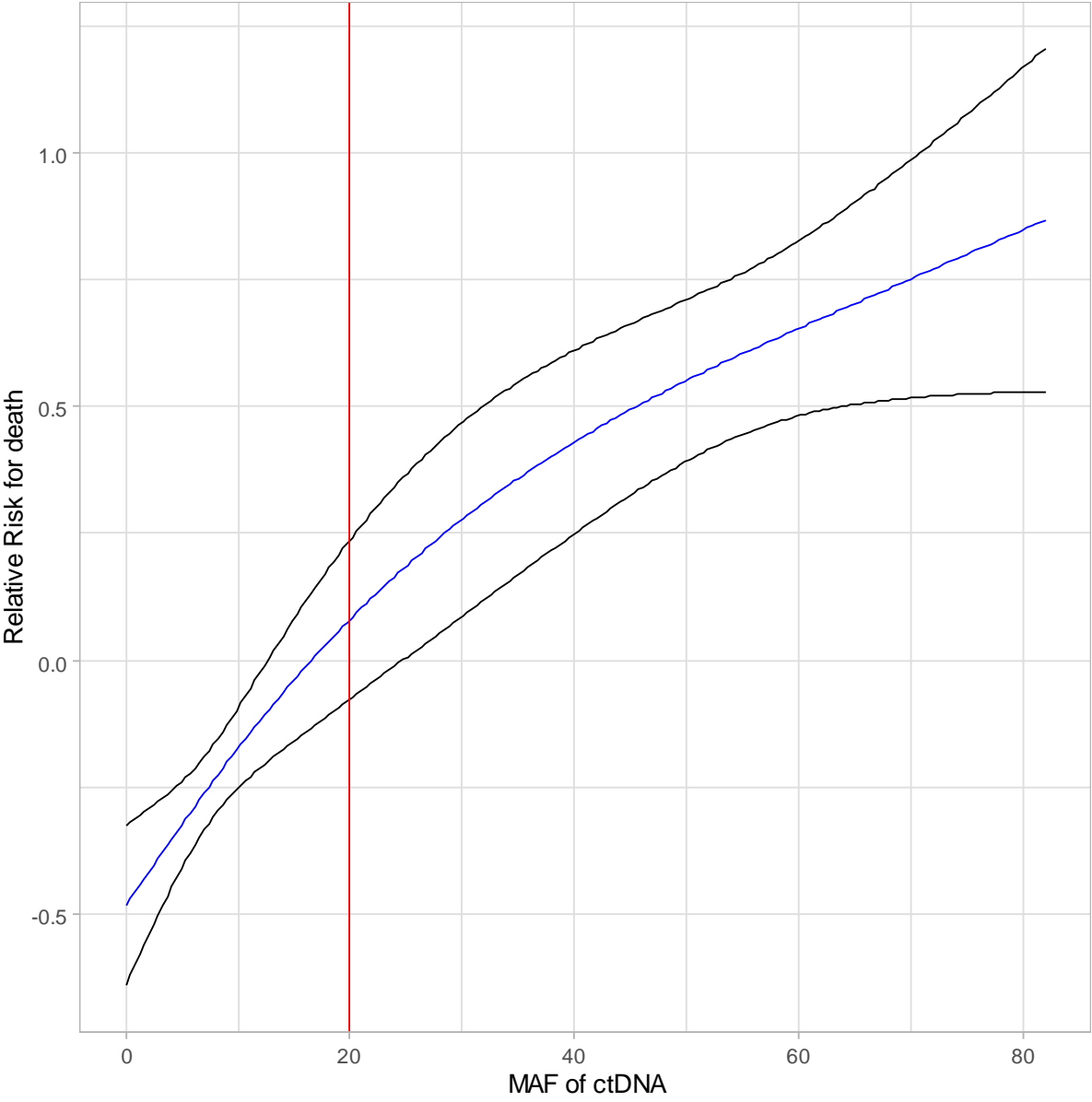


Figure 1c: Kaplan-Meier estimation of overall survival according to the defined threshold of the MAF of ctDNA (<20% vs ≥20%)

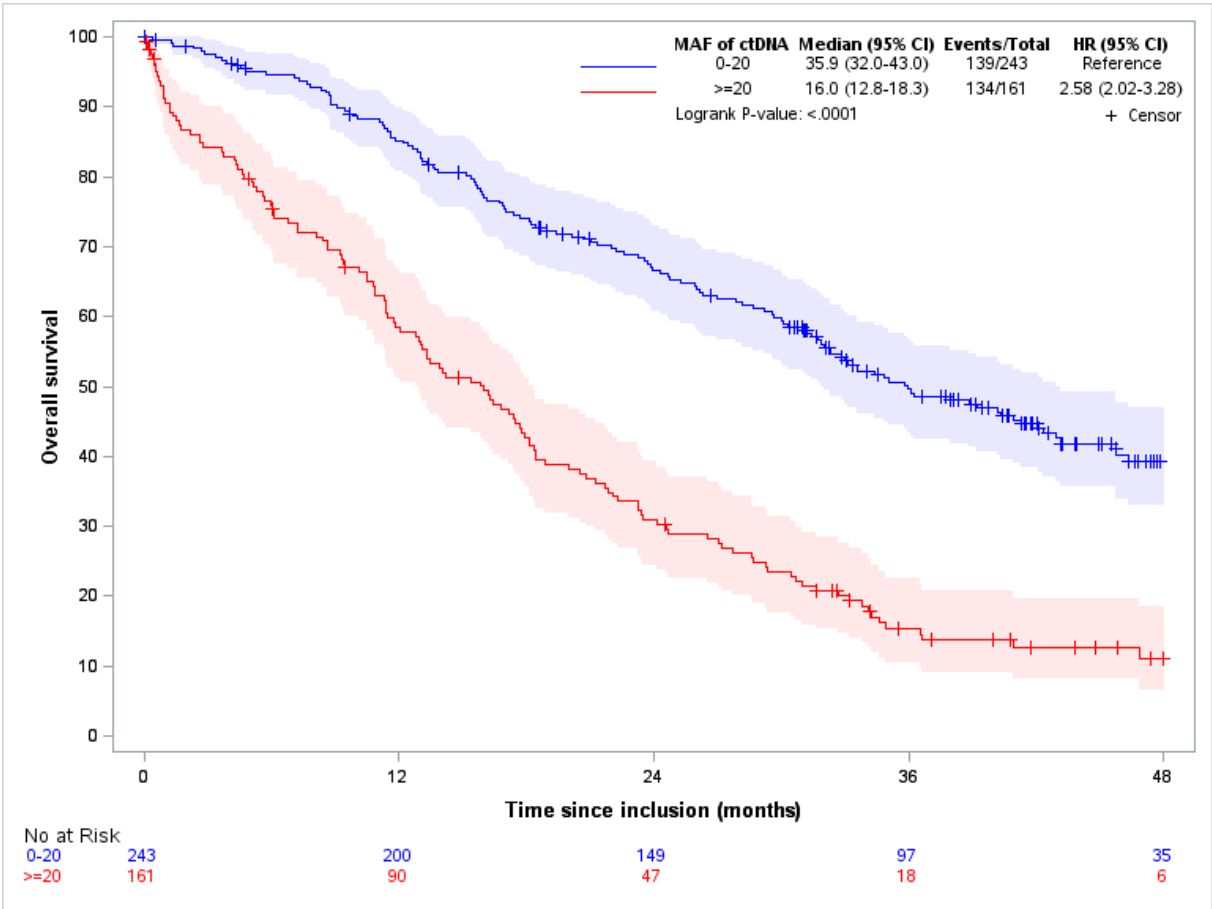
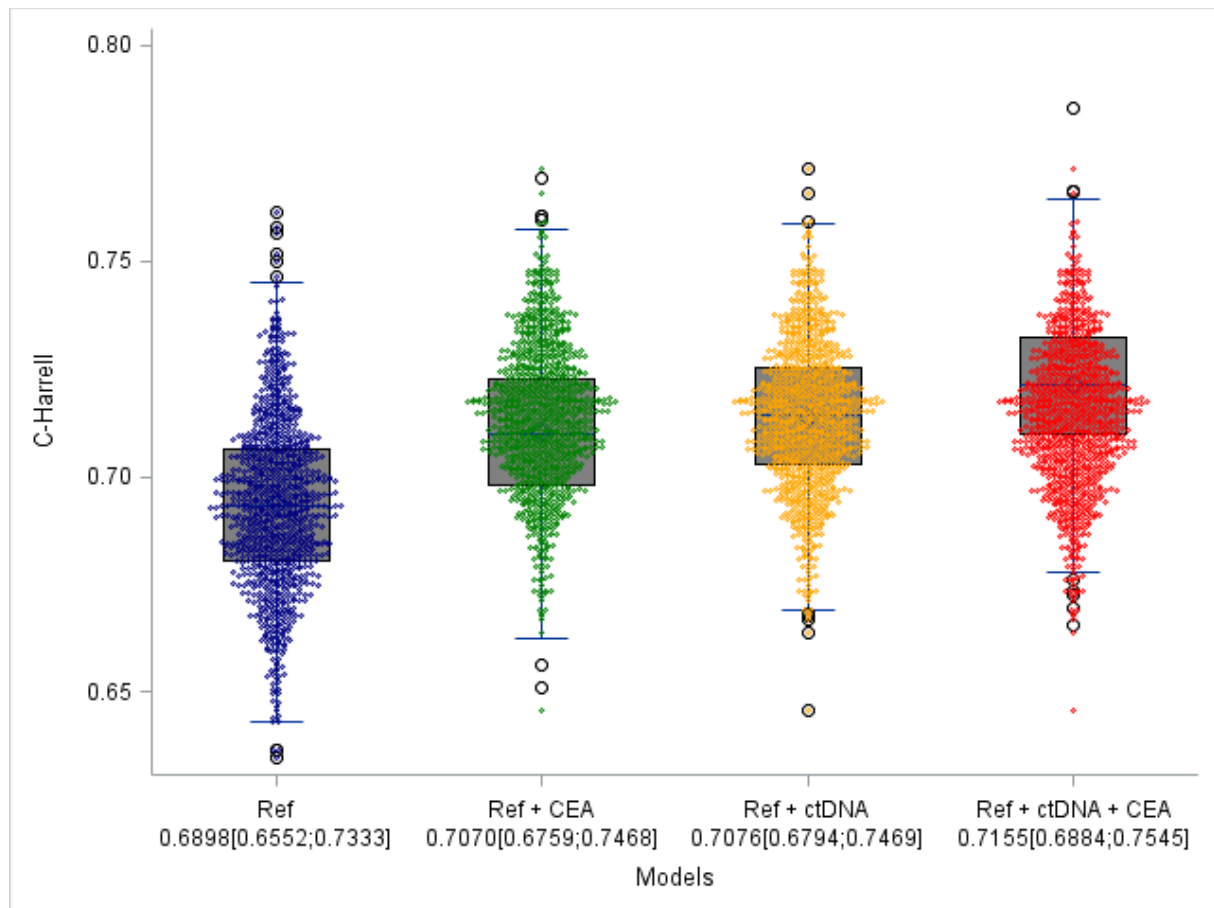


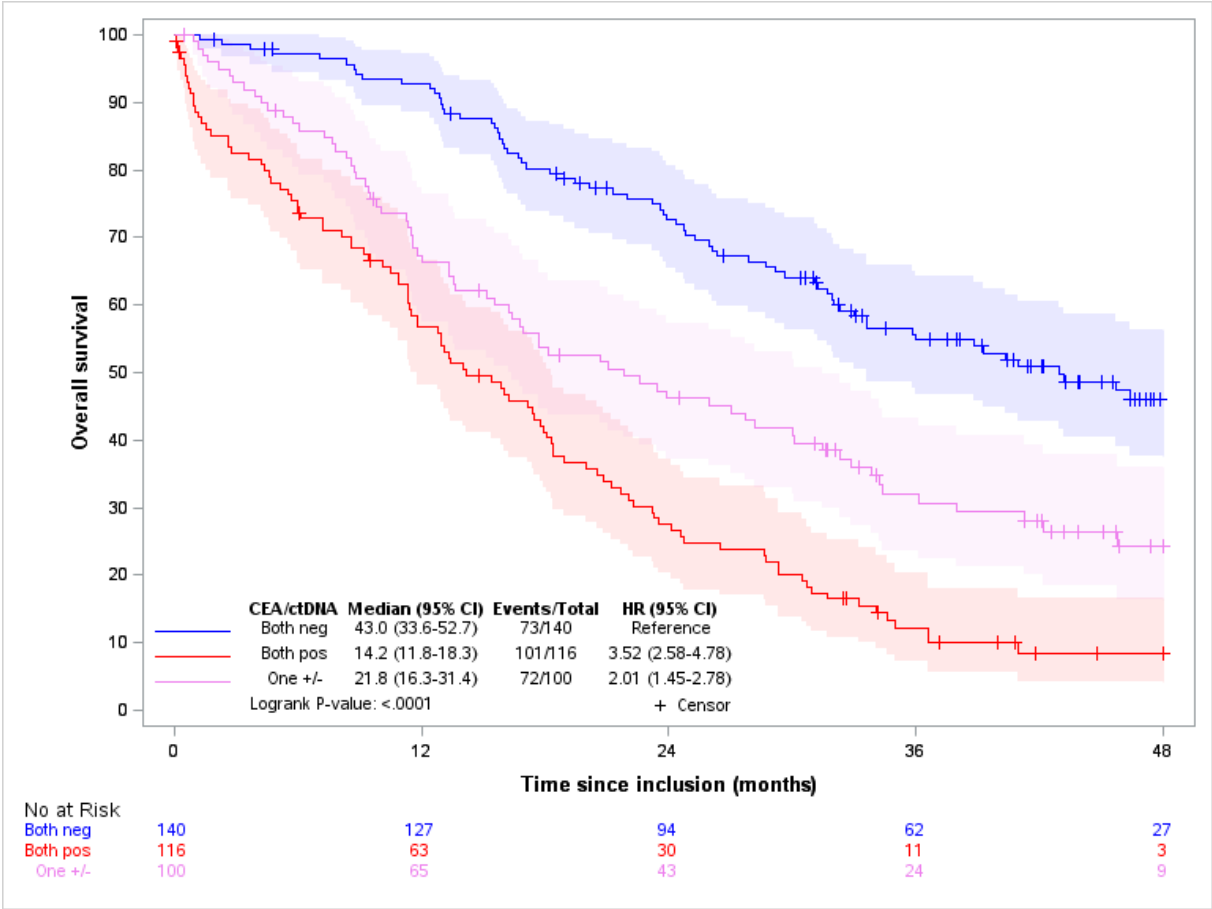
Figure 2: Prognostic analyses combining CEA and ctDNA MAF levels

Figure 2a: Bootstrap procedure (N=1000) to estimate discrimination index (C-Harrell) for death in the risk model with or without the addition of CEA and/or ctDNA



The variables included in the reference risk model are: Age (<70 vs ≥70); Köhne score; Resectability of metastases; *RAS/BRAF* status

Figure 2b: Overall survival of the three subgroups of patients defined by the combination of CEA level (< or ≥ 30 ng/mL) and the MAF of ctDNA (< or ≥ 20%) at baseline



Both neg: CEA < 30 ng/mL and MAF of ctDNA < 20%

One +/-: CEA ≥ 30 ng/mL or MAF of ctDNA ≥ 20%

Both pos: CEA ≥ 30 ng/mL and MAF of ctDNA ≥ 20%