Circulating tumor DNA is prognostic and potentially predictive of eryaspase efficacy in second-line in patients with advanced pancreatic adenocarcinoma

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Statement of translational relevance

Eryaspase is composed of L-asparaginase encapsulated in erythrocytes. It targets asparagine used by non-canonical pathways constitutively activated by KRAS signaling pathway in 80 to 90% of pancreatic cancers. In combination with chemotherapy, eryaspase was associated with a promising activity in a randomized phase 2 trial. Circulating tumor DNA (ctDNA) was assessed from plasma samples of patients included in this trial.

At baseline, ctDNA was detectable in two thirds of patients and was a strong prognostic factor. Early change of ctDNA level was significantly correlated with all oncologic outcomes. A significant interaction was observed between the presence of ctDNA and eryaspase efficacy.

We confirm in a phase 2 trial the interest of ctDNA, at baseline and in monitoring under treatment. Moreover, presence of ctDNA could be a predictive biomarker of eryaspase efficacy and had to be assessed in the ongoing phase 3 trial.

Abstract

Background:

Eryaspase is composed of L-asparaginase encapsulated in erythrocytes and has demonstrated significant efficacy in a randomized phase 2 trial. We assessed the prognostic and predictive value of circulating tumor DNA (ctDNA) in patients plasma included in this trial.

Patients and methods:

Samples prospectively collected pre-treatment were centrally analyzed by next-generation sequencing. Prognostic values of baseline ctDNA and ctDNA early changes between day 0 and 28 were assessed in both arms combined on objective response rate (ORR), progression free survival (PFS) and overall survival (OS); three groups were defined: negative ctDNA (Neg), ctDNA responders (Resp) and ctDNA non-responders (NResp). Predictive value of ctDNA for eryaspase efficacy was investigated.

Results:

CtDNA was positive at baseline in 77 patients out of the 113 tested patients (68%). Detectable ctDNA was an independent negative prognostic factor for OS (4.6 vs 8.8 months; p=0.0025) and PFS (1.6 vs 3.3 months; p=0.00043). Early change in ctDNA levels was correlated with ORR (20 %, 26%, 0%; p<0.04), PFS (3.7, 3.4, 1.6 months; p<0.0001) and OS (11.7, 6.5, 4.3 months; p<0.0001) according to the three defined groups (Neg, Res, NResp, respectively). In patients with ctDNA detectable at baseline, eryaspase was associated with better PFS (HR=0.53; 95% CI: 0.3-0.94) and OS (HR=0.52; 95% CI: 0.29-0.91).

Conclusion:

We confirm from a prospective randomized trial that 1/ the presence of ctDNA at baseline is a major prognostic factor, 2/ the early change of ctDNA correlates with treatment outcome and 3/ the ctDNA could be a predictive biomarker of eryaspase efficacy.

Trial registration: NCT02195180

Keywords: L-asparaginase, circulating tumor DNA, phase 2, prognostic, predictive

Introduction

Pancreatic adenocarcinoma (PAC) incidence increases in many countries and is expected to become the second leading cause of cancer death in 2030 (1). Despite some progress, its prognosis remains poor with five-year survival rate of 9% all-stages combined (2). During the last 10 years, two chemotherapy regimens have demonstrated a clinical benefit in comparison to gemcitabine alone in first-line in metastatic patients: the FOLFIRINOX and the gemcitabine plus nab-paclitaxel regimen (3,4). The benefit of a second-line chemotherapy has been formally demonstrated in the CONKO-003 trial (5), and the combinations of 5-Fluorouracil (5-FU) plus nanoliposomal irinotecan and of 5-FU plus oxaliplatin have been validated as standard treatments after progression under a gemcitabine based first-line chemotherapy (6,7).

New drugs developments are needed to improve our therapeutic possibilities and increase the overall survival of patients. One of the promising areas of research is to target some metabolites, such as glutamine or asparagine, used by non-canonical pathways constitutively activated by KRAS signaling pathway in 80 to 90% of pancreatic cancers (8,9).

Eryaspase is a novel approach to deliver asparaginase that is encapsulated within erythrocytes and administered as intravenous infusion. Eryaspase is well tolerated as monotherapy (10), and in combination with chemotherapy (gemcitabine or FOLFOX) in a randomized phase 2b trial (11). Significant increases of progression free survival (PFS) (HR=0.56; 95% CI: 0.37-0.84; p=0.005), and overall survival (OS) (HR=0.60; 95% CI: 0.41-0.87; p=0.008), and a good safety profile were observed. A randomized phase 3 trial is currently ongoing (EndraCT: 2018-000572-15).

Circulating tumor DNA (ctDNA) can be applied to monitor response and resistance to systemic therapy and to capture tumor heterogeneity better than tissue biopsies (12). Its

testing is highly specific but its sensitivity is influenced by technical issues and by tumor characteristics (12-15). In patients with PAC at diagnosis, the detection of ctDNA and the level of ctDNA if detectable are highly prognostic all stages combined (15,16). Moreover, preliminary data suggests that the evolution of ctDNA under chemotherapy may be an early predictive biomarker of treatment efficacy (15,17).

In this study, we investigated the prognostic and predictive value of ctDNA in the randomized phase 2b trial that compared chemotherapy with or without eryaspase in second-line treatment of patients with advanced PAC.

Patients and Methods

Study design, patients and sample collection

This multicenter, open-label, randomized phase 2b trial was conducted by the GERCOR group ("Groupe coopérateur multidisciplinaire en Oncologie") and was sponsored by Erytech Pharma (NCT02502656). All the patients gave their written informed consent. The trial was conducted in accordance with the protocol and principles of International Conference of Harmonization Good Clinical Practices and Declaration of Helsinki and was approved by an independent ethics committee. Details of this study have been previously published (11). Briefly, eligible patients were randomized in a 2:1 ratio to receive eryaspase plus chemotherapy (gemcitabine or mFOLFOX6 according to previously administered first-line) or chemotherapy alone. Tumor assessments were done every 8 weeks using RECIST 1.1 criteria.

As part of the study, plasma samples of included patients were collected at day 1 of first cycle (baseline), second cycle and third cycle.

Circulating tumor DNA assessment

ctDNA detection was performed by NGS as previously described (15). Briefly, Circulating cell-free DNA was extracted from plasma by using the Maxwell® RSC ccfDNA Plasma Kit (Promega, France) and sequenced using the AmpliSeq Colon and Lung Cancer Panel V2 (22 genes). Samples were analyzed after BAM recalibration using a specific algorithm developed to detect allelic ratios <2%, the BPER method.

Statistical analyses

The analysis plan included the evaluation of objective response rate (ORR), PFS and OS in the intent to treat (ITT), and as a function of the treatment arm (eryaspase vs control).

The median (range) or mean (standard deviation, SD) and frequency (percentage) were used to describe continuous and categorical variables, respectively. Medians and proportions were compared using the Wilcoxon-Mann-Whitney and chi-square test, respectively, or with Fisher's exact test if appropriate. PFS and OS were estimated using the Kaplan-Meier method. As exploratory purpose univariate and multivariate Cox analyses were done to estimate hazard ratio with 95% confidence interval (CI).

For quantitative analysis of ctDNA we used variant allelic fraction (VAF). In case of several mutations identified in the same patient, the highest value was retained. The prognostic value of ctDNA was assessed by a Cox model. To draw survival curves, ctDNA value was divided in VAF tertiles.

To assess the early variation of ctDNA, we calculated a ratio between the VAF measured at day 1 of first cycle (day 1) and at day 1 of the second cycle (day 28). We used the maximal VAF (maxVAF) at day 1 of first cycle (P1); the corresponding mutation was selected and if the mutation was present in the plasma sampling at day 1 of the second cycle (P2) the ratio was calculated as follow: ($maxVAF\_P1_{mutA}$-VAF_P2_{mutA}) / $maxVAF\_P1_{mutA}$. If the mutation corresponding to the maximal VAF at P1 was not present at P2 and if one or more mutations was present at P2, the common mutations between the 2 time points were selected and the mutation with the highest VAF at P2 was used to calculate the ratio as follow: (VAF_P1_{mutA}-VAF_P2_{mutA})/ VAF_P1_{mutA} (this type of calculation was used in 2 cases). If no mutation was present at P2 the ratio was calculated as follow: $maxVAF\_P1_{mutA}$/$maxVAF\_P1_{mutA}$ leading to a ratio at 1 (this type of calculation was used in 11 cases). If no mutation was present at P1 and one or more mutation was present at P2 the ratio was calculated at follow: (0-$maxVAF\_P2_{mutA}$)/ VAF_P2_{mutA} and lead to a ratio of -1 (this type of calculation was used in

4 cases). In the cases with 2 different unique mutations present at the 2 time points, the ratio was calculated as follow VAF_P1_{mutA}-$VAF\_P2_{mutB}/VAF\_P1_{mutA}$ (this type of calculation was used in 1 case). Based on this ratio, we defined 3 groups of patients: patients with no detectable ctDNA at P1 and P2 were classified in a "negative ctDNA" group; patients with ctDNA at least P1 or P2 were divided in 2 groups according to the ratio median value. For both arms combined, prognostic value of defined groups was assessed according to RECIST criteria (ORR and disease control rate), then on PFS and OS.

Finally, we evaluated the effect of ctDNA at baseline on efficacy of treatment by assessing an interaction between the presence of ctDNA and survival (OS and PFS) using a univariate and multivariate analyses. A significant p-value was defined as a p-value <0.01 at the exception of interaction analyses. A significant interaction was defined by a p-value <0.05, and an interesting interaction by a p-value <0.1. All statistical analyses were performed with R survival package.

Results

Patients' characteristics

At least one plasma sample was available in 122 of the 141 patients (87%). Plasma samples were available at baseline in 117 patients and at one month (day 1 of 2nd cycle) in 88 patients. Characteristics at baseline of the 122 patients with at least one plasma sample available were not different of those of the 141 patients included in the phase IIb trial (see supplementary Data File S1). OS and PFS of the patients with or without available plasma sample were not different (see supplementary Data File S2). Similar to the results in the ITT population (N=141), eryaspase was associated with a significant improvement of both OS and PFS in this subgroup of 122 patients (see supplementary Data File S2).

ctDNA analysis at baseline

For baseline time point, failure of sequencing was observed in 4 patients either due to too low amount of DNA or non-interpretable sequencing. CtDNA was detectable by the presence of at least one mutated gene in 77 of 113 patients (68.1%). Among those, the most frequent mutated genes were *KRAS* (n=64, 83%) and *TP53* (n=38, 49%). The number of mutated genes identified was 4 in one patient (1%), 3 in 9 patients (12%), two in 33 patients (43%) and one in 34 patients (44%). The most frequent combination of mutations, *KRAS* and *TP53*, was observed in 34 patients (44%).

No difference in baseline characteristics was observed between patients with or without detectable ctDNA (see supplementary Data File S3).

Prognostic value

Among the 113 patients for whom NGS analysis was available at baseline, the presence of ctDNA was associated with significant shorter OS (median 4.6 vs 8.8 months; p=0.0025) and PFS (median 1.6 vs 3.3 months; p=0.00043). The hazard ratio (HR) adjusted to gender, CA19.9 at baseline, treatment arm, number of metastatic site and delay before the inclusion were 1.92 (95% CI: 1.18-3.1) and 2.13 (95% CI: 1.3-3.5) for OS and PFS, respectively (Figure 1A and 1B). ORR was not significantly different among patients with or without detectable ctDNA: 10.4% (8/77) vs 13.9% (5/36), respectively.

The mean of the maximum of the variant allelic fraction (VAF) at baseline was 0.097 (median: 0.025; range [0-0.74]). The maximal VAF frequency observed at baseline was significantly negatively correlated with OS (HR=23.9; 95% CI: 6.7-84.6; p<0.0001) and PFS (HR=8.27; 95% CI: 2.4-28.3; p=0.0008) in univariate analysis. In multivariate analysis, a significant correlation was observed with OS (HR=16.8; 95% CI: 3.83-73.58; p=0.00018) and a clear trend with PFS (HR=5.66; 95% CI: 1.43-22.39; p=0.0135) (see supplementary Data File S4). Prognostic value of ctDNA at baseline was then analyzed by tertiles according to VAF frequency (cut points: 0.007 and 0.0683). Characteristics of patients of three tertiles are described in supplementary Data File S5. Higher tertiles of VAF frequency was prognostic of OS and PFS (Figure 2A and 2B).

Quantitative monitoring of ctDNA under treatment

The mean of the VAF ratio between P1 and P2 was 0.12 (IQR: 1.06) and the median 0.28 (range [-5.33-1]). Twenty-five patients were classified in the “negative ctDNA” group (no ctDNA at P1 and P2); 23 in the “ctDNA responders” group and 23 in the “ctDNA non-responders” group.

ORR was correlated with the groups defined by ctDNA variation: 20 % (5/25), 26% (6/23) and 0% (0/23) in “negative ctDNA”, “ctDNA responders” and “ctDNA non-responders”

groups (p<0.04), respectively. Significant correlation was observed for disease control rate: 68 % (n=17/25), 61% (14/23) and 22% (5/23) in “negative ctDNA”, “ctDNA responders” and “ctDNA non-responders” groups (p=0.002), respectively.

For both arms combined, the groups defined by ctDNA variation were significantly correlated with OS and PFS (Figure 3A and 3B).

Plasma samples were available before the first day of the three cycles in 40 patients. Patients who had a disease control had more frequently a negative ctDNA or a decrease of the maximal VAF after treatment initiation (supplementary Data File S6).

ctDNA and treatment arm

For OS, an interesting interaction between the presence of ctDNA at baseline and eryaspase efficacy was observed in univariate (HR=0.39; 95% CI: 0.14-1.09; p=0.073) and multivariate (HR=0.35; 95% CI: 0.12-1.05; p=0.060) analyses. For PFS, there was a trend for an interesting interaction in univariate analysis (HR=0.47; 95% CI: 0.18-1.22; p=0.122) and in multivariate (HR=0.42; 95% CI: 0.15-1.20; p=0.105) analysis (see supplementary Data File S7).

Among patients with detectable ctDNA at baseline, eryaspase was associated with a not significant increase in disease control rate (47% (n=15/32) vs 29% (n=4/14); p=0.3) and a significant increase of OS (HR=0.52; 95% CI: 0.29-0.91: p<0.001) and PFS (HR=0.53; 95% CI: 0.3-0.94; p<0.001). No significant difference of treatment efficacy was observed in the subgroup of patients with unknown ctDNA status (Figure 4A and 4B).

No correlation was observed between asparagine synthetase (ASNS) expression level and ctDNA status. Among patients with negative and positive ctDNA at baseline, null-low (0-1) ASNS expression rates were 72% (n=26/36) and 68% (n=52/77) (p=0.776), respectively.

Discussion

The aim of this exploratory translational study was to evaluate the prognostic and predictive value of ctDNA from plasma samples collected during the randomized phase 2 trial which has assessed eryaspase efficacy in second-line in patients with advanced PAC. For this, we used a NGS method adapted to plasma analysis and allowing to search the main mutated genes in PAC (15). The prognostic value of ctDNA at diagnosis (baseline) is now well established in several cancer types, and more specifically in PAC, irrespective of the stage of the disease (13-16,18). The rate of detectable ctDNA at diagnosis accounts for approximately two thirds of patients with metastatic disease (15,17-20). The current study represents the largest cohort investigating the prognostic and predictive value of quantitative ctDNA in second-line setting. The prevalence rate of 68% is similar to that found in other studies of metastatic PAC. To our knowledge, the features of metastatic PAC patients with undetectable ctDNA are not well described, unlike metastatic colorectal cancer for which metastatic spread to the liver is clearly correlated to detectable ctDNA (21). The detection of ctDNA seems correlated with differentiation grade and CA 19-9 level but not clearly with tumor burden (15). Given the strong prognostic value of ctDNA at baseline, future studies should aim to better define the subset of patients with undetectable ctDNA, with the goal of stratifying future trials based on ctDNA content.

In addition to its prognostic value at diagnosis, the value of the dynamic change of ctDNA during follow-up is very promising (12-14). Indeed, the effect of chemotherapy on ctDNA levels could inform on treatment efficacy (15,20). In a prospective cohort of patients with advanced PAC, a significant correlation between the kinetics of ctDNA, from day 0 to day 14, and objective response rate was reported (17). Moreover, an increase in ctDNA level under treatment indicated progression with a sensitivity of 83% and specificity of 100%. Changes in

ctDNA levels appeared more informative than changes in more "classical" biomarkers such as CA 19-9 and CEA (17). In our study, we assessed the value of early changes, between days 0 and 28, in ctDNA levels and identified three distinct groups that were strongly correlated with ORR, PFS and OS. Generally, only half of PAC patients are able to receive second line therapy (3,4,7), therefore, it is prudent to argue that identifying early disease progression based on increasing levels of ctDNA and subsequently instating salvage therapy before objective disease progression may have a positive effect on OS and improving the patients outcome.

The main strengths of this exploratory study are that approximately 85% of the patients in this trial had analyzable ctDNA samples, confirming the utility of our approach and the correlation with efficacy indicators in the ITT population. While our results are promising, there are still several outstanding issues that should be addressed in a prospective clinical trial, such as the optimal timing for collecting ctDNA samples, the ctDNA threshold and the different methods available to evaluate ctDNA. Furthermore, the clinical utility of using early evolution of ctDNA during treatment follow-up should also be addressed in future trials.

The eryaspase benefit appeared most significant for OS and PFS in the subgroup of patients with detectable ctDNA, which is also the subgroup of patients with the worst prognosis. This result could be explained either by an efficacy of eryaspase on circulating tumor cells (potentially correlated to ctDNA), or by an increase anticancer drug delivery in the subgroup of pancreatic adenocarcinomas with ctDNA positivity. Given this correlation, these results provide further support for the role of targeting metabolic pathways as a novel therapeutic modality in PAC. Interestingly, to our knowledge no specific therapy has demonstrated efficacy in a subgroup defined by the presence of ctDNA. PAC is characterized by extensive reprogramming of cellular metabolism, specifically a robust glycolytic activity and glutamine addiction (21), which plays a role in controlling proliferation of cancer cells and enabling

invasion and metastasis in a nutrient-poor, hypoxic microenvironment. These metabolic switches are driven by the acquisition of activating *KRAS* mutations (22). Therefore, eryaspase provides a novel therapeutic target for the treatment of PAC (10,11). The current results provide a rationale for utilizing ctDNA as a potential marker of response. At the opposite, no benefit of eryaspase was observed in the subgroup of patients with undetectable ctDNA at baseline. This observation could be due to the small number of patients of this subgroup (n=36; 8 vs 28). It is therefore important to confirm these findings in the ongoing phase 3 trial. ASNS expression was not predictive of eryaspase efficacy in the phase 2 trial, and no correlation was found between ASNS expression and ctDNA status.

In conclusion, we confirm that the presence of ctDNA at baseline is a prognostic factor in patients with advanced PAC. The feasibility of this approach and its potential prognostic value provides a rationale for stratifying patients in future clinical trials. Our results suggest that presence of ctDNA could be a predictive biomarker of eryaspase efficacy. Taken together, the detection of ctDNA in approximately two thirds of patients with metastatic PAC and eryaspase being associated with a good safety profile, argue for future first-line development of this combination in the event of a positive phase 3 results.

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Figure legends

Figure 1A. Overall survival curves according to the presence or absence of ctDNA at baseline.

Forest Plot of the ctDNA presence effect on overall survival with Hazard Ratio (95% Confidence Interval) adjusted on Age, Gender, CA19.9 levels at baseline, Treatment Arm, Number of metastatic sites, delay before inclusion.

Figure 1B. Progression free survival curves according to the presence or absence of ctDNA at baseline.

Forest Plot of the ctDNA presence effect on progression free survival with Hazard Ratio (95% Confidence Interval) adjusted on Age, Gender, CA19.9 levels at baseline, Treatment Arm, Number of metastatic sites, delay before inclusion.

Figure 2A. Overall survival curves according to the tertiles defined by the VAF frequency of ctDNA.

Forest Plot of the ctDNA level tertiles effect on overall survival with Hazard Ratio (95% Confidence Interval) adjusted on Age, Gender, CA19.9 levels at baseline, Treatment Arm, Number of metastatic sites, delay before inclusion.

Figure 2B. Progression free survival curves according to the tertiles defined by the VAF frequency of ctDNA.

Forest Plot of the ctDNA level tertiles effect on progression free survival with Hazard Ratio (95% Confidence Interval) adjusted on Age, Gender, CA19.9 levels at baseline, Treatment Arm, Number of metastatic sites, delay before inclusion.

Figure 3A. Overall survival according to the groups defined by ctDNA variation between first and second cycle.

Forest Plot of the ctDNA variation effect on overall survival with Hazard Ratio (95% Confidence Interval) adjusted on Age, Gender, CA19.9 levels at baseline, Treatment Arm, Number of metastatic sites, delay before inclusion.

Figure 3B. Progression free survival according to the groups defined by ctDNA variation between first and second cycle.

Forest Plot of the ctDNA variation effect on progression free survival with Hazard Ratio (95% Confidence Interval) adjusted on Age, Gender, CA19.9 levels at baseline, Treatment Arm, Number of metastatic sites, delay before inclusion.

Figure 4A. Overall survival curves according to ctDNA at baseline and treatment arm.

Hazard ratio adjusted on age, gender, Ca 19-9 at baseline (elevated vs normal), treatment arm, number of metastatic site and delay before inclusion.

Figure 4B. Progression free survival curves according to ctDNA at baseline and treatment arm.

Hazard ratio adjusted on age, gender, Ca 19-9 at baseline (elevated vs normal), treatment arm, number of metastatic site and delay before inclusion.

Figure 1B

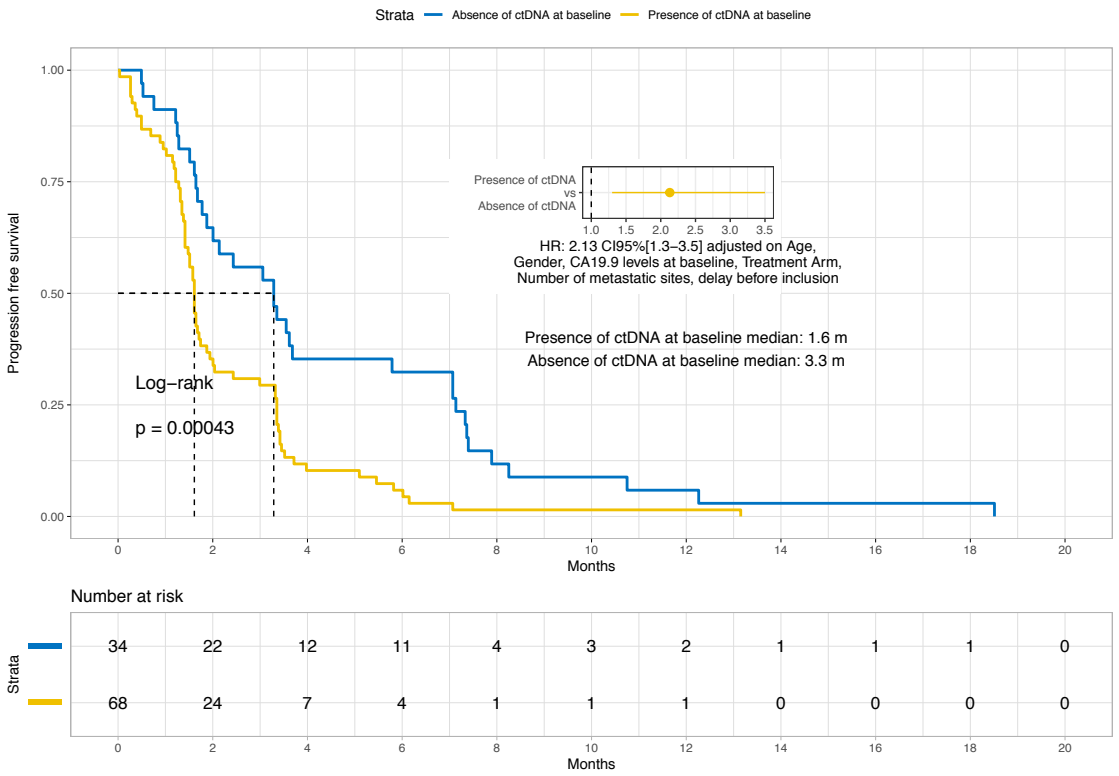

Strata
Absence of ctDNA at baseline
Presence of ctDNA at baseline
Progression free survival
1.00
0.75
0.50
0.25
0.00
Presence of ctDNA
vs
Absence of ctDNA
1.0 1.5 2.0 2.5 3.0 3.5
HR: 2.13 CI95%[1.3–3.5] adjusted on Age,
Gender, CA19.9 levels at baseline, Treatment Arm,
Number of metastatic sites, delay before inclusion
Presence of ctDNA at baseline median: 1.6 m
Absence of ctDNA at baseline median: 3.3 m
Log−rank
p = 0.00043
0 2 4 6 8 10 12 14 16 18 20
Months
Number at risk
Strata
34 22 12 11 4 3 2 1 1 1 0
68 24 7 4 1 1 1 0 0 0 0
0 2 4 6 8 10 12 14 16 18 20
Months

Figure 2A

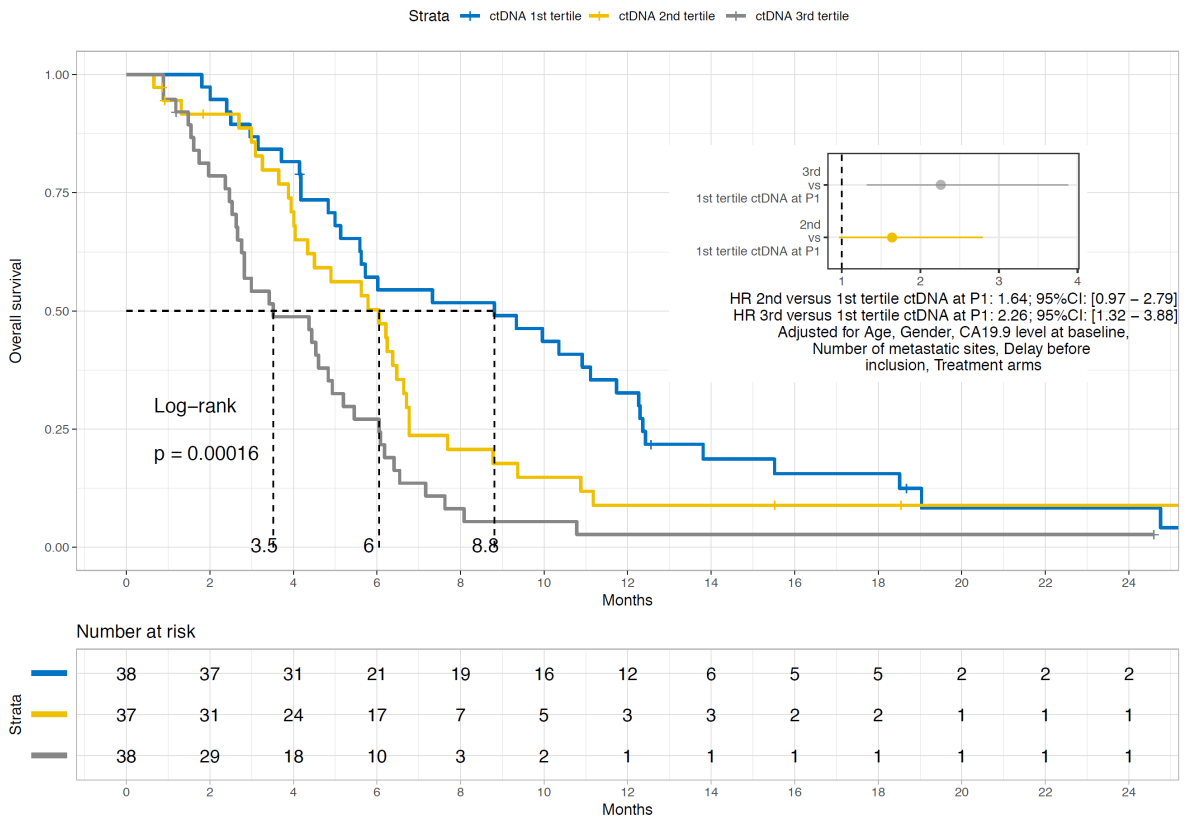

Strata
ctDNA 1st tertile
ctDNA 2nd tertile
ctDNA 3rd tertile
Overall survival
1.00
0.75
0.50
0.25
0.00
Log−rank
p = 0.00016
3.5
6
8.8
3rd vs 1st tertile ctDNA at P1
2nd vs 1st tertile ctDNA at P1
HR 2nd versus 1st tertile ctDNA at P1: 1.64; 95%CI: [0.97 – 2.79]
HR 3rd versus 1st tertile ctDNA at P1: 2.26; 95%CI: [1.32 – 3.88]
Adjusted for Age, Gender, CA19.9 level at baseline,
Number of metastatic sites, Delay before
inclusion, Treatment arms
Months
Number at risk
38 37 31 21 19 16 12 6 5 5 2 2 2
37 31 24 17 7 5 3 3 2 2 1 1 1
38 29 18 10 3 2 1 1 1 1 1 1 1
Strata
Months

Figure 2B

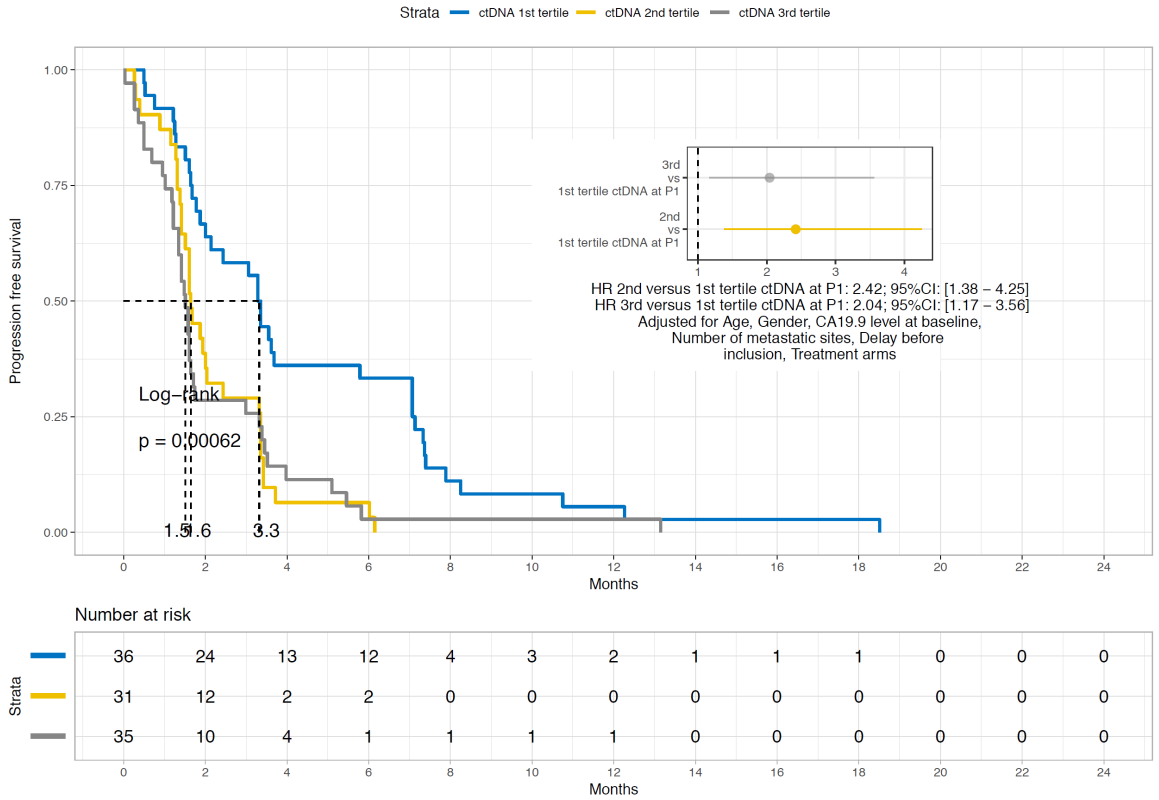
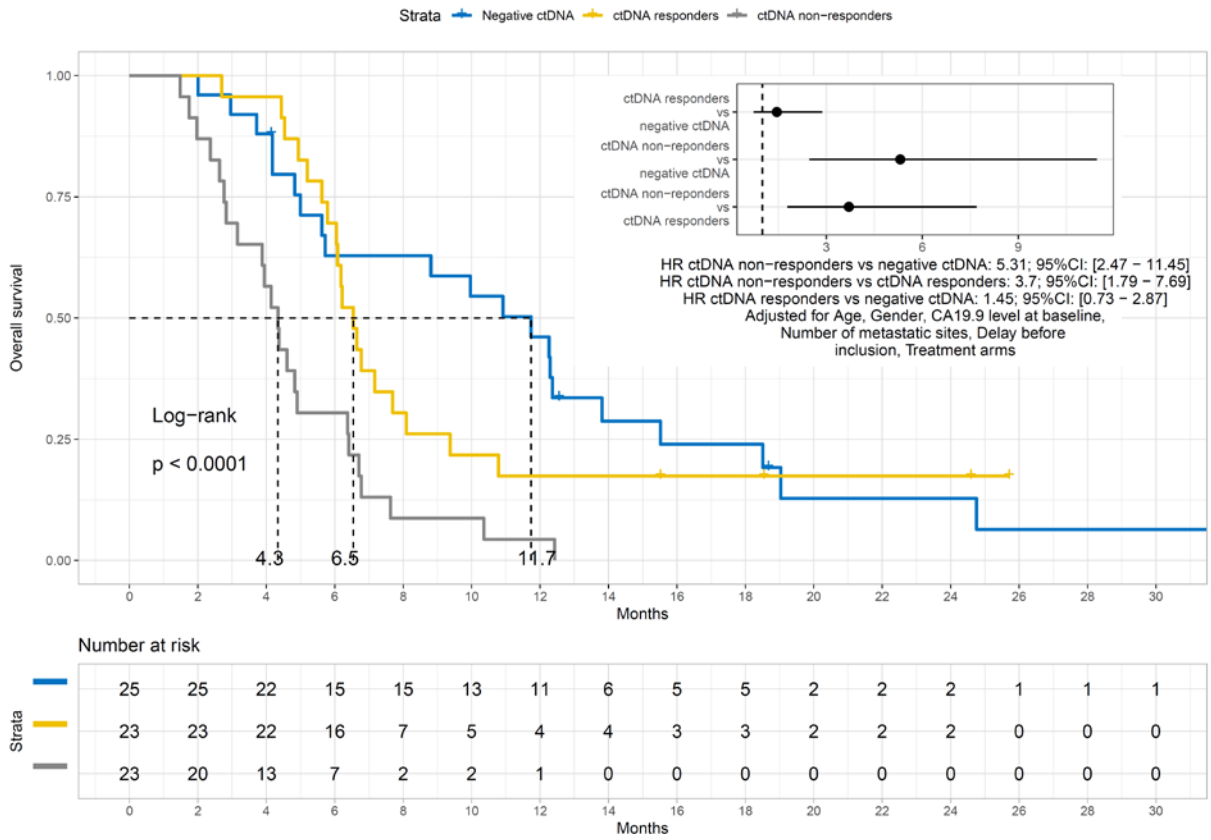
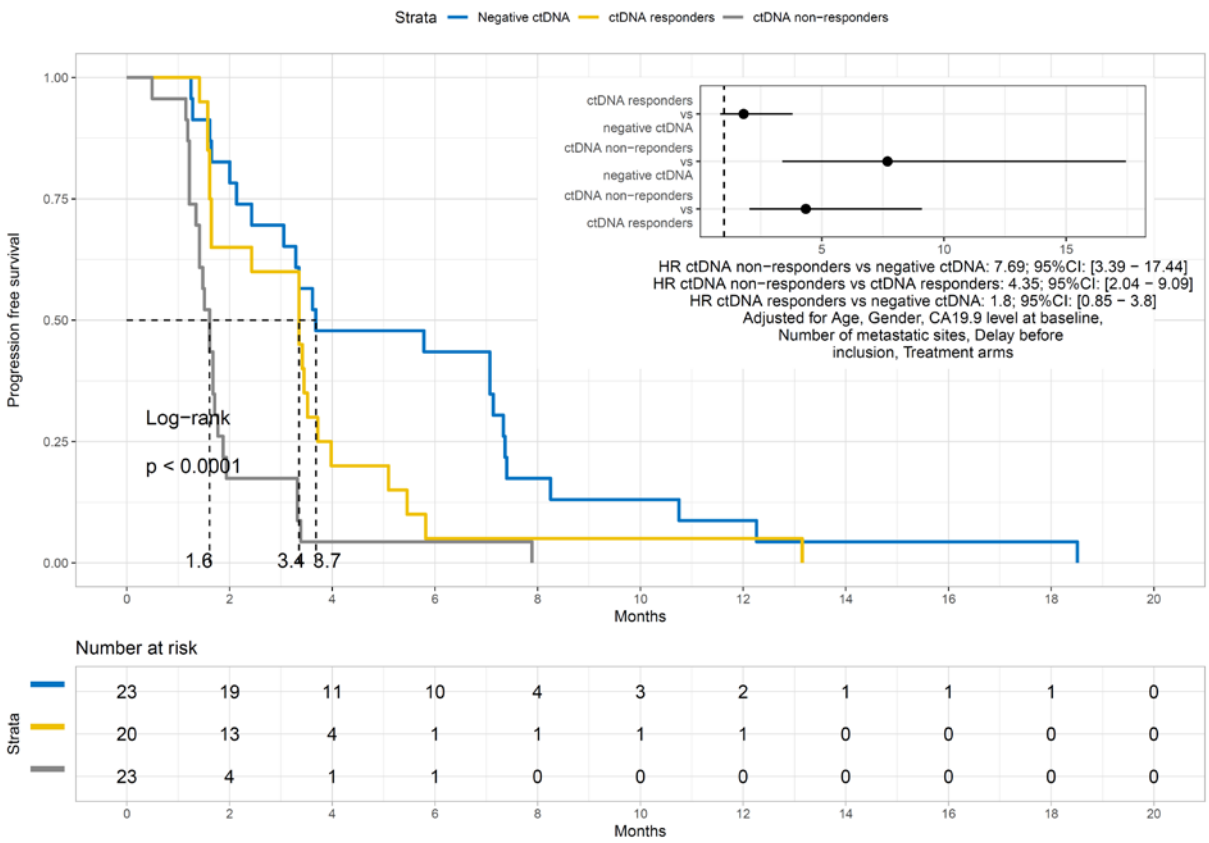

Strata
ctDNA 1st tertile
ctDNA 2nd tertile
ctDNA 3rd tertile
Progression free survival
1.00
0.75
0.50
0.25
0.00
Log−rank
p = 0.00062
1.5
1.6
3.3
3rd vs 1st tertile ctDNA at P1
2nd vs 1st tertile ctDNA at P1
HR 2nd versus 1st tertile ctDNA at P1: 2.42; 95%CI: [1.38 – 4.25]
HR 3rd versus 1st tertile ctDNA at P1: 2.04; 95%CI: [1.17 – 3.56]
Adjusted for Age, Gender, CA19.9 level at baseline,
Number of metastatic sites, Delay before
inclusion, Treatment arms
Months
Number at risk
Strata
36 24 13 12 4 3 2 1 1 1 0 0 0
31 12 2 2 0 0 0 0 0 0 0 0 0
35 10 4 1 1 1 1 0 0 0 0 0 0
0 2 4 6 8 10 12 14 16 18 20 22 24
Months

Figure 3

A/


Strata
Negative ctDNA
ctDNA responders
ctDNA non−responders
Overall survival
1.00
0.75
0.50
0.25
0.00
Log−rank
p < 0.0001
4.3
6.5
11.7
ctDNA responders vs negative ctDNA
ctDNA non−reponders vs negative ctDNA
ctDNA non−reponders vs ctDNA responders
HR ctDNA non−responders vs negative ctDNA: 5.31; 95%CI: [2.47 − 11.45]
HR ctDNA non−responders vs ctDNA responders: 3.7; 95%CI: [1.79 − 7.69]
HR ctDNA responders vs negative ctDNA: 1.45; 95%CI: [0.73 − 2.87]
Adjusted for Age, Gender, CA19.9 level at baseline, Number of metastatic sites, Delay before inclusion, Treatment arms
Months
Number at risk
Strata
25 25 22 15 15 13 11 6 5 5 2 2 2 1 1 1
23 23 22 16 7 5 4 4 3 3 2 2 2 0 0 0
23 20 13 7 2 2 1 0 0 0 0 0 0 0 0 0
Months

B/



Number at risk

Strata	0	2	4	6	8	10	12	14	16	18	20
Negative ctDNA	23	19	11	10	4	3	2	1	1	1	0
ctDNA responders	20	13	4	1	1	1	1	0	0	0	0
ctDNA non−responders	23	4	1	1	0	0	0	0	0	0	0

Months

Figure 3A

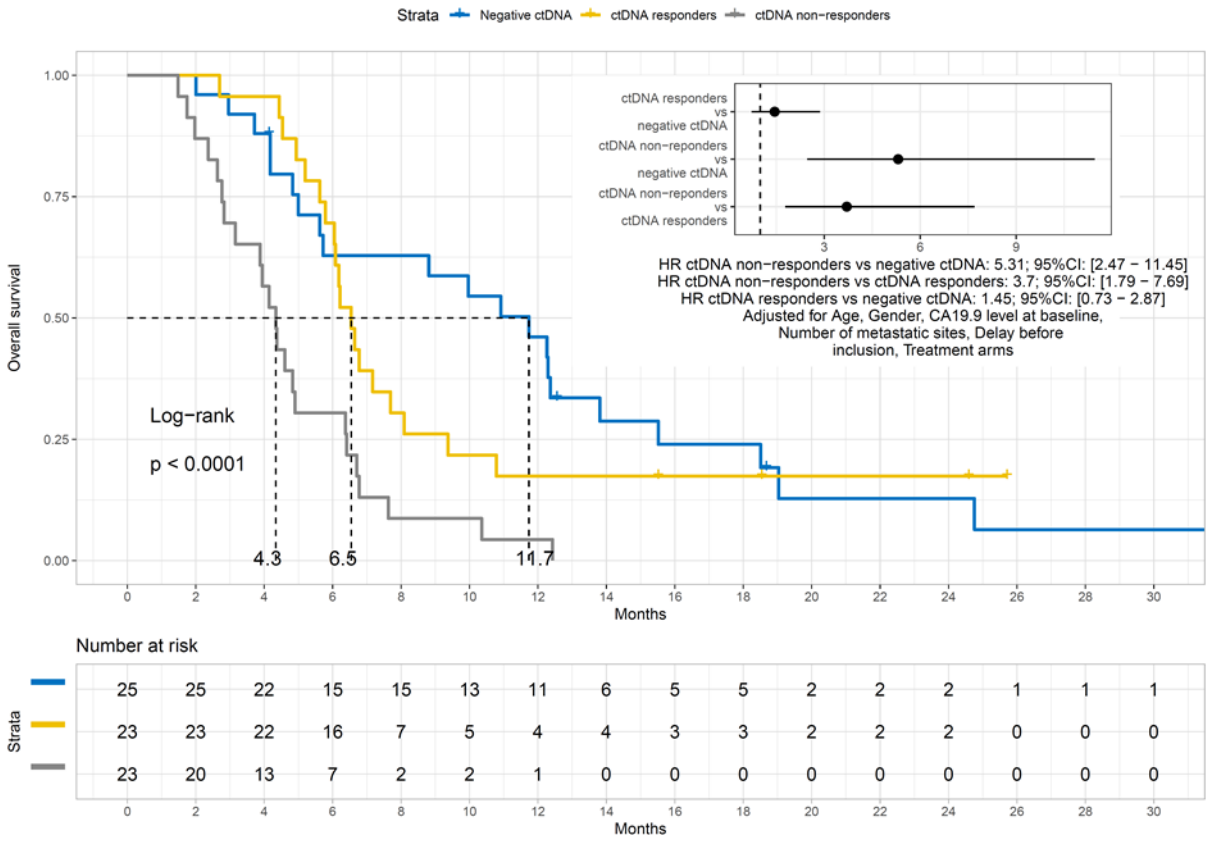

Strata
Negative ctDNA
ctDNA responders
ctDNA non−responders
Overall survival
1.00
0.75
0.50
0.25
0.00
Log−rank
p < 0.0001
4.3
6.5
11.7
ctDNA responders vs negative ctDNA
ctDNA non−reponders vs negative ctDNA
ctDNA non−reponders vs ctDNA responders
HR ctDNA non−responders vs negative ctDNA: 5.31; 95%CI: [2.47 − 11.45]
HR ctDNA non−responders vs ctDNA responders: 3.7; 95%CI: [1.79 − 7.69]
HR ctDNA responders vs negative ctDNA: 1.45; 95%CI: [0.73 − 2.87]
Adjusted for Age, Gender, CA19.9 level at baseline, Number of metastatic sites, Delay before inclusion, Treatment arms
Months
Number at risk
Strata
25 25 22 15 15 13 11 6 5 5 2 2 2 1 1 1
23 23 22 16 7 5 4 4 3 3 2 2 2 0 0 0
23 20 13 7 2 2 1 0 0 0 0 0 0 0 0 0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Months

Figure 3B

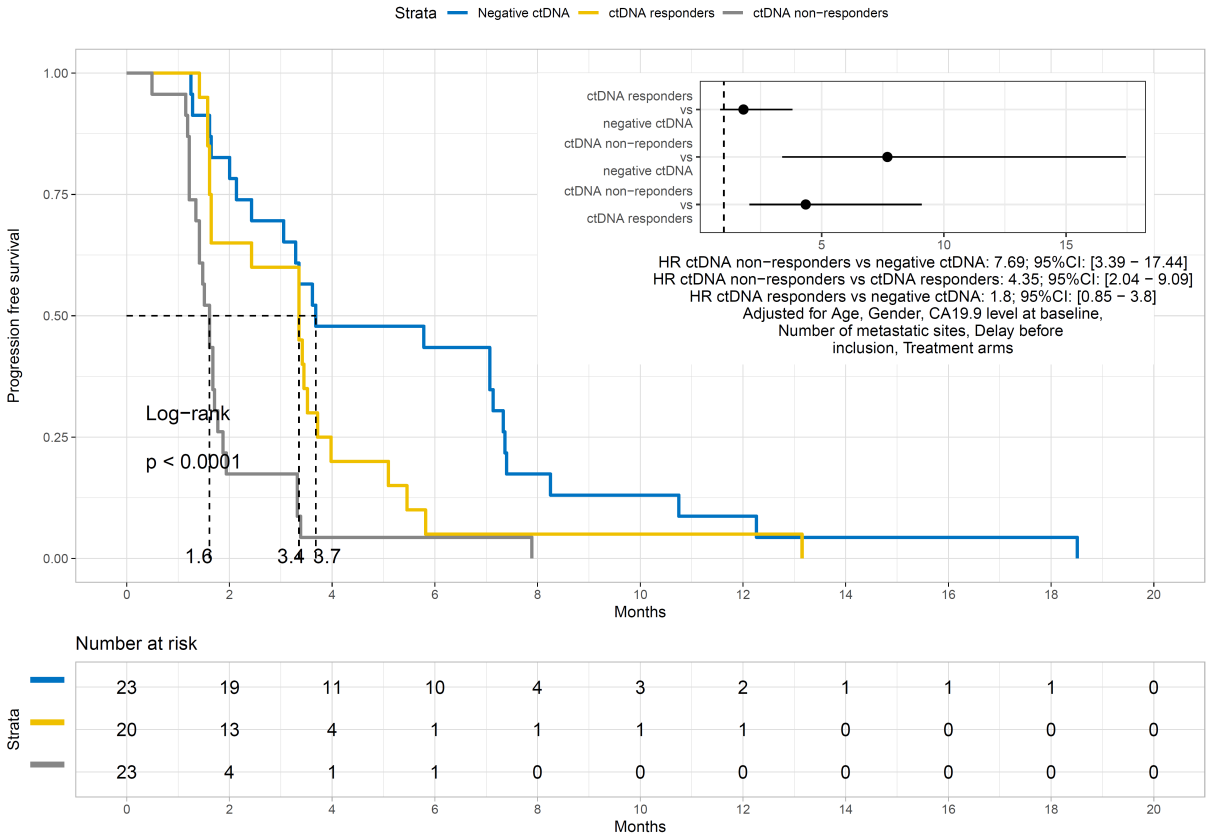

Strata
Negative ctDNA
ctDNA responders
ctDNA non−responders
Progression free survival
1.00
0.75
0.50
0.25
0.00
Log−rank
p < 0.0001
1.6
3.4
8.7
ctDNA responders vs negative ctDNA
ctDNA non−reponders vs negative ctDNA
ctDNA non−reponders vs ctDNA responders
5
10
15
HR ctDNA non−responders vs negative ctDNA: 7.69; 95%CI: [3.39 − 17.44]
HR ctDNA non−responders vs ctDNA responders: 4.35; 95%CI: [2.04 − 9.09]
HR ctDNA responders vs negative ctDNA: 1.8; 95%CI: [0.85 − 3.8]
Adjusted for Age, Gender, CA19.9 level at baseline,
Number of metastatic sites, Delay before
inclusion, Treatment arms
0
2
4
6
8
10
12
14
16
18
20
Months
Number at risk
Strata
23 19 11 10 4 3 2 1 1 1 0
20 13 4 1 1 1 1 0 0 0 0
23 4 1 1 0 0 0 0 0 0 0
Months

Figure 4A

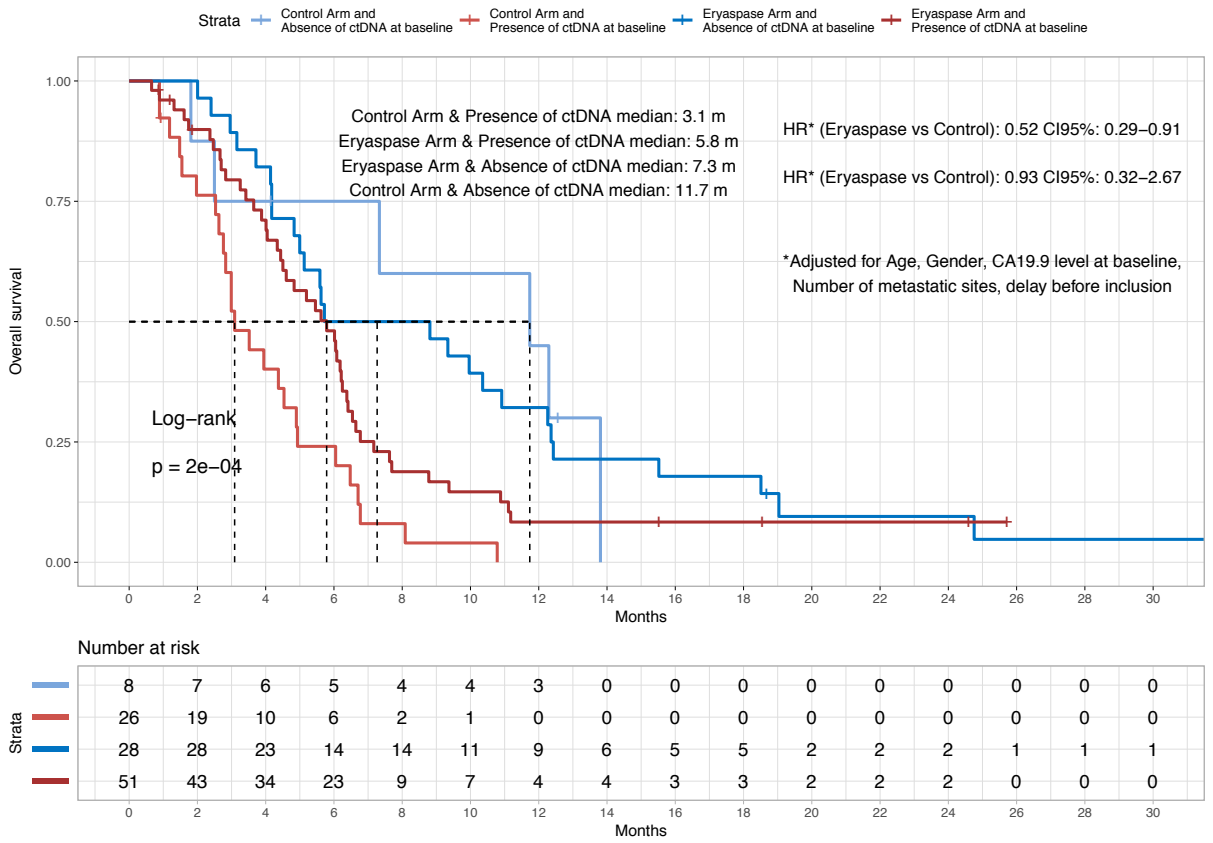
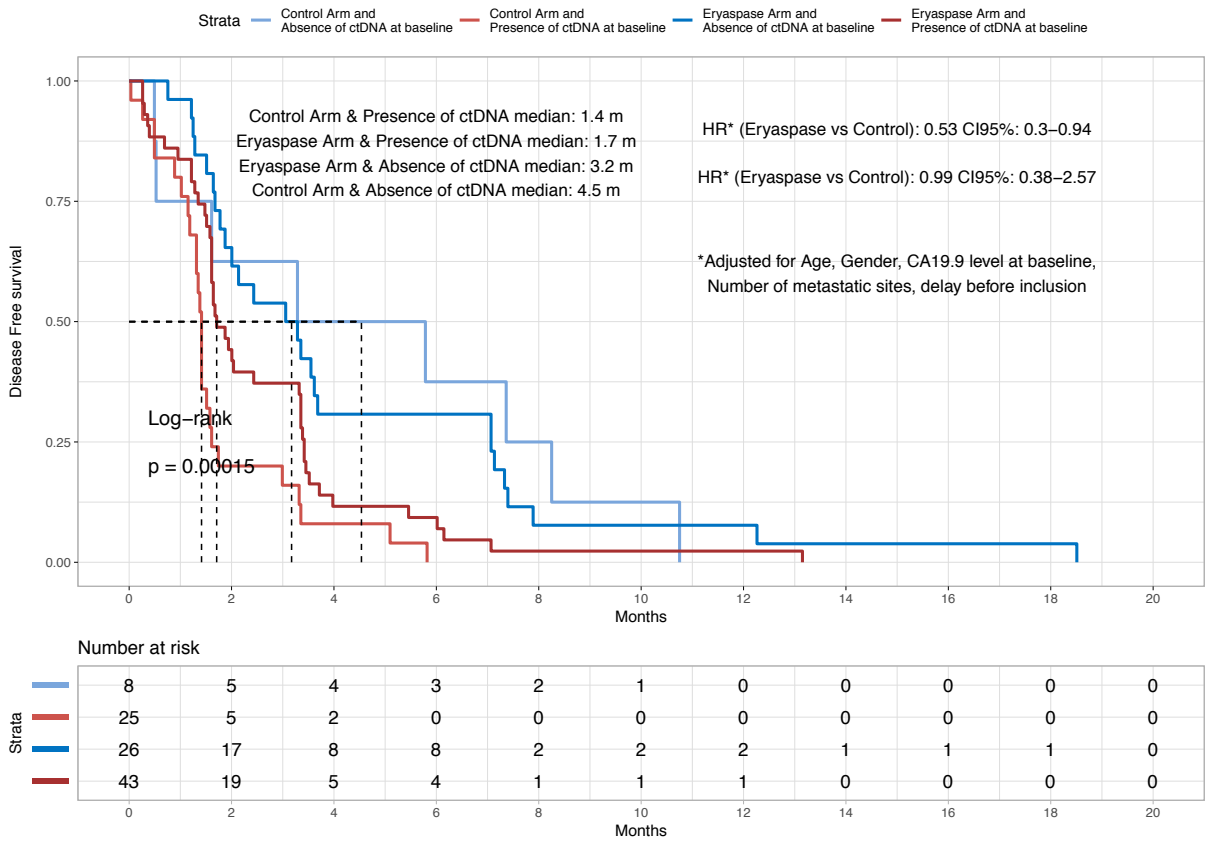

Strata
Control Arm and Absence of ctDNA at baseline
Control Arm and Presence of ctDNA at baseline
Eryaspase Arm and Absence of ctDNA at baseline
Eryaspase Arm and Presence of ctDNA at baseline
Overall survival
1.00
0.75
0.50
0.25
0.00
Control Arm & Presence of ctDNA median: 3.1 m
Eryaspase Arm & Presence of ctDNA median: 5.8 m
Eryaspase Arm & Absence of ctDNA median: 7.3 m
Control Arm & Absence of ctDNA median: 11.7 m
HR* (Eryaspase vs Control): 0.52 CI95%: 0.29–0.91
HR* (Eryaspase vs Control): 0.93 CI95%: 0.32–2.67
*Adjusted for Age, Gender, CA19.9 level at baseline, Number of metastatic sites, delay before inclusion
Log−rank
p = 2e−04
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Months
Number at risk
Strata
8 7 6 5 4 4 3 0 0 0 0 0 0 0 0 0
26 19 10 6 2 1 0 0 0 0 0 0 0 0 0 0
28 28 23 14 14 11 9 6 5 5 2 2 2 1 1 1
51 43 34 23 9 7 4 4 3 3 2 2 2 0 0 0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Months

Figure 4B

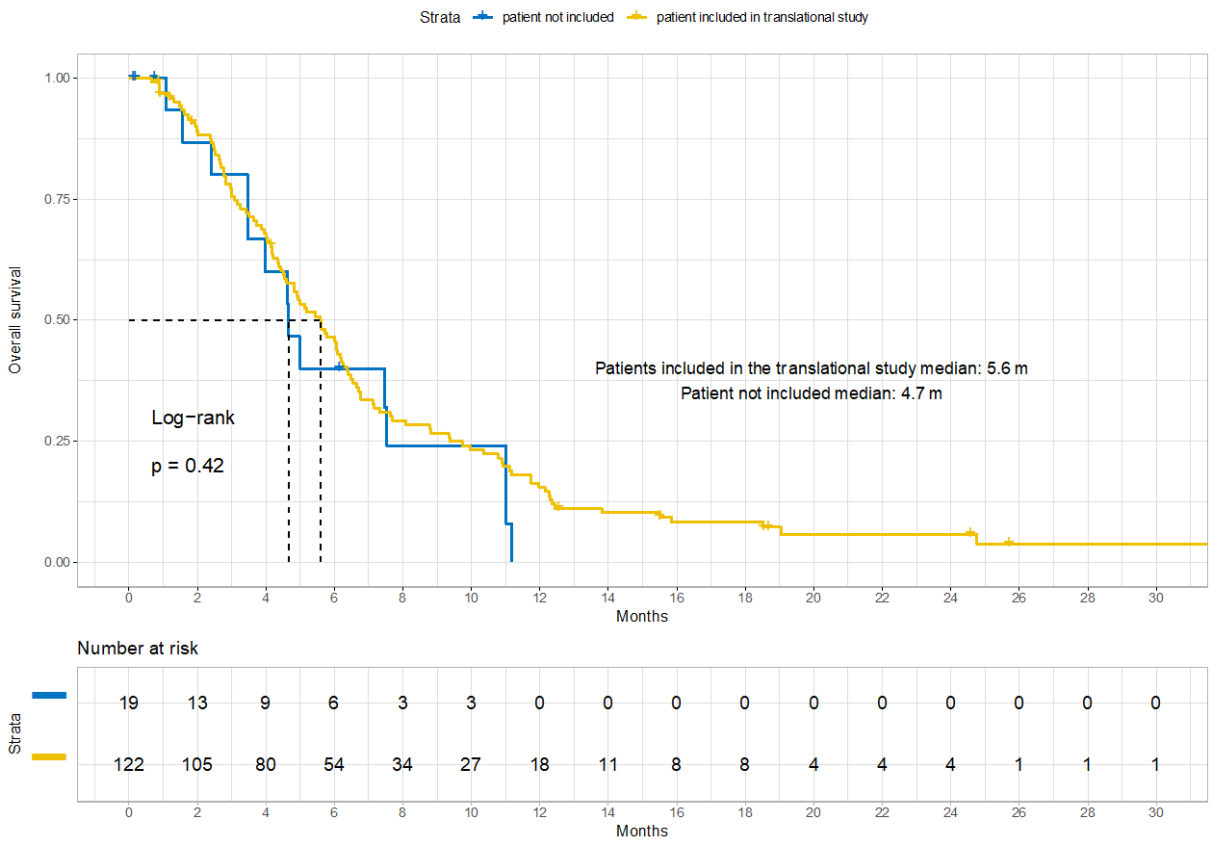

Strata
Control Arm and Absence of ctDNA at baseline
Control Arm and Presence of ctDNA at baseline
Eryaspase Arm and Absence of ctDNA at baseline
Eryaspase Arm and Presence of ctDNA at baseline
Disease Free survival
1.00
0.75
0.50
0.25
0.00
Control Arm & Presence of ctDNA median: 1.4 m
Eryaspase Arm & Presence of ctDNA median: 1.7 m
Eryaspase Arm & Absence of ctDNA median: 3.2 m
Control Arm & Absence of ctDNA median: 4.5 m
HR* (Eryaspase vs Control): 0.53 CI95%: 0.3–0.94
HR* (Eryaspase vs Control): 0.99 CI95%: 0.38–2.57
*Adjusted for Age, Gender, CA19.9 level at baseline, Number of metastatic sites, delay before inclusion
Log−rank
p = 0.00015
0 2 4 6 8 10 12 14 16 18 20
Months
Number at risk
Strata
8 5 4 3 2 1 0 0 0 0 0
25 5 2 0 0 0 0 0 0 0 0
26 17 8 8 2 2 2 1 1 1 0
43 19 5 4 1 1 1 0 0 0 0
0 2 4 6 8 10 12 14 16 18 20
Months

Supplementary Data File S1: Description of the studied population

	Patients included in translational study (n=122)	Patients included in the phase IIb trial (n=141)
Age		
Mean (SD)	62.4 (9.48)	62.6 (9.72)
Median [Min, Max]	63.0 [37.0, 84.0]	63.0 [37.0, 84.0]
Gender		
F	52 (42.6%)	58 (41.1%)
M	70 (57.4%)	83 (58.9%)
Treatment Arm intent to treat		
Control arm	36 (29.5%)	46 (32.6%)
Eryaspase arm	86 (70.5%)	95 (67.4%)
CA19.9 Normal vs Elevated		
Normal	22 (18.0%)	25 (17.7%)
Elevated	88 (72.1%)	97 (68.8%)
Missing	12 (9.8%)	19 (13.5%)
Time from initial diagnosis to randomization		
Mean (SD)	11.0 (10.1)	10.7 (9.71)
Median [Min, Max]	8.10 [2.50, 86.9]	8.08 [2.50, 86.9]
Number of metastatic sites		
Mean (SD)	1.37 (0.645)	1.39 (0.674)
Median [Min, Max]	1.00 [0.00, 5.00]	1.00 [0.00, 5.00]
Number of metastatic sites		
1 or 2 sites	117 (95.9%)	134 (95.0%)
more than 2 sites	5 (4.1%)	7 (5.0%)

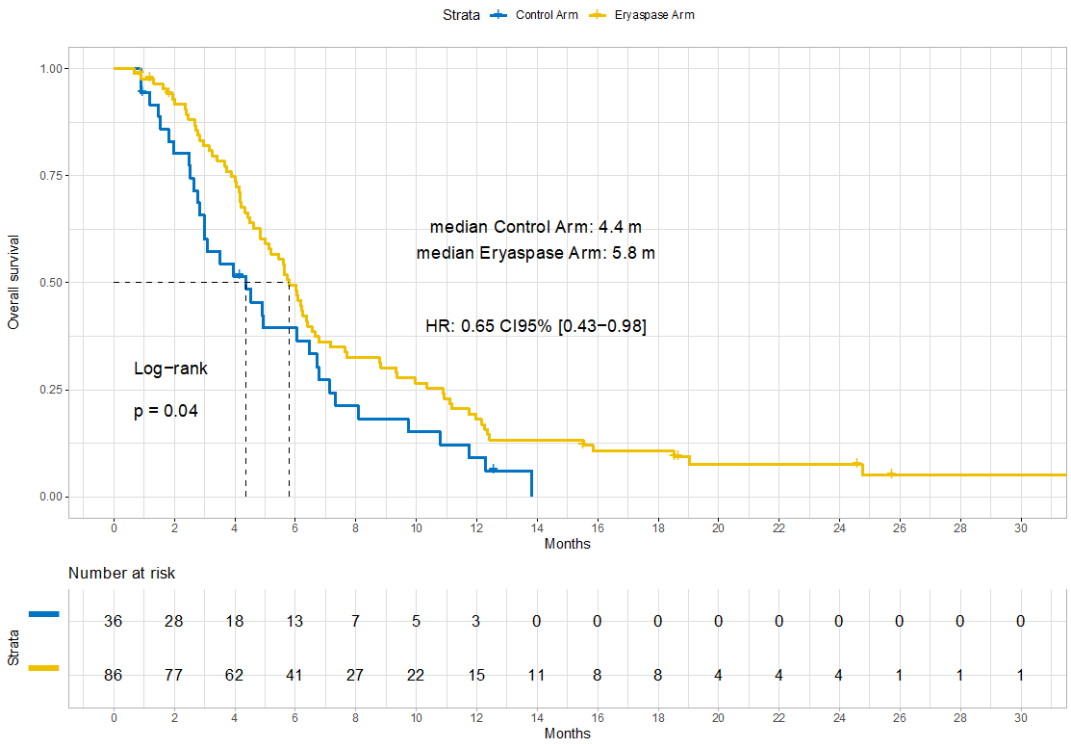
Supplementary Data File S2

Figure S2a: Overall survival of patients with or without at least one available plasma sample.



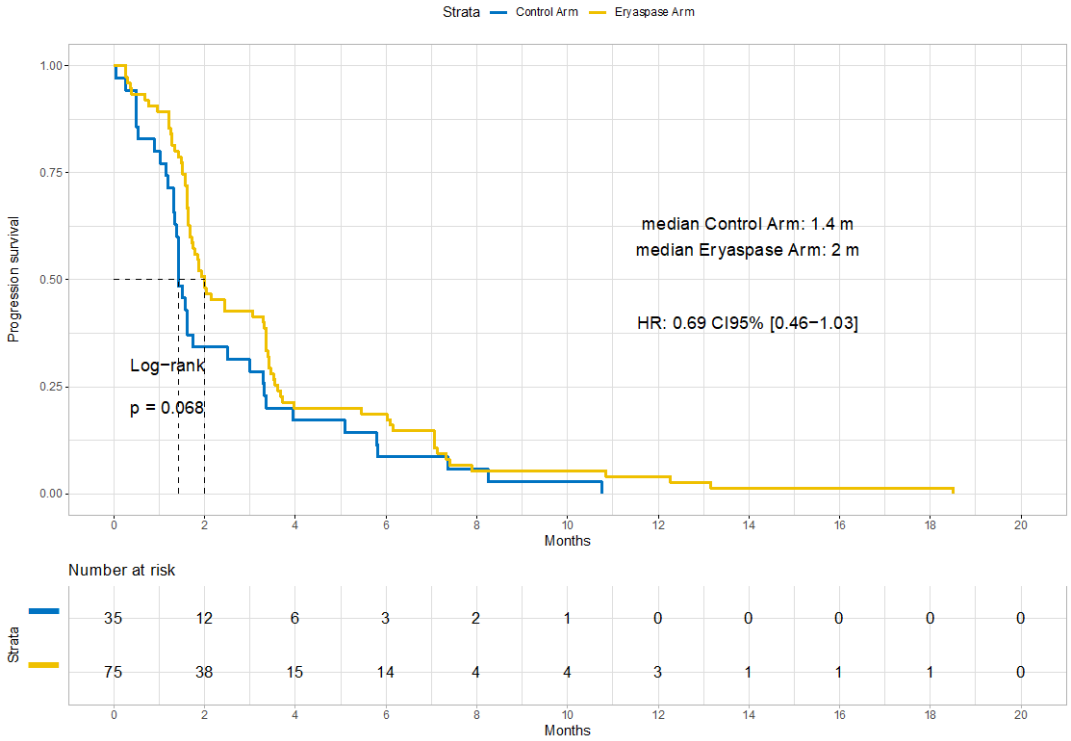
Median overall survivals were of 5.6 and 4.7 months for patients with and without at least one available plasma sample (p=0.52), respectively. Median progression free survivals were of 1.8 and 2.1 months for patients with and without at least one available plasma sample (p=0.61), respectively.

Figure S2b: Overall survival curves according to treatment arms for the 122 patients with at least one available plasma sample.



Median overall survivals were of 5.8 and 4.4 months in the eryaspase arm (n=86) and control arm (n=36), respectively (HR=0.65; 95% CI: 0.43-0.98; p=0.04).

Figure S2c: Progression free survival curves according to treatment arms for the 122 patients with at least one available plasma sample.



Median progression free survivals were of 2.0 and 1.4 months in the eryaspase arm (n=75) and control arm (n=35), respectively (HR=0.69; 95% CI: 0.46-1.03; p=0.068).

Supplementary Data File S3: Patients characteristics at baseline according the presence or not of ctDNA

	No detectable ctDNA (n=36)	Detectable ctDNA (n=77)	Overall (n=113)
Age			
Mean (SD)	61.4 (9.70)	62.3 (8.93)	62.6 (9.72)
Median [Min, Max]	62.5 [42.0, 80.0]	63.0 [37.0, 84.0]	63.0 [37.0, 84.0]
Gender			
F	15 (41.7%)	34 (44.2%)	58 (41.1%)
M	21 (58.3%)	43 (55.8%)	83 (58.9%)
Treatment Arm intend to treat			
Control	8 (22.2%)	26 (33.8%)	46 (32.6%)
Eryaspase	28 (77.8%)	51 (66.2%)	95 (67.4%)
CA19.9 Normal vs Elevated			
Normal	5 (13.9%)	14 (18.2%)	25 (17.7%)
Elevated	26 (72.2%)	57 (74.0%)	97 (68.8%)
Missing	5 (13.9%)	6 (7.8%)	19 (13.5%)
Time from initial diagnosis to randomization			
Mean (SD)	12.9 (14.2)	9.96 (7.40)	10.7 (9.71)
Median [Min, Max]	9.92 [2.76, 86.9]	7.98 [2.50, 47.9]	8.08 [2.50, 86.9]
Number of metastatic sites			
Mean (SD)	1.44 (0.558)	1.38 (0.689)	1.39 (0.674)
Median [Min, Max]	1.00 [1.00, 3.00]	1.00 [1.00, 5.00]	1.00 [0.00, 5.00]
Number of metastatic sites			
1 or 2 sites	35 (97.2%)	73 (94.8%)	134 (95.0%)
more than 2 sites	1 (2.8%)	4 (5.2%)	7 (5.0%)

Supplementary Data File S4: Multivariate analysis of prognostic factors at baseline

Factors	estimate	std.error	statistic	p.value	conf.low	conf.high
Overall Survival						
maximal VAF frequency observed at baseline*	16.80	0.75	3.74	0.00018	3.83	73.58
Treatment Arm: Eryaspase vs Control	0.97	0.26	-0.10	0.92	0.58	1.63
Age*	0.98	0.01	-1.31	0.20	0.96	1.01
Gender: Male vs Female	0.87	0.23	-0.62	0.53	0.56	1.35
CA19.9 levels at baseline: Normal vs Elevated	0.86	0.30	-0.50	0.61	0.48	1.55
Delay before inclusion*	1.00	0.01	-0.44	0.66	0.97	1.02
Number of metastatic sites*	0.98	0.17	-0.09	0.93	0.71	1.37
Progression Free Survival						
maximal VAF frequency observed at baseline*	5.66	0.70	2.47	0.0135	1.43	22.39
Treatment Arm: Eryaspase vs Control	0.87	0.25	-0.57	0.56	0.53	1.42
Age*	1.00	0.01	-0.37	0.71	0.97	1.02
Gender: Male vs Female	0.93	0.22	-0.30	0.76	0.60	1.45
CA19.9 levels at baseline: Normal vs Elevated*	0.82	0.29	-0.69	0.48	0.46	1.44
Delay before inclusion*	1.00	0.01	0.10	0.92	0.98	1.03
Number of metastatic sites*	1.07	0.19	0.35	0.73	0.74	1.54

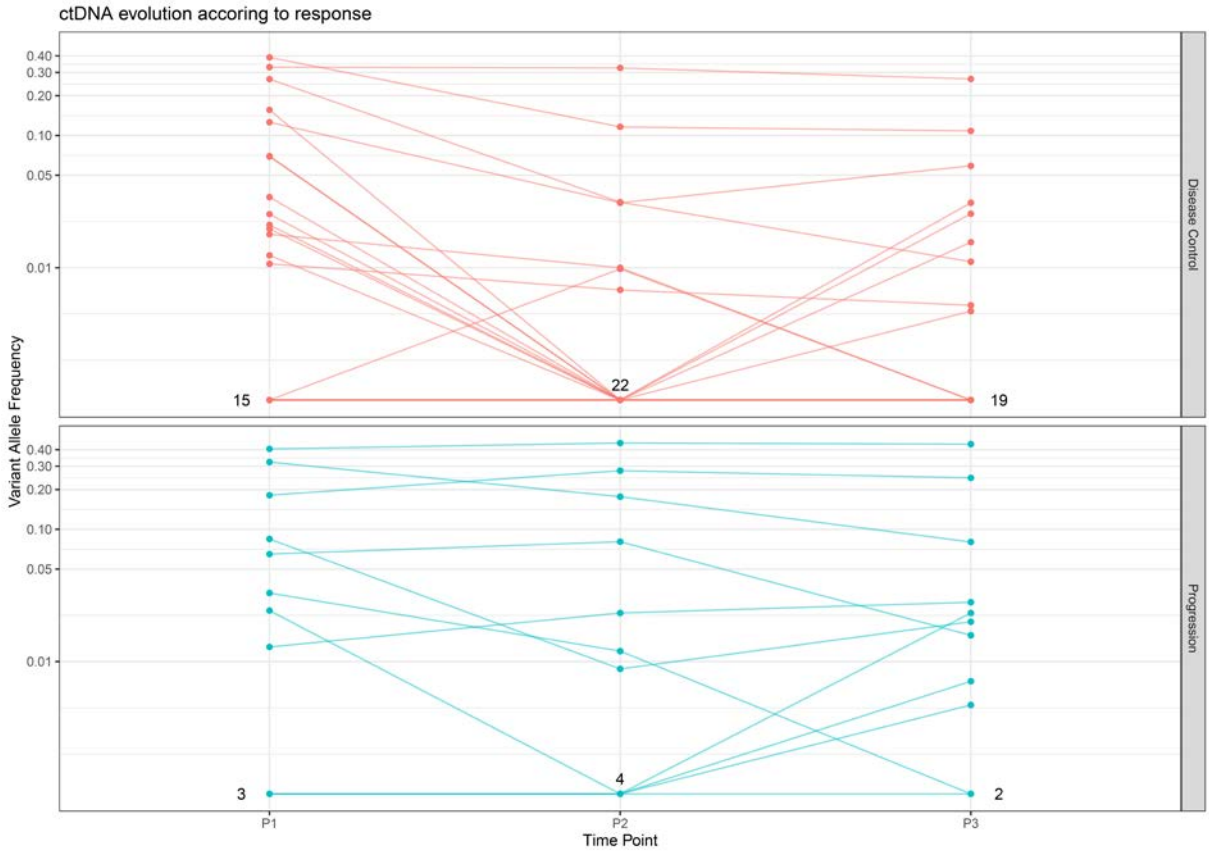
Abbreviation: VAF: variant allelic fraction

* Continuous variables

Supplementary Data File S5: Description of patients' characteristics according to tertiles of ctDNA VAF frequency at baseline

	1st tertile (n=38)	2nd tertile (n=37)	3rd tertile (n=38)
Age			
Mean (SD)	61.6 (9.57)	61.8 (9.56)	62.6 (8.51)
Median [Min, Max]	62.5 [42.0, 80.0]	64.0 [37.0, 78.0]	62.5 [42.0, 84.0]
Gender			
Female	17 (44.7%)	16 (43.2%)	16 (42.1%)
Male	21 (55.3%)	21 (56.8%)	22 (57.9%)
Treatment Arm intent to treat			
Control	8 (21.1%)	9 (24.3%)	17 (44.7%)
Eryaspase	30 (78.9%)	28 (75.7%)	21 (55.3%)
CA19.9 Normal vs Elevated			
Normal	6 (15.8%)	6 (16.2%)	7 (18.4%)
Elevated	27 (71.1%)	31 (83.8%)	25 (65.8%)
Missing	5 (13.2%)	0 (0%)	6 (15.8%)
Time from initial diagnosis to randomization			
Mean (SD)	12.8 (14.0)	10.1 (8.33)	9.69 (6.33)
Median [Min, Max]	9.92 [2.76, 86.9]	7.98 [2.50, 47.9]	7.90 [2.99, 31.4]
Number of metastatic sites			
Mean (SD)	1.45 (0.555)	1.35 (0.588)	1.39 (0.790)
Median [Min, Max]	1.00 [1.00, 3.00]	1.00 [1.00, 3.00]	1.00 [1.00, 5.00]
Number of metastatic sites			
1 or 2 sites	37 (97.4%)	35 (94.6%)	36 (94.7%)
more than 2 sites	1 (2.6%)	2 (5.4%)	2 (5.3%)

Supplementary Data File S6: Evolution of the maximal variant allelic fraction during the first three cycles in the 40 patients with all three available points.



For each graph, the number on the bottom line corresponds to the number of patients with non-detectable (negative) ctDNA at each point.

Supplementary Data File S7: Interaction between the presence of ctDNA at baseline and eryaspase efficacy on PFS and OS

Factors	estimate	std.error	statistic	p.value	conf.low	conf.high
Overall Survival						
Univariate analysis						
ctDNA present versus ctDNA absent	3.94	0.47	2.94	0.00332	1.58	9.84
Eryaspase arm versus control arm	1.22	0.46	0.43	0.66377	0.50	2.98
ctDNA present:Eryaspase arm (interaction)	0.39	0.52	-1.79	0.07346	0.14	1.09
Multivariate analysis						
ctDNA present versus ctDNA absent	4.16	0.50	2.87	0.00415	1.57	11.01
Eryaspase arm versus control arm	1.50	0.48	0.85	0.39363	0.59	3.83
Age	0.98	0.01	-1.83	0.06698	0.96	1.00
Gender: Male vs Female	0.75	0.22	-1.30	0.19323	0.48	1.16
CA19.9 level at baseline Elevated versus Normal	0.85	0.29	-0.57	0.56981	0.47	1.51
Delay before inclusion	0.99	0.01	-0.78	0.43433	0.97	1.01
Number of metastatic site	1.04	0.18	0.24	0.80923	0.74	1.48
ctDNA present:Eryaspase arm (interaction)	0.35	0.55	-1.88	0.06012	0.12	1.05
Progression Free Survival						
Univariate analysis						
ctDNA present versus ctDNA absent	3.81	0.43	3.12	0.00181	1.64	8.84
Eryaspase arm versus control arm	1.12	0.41	0.27	0.78964	0.50	2.49
ctDNA present:Eryaspase arm (interaction)	0.47	0.49	-1.54	0.12237	0.18	1.22
Multivariate analysis						
ctDNA present versus ctDNA absent	3.93	0.47	2.92	0.00345	1.57	9.82
Eryaspase arm versus control arm	1.31	0.45	0.61	0.54264	0.55	3.15
Age	0.99	0.01	-0.76	0.44972	0.96	1.02
Gender: Male vs Female	0.86	0.23	-0.67	0.50562	0.55	1.34
CA19.9 level at baseline Elevated versus Normal	0.87	0.29	-0.47	0.64139	0.49	1.55
Delay before inclusion	1.00	0.01	0.08	0.93554	0.97	1.03
Number of metastatic site	1.03	0.19	0.17	0.86501	0.72	1.49
ctDNA present:Eryaspase arm (interaction)	0.42	0.53	-1.62	0.10507	0.15	1.20