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Oncology

Circulating tumor DNA in unresectable pancreatic cancer is a strong predictor of first-line treatment efficacy: The KRASCIPANC prospective study



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ABSTRACT

Background: There is no robust predictor of response to chemotherapy (CT) in unresectable pancreatic adenocarcinomas (UPA). The objective of the KRASCIPANC study was to analyze the kinetics of cell-free DNA (cfDNA)/circulating tumor DNA (ctDNA) as a predictor of response to CT in UPA.

Methods: Blood samples were collected just before first CT and at day 28. The primary endpoint was the kinetics of KRAS-mutated ctDNA by digital droplet PCR between D0 and D28 as a predictor of progression-free survival (PFS).

Results: We analyzed 65 patients with a KRAS-mutated tumor. A high level of cfDNA and KRAS-mutated ctDNA at D0, as well as the presence of KRAS-mutated ctDNA at D28, were strongly associated with lower centralized disease control rate (cDCR), shorter cPFS and OS in multivariate analysis. A score combining cfDNA level at diagnosis \geq or $<$ 30 ng/mL and presence or not of KRAS-mutated ctDNA at D28 was an optimal predictor of cDCR (OR=30.7, IC95% 4.31–218 $P=$.001), PFS (HR=6.79, IC95% 2.76–16.7, $P<$.001) and OS (HR=9.98, IC95% 4.14–24.1, $P<$.001).

Conclusion: A combined score using cfDNA level at diagnosis and KRAS-mutated ctDNA at D28 is strongly associated with patient survival/response to chemotherapy in UPA.

Trial Registration: ClinicalTrials.gov Identifier: NCT04560270

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1. Introduction

By 2030, pancreatic adenocarcinoma (PA) will have become the second leading cause of death from cancer [1,2]. In most cases PAs are unresectable at diagnosis and are treated by palliative chemotherapy [3]. Despite improved prognosis for unresectable PA (UPA), it remains poor, with median overall survival (OS) of 12

months [4]. A minority of patients with UPA can receive second-line chemotherapy due to rapid deterioration of performance status [5].

Carcinogenesis of PA and its pre-cancerous lesions, pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN) and mucinous cystadenomas (MC) are well-known [6,7]. More than 80% of PAs have a KRAS gene mutation, mainly in codons 12 and 13, which is an early event [8,9,10]. Mutation of *Guanine nucleotid binding protein (GNAS)* gene at codon 201 has been identified, primarily in IPMN (\approx 65%) and subsequently in PA derived from IPMN [11,12]. KRAS and GNAS mutations are not mutually exclusive [12,13].

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Circulating tumor DNA (ctDNA), also called "liquid biopsy", emerges as a prognosis marker insofar as it accurately reflects real-time tumor burden [8]. Circulating cell-free DNA (cfDNA), which includes both DNA released by non-tumoral cells and ctDNA, has likewise been associated with prognosis in many cancers [14]. Rates of ctDNA among cfDNA range from 0.001% to 90%, mostly according to stage of disease and type of cancer [15]. ctDNA provides early prediction treatment response before radiologic evaluation at 2 or 3 months in many tumors, including metastatic colorectal cancer (mCRC). The PLACOL study demonstrated that decreased ctDNA at 2 or 4 weeks is associated with longer median progression-free survival (PFS) [16]. In PA, presence of ctDNA has been associated with a poor prognosis [17]. Only small series have evaluated ctDNA kinetics as an early predictor of response to chemotherapy in UPA [18–20].

In UPA, early predictors of chemoresistance to first-line regimen remain to be determined and represent a major challenge. Consequently, in the prospective KRASCIPANC trial (KRAS Circulating tumor DNA in PANcreatic cancer) we analyzed the kinetics of cfDNA/ctDNA as an early predictor of response to chemotherapy in UPA.

2. Patients and methods

2.1. Population

The main inclusion criteria were patients older than 18 years with histologically proven UPA treated by first-line palliative chemotherapy. The non-inclusion criterion was patients with other malignancies within the previous five years. All the patients signed an informed consent form before inclusion in the KRASCIPANC prospective study.

Treatment efficacy was evaluated using chest-abdomen-pelvis computed tomography-scans (CT-scans) at baseline and every 2- or 3-months during treatment. All CT-scans were centrally reviewed by an expert digestive radiologist, blinded to treatment efficacy, to determine response to treatment according to the RECIST criteria version 1.1.

Tumor tissues were collected to determine both *KRAS* and *GNAS* mutational status. Blood samples for cfDNA and ctDNA analyses were collected just before the first chemotherapy course and at different time points until disease progression at days (D) 14, 28 and once a month up to 6 months, always just before a chemotherapy course.

Protocol was approved by the French ethics committee "Comité de Protection des Personnes Ouest III" (DC 2016–2631) and was registered at the ClinicalTrials.gov database (NCT04560270).

2.2. DNA extraction and detection of *KRAS* and *GNAS* mutated DNA

Blood sample was collected in EDTA tubes, centrifuged at 1500xg for 10 min within 3 h of collection to collect the plasma. It was then stored at -80°C . After a 2nd centrifugation at 10 000xg for 10 min at 4°C , the QIAamp Circulating Nucleic Acid kit (Qiagen, Valencia, CA) was used to isolate cfDNA from plasma sample according to the manufacturer's instructions, as previously described [21]. DNA extraction from formalin-fixed, paraffin-embedded (FFPE) pancreatic lesions was performed using KAPA Express Extract Kits® (KAPA Biosystems, CliniSciences, Nanterre, France). Quantification of DNA and cfDNA was determined using the Quantus® fluorimeter and cfDNA quantification was expressed in ng/mL of plasma.

KRAS and *GNAS* mutational status were determined using ddPCR (Bio-Rad QX200 Droplet Digital PCR System®, BioRad, Hercules, California, United-States), as previously describe [21]. Determination of *KRAS* and *GNAS* mutations was performed on one type

of tumor tissue, either primary tumor or metastasis according to the material used to perform the initial tumor diagnosis. The *KRAS* mutations were analyzed simultaneously using the multiplex ddPCR™ *KRAS* G12/G13 Screening Kit (p.G12V, p.G12D, p.G12R, p.G12C, p.G12A, p.G12S, and p.G13D; BioRad, Hercules, California, United-States) while R201H and R201C *GNAS* mutations were analyzed separately using simplex kits (#dHsaMDV2516796 and #dHsaMDV2510562 respectively; Biorad). Approximately 10 ng of DNA/cfDNA were analyzed using the QX200 Droplet Digital PCR platform (Bio-Rad) as previously described [21] according to the manufacturer's standard protocol. Results were analyzed and interpreted using QuantaSoft™ software version 1.7.4.0917 (BioRad) (Fig. 1). Results were expressed by mutated allelic frequency (MAF) which is the percentage of *KRAS* mutated DNA among the cfDNA. Limit of detection at 0.1% was previously determined at our laboratory, performing validation standard procedure (Supplementary Figure 1).

The same technique was used for circulating DNA and for tissue DNA (see Fig. 1).

2.3. Statistical analysis

Due to no data in literature regarding ctDNA as a predictor of the response to chemotherapy in UPA no patient number calculation was possible. Indeed, all consecutive patients were included prospectively between 2016 and 2019 in this pilot study to have at least fifty evaluable patients, which permitted inclusion of both responder and non-responder patients to evaluate predictive value of ctDNA.

All characteristics are described on the overall population and all sub-population analyzed. Descriptive statistics included mean and/or median and extreme for the quantitative variables and frequencies and percentages for qualitative variables. Baseline comparisons used Mann-Whitney nonparametric test for quantitative variables, and Fisher's exact test for qualitative variables. For survival analysis, the cutoff date used was September 30, 2021. Median follow-up was determined using inverse Kaplan-Meier method.

The primary endpoint was to evaluate variation of *KRAS*-mutated ctDNA between day 28 and day 0 (in MAF) as a predictor of PFS. It was calculated for each chemotherapy line from the date of the first day of treatment to progression and/or death, whichever occurred first. Progression was defined according to RECIST 1.1 criteria both by investigator and centralized review by an expert digestive radiologist blinded to treatment efficacy. Patients alive or without progression were censored at the date of the latest news. Univariate and multivariate associations with progression rates were analyzed by logistic regression with adjusted OR (aOR) and 95% confidence interval. Survival curves were estimated using the Kaplan-Meier method and compared using the likelihood-ratio test in univariate or multivariate Cox proportional hazards models with adjusted HR (aHR) and 95% confidence interval. Multivariate logistic or Cox regression modeling initially selected significant variables at the $p < .20$ level in univariate analysis, excluding those with collinearities or excessive missing rate. Ascending stepwise entry then sequentially included variables at $p < .10$ threshold. Selection in the final model was restricted to variables that satisfied the $p < .05$ significance level.

Quantitative variables were compared using Mann-Whitney nonparametric test and qualitative variables using Fisher's exact test. Correlation analysis was performed using Spearman rank correlation (rS) test, nonparametric Kruskal-Wallis ANOVA or two-sample Wilcoxon test and Fisher's exact test according to the type of variables.

Secondary endpoints were disease control rate (DCR), PFS by investigator and overall survival (OS). DCR was defined as complete

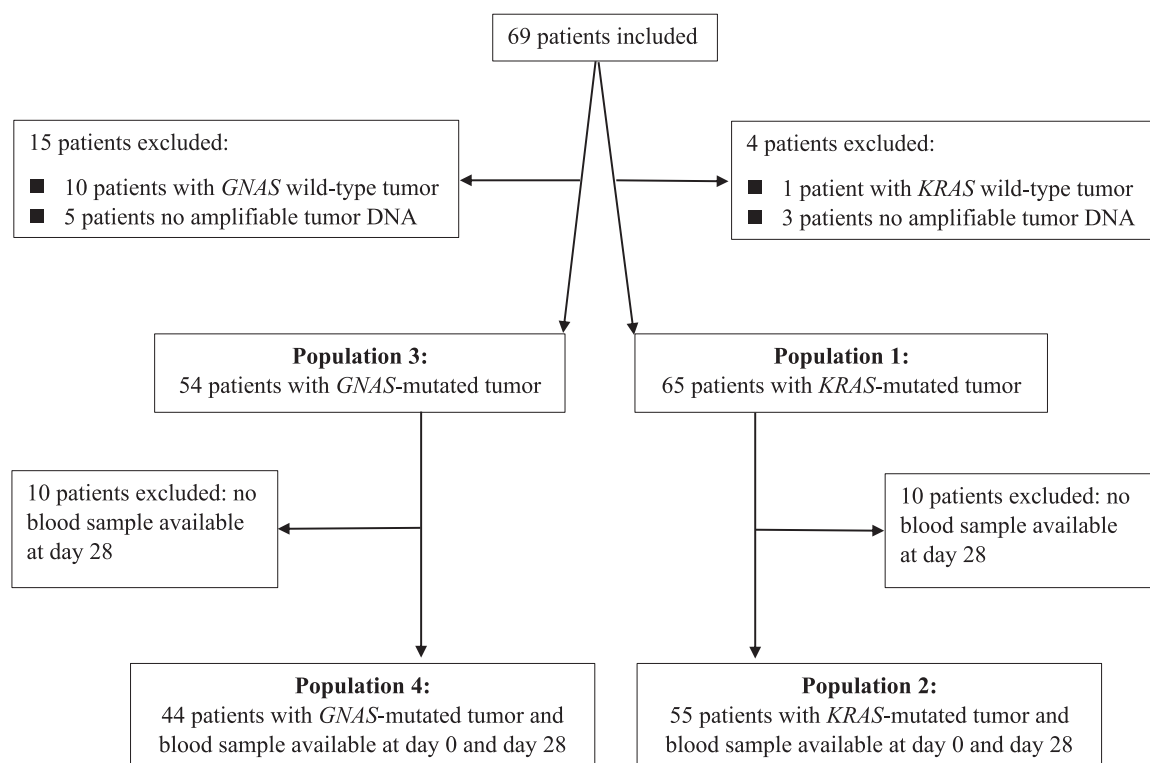


Fig. 1. flowchart.

response, partial response or stability according to RECIST 1.1 criteria. OS was defined by patient survival since the date of diagnosis to death regardless of the cause. Patients alive at the date of the latest news were censored. Prognostic value of cfDNA, *KRAS*-mutated ctDNA and *GNAS*-mutated ctDNA at D0 were also evaluated, as were variations of cfDNA, *KRAS*-mutated ctDNA and *GNAS*-mutated ctDNA between D0 and D28.

Finally, others predictive/prognosis factors of RR, PFS and OS were evaluated in univariate and multivariate analysis using Cox regression model. Performance of ctDNA and cfDNA values as predictors of treatment efficacy was evaluated using AUC (Area Under the Curve) and ROC (Receiver Operating Characteristics) curves. Identification of optimal cut-off values was based on Youden's J index (sensitivity + specificity - 1).

For each comparison, a P value less than 0.05 was considered as significant. All tests were bilateral. The analyses were performed with SAS software version 9.4 (SAS Institute, Cary, NC, US).

3. Results

3.1. Study population

All in all, 69 patients were included in this prospective study between 2016 and 2019. Since the primary endpoint evaluated *KRAS*-mutated ctDNA, the study population focused on patients with *KRAS*-mutated tumors, called "Population 1" ($n = 65$) (Fig. 1). Median age was 66.2 years and 82% were ECOG PS 0 or 1 (Table 1). The primary tumor site was most often the head of the pancreas (62%) and a majority had a metastatic disease (60%). The first-line chemotherapy received by the patients was 5-Fluoro-Uracile-based chemotherapy (5-FU) for 52% (Folfinrox or Folfox every 14 days) and gemcitabine-based chemotherapy for 48% (gemcitabine plus nab-paclitaxel at D0, D8, D15 every 4 weeks, gemcitabine alone at D0, D8, D15 every 4 weeks or Gemox every 14 days).

In "Population 1," median follow-up was 39.1 months. Median OS was 11.8 months (95%CI, 8.3–16.0) (Supplementary Figure 2A). Median PFS according to the local investigator was 6.7 months and median PFS according to independent centralized review (cPFS) was 6.3 months (95%CI, 2.6–8.4) (Supplementary Figure 2B). DCR according to independent centralized review (cDCR) was 54%.

A second population, called "Population 2" ($n = 55$), was used to analyze ctDNA kinetics, which included only patients with *KRAS*-mutated tumor and available blood samples at day 0 and 28 for ctDNA tests (Fig. 1). Populations 1 and 2 differed as regards presence of liver metastases, albumin, bilirubin and CA 19-9 levels and first-line chemotherapy regimen (Supplementary Table 1).

3.2. *KRAS* tumor mutational status and circulating DNA at baseline

Concerning *KRAS*-mutated tumor, median allelic frequency (MAF) of *KRAS* mutation was 10.4%, ranging from 0.15% to 68.3%. In population 1, baseline median level of cfDNA was 14.2 ng/mL (range: 2.4–339.0 ng/mL) (Table 1). Levels of cfDNA were higher in females, in patients with liver metastases, with lower levels of albumin and higher levels of LDH, CA19.9 and CEA (Supplementary Table 2).

KRAS-mutated ctDNA at baseline was detectable in 62% of patients, 42% for locally advanced UPA and 74% for metastatic UPA. MAF of *KRAS*-mutated ctDNA was 0.26% (range: 0.0%–63.9%) (Table 1). As for cfDNA, *KRAS*-mutated ctDNA level was associated with liver metastases at diagnosis (Supplementary Table 2). *KRAS*-mutated ctDNA level was associated with the cfDNA level at baseline ($r_s=0.45$, $P<.001$) (data not shown). Among the 26 patients with locally advanced pancreatic adenocarcinoma, only 42.3% had *KRAS*-mutated ctDNA detectable at baseline as compared to 74.4% in patients with metastatic pancreatic adenocarcinoma ($n = 39$). Median CA 19.9 levels were lower in patients with undetectable *KRAS*-mutated ctDNA (395 UI/L) as compared to patients with detectable *KRAS*-mutated ctDNA (1829 UI/L) (Supplementary Table 2).

Table 1
Patients, tumors and circulating DNA characteristics.

	Population 1 (n = 65)	Population 2 (n = 55)
Variables at baseline		
Median age (years, range)	66.2 [37.5–85.3]	67.4 [37.5–85.3]
Gender: Female	34 (52%)	30 (55%)
ECOG PS: 2 (vs. 0–1)	12 (18%)	8 (15%)
Primary tumor site		
Head	40 (62%)	33 (60%)
Body	17 (26%)	15 (27%)
Tail	8 (12%)	7 (13%)
Tumor stage: Metastatic (vs. locally advanced)	39 (60%)	31 (56%)
Metachronous metastasis (vs. synchronous)	4/39 (10%)	4/31 (13%)
Number of metastatic sites > 1 (vs.1)	15/39 (38%)	12/31 (39%)
Liver metastasis at diagnosis		
Albumin	32 (49%)	24 (44%)
Median (g/L, range)	39.0 [22.1–50.0]	39.8 [22.1–50.0]
LDH	n = 45	n = 39
Median (U/L, range)	343 [215–1101]	345 [240–1101]
Bilirubin (ULN=17)	n = 63	n = 54
Median (umol/L, range)	9 [2–300]	9 [2–300]
CA 19–9 (ULN=27)	n = 64	n = 54
Median (U/L, range)	783 [1–474,600]	517 [1–474,600]
CEA (ULN=5)	n = 61	n = 52
Median (ng/mL, range)	5.9 [0.8–1870]	6.3 [0.8–1870]
First-line CT		
5FU-based ^a	34 (52%)	33 (60%)
Gemcitabine-based ^b	31 (48%)	22 (40%)
Centrally reviewed response at M3		
Partial response	1 (2%)	1 (2%)
Stable disease	34 (52%)	34 (62%)
Disease progression	30 (46%)	20 (36%)
cfDNA at D0		
Median (ng/mL, range)	14.2 [2.4–339]	13.3 [2.4–323]
≥ 30 ng/mL	24 (37%)	17 (31%)
KRASmut ctDNA at D0		
Median allelic frequency (% range)	0.26 [0–63.9]	0.15 [0–63.9]
≥ 0.35%	28 (43%)	20 (36%)
Detectable	40 (62%)	32 (58%)

Population 1: patients with KRAS-mutated tumors; Population 2: patients with KRAS-mutated tumor and available blood samples at day 0 and 28 for ctDNA tests. ECOG-PS: Eastern Cooperative Oncology Group - Performance Status; ULN: upper limit of normal; LDH: lactate dehydrogenase; CEA: carcinoembryonic antigen; CT: chemotherapy; M3: 3 months; vs.: versus; cfDNA: cell-free DNA; ctDNA: circulating tumor DNA; CA 19–9: carbohydrate antigen 19–9.

^a Folfirinax (n = 34), Folfax (n = 2).

^b Gemcitabine-Abraxane (n = 27), Gemox (n = 1), Gemcitabine (n = 5).

3.3. Prognostic value of circulating DNA at baseline

A high level of cfDNA at baseline, as a continuous variable, was strongly associated with lower cDCR ($P=.005$), shorter cPFS ($P=.001$) and OS ($P<.001$) (data not shown). Using a cut-off at 30 ng/mL (most relevant binary threshold using Youden's J index), patients with cfDNA ≥ 30 ng/mL had significantly shorter OS in comparison with patients with cfDNA under 30 ng/mL, median OS was 4.5 versus 16.6 months (HR=3.81; 95%CI, 2.15–6.75; $P<.001$) (Fig. 2A). Using the same cut-off at 30 ng/mL cPFS was 2.5 months and 9.6 months (HR=4.25; 95%CI, 2.25–8.03; $P<.001$) (Fig. 2B). cDCR was 25% and 71% in patients with cfDNA above or below 30 ng/mL respectively (OR=7.25; 95%CI 2.31–22.7; $P<.001$).

Level of KRASmut ctDNA at baseline was associated with lower cDCR ($P=.01$) and OS ($P=.003$) and a trend toward shorter cPFS ($P=.06$) (data not shown). The level of KRASmut ctDNA using a cut-off at 0.35% (most relevant binary threshold using Youden's J index) was associated with cDCR (70% versus 32%, OR=4.99; 95%CI, 1.73–14.4; $P=.002$), cPFS (2.5 versus 9.1 months) (HR=2.14; 95%

CI, 1.24–3.68; $P=.008$) and OS (4.6 months versus 16.0 months, HR=2.06; 95% CI, 1.22–3.48; $P=.008$) (Supplementary Table 3 and Supplementary Figure 3).

3.4. GNAS mutational status in tumor and circulating DNA

For GNAS mutation, 84% of tumors were mutated, called “Population 3” (Fig. 1). There were 70% of GNAS R201H mutations and 65% of GNAS R201C mutations on primary tumor and 57% of tumors had the two mutations. Among the KRAS-mutated tumors a GNAS mutation was present in 80% and 100% in KRAS wild-type tumors. In fact, there was no wild-type tumor for both KRAS and GNAS. There was no significant difference between Population 3 and the overall population except for CA19.9 and CEA levels (Supplementary Table 4). MAF of GNAS mutations in tumors was 0.25% (range 0–5.30%) and 0.26% (range 0–8.40%) respectively for R201H and R201C mutations.

GNASmut ctDNA at baseline was detectable in 33% of patients with GNAS-mutated tumors. Even if some patients had 2 GNAS mutations in primary tumor, only one was detected in the ctDNA, 13 R201H and 5 R201C. GNASmut ctDNA level was not associated with patient and tumor characteristics (Supplementary Table 2). GNASmut ctDNA level was correlated with the level of cfDNA ($r_s=+0.32$, $P=.01$) but not with the rate of KRASmut ctDNA ($r_s=+0.21$, $P=.12$) (data not shown). Among patients with detectable KRASmut ctDNA, 35% had detectable GNASmut ctDNA, and among patients without detectable KRASmut ctDNA, only 8% patients had detectable GNASmut ctDNA (Supplementary Table 5).

A high level of GNASmut ctDNA at baseline was not associated with shorter cDCR ($P=.37$), cPFS ($P=.68$) or OS ($P=.28$). Initial detection of GNASmut ctDNA was associated with a trend toward lower cDCR ($P=.08$), cPFS ($P=.07$) and OS ($P=.006$) (Supplementary Figure 4).

3.5. Circulating DNA kinetics and its prognostic value

Median change of cfDNA between baseline and D28 was +57% (range –86% to +842%). KRASmut ctDNA was detectable in 40% of patients at D28. As for KRASmut ctDNA at diagnosis, level of KRASmut ctDNA at D28 was associated with liver metastasis and tumor markers (Supplementary Table 2). Among the 33 patients with undetectable KRASmut ctDNA at D28, 30 had disease control with chemotherapy (91%). MAF change of KRASmut ctDNA between baseline and D28 was –68% (range –100% to +100%). GNASmut ctDNA was detectable in 30% of patients at D28 and MAF change of GNASmut ctDNA between baseline and D28 was 0% (range –100% to +171%).

Decreased cfDNA between baseline and D28 was associated not with cDCR ($P=.65$) and cPFS ($P=.15$) but only with OS ($P=.05$) (Table 2). Decreased ctDNA KRASmut was not associated with cDCR, cPFS or OS, whereas presence of ctDNA KRASmut at D28 was strongly associated with lower cDCR ($P<.001$) and lower cPFS ($P=.003$) and OS ($P=.004$) (Figs. 2C and D) (Table 2).

Presence of ctDNA GNASmut at D28 or kinetics of ctDNA GNASmut were not associated with survival outcome.

3.6. Predictive factors of treatment response and survival

At baseline, in multivariate analysis only level of cfDNA ($P=.003$) and KRASmut ctDNA ($P=.01$) were associated with cDCR (Supplementary Table 3). Level of cfDNA ($P<.001$) and KRASmut ctDNA ($P=.03$) were associated with cPFS and ECOG-PS ($P=.04$), level of cfDNA ($P<.001$) and KRASmut ctDNA ($P=.008$) were associated with OS.

When including kinetic markers, in univariate analysis, ECOG-PS, albumin, cfDNA at diagnosis, KRASmut ctDNA at diagnosis and

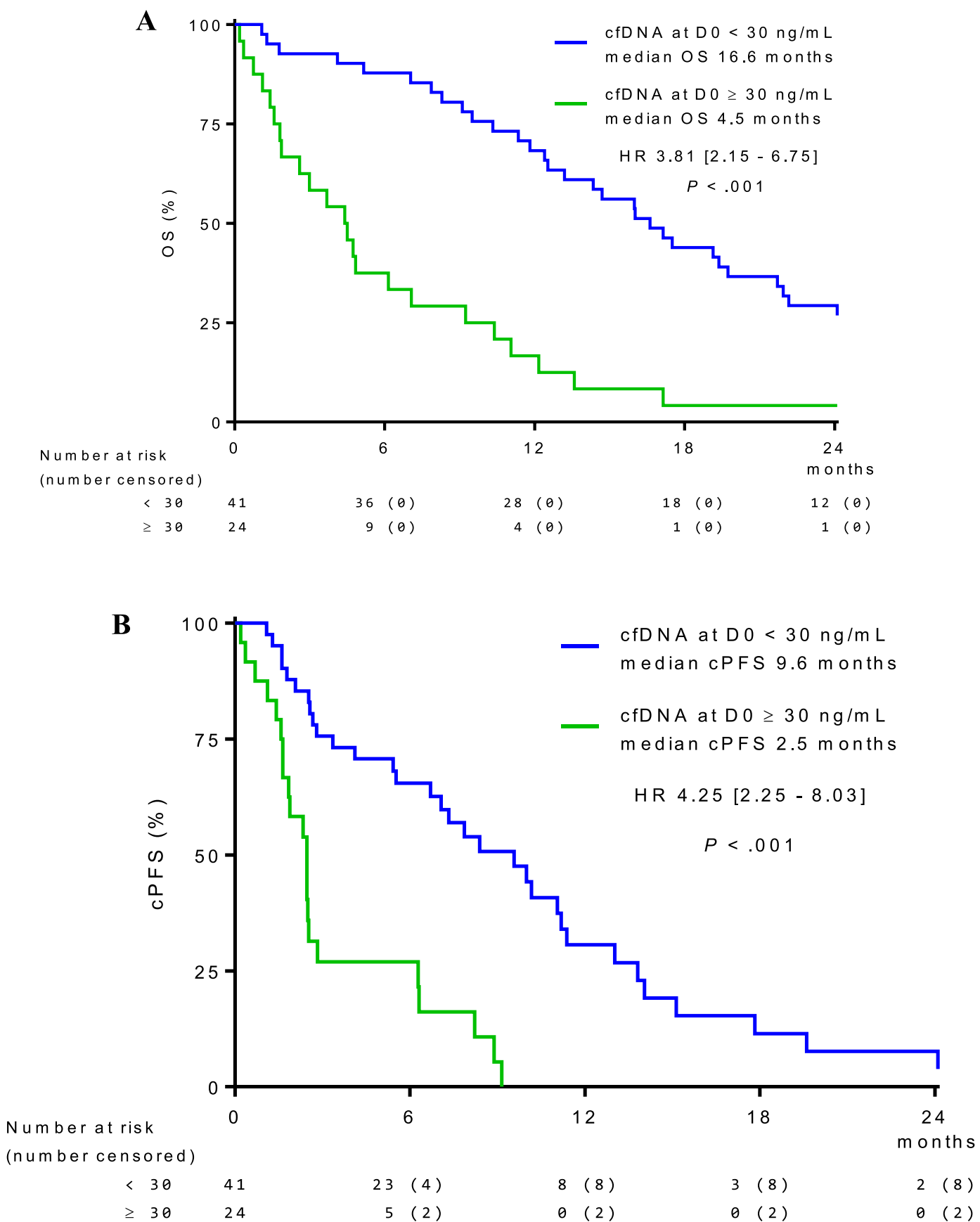


Fig. 2. Relation between levels of baseline cell-free DNA (cfDNA) (2A and 2B) and presence of KRAS-mutated circulating tumor DNA (ctDNA) at D28 (2C and 2D) with overall survival (OS) and progression-free survival according to centralized review (cPFS). A. Association with level of baseline cell-free DNA (cfDNA) and overall survival (OS). B. Relation with level of baseline cell-free DNA (cfDNA) and progression-free survival according to centralized review (cPFS). C. Association with level at D28 of KRAS-mutated circulating tumor DNA (ctDNA) and overall survival (OS). D. Relation with level at D28 of KRAS-mutated circulating tumor DNA (ctDNA) and progression-free survival according to centralized review (cPFS).

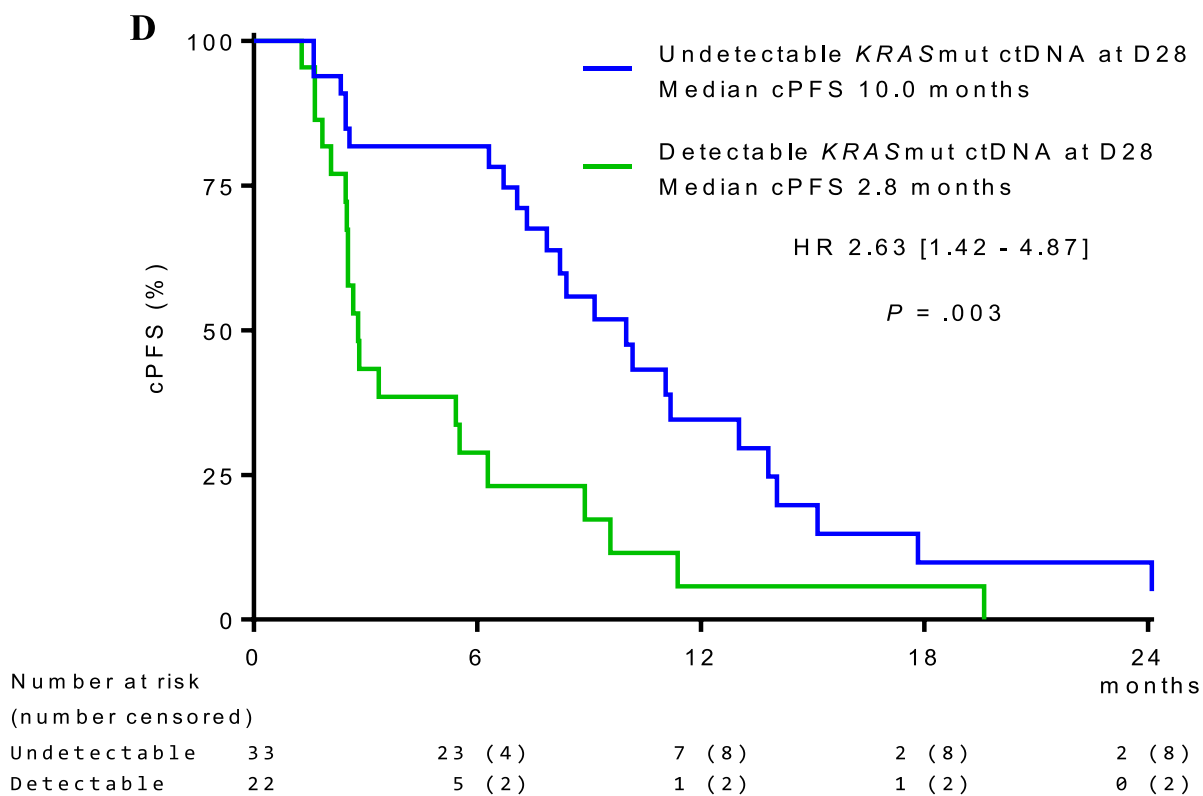
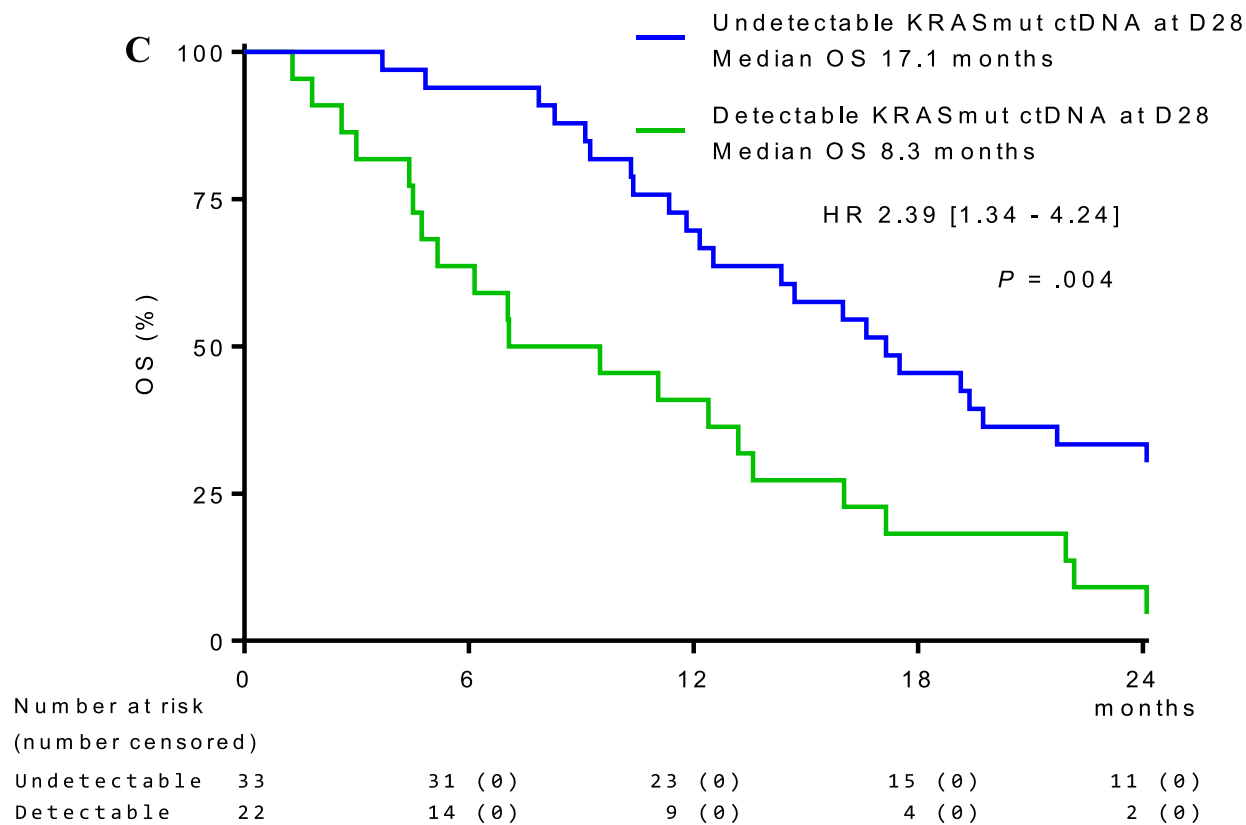


Fig. 2. Continued

Table 2
Predictive factors of disease control rate, progression-free survival and overall survival (population 2).

	n	Centralized DCR at 3 months (event P or D)		Centralized PFS		OS	
		Univariate Logistic regression OR [95% CI] <i>p</i> ^a	Multivariate Logistic regression aOR [95% CI] <i>p</i> ^a	Univariate Cox regression HR [95% CI] <i>p</i> ^a	Multivariate Cox regression aHR [95% CI] <i>p</i> ^a	Univariate Cox regression HR [95% CI] <i>p</i> ^a	Multivariate Cox regression aHR [95% CI] <i>p</i> ^a
Age: ≥65 vs <65	55	0.35 [0.11 – 1.08]	–	0.91 [0.50 – 1.67]	–	1.14 [0.64 – 2.04]	–
Gender: Male vs Female	55	1.33 [0.44 – 4.02]	–	1.04 [0.57 – 1.89]	–	0.99 [0.56 – 1.74]	–
ECOG-PS: >1 vs 0–1	55	1.94 [0.43 – 8.78]	–	1.56 [0.72 – 3.40]	–	2.85 [1.28 – 6.31]	2.48 [1.01 – 6.06]
Primary tumor site: Body or Tail vs Head	55	1.39 [0.45 – 4.23]	–	1.22 [0.65 – 2.28]	–	1.39 [0.78 – 2.45]	–
Tumor stage metastatic vs locally advanced	55	2.47 [0.77 – 7.91]	NS	0.89 [0.48 – 1.67]	–	1.09 [0.62 – 1.91]	–
Liver metastasis at diagnosis: Yes vs No	55	2.88 [0.92 – 8.94]	NS	1.18 [0.64 – 2.16]	–	1.45 [0.82 – 2.55]	NS
Albumin initial rate: ≤ 39 vs > 39	52	1.33 [0.43 – 4.13]	–	1.51 [0.82 – 2.79]	NS	2.30 [1.25 – 4.20]	NS
LDH initial rate: > 342 vs ≤ 342	39	1.87 [0.48 – 7.25]	–	2.45 [1.14 – 5.26]	–	1.47 [0.73 – 2.97]	–
Bilirubin initial rate: > 9 vs ≤ 9	54	0.84 [0.27 – 2.59]	–	0.77 [0.42 – 1.41]	–	1.63 [0.91 – 2.91]	NS
CA 19–9 level: > 783 vs ≤ 783	54	1.20 [0.39 – 3.69]	–	1.02 [0.55 – 1.87]	–	1.28 [0.72 – 2.29]	–
CEA level: > 5.9 vs ≤ 5.9	52	2.53 [0.77 – 8.34]	NS	1.64 [0.88 – 3.06]	NS	1.78 [0.98 – 3.22]	NS
First-line CT: 5FU-based vs gemcitabine-based	55	1.00 [0.33 – 3.07]	–	1.51 [0.81 – 2.81]	–	1.39 [0.77 – 2.50]	–
Initial level of cfDNA (≥30 ng/mL)	55	5.91 [1.70 – 20.5]	4.81 [1.22 – 18.9]	3.97 [1.92 – 8.19]	3.34 [1.59 – 7.00]	3.62 [1.91 – 6.88]	3.71 [1.85 – 7.45]
Level of cfDNA at D28 (> median=22.1 ng/mL)	55	1.06 [0.35 – 3.18]	–	0.91 [0.50 – 1.65]	–	1.02 [0.58 – 1.80]	–
Change of cfDNA between D0 and D28 (> median=+57%)	55	0.77 [0.26 – 2.33]	–	0.63 [0.34 – 1.18]	–	0.56 [0.32 – 1.00]	NS
Initial KRASmut ctDNA ≥ 0.35%	55	3.53 [1.10 – 11.3]	–	1.80 [0.97 – 3.32]	NS	1.71 [0.95 – 3.08]	NS
Presence of KRASmut ctDNA at D28	55	7.88 [2.28 – 27.2]	6.72 [1.81 – 24.9]	2.63 [1.42 – 4.87]	2.21 [1.17 – 4.15]	2.39 [1.34 – 4.24]	2.37 [1.28 – 4.36]
Change of KRASmut ctDNA between D0 and D28 (> median=–68%)	55	0.69 [0.23 – 2.08]	–	1.24 [0.66 – 2.35]	–	1.01 [0.57 – 0.176]	–
Presence of GNASmut ctDNA at D0	55	4.00 [1.09 – 14.7]	NS	2.09 [1.05 – 4.12]	NS	3.57 [1.84 – 6.95]	3.74 [1.77 – 7.87]
Presence of GNASmut ctDNA at D28	44	0.99 [0.26 – 3.74]	–	1.06 [0.54 – 2.08]	–	1.01 [0.51 – 1.99]	–
Change of GNASmut ctDNA between D0 and D28 (> median=0%)	44	1.19 [0.31 – 4.60]	–	1.19 [0.60 – 2.37]	–	1.08 [0.54 – 2.19]	–
Kinetic of CA 19–9 between D0 and D28 Increase vs decrease	47	2.38 [0.71 – 7.92]	–	1.53 [0.78 – 3.01]	–	1.31 [0.71 – 2.40]	–

ECOG-PS: Eastern Cooperative Oncology Group - Performance Status; LDH: lactate dehydrogenase; cfDNA: cell-free DNA; ctDNA: circulating tumor DNA; CEA: carcinoembryonic antigen; CA 19–9: carbohydrate antigen 19–9; SD: stable disease; PR: partial response; Ref: reference; OR: odds ratio; aOR: multivariate adjusted odds ratio; HR: hazard ratio; aHR: multivariate adjusted hazard ratio; P: progression; D: death; NS: not significant.

“–” are variables not included in multivariate analysis because there were not significant in univariate analysis.

“NS” are variables included in multivariate analysis but with not significant results.

KRASmut ctDNA at D28 were associated with cDCR, cPFS and/or OS (Table 2). In multivariate analysis, level of cfDNA at diagnosis (*P*=.025, 0.001 and <0.001) and presence of KRASmut ctDNA at D28 (*P*=.004, 0.015 and 0.006) were associated with cDCR, cPFS and OS, respectively. Kinetic of CA19.9 was not associated with cPFS or OS.

As the most robust predictive markers of response to treatment and survival were level of cfDNA level at diagnosis and presence of ctDNA KRASmut at D28, we built a combined score with cfDNA at baseline and KRASmut ctDNA at D28 with 3 groups as follows, “best”: cfDNA at D0 <30 ng/mL and no KRASmut ctDNA at D28; “mid”: cfDNA at D0 ≥30 ng/mL or presence of KRASmut ctDNA at D28; “worst”: cfDNA at D0 ≥30 ng/mL and presence of KRASmut

ctDNA at D28. Patients with best, mid and worst combined score had cDCR of 88%, 53% and 20%, respectively (*P*<.001) (Fig. 3A). Median OS rates were 19.3, 12.4 and 4.6 months (*P*<.001) (Fig. 3B) and median cPFS rates were 11.1, 5.4 and 2.5 months (*P*<.001), respectively (Fig. 3C).

4. Discussion

In this prospective study we demonstrated that cfDNA, KRAS-mutated ctDNA and GNAS-mutated ctDNA at diagnosis in patients with UPA have a high prognostic value. In addition, KRAS-mutated ctDNA at D28 was a predictor of response to first-line chemotherapy in terms of PFS and DCR. Finally, we determined a combined

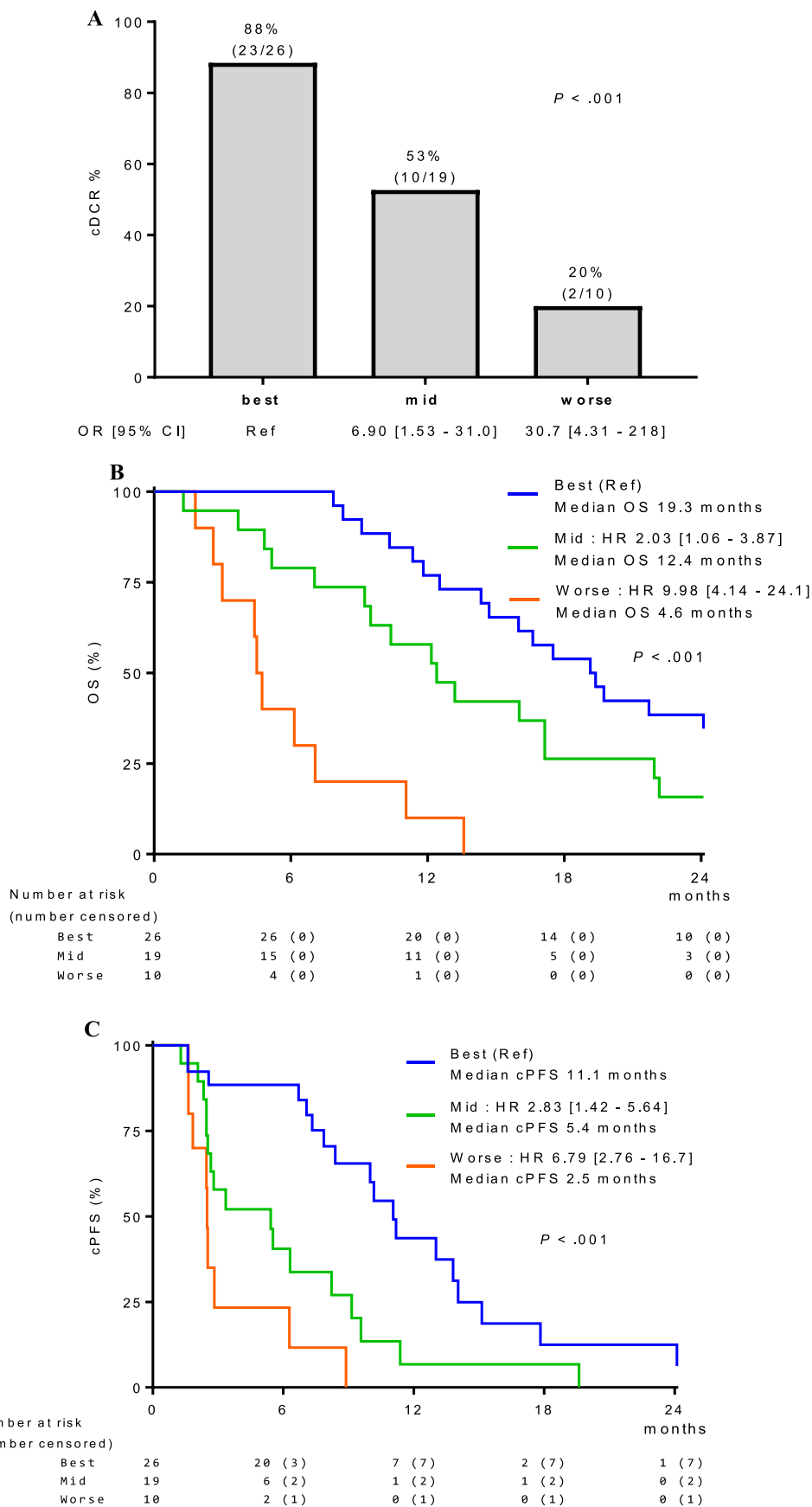


Fig. 3. Association combined score with level of cfDNA at baseline and level of KRASmut ctDNA at D28 and centralized disease control rate (cDCR), overall survival (OS) and progression-free survival according to centralized review (cPFS). Combined score “best”: cfDNA at D0 <30 ng/mL and no KRASmut ctDNA at D28; Combined score “mid”: cfDNA at D0 ≥30 ng/mL or KRASmut ctDNA at D28 >0%; Combined score “worse”: cfDNA at D0 ≥30 ng/mL and KRASmut ctDNA at D28 >0%. A. Centralized disease control rate according to the combined score. cDCR: centralized disease control rate; S: stable disease; PR: partial response; Ref: reference; OR: odds ratio. B. Overall survival according to the combined score. C. Centralized review progression-free survival according to the combined score.

score able to predict early response to chemotherapy with high efficiency, combining level of cfDNA at diagnosis and presence or not of *KRAS*mut ctDNA at D28. In the best group, PFS was 11.1 months, OS 19.3 months and DCR 88% as compared to 2.5 months, 4.6 months and 20% in the worst group.

In our study, patient characteristics were similar as compared to populations in randomized trials [4,22]. Median PFS of 6.3 months and median OS of 11.8 months are concordant with data in the literature for patients with UPA treated with first-line chemotherapy [4,22]. Only 2% of patients in our study had partial response, which is quite low as compared to previous studies, 31.6% with FOLFIRINOX and 23% with gemcitabine plus nab-paclitaxel [4,22]. We have no explanation for this result, but all imaging was reviewed by an expert digestive radiologist. Patients with locally advanced tumor (40%) and metastatic tumor (60%) were analyzed together, which has an impact on survival results since OS of patients with locally advanced UPA is around 15 months versus 11 months for patients with metastatic UPA [4,23]. Most UPAs are *KRAS*-mutated (94%), in accordance with recent series using ddPCR or NGS (from 75% to 100%) [13,17,19,24–27]. In our KRASCIPANC study, while the type of *KRAS* mutation was not available using multiplex ddPCR, the spectrum of the *KRAS* mutation in UPA is already well-known and the aim of our work was to determine the prognostic value of circulating *KRAS*mut ctDNA, whatever the type of *KRAS* mutation [8]. In our series, 84% of patients had a *GNAS*-mutated tumor, which seems higher than results in recent studies, probably because of higher sensitivity of ddPCR than NGS, which was used in the few studies already published [11–13].

Few studies have assessed prognostic value of cfDNA level in patients with UPA, especially without considering other prognostic factors. In our study, median cfDNA level was 14.2 ng/mL as compared to 8.7 to 105.8 ng/mL in other recent series [17,26,28,29] and was higher in patients with liver metastases and in females, as previously described [26,28,30,31]. cfDNA level was associated with high levels of LDH, CA19.9 and CEA, which probably reflects the aggressiveness of the disease and rapid tumor cell turnover [32,33]. Moreover, baseline cfDNA was significantly associated with OS, PFS and DCR, as previously shown in the literature [27,28].

*KRAS*mut ctDNA at baseline was detectable in 62% of patients with *KRAS*-mutated UPA in our series. This result is concordant with other studies with a detection rate of *KRAS*mut ctDNA ranging from 56 to 86% [17–19,26,29]. Analysis of locally advanced and metastatic UPA together probably explains that our detection rate of *KRAS*mut ctDNA was within the lowest range. Although we do not have the statistical power to separately analyze metastatic versus locally advanced tumors, there is no scientific rationale to suggest that tumor stage can influence the predictive value of treatment efficacy of circulating DNA. Recent studies confirm that cfDNA/ctDNA are also associated with treatment response in borderline/locally advanced pancreatic adenocarcinoma [34,35]. Sensitivity to detect ctDNA in UPA using multiple mutation panels or methylation assay with NGS or ddPCR has not provided higher sensitivity (48 to 68%) [17,20,36]. As expected, we observed a strong association between *KRAS*mut ctDNA and cfDNA levels. In our study, *KRAS*mut ctDNA rate was strongly associated with PFS and OS, in accordance with other series [17,18,20,36].

*GNAS*mut ctDNA at baseline was detectable in only 33% of patients with *GNAS*-mutated tumors and was not associated with survival. In the literature, presence of *GNAS* mutations in cfDNA has been evaluated only in patients with IPMN and was significantly higher than in those with other pancreatic cystic neoplasms (32% versus 0%) [37]. In our study, the rate of detectable *GNAS*mut ctDNA was low probably due to the low MAF of *GNAS* mutation in tumors (0.25% and 0.26% for R201H and R201C mutations, respectively) as compared with the MAF of *KRAS* mutations in tumors (10.4%). In the literature, there are no data concerning the prog-

nostic value of *GNAS*-mutated ctDNA. *GNAS* mutations are probably passenger mutations with no prognostic impact and do not reflect the aggressiveness of the tumor [38,39].

Decreased cfDNA between baseline and D28 was not correlated with survival. Literature data of cfDNA kinetic are lacking but in one published series there was no significant change of cfDNA level during treatment [40]. More surprisingly, decreased *KRAS*mut ctDNA was not correlated with survival, but as shown in the Bernard et al. study, serial ctDNA level did not correlate significantly with presence or absence of progression [41]. Some other studies found a significant association of ctDNA kinetics with PFS and OS [18,19,25]. Nevertheless, in our study the detection or non-detection of *KRAS*mut ctDNA at D28 was strongly associated with DCR, PFS and OS. Indeed, median OS was 2.8 versus 10.0 months in patients with or without *KRAS*mut ctDNA, respectively. This result is in accordance with other studies [27,42].

As already published, ECOG-PS, tumor stage, liver metastasis, albumin, bilirubin and CA19–9 levels were associated with DCR, PFS and/or OS [4,27,43]. In our study in multivariate analysis cfDNA at diagnosis, presence of *KRAS*mut ctDNA at D28 was the only factor associated with DCR, PFS and OS. No study has previously performed a multivariate analysis to demonstrate that cfDNA/ctDNA outperformed common prognostic factors in UPA. Finally, we built a combined score using level of cfDNA at diagnosis and presence or absence of *KRAS*mut ctDNA at D28 to predict early response to chemotherapy. In the best group PFS was 11.1 months and DCR 88%, as compared to 2.5 months and 20% in the worst group. None of the previously published scores/ctDNA cut-offs presented performance as likely as ours to predict survival and response to treatment [18,19,25,27,34,36,42].

While our results are very promising, there remain some issues to assess before use in routine clinical practice. KRASCIPANC is a prospective study, but the population was heterogeneous regarding first-line treatment, and we analyzed locally advanced or metastatic stages together, which could cause difficulties of interpreting the survival results. By contrast to NGS, ddPCR has higher sensitivity but is not able to analyze a large panel of molecular alterations. Nevertheless, more than 90% of UPAs are *KRAS*-mutated. By contrast to all previously published series, the main strengths of the KRASCIPANC study consist in its assessment of both cfDNA and *KRAS*mut ctDNA and in its performance of a kinetic analysis of ctDNA with multivariate analyses. To our knowledge, our study is the only one to have built a combined score with cfDNA and ctDNA, which enables robust prediction of treatment efficacy.

To conclude, our combined score, using cfDNA at baseline and ctDNA at day 28, is one of the most robust parameters to predict treatment response in UPA, as opposed to common predictors such as CA19.9 or cfDNA levels alone. While the score requires validation in an independent series, it may soon be used to optimize first-line treatment in UPA. Earlier than CT-scan, the combined score can detect patients who will have disease progression. Indeed, based on KRASCIPANC results, we have planned a prospective phase II trial where patients with metastatic UPA are randomized between continuation of first-line chemotherapy versus switching to second-line treatment before the CT-scan results, according to cfDNA/ctDNA results, the objective being to increase overall survival.

Ethics statement

Approval of the research protocol by an Institutional Reviewer Board

Protocol was approved by the French ethics committee “Comité de Protection des Personnes Ouest III” (DC 2016–2631). The study was conducted in accordance with the precepts of the Helsinki declaration.

Informed consent

Written informed consent was obtained from all patients before study entry.

Registry and the Registration No. of the study/trial

The study was registered in ClinicalTrials.gov with the number NCT04560270.

Data availability statement

David Tougeron and Camille Evrard had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Data are available upon reasonable request.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author contributions

Concept and design: David Tougeron and Camille Evrard

Acquisition, analysis, or interpretation of data: all authors

Drafting of the manuscript: Camille Evrard, David Tougeron and Pierre Ingrand

Critical revision of the manuscript for important intellectual content: all authors

Statistical analysis: Pierre Ingrand, David Tougeron and Camille Evrard

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.dld.2023.03.011](https://doi.org/10.1016/j.dld.2023.03.011).

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