SOMATO PUBLICATIONS ISSN: 2643-2609

Annals of Gastroenterology and Digestive Disorders

Research Article

Molecular Profiling and Prognostic Value of KRAS Gene Mutations in Tumor Samples and Liquid Biopsies from Patients with Pancreatic Ductal Adenocarcinoma

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Received: 28 November 2022; Accepted 25 December 2022; Published: 28 December 2022

Citation of this article: Morão, B., Oliveira, B., Costa, B., Saraiva, S., Fidalgo, C., Faias, S., et al. (2022) Molecular Profiling and Prognostic Value of KRAS Gene Mutations in Tumor Samples and Liquid Biopsies from Patients with Pancreatic Ductal Adenocarcinoma. Ann Gastroenterol Dig Dis, 5(1): 01-15.

Abstract

Introduction: The lack of sensitive, cheap, and fast molecular technologies for detecting KRAS mutations in pancreatic ductal adenocarcinoma (PDAC) patients has hampered its translation into clinical practice. We validated the fast single-step methodology based on amplification-refractory mutation system(ARMS) coupled with high resolution melting analysis(HRMA) to assess KRAS mutations and its prognostic value in PDAC patients.

Methods: Prospective multicentric cohort study including PDAC patients with codon-12 KRAS mutational status determined by Sanger Sequencing (SS) on tumor and plasma samples collected at diagnosis. ARMS/HRMA was further applied to assess the two most frequent mutations (G12D/G12V). Primary endpoint was the frequency of KRAS mutations by both techniques and their concordance. Secondary endpoints were the association of KRAS mutations with progression free survival (PFS) and overall survival (OS).

Results: Of 55 patients, 29 underwent resection surgery and 26 underwent palliative chemotherapy (ChT) or best supportive care. ARMS/HRMA was performed in 38/55 tumor samples (6 with G12R/G12C mutations by SS and in 11 there was no DNA left after SS). SS detected KRAS mutations in 23/38(60%) and ARMS/HRMA in 33/38(87%) tumor samples (G12D=18, G12V=12, G12V/G12D=3). DNA was successfully extracted in 32/55 plasma samples: SS genotyping was conclusive in 22/32(69%) and only detected a G12V mutation, while ARMS/HRMA was conclusive in all samples and detected mutations in 7 (G12V=3, G12D=3, G12V/G12D=1). Operated patients with KRAS-mutated tumors had lower PFS (5 vs 13 months, $p<0.01$) and OS (9 vs 14 months, $p<0.01$). ChT treated patients with KRAS-mutated ctDNA had lower PFS (1 vs 6 months, $p=0.02$) and lower OS (1 vs 8 months, $p<0.01$).

Conclusions: ARMS/HRMA seems to be highly sensitive and more accurate than SS to assess KRAS mutations in PDAC tumor and plasma samples, with advantages of fastness and low cost. KRAS mutations were associated with lower OS and PFS. Future studies should corroborate our findings and compare ARMS/HRMA to other molecular techniques.

Keywords: Pancreatic cancer, circulating tumor DNA, ctDNA, liquid biopsy, KRAS, genotyping, amplification-refractory mutation system, ARMS/HRMA, prognosis

Key Summary

- We validated the Amplification-refractory mutation system (ARMS) coupled with high resolution melting analysis (HRMA) technology to assess tumor and ctDNA codon-12 KRAS mutations and its prognostic value in a small cohort of PDAC patients
- ARMS/HRMA technology seems to be a faster, more accurate and cheaper diagnostic tool to assess KRAS mutations in PDAC tumor samples and liquid biopsies, with a higher sensitivity compared to Sanger Sequencing
- Tumor and ctDNA codon-12 KRAS mutations were associated with lower OS and PFS, namely the G12D mutation
- Future studies should corroborate our results and compare ARMS/HRMA to other molecular techniques

Introduction

Current trends suggest that pancreatic cancer (PC) will become the second leading cause of cancer-related deaths and the most lethal digestive cancer by 2030 [1,2], with pancreatic ductal adenocarcinoma (PDAC) accounting for 85% of cases. The 5-year survival rate is only 9% if all stages combined [3]. This discouraging scenario is mainly explained by a late diagnosis and the lack of an effective therapy. Surgical resection followed by adjuvant chemotherapy (ChT), the only potentially curative therapy, is only possible in a minority of patients. Novel biomarkers to be used as diagnostic, predictive tools of response and targets to tailored therapy, may contribute to improve the prognosis [4].

KRAS mutation occurs early in pancreatic carcinogenesis, and is present in at least 80% of PDAC [5,6]. Over 90% of activating mutations are found at codon 12 of the oncoprotein (p.G12D, p.G12V, p.G12R, pG12C or p.G12A) corresponding to single-nucleotide mutations at the cDNA sequence (c.35G>A, c.35G>T, c.34G>C, c.34G>T or c.35G>C, respectively) [7,8]. Although there are mixed findings in the literature, several studies have already demonstrated the prognostic value of *KRAS* mutations in tumor samples and liquid biopsies in patients with PDAC [9]. Tumor KRAS G12D mutation has been shown to be an independent predictor of worse overall survival in patients with PDAC, while KRAS G12V mutation was associated with better tumor regression following chemotherapy and radiotherapy in borderline and locally advanced PDAC [10-12]. However, there are conflicting findings and KRAS G12V-mutant tumors have also been proposed has having worse outcomes than other subtypes [13,14]. Additionally, the presence and levels of circulating tumor DNA (ctDNA) harboring *KRAS* mutations have been shown to be a predictor of worse overall survival (OS) in patients with PDAC and changes in its concentration during ChT were better to predict response to treatment than changes in CA 19.9 [15-18]. In one of these studies, levels of ctDNA correlated with disease status, with higher levels found in PDAC patients than in healthy controls; furthermore, ctDNA levels lowered after resection surgery, suggesting that ctDNA levels may reflect disease burden [18]. Most recently, treatment with a KRAS G12C selective inhibitor in patients with locally advanced or metastatic PDAC resulted in clinically meaningful responses (objective response 21%, disease control 84%, median duration of response 6 months) [19], adding to the importance of assessing *KRAS* mutational status in PDAC.

Despite recent advances, the detection ctDNA is still demanding due to its low abundance in peripheral blood, and high-sensitivity molecular techniques are needed [20]. The lack of standard methods for detection of ctDNA readily available in clinical practice and at a low-cost, have prevented wider use of ctDNA technology [21].

The purpose of this study was: 1) To further validate a single-step methodology based on Amplification-Refractory Mutation System (ARMS) coupled with High-Resolution Melting Analysis (HRMA) to assess codon 12 KRAS mutations in a cohort of PDAC patients with stored tumor and plasma samples 2) To assess its prognostic value regarding disease progression and all-cause mortality.

Materials and Methods

Study design and inclusion criteria

We conducted a longitudinal, multicentric, prospective cohort study including patients with newly diagnosed and histologically proven PDAC treated at the referral center Hospital Beatriz Ângelo (Loures, Portugal) and Hospital da Luz (Lisbon, Portugal), between October 2017 and May 2021. According to tumor staging and performance status (PS) patients were either referred for direct surgery or for ChT. In the latter, an endoscopic ultrasound-guided fine-needle biopsy (EUS-FNB) was performed to confirm the histological type of the tumor.

We included adult patients (18 years old or older) with newly diagnosed PDAC who were treated in both hospitals, with available tumor and blood samples collected before therapy and with the ability of giving written informed consent. Patients previously treated with surgery, chemotherapy or radiotherapy; those referred to neoadjuvant or palliative chemotherapy but with a biopsy performed at another institution; those unable to undergo surgery or EUS-FNA for histologically proven PDAC; and those who underwent direct surgery with a pancreatic tumor other than

PDAC were excluded from this study. The study protocol was approved by both Hospitals ethical committees. Informed written consent was obtained from all the patients.

During the study period, 256 patients with PDAC were treated at both institutions (Figure 1). We were able to collect tumor and plasma samples at diagnosis from 95 patients that were stored for later analysis. Of these, 55 PDAC patients had available fresh frozen tumor and plasma samples and were included in the present study. Tumor samples were collected during surgery in 28 patients (patients referred for direct surgery) and by EUS-FNB in 27 patients (patients referred for neoadjuvant ChT). We could extract tumor DNA for mutational analysis by SS in all 55 cases, but we did not have enough tumor DNA sample left for further ARMS/HRMA analysis in 11 patients due to cell paucity (6 samples had been collected by EUS-FNB and 5 samples during surgery).

SS of the tumor sample was performed in all 55 patients. For comparison and validation of ARMS/HRMA technology, the 2 most frequent KRAS mutations in codon 12 (G12D and G12V) were analyzed. As 11 patients were excluded from analysis because there was no tumor DNA sample left, and 6 out of 55 samples had a different G12R or G12C mutations, a population of 38 patients was used to compare the performance of SS and ARMS/HRMA technology.

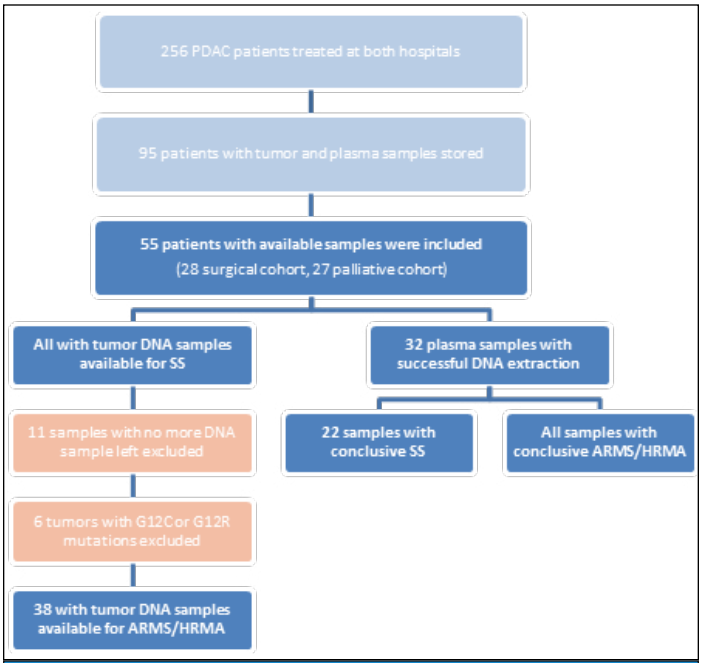

Figure 1: Workflow diagram of patients included.

Tumor sample collection

A tumor sample was collected at diagnosis before any treatment, in one of the following settings: 1) during resection surgery in patients who underwent direct surgery, or 2) during EUS-FNB in patients undergoing neoadjuvant or palliative ChT as first-line treatment. During the preparation of the surgical specimen, a 5 mm of the tumor mass was collected into a sterile 1-ml Eppendorf kept at 4ºC, immediately frozen in liquid nitrogen and stored at -80ºC for later molecular analysis. EUS was performed under intravenous propofol anesthesia using a curved linear array echo-endoscope by Olympus connected to Aloka ultrasound device. EUS-FNB was performed using a 22-gauge needle. A minimum of two needle passes were performed for diagnosis. Sample adequacy was either evaluated by Rapid On-site Evaluation by a pathologist at the endoscopy room or by Macroscopic On-Site evaluation. When the sample obtained was considered to be inadequate for diagnosis, an extra needle pass was performed to collect material for the study, never exceeding a total of five passes in order avoid adverse events. Core-biopsy samples were transferred into Cytolyt media and were used for histological diagnosis according to usual clinical practice.

Blood sample collection

Blood samples were collected at diagnosis before any treatment. A small amount of blood (8mL) was collected into 2 EDTA blood tubes and immediately kept at 4ºC; they were transported to the laboratory at 4ºC in a maximum period of 4 hours (ideally 2 hours). Blood components were then separated by centrifugation at 1000G at 4ºC and placed in cryotubes: 4 containing plasma and 1 containing red blood cells. Blood components in cryotubes were fresh frozen in liquid nitrogen and stored and kept at -80ºC for later analysis.

KRAS mutation analysis

Materials and reagents: For DNA Isolation the High Pure PCR

Template Preparation Kit (Roche, Basel, Switzerland) was used. $MgCl_2$, dNTPs, NZYTaq II DNA Polymerase and NZYSpeedy qPCR Green Master Mix (2x) were purchase from NzyTech, Lisbon, Portugal. DMSO was purchase from Sigma-Aldrich, St. Louis, Missouri, USA. The DNA size marker GeneRulerTM DNA Ladder Mix was purchased from Fermentas (Burlington, Canada). GelRed® Nucleic Acid Gel Stain was purchased to Biotium (California, USA). All the primers were purchased from STAB VIDA, Lda (Caparica, Setúbal, Portugal).

DNA Extraction: DNA extraction from PDAC patients' tumor and plasma samples was performed using the High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions (from solid tissue and whole blood/plasma). AASSElution volume was altered to 50 µL for tumors and 30 µL for plasmas. DNA purity and yield were quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

PCR amplification to assess KRAS exon 2 mutational status with SS: S50 ng of tumor/ plasma DNAs were used for PCR amplification using 0.12 µM of each primer (*KRAS* forward: 5′-GGTGGAGTATTTGATAGTGTA-3′; *KRAS* reverse: 5′- TGGACCCTGACATACTCCCAAG -3′), 2 mM of $MgCl_2$ (NzyTech, Lisbon, Portugal), 0.8 mM of dNTPs Mix (NzyTech, Lisbon, Portugal) and 0.15 U of NZYTaq II DNA Polymerase (NzyTech, Lisbon, Portugal) for a final volume of 20 µL of reaction. Reactions were performed on a MyCycler Thermocycler (Bio-Rad, Hercules, California, USA) using a denaturing step of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 61°C for 30 s for tumor and 53°C for 30 s for plasma samples, and 72°C for 20 s. For each plasma samples four independent amplification reactions were performed, concentrated in a SpeedVac (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and resuspended in 15 µL of DEPC-treated water. PCR products were then direct sequenced by STAB VIDA (Caparica, Setubal, Portugal), and the chromatograms analyzed using FinchTV software (Geospiza, Inc) for sequence characterization and identification of possible mutations in exon 2.

ARMS-HRMA for detection of KRAS G12V and G12D mutations: Tumor and plasma DNA samples were analyzed for the G12D and G12V KRAS point mutations through ARMS/HRMA (amplicon size 96 bp). The reaction mixture consisted of 4 ng of template DNAs, 0.3 µM of allele specific forward primers and a common reverse primer (*KRAS* Forward G12V 5′-CTTGTGGTAGTTGGAGCTTT-3′; *KRAS* Forward G12D 5′-CTTGTGGTAGTTGGAGCTTA-3′; *KRAS* Reverse 5′-CTCTATTGTTGGATCATATTCG-3′), 2% (v/v) of DMSO (Sigma-Aldrich, St. Louis, Missouri, USA), 1x of NZYSpeedy qPCR Green Master Mix (2x) (NzyTech, Lisbon, Portugal) in a final reaction volume of 10 µL.

ARMS-HRMA reactions were performed on a Corbett Rotor-Gene 6000 (Qiagen, Hilden, Germany) thermocycler. Briefly, an initial denaturation at 95°C for 3 min, 10 cycles of 95°C for 30 s, 52°C for 15 s for G12V mutation and 54°C for 15 s for G12D mutation, and 72°C for 10 s; followed by 25 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 10 s. After ARMS reaction, amplification products were assessed by HRMA with an initial increase of temperature from 45°C to 95°C, rising at 0.2°C per step/wait 5 sec each step. The resulting derivative plot was generated using Rotor-Gene 6000 Series Software 1.7 (Qiagen, Hilden, Germany).

Variables and endpoints

Baseline demographic and clinical data included gender, age, date of diagnosis, Eastern Cooperative Oncology Group (ECOG) performance status, serum CA 19.9 level, disease stage according to American Joint Committee on Cancer (AJCC) TNM classification and resectability status according to National Comprehensive Cancer Network (NCCN). During follow-up, the following variables were collected: occurrence and date of disease progression (either locally or metastatic) detected on computed tomography or magnetic resonance cholangiopancreatography, and date of last follow-up or death. The primary endpoint was the frequency of codon 12 KRAS mutations detected in the primary tumor and liquid biopsies of patients with PDAC by ARMS/HRMA technology and its concordance with Sanger sequencing. The secondary endpoints were the association of *KRAS* mutations in tumor and liquid biopsies with the occurrence of disease progression and death during

follow-up. Disease progression was defined by radiological and/or serologic criteria. The former was considered as the appearance of new infiltrative disease on CT or MRI during follow-up, either locally or metastatic. The latter was defined as CA 19-9 elevation in association with clinical deterioration, despite no radiological evidence of disease progression.

Follow-up and treatment

After histological confirmation of PDAC, patients were routinely managed according to the standard of care and international guidelines. The *KRAS* status in tumor and plasma samples of each patient was only evaluated later, and as such, did not influence therapeutic decision.

Statistical analysis

Data was processed and analyzed with IBM SPSS Statistics 22® (Statistical Package for Social Sciences, IBM®, Armonk, NY). Categorical variables were described through absolute and relative frequencies and continuous variables as mean and standard deviation, median, minimum, and maximum. Hypotheses about the distribution of categorical variables were tested by using the Chi-square test. Hypotheses about the distribution of continuous variables were tested by using the independent sample t-test/one-way Anova or nonparametric Mann-Whitney and Kruskal-Wallis test, depending on if normal or non-normal distribution, respectively, and considering the nature of the hypothesis. All hypotheses were tested at 5% level of significance. Survival analysis was performed using the Kaplan-Meier method, and comparison of survival rates between the group was performed using the log-rank test. Overall survival (OS) was defined as the length of time from the date of diagnosis that patients are still alive, taking into consideration death by any cause. Progression free survival (PFS) was defined as the length of time during and after treatment that patients lived with the disease, until the occurrence of either disease progression or death.

Results

Population baseline characteristics

Patients' characteristics according to tumor *KRAS* genotype are shown in Table 1. Among the 55 patients included, 32 (58%) presented a mutation in codon 12 of KRAS protein by SS. The most frequent mutation was c.35G>A (G12D; 15 patients; 27%), followed by c.35G>T (G12V; 11 patients; 20%), c.34G>C (G12R; 4 patients; 7%) and c.34G>T (G12C; 2 patients; 4%). No other *KRAS* mutations were found. Baseline characteristics did not differ between patients with wild-type and mutated *KRAS*, neither between the different *KRAS* mutations, namely sex distribution, age, performance status score, TNM stage, clinical stage, and general treatment (Table 1).

Patient management according to clinical staging is summarized in Figure 2. All 7 patients with borderline resectable disease underwent neoadjuvant ChT with FOLFIRINOX regimen. Of these patients, only 3 (43%) were operated; the remaining four were kept on palliative ChT because of disease progression or because they no longer had surgical conditions. Of the 28 patients with

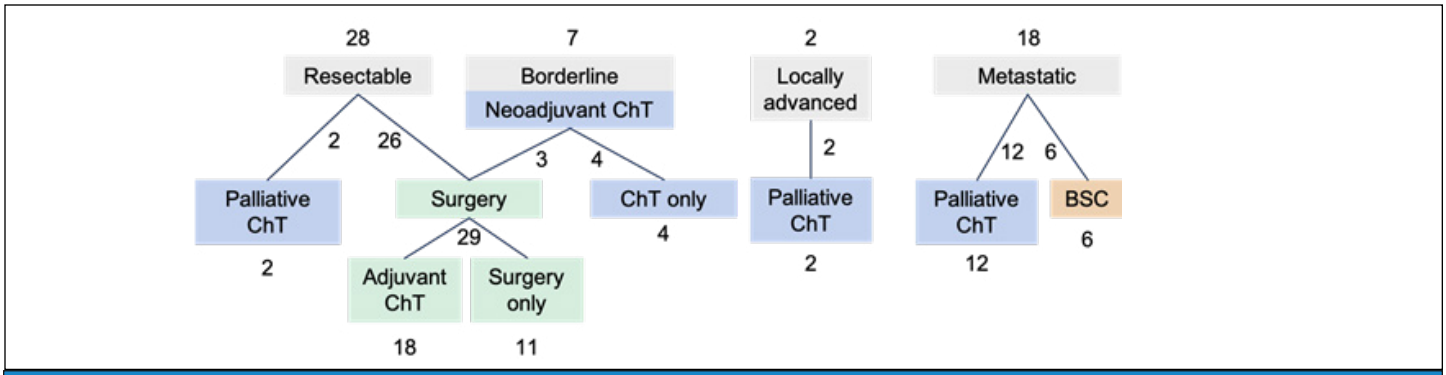

Figure 2: Patient management according to clinical stage. Numbers represent number of patients.
Abbreviations: ChT – Chemotherapy; BSC – Best Supportive Care

Table 1: Baseline patient's characteristics according to tumor *KRAS* genotype.

	Total	KRAS-wt	KRAS-mut	P	G12D	G12V	G12R	G12C	p
Gender									
Male	28	14	14	0.21	6	7	1	0	0.19
Female	27	9	18		9	4	3	2	
Age, years (median) Mean±std.dev	70 70±9	70 70±7	70 69±10	0.90	76 73±11	68 66±11	70 66±4	65 65±9	0.34
ECOG PS score									
≤ 1	50	21	29	0.93	13	10	4	2	0.70
> 1	5	2	3		2	1	0	0	
CA 19-9, UI/L									
Within normal range	17	5	12	0.24	7	2	2	1	0.56
Elevated (>37 UI/L)	32	15	17		7	7	2	1	
Mean±Std.dev	3043±15554	998±2788	4453±20109	0.99	265±358	12957±35969	1890±2516	628±884	0.35
Median (IQR)	136 (1-108848)	98 (1-12547)	230 (1-108848)		70 (1-1049)	1120 (1-108848)	1117 (5-5321)	628 (3-1253)	
TNM stage									
I	12	6	6	0.59	5	1	0	0	0.28
II	13	4	9		2	4	2	1	
III	11	6	5		4	1	0	0	
IV	19	7	12		4	5	2	1	
Clinical stage									
Resectable	28	12	16	0.99	9	5	1	1	0.43
Borderline resectable	7	3	4		3	0	1	0	
Locally advanced	2	1	1		0	1	0	0	
Metastatic	18	7	11		3	5	2	1	
Treatment									
Surgery ± ChT	29	14	15	0.40	8	5	1	1	0.74
Only ChT (palliative)	20	6	14		6	4	3	1	
Best supportive care	6	3	3		1	2	0	0	

Abbreviations: KRAS-wt – KRAS wild-type; KRAS-mut – KRAS-mutated; ECOG PS – performance status; ChT – chemotherapy; p – p value

resectable disease, two were unfit for surgery and were also offered palliative ChT.

First-line adjuvant ChT regimens included gemcitabine monotherapy in 5 (28%) patients, gemcitabine plus capecitabine in 5 (28%) patients, FOLFIRINOX in 6 (33%) patients and capecitabine monotherapy in 2 (11%) patients. Of the 20 patients with locally advanced (unresectable) or metastatic disease, only 14 (70%) were treated with palliative ChT due to poor performance status in the remaining six. These six patients had a rapid deterioration and received best supportive care only. First-line palliative ChT regimens included FOLFIRINOX in 8 (57%) patients, gemcitabine plus nab-paclitaxel in 4 (29%) patients and gemcitabine monotherapy in 2 (14%) patients.

Performance of ARMS/HRMA technique compared to sanger sequencing

Tumor samples: ARMS is based on the use of primers whose 3´

Table 2: Cross-tabulation of tumor KRAS genotype according to ARMS/HRMA and SS.

		Sanger sequencing			Total
		WT	G12V	G12D	
ARMS/ HRMA	WT	5	0	0	5
	G12V	4	8	0	12
	G12D	5	0	13	18
	G12V/ G12D	1	2	0	3
Total		15	10	13	38

Table 3: Cross-tabulation of plasma KRAS genotype according to ARMS/HRMA and SS.

		Sanger sequencing		Total
		WT	G12V	
ARMS/ HRMA	WT	15	0	15
	G12V	2	1	3
	G12D	3	0	3
	G12V/G12D	1	0	1
Total		21	1	22

Table 4: Tumor KRAS genotype in the surgical cohort.

		N	%
KRAS genotype	Wild-type	14	49
	G12D	8	28
	G12V	5	17
	G12R	1	3
	G12C	1	3
Total		29	100

nucleotides are allele specific. Nevertheless, in the case of heterozygous samples (G12V/wt or G12D/wt), ARMS amplification as a stand-alone technique still shows amplification with both nucleotide specific primers (G12V mutated primer or G12D mutated primer) and WT primer. Additionally, the specificity and sensitivity of the ARMS reaction is dependent on the nucleotide that causes the mismatch; there are pairs of mismatches that cause higher destabilization of the polymerase, such as the one present in G12D mutation (GGT→GAT). On the contrary, for the G12V mutation (GGT→GTT) the destabilization caused by the mismatch is lower, meaning the polymerase still manages to amplify in the presence of WT samples. To avoid this type of issues, HRMA was immediately performed on the ARMS products (same tube without opening it) for an undoubtedly discrimination between genotypes, mainly the heterozygous G12V. In fact, HRMA is a more recent method that stands out for being fast and cost-effective for high-throughput mutation screening [22,23]. HRMA is based on the dissociation behaviour of DNA when subjected to increasing temperatures, in the presence of saturating fluorescent dyes with greater affinity for double-strand DNA than for single-strand DNA [22-24]. HRMA has been shown to detect between 3% to 10% of mutant DNA in a background of wild-type DNA [22]. In this regard, ARMS/HRMA was optimized to detect the most frequent mutations in KRAS (G12D and G12V). After excluding patients harbouring G12C and G12R mutations (6 out of 55 tumors) and 11 samples with no DNA available after SS, a population of 38 patients was used to compare SS and ARMS/HRMA techniques. Results of SS and ARMS/HRMA were successfully obtained from all the 38 tumor samples and are summarized in table 2. SS detected *KRAS* mutations in 23 (60%) patients: G12D in 13 and G12V in 10 patients. ARMS/HRMA was able to detect the same *KRAS* mutations detected by SS and an additional 10 mutated samples, with an overall of 33 *KRAS* mutated (87%) patients: G12D in 18, G12V in 12 and both G12V/G12D in 3 patients. There was a good agreement between the two techniques (kappa 0.215, p<0.001).

Liquid biopsies (Plasma samples): Regarding liquid biopsies, we were able to extract DNA from plasma samples to determine *KRAS* genotype by SS and ARMS/HRMA in 32 patients. As SS was not conclusive for 10 patients due to poor sequencing quality, only the results for the remaining 22 patients were further compared. SS was able to detect a single KRAS G12V mutation in 1 (4.5%) patient. ARMS/HRMA was successful in detecting *KRAS* mutation in the same patient (from SS) and in 6 additional patients (total of 32%): G12V in 3, G12D in 3 and G12V/G12D in 1 patient. Results are summarized in table 3. There was a good agreement between the two techniques (kappa 0.265, p<0.001).

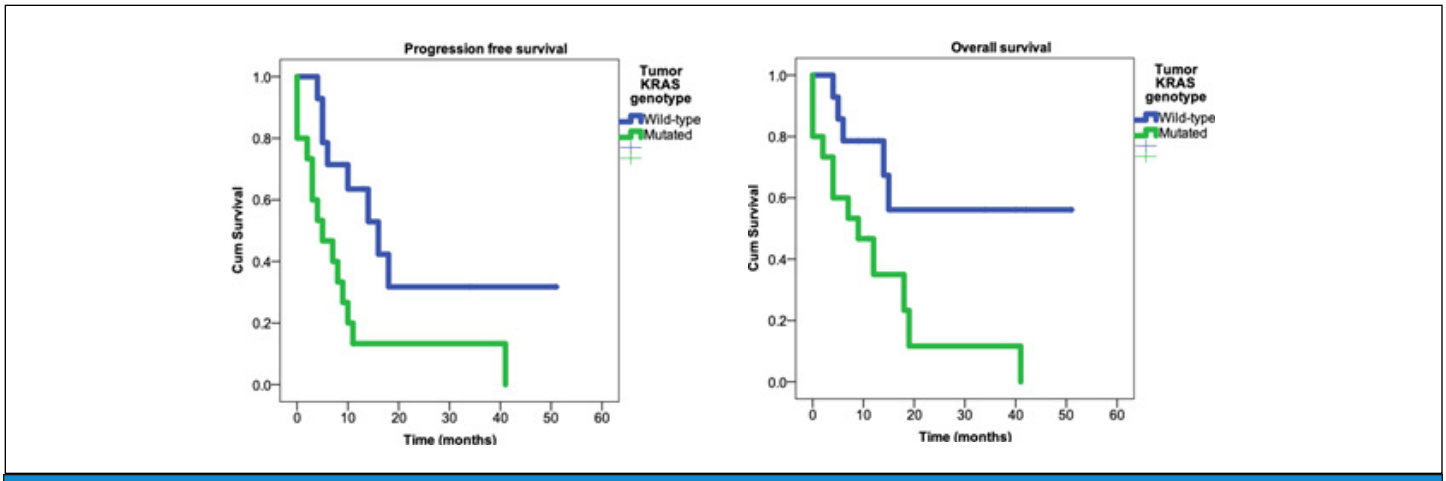

Figure 3: Kaplan-Meier curve for progression free survival (left, p<0.01) and overall survival (right, p<0.01) in the surgical cohort, according to tumor KRAS mutational status.

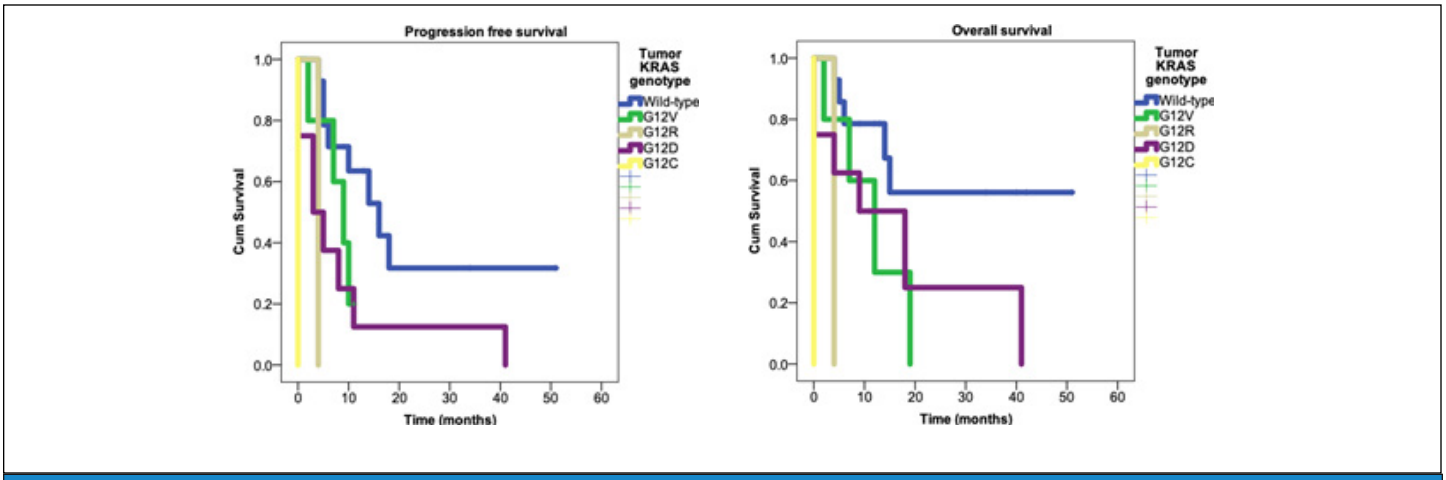

Figure 4: Kaplan-Meier curve for progression free survival (left, p<0.01) and overall survival (right, p<0.01) in the surgical cohort, according to tumor KRAS different mutations.

Prognostic value of tumor KRAS mutations: Mean follow-up time of the overall population was 13±13 months (median 9 months, ranging from 0 to 51 months). In order to assess the prognostic value of *KRAS* mutations, we considered two cohorts of patients according to treatment performed, either surgical resection or palliative chemotherapy/best supportive care.

Surgical cohort: Considering only operated patients (Table 4), we observed that 49% of operated patients (14/29) had a WT tumor. Also, those with tumors harboring *KRAS* mutation had higher mortality (80% vs 36%, p=0.02) – Table 5. No significant differences were found regarding disease progression and/or according to different *KRAS* mutations.

In a survival analysis (Figure 3), *KRAS*-mutated patients had a lower PFS (median 5 vs 13 months, p<0.01), with a lower median PFS in patients harboring G12C, G12R and G12D mutations (G12C=0 vs G12R=4 vs G12D=4 vs G12V=9, p<0.01) – Figure 4. Similarly, *KRAS*-mutated patients had a lower OS (median 9 vs 14 months, p<0.01), with a lower median OS in patients harbor-

Table 5: Events during follow-up in the surgical cohort according to tumor KRAS genotype.

	Total	KRAS-wt	KRAS-mut	p	G12V	G12R	G12D	G12C	p
Progression	11	4 (29%)	7 (47%)	0.32	2 (40%)	0 (0%)	5 (63%)	0 (0%)	0.33
Death	17	5 (36%)	12 (80%)	0.02	4 (80%)	1 (100%)	6 (75%)	1 (100%)	0.8

Abbreviations: KRAS-wt – KRAS wild-type; KRAS-mut – KRAS-mutated; p – p value. Results in N (%).

ing G12C, G12R and G12D mutations (G12C=0 vs G12R=4 vs G12D=9 vs G12V=11, p<0.01) – Figure 4.

Palliative and BSC cohort: Regarding the cohort of patients submitted to palliative ChT or BSC (Table 6), we observed that 35% (9/26) had WT tumors. Patients with *KRAS* mutated tumors tended to have less disease progression than patients who were *KRAS* wild-type (41% vs 78%, p=0.07) – Table 7. No significant differences were found regarding mortality rate or different *KRAS* mutations.

In a survival analysis, no significant differences were found in PFS and OS between *KRAS*-mutated and *KRAS*-WT, neither between different *KRAS* mutations.

Prognostic value of plasma KRAS mutations

Surgical cohort: In patients submitted to surgery (Table 8), those with ctDNA detectable *KRAS* mutation had a lower rate of disease progression (0% vs 50%, p=0.04). No significant differences were observed regarding mortality rate or between different *KRAS* mutations, although the two patients that died had a ctDNA G12D mutation (Table 9).

In a survival analysis, no significant differences were found in PFS and OS between patients with ctDNA *KRAS* mutation and *KRAS*-WT, neither between different *KRAS* mutations.

Palliative and BSC cohort: Regarding the cohort of patients treated with palliative ChT or BSC (Table 10), no significant differences were found in disease progression and mortality rates between patients with ctDNA KRAS mutation compared to KRAS-WT (Table 11). Similarly, no differences were found between different KRAS mutations.

In a survival analysis, patients who had ctDNA with KRAS mutation had a lower PFS (median 1 vs 6 months, p=0.02) and lower OS (median 1 vs 8 months, p<0.01) – Figure 5. When considering different KRAS mutations no significant differences in PFS and OS were observed between patients harboring G12D (median 1 month) or G12V (median 1 month) mutations; once again, patients with any of these two mutations had a tendency for a lower PFS (median 6 months, p=0.05) and a lower OS (median 8 months, p=0.02) – Figure 6.

Discussion

In the present study, we prospectively evaluated a group of 55 patients with newly diagnosed PDAC, to clarify the prognostic value of *KRAS* mutations. Furthermore, to determine the *KRAS* mutational status we applied and validated a technology based on ARMS coupled with HRMA and compared the results with SS.

We demonstrated that ARMS/ HRMA technique is more sensitive than the gold standard Sanger Sequencing to detect *KRAS* mutations both in tumours and plasma of patients with PDAC. Besides its higher sensitivity, ARMS/HRMA is a faster and cheaper technology, able to provide *KRAS* mutational status in a 6-hour period. Additionally, we observed that these mutations seem to have a prognostic value both in operated and chemotherapy treated patients.

The prognosis of PDAC remains very poor with only 9% of pa-

Table 6: Tumor KRAS genotype in the palliative/BSC cohort.

		N	%
KRAS genotype	**Wild-type**	9	35
	G12D	7	27
	G12V	6	23
	G12R	3	11
	G12C	1	4
Total		26	100

Table 7: Events during follow-up in the palliative/BSC cohort according to tumor KRAS genotype.

	Total	KRAS-wt	KRAS-mut	P	G12V	G12R	G12D	G12C	p
Progression	14	7 (78%)	7 (41%)	0.07	2 (33%)	2 (67%)	3 (43%)	0 (0%)	0.47
Death	23	8 (89%)	15 (88%)	0.96	5 (83%)	2 (67%)	7 (100%)	1 (100%)	0.38

Abbreviations: KRAS-wt – KRAS wild-type; KRAS-mut – KRAS-mutated; p – p value. Results in N (%).

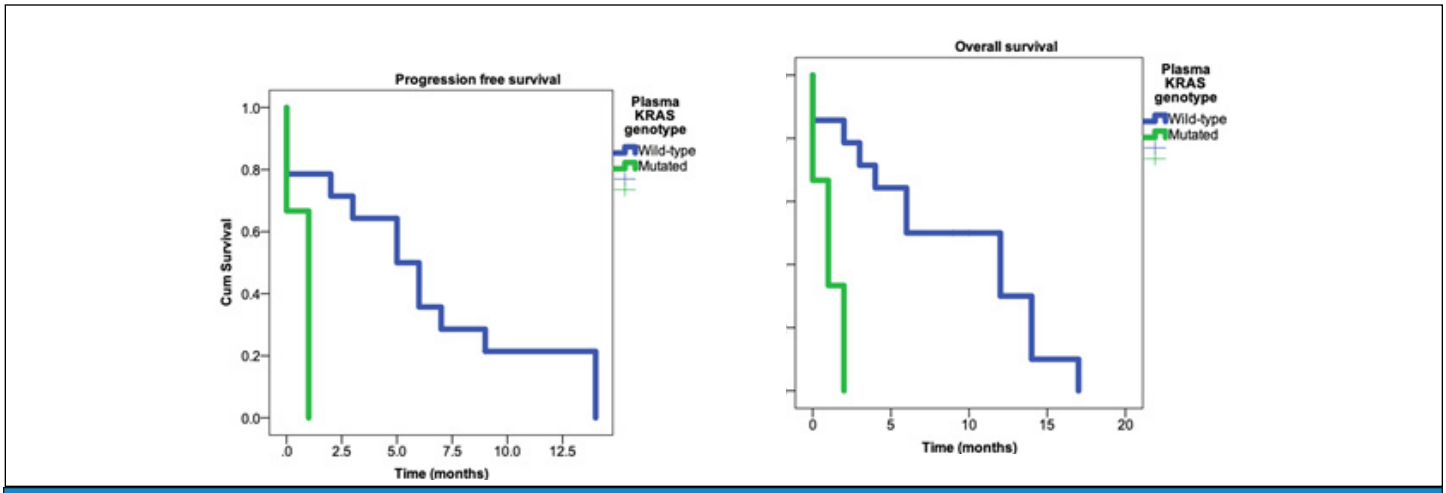

Figure 5: Kaplan-Meier curve for progression free survival (left, p=0.02) and overall survival (right, p<0.01) in the palliative/BSC cohort, according to plasma KRAS mutational status.

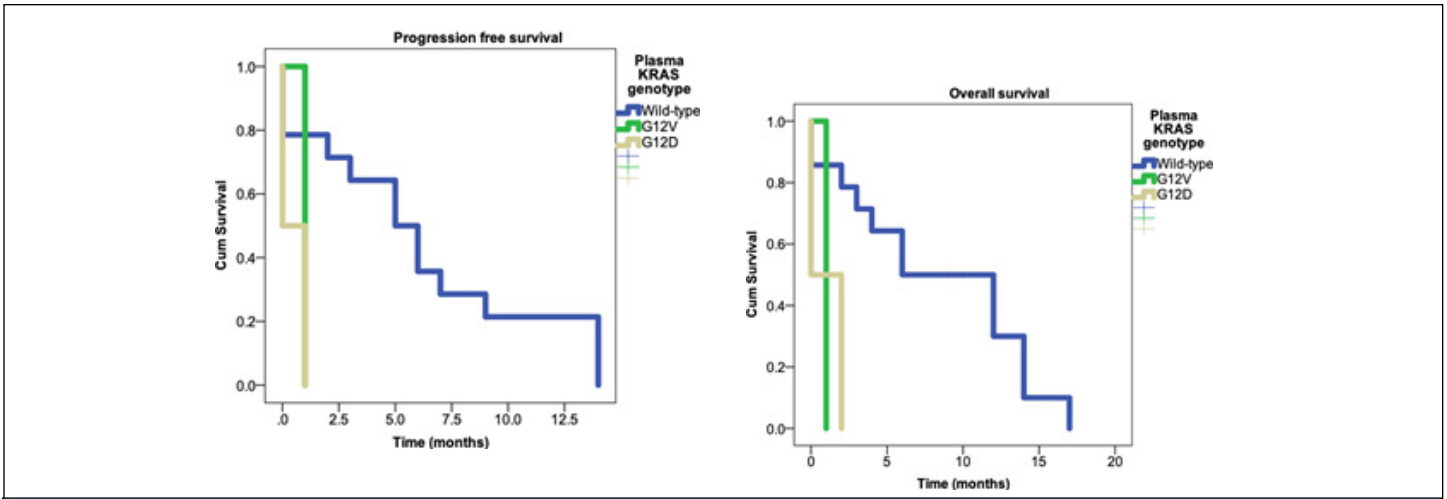

Figure 6: Kaplan-Meier curve for progression free survival (left, p=0.05) and overall survival (right, p=0.02) in the palliative/BSC cohort, according to plasma KRAS different mutations.

tients being alive at 5 years. Besides a late diagnosis in most of the cases, very little advances have been made in tailored therapies and all tumours are treated equally irrespective of their molecular characteristics. Currently, therapeutic decisions are based solely on cTNM staging despite previous studies showing the prognostic value of the type of *KRAS* mutations, an early and almost universal event in pancreatic carcinogenesis. Namely, *KRAS* G12D muta-

Table 8: Plasma KRAS mutational status in the surgical cohort.

		N	%
KRAS genotype	Wild-type	11	73
	G12D	1	7
	G12V	2	13
	G12V/G12D	1	7
Total		15	100

Table 9: Events during follow-up in the surgical cohort according to plasma KRAS genotype.

	Total	KRAS-wt	KRAS-mut	p	G12V	G12D	G12V/ G12D	p
Progression	5	5 (50%)	0 (0%)	0.04	0 (0%)	0 (0%)	0 (0%)	0.27
Death	6	4 (36%)	2 (50%)	0.64	0 (0%)	1 (100%)	1 (100%)	0.12

Abbreviations: KRAS-wt – KRAS wild-type; KRAS-mut – KRAS-mutated; p – p value. Results in N (%).

Table 10: Plasma KRAS mutational status in the palliative/BSC cohort.

		N	%
KRAS genotype	Wild-type	14	82
	G12V	1	6
	G12D	2	12
Total		17	100

tion has been shown to be an independent predictor of worse survival whereas G12V mutations might identify those tumours that would respond better to chemo/radiotherapy [10–12]. One of the major reasons for molecular analysis not being considered in the therapeutic decisions of these patients, is certainly linked to the complexity and costs of molecular analysis which is incompatible with having available results in a short period of time for clinical decisions.

In this study, we validated a technology based on ARMS coupled with HRMA for a cohort of PDAC patients with stored tumor and plasma samples. ARMS/HRMA demonstrated to be a more sensitive technique with 100% specificity, being able to detect *KRAS* mutations in tumor samples in 10 additional patients (overall in 87% of patients) compared to SS (only detected *KRAS* mutations in 60%). This prevalence of *KRAS* mutations detected by ARMS/HRMA is concordant to what is described in the literature using Next Generation Sequencing (NGS) or Droplet Digital PCR [5,6] but this technology is significantly faster and cheaper than NGS.

In regard to its prognostic value, we observed that in the surgical cohort, *KRAS* mutations in the tumor were associated with a lower PFS (median 5 vs 13 months, $p<0.01$) and lower OS (median 9 vs 14 months, $p<0.01$) compared to *KRAS*-WT; furthermore, G12C, G12R and G12D mutations were associated with lower survival times compared to G12V mutation ($p<0.01$). This discriminant value was not observed in chemotherapy treated patients.

In our perspective, these are important findings because at the present moment, although few PDAC patients go for direct surgery, this decision is mostly taken based on criteria of vascular invasion observed on CT/MRI. However, some patients, despite having resectable tumors, relapse shortly after. As pancreatic surgery is still an aggressive procedure with significant morbidity even in high volume centers, having the ability of selecting patients whose tumors have a higher probability of relapsing, could be additional decision criteria. In contrast, in the present study, tumors with *KRAS* mutations do not seem to have a worse prognosis when treated with chemotherapy. It will be important to confirm these observations in a larger sample of patients as well as try to understand whether specific mutations respond better to specific regimens of chemotherapies.

Liquid biopsy is an emerging technology that allows the non-invasive sampling of tumoral genetic material in circulation, and therefore is a promising tool to be used as a prognostic biomarker and to guide therapy [25]. Different types can be considered - circulating DNA, exosomes and circulating tumors cells. The latter are certainly the most sensitive but the more difficult to implement in clinical practice due to the complex and expensive technology involved. Although the sensitivity is much lower when using ctDNA, in a recent meta-analysis, both the presence of mutated ctDNA and higher concentrations of ctDNA were predictors of overall survival and progression free survival in PDAC [26]. Furthermore, ctDNA could potentially be used as a tool for disease monitoring since its detection is associated with the presence of micrometastases and could predict disease relapse before it becomes detectable by imaging [27,28].

Since it is well known that ctDNA has very low concentrations in plasma samples, a highly sensitive molecular analysis is needed to provide robust results [20]. In the present study, ARMS/HRMA

Table 11: Events during follow-up in the palliative/BSC cohort according to plasma KRAS genotype.

	Total	KRAS-wt	KRAS-mut	p	G12V	G12D	p
Progression	10	9 (64%)	1 (33%)	0.73	0 (0%)	1 (50%)	0.37
Death	15	12 (86%)	3 (100%)	0.36	1 (100%)	2 (100%)	0.66

Abbreviations: KRAS-wt – KRAS wild-type; KRAS-mut – KRAS-mutated; p – p value. Results in N (%).

was conclusive in all plasma samples analyzed (100% success rate) while SS had poor sequencing quality in 10 samples (69% success rate). Once again, ARMS/HRMA detected *KRAS* mutations in 7 patients (overall in 32% of patients) compared to SS which only detected *KRAS* mutations in one patient (4.5%). However, in our cohort the prevalence of *KRAS* mutation in ctDNA of PDAC patients is somewhat below to what has been reported in the literature (ranging from 44% to 67%) [29-32]. This can be due to the fact that we only analyzed 2 of the most frequent *KRAS* mutations; furthermore, different molecular technologies were used, as most studies used digital PCR or NGS, with no published reports of ARMS performance in this setting so far. Sample processing and storage might also be of paramount importance and could be further optimized. Despite the low prevalence of *KRAS* ctDNA mutation in our cohort detected by ARMS/HRMA, and considering only patients treated with chemotherapy, we observed that the presence of ctDNA harboring *KRAS* mutation at diagnosis was associated with lower PFS (median 1 vs 6 months, p=0.02) and lower OS (median 1 vs 8 months, p<0.01), with no significant differences being observed between G12D and G12V mutations. In the context of patients undergoing ChT, the use of liquid biopsies longitudinally could be particularly useful for assessing response to therapy. Our analysis was limited to ctDNA assessment only at diagnosis, but recently Kruger et al. have demonstrated that changes in *KRAS*-mutated ctDNA levels during ChT in 54 patients with advanced PDAC were more rapid and pronounced than traditional biomarkers. An early decrease in its levels was an indicator of early response to therapy and repeated measures during follow-up were superior to CA 19.9 in detecting disease progression [29]. Future studies should compare the performance of ARMS/HRMA with other molecular technologies in plasma samples as well as exploring the association of different types of mutations and response to chemotherapy.

In the surgical cohort, ctDNA detection showed no prognostic value which may be explained by our reduced sample size. In this context, a recent study including 112 patients has demonstrated that PDAC patients with detectable ctDNA harboring *KRAS* mutations pre- and/or post-surgery had lower recurrence-free survival and lower OS, with recurrence of disease occurring in all patients with ctDNA detectable after resection surgery [30]. These findings should be corroborated by further studies, so treatment intensification strategies with more effective ChT regimens could be adopted in the presence of detectable ctDNA.

Two major limitations of our study are the reduced sample size (even more reduced due to the number of samples with available DNA to be used simultaneously in SS and ARMS/HRMA) and the subsequent reduced number of events during follow-up, which may have limited the statistical power of the data analysis. To have an acceptable sample size and get robust results, a prospective multicentric study including more patients would be needed.

Conclusion

In conclusion, ARMS/HRMA technique seems to be a fast, accurate, cheaper and reliable diagnostic tool to assess *KRAS* mutations in PDAC tumor and plasma samples, with a higher sensitivity compared to SS. In our cohort, tumor and plasma codon 12 *KRAS* mutations in PDAC patients were associated with lower OS and PFS, more importantly the G12D mutation. The detection of *KRAS* mutations in tumor and plasma samples could help to guide therapeutic decisions in patients with PDAC. Future studies should corroborate our results regarding ARMS/HRMA and compare it to other molecular techniques, so that in the near future we could have a tool to access *KRAS* mutational status in PDAC patients readily available in routine clinical practice.

Acknowledgements

This work was supported by a grant from Grupo Luz Saúde, and is financed by national funds from FCT - Fundação para a Ciência e a Tecnologia, I.P., in the scope of the project UIDP/04378/2020 and UIDB/04378/2020 of the Research Unit on Applied Molecular Biosciences - UCIBIO and the project LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy - i4HB. FCT-MCTES is also acknowledged for 2020.07660.BD for BO.

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