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Detection of mutated KRAS, TP53, CDKN2A, and SMAD4 in tumor cell-free DNA of Brazilian pancreatic adenocarcinoma patients using next-generation sequencing

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Pancreatic adenocarcinoma (PDAC) is one of the most lethal cancer types worldwide, with early diagnosis being challenging due to nonspecific symptoms that are often noticed only when the tumor reaches a large size. Liquid biopsy has emerged as a promising technology in oncology, where the identification of tumoral cell-free DNA (cfDNA) using sensitive methodologies can identify genetic mutations, allowing for tumor diagnosis, prognosis, and therapeutic approaches. However, the cfDNA extracted yields represent the first challenge, highlighting the need for cfDNA concentration. Here, using the NGS approach, we detected mutations in *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* in the cfDNA of 55 PDAC patients, using a total of 133 blood samples. After that, we identified 11 pathogenic variants across 13 cfDNA samples from 11 patients: 9 in *KRAS*, 4 in *TP53*, and 4 in *SMAD4*. Additionally, we discovered a *SMAD4* variant not previously associated with PDAC, and significantly correlated survival. Our data describes and discusses the properties of cfDNA extraction and yield for NGS and its clinical application. We demonstrate that liquid biopsy using NGS for cfDNA targeting *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* represents a promising alternative for the early diagnosis and genetic profiling of PDAC, with implications for prognosis and targeted therapy.

Keywords PDAC, Pancreatic cancer, Liquid biopsy, CfDNA, NGS

Pancreatic cancer is one the leading causes of cancer-related death, with an estimated 66,440 new cases and 51,750 deaths in the United States in 2024, accounting for 3.3% of new cases and 8.5%[1], which is represented in 90% by pancreatic adenocarcinoma (PDAC)[2]. The disease is responsible for 510,992 new cases and 467,409 deaths worldwide[3], and in Brazil, an estimated 10,980 new cases of pancreatic cancer were reported in 2022, with 11,974 deaths recorded in 2021[4]. PDAC is often diagnosed at a late stage, primarily due to its nonspecific symptoms, including jaundice, weight loss, fatigue, abdominal or back pain, and nausea, which lead to delayed patient presentation to the healthcare system[5,6]. The American Cancer Society reported that survival rate reached 13% across all races and ethnicities for patients diagnosed between 2013 and 2019[7].

PDAC patients frequently exhibit somatic mutations in four genes: the oncogene *KRAS*, present in approximately 90% of cases, and the tumor suppressor genes *TP53* (80%), *CDKN2A* (60%), and *SMAD4* (40%)[8,9]. *KRAS* mutations are among the earliest genetic events in PDAC development[10–12], followed by inactivation of

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tumor suppressor gene *CDKN2A*[13,14]. Inactivation of both *TP53* and *SMAD4* are late events in carcinogenesis, often associated with features of carcinoma in situ and metastasis[15–17].

Diagnosis for PDAC generally combines clinical findings with laboratory tests and imaging examinations[5,18], where carbohydrate antigen 19 – 9 (CA19-9) is the most commonly used, though its sensitivity and specificity as a screening, early diagnostic, and prognostic marker remain controversial[19,20]. Imaging modalities include endoscopic ultrasound (EUS), computed tomography (CT), and magnetic resonance imaging (MRI); however, these methods are limited by tumor size, as small tumors are often undetectable.

Cell-free DNA (cfDNA) is emerging as a promising clinical tool for cancer diagnosis and prognosis evaluation. These DNA fragments can be detected in blood, urine, saliva, and other extracellular fluids[21–25], generated by apoptosis, necrosis, and active secretion[26–28]. A subfraction of cfDNA corresponds to tumor cell-free DNA (ctDNA), which is released from tumor cells and serves as a primary target for diagnostic and prognostic applications in oncology.

The detection of ctDNA in the bloodstream using molecular techniques offers a practical and promising approach for PDAC diagnosis and prognostic evaluation within a Brazilian clinical setting. Therefore, in this study, we evaluated ctDNA from a clinical cohort of PDAC patients, employing next-generation sequencing (NGS) to detect mutations in *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*, as well as digital PCR (dPCR) for key *KRAS* mutations, showing the correlation of our findings with CA19-9 levels and clinical data, particularly within a sequential cohort of PDAC patients. Considering this, we share the experience of ctDNA detection properties, challenges, and opportunities, providing data and evidence to its optimization and implementation as a clinical application.

Results

Cohort

The study included 55 PDAC patients, of whom a total of 133 blood samples were collected, with serial samples obtained based on the patient's clinical progression and outcomes. Figure 1 illustrates the number of samples per patient, highlighting that 20 patients provided one sample each, while four patients contributed six samples each. The patients were born in 10 of the 27 Brazilian federation states, with São Paulo and Bahia represented by 32 and 8 patients, respectively.

The demographic and epidemiological data are shown in Fig. 2; Table 1. The mean age at diagnosis was 64.09 ± 11.56 years, with body mass index (BMI) ranging from 15.16 to 39.44. Most patients were female, with 47.27% self-identifying as white and 40% as brown (a person of mixed European and African descent). Smoking and alcohol use were categorized as past use, current use, and never used. Additionally, 32.72% of patients had diabetes, and 58.18% had a family history of cancer in first-degree relatives (parents, siblings, and children), with three patients reporting a family history of pancreatic cancer.

cfDNA Extraction and Quantification Figure 3 presents a flowchart for the 133 plasma samples, illustrating the cfDNA extraction yield and indicating which samples could be used directly for NGS library construction and which required concentration using a Speed-Vac concentrator.

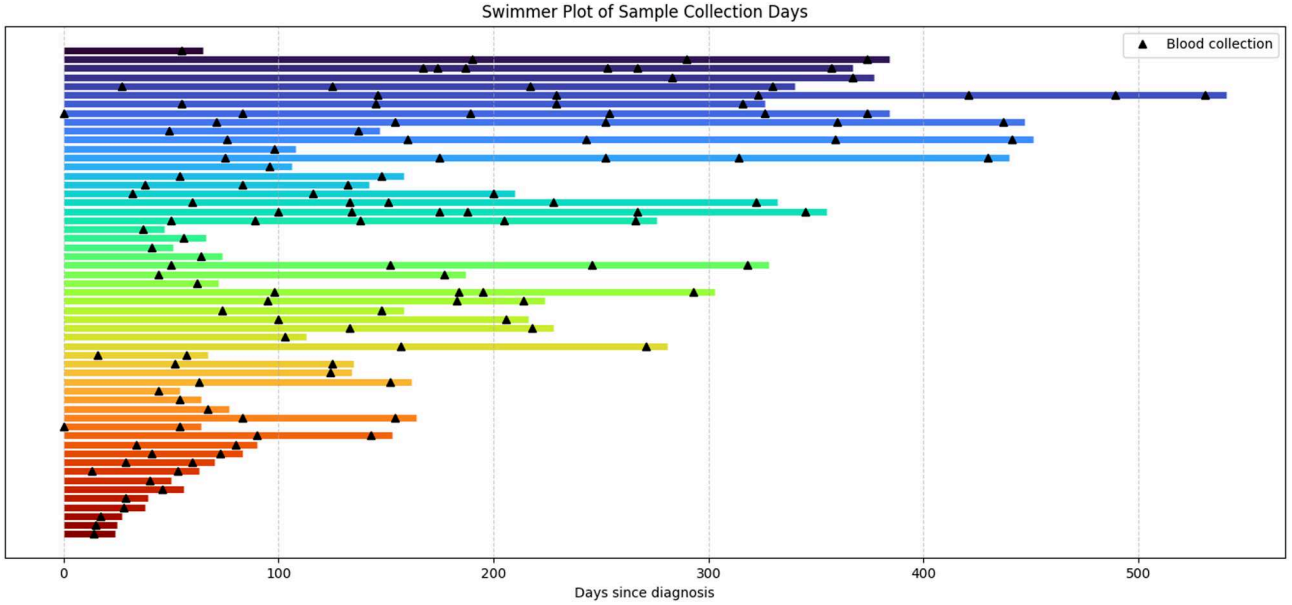

Fig. 1. Number of patients correlated with samples per patient enrolled in the project, with sample count varying based on diagnosis date. Day 0 corresponds to the diagnosis date, and triangles represent blood sample collection.

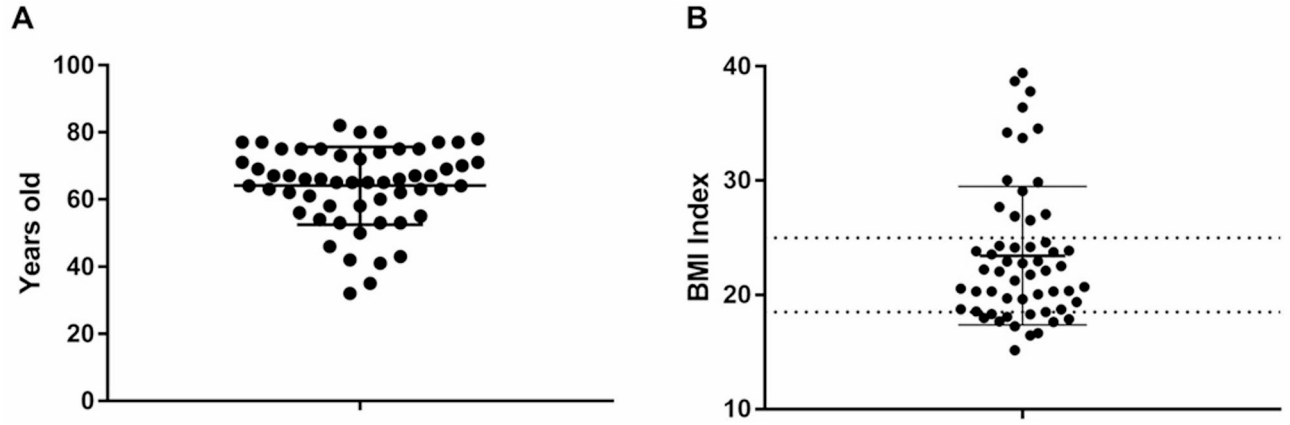

Fig. 2. Epidemiological data at diagnosis. (**A**) Age at diagnosis for 55 PDAC patients enrolled in the project, with a mean age of 64.09 years. (B) Body-mass index (BMI) at diagnosis from all 55 PDAC patients, with horizontal lines indicating the normal BMI range of 18–25. Most patients were included in the normal range, while 14 had a BMI above the upper limit.

Gender		Self-reported ethnicity			Smoking			Alcohol usage		
Male	Female	White	Black	Brown	Past	Current	Never	Past	Current	Never
41.82%	58.18%	47.27%	12.73%	40%	35.86%	7.54%	56.60%	18.87%	11.33%	69.80%

Table 1. Epidemiological and demographic data of enrolled PDAC patients, showing that most are women and self-declared as white. Most patients reported never smoking or consuming alcohol.

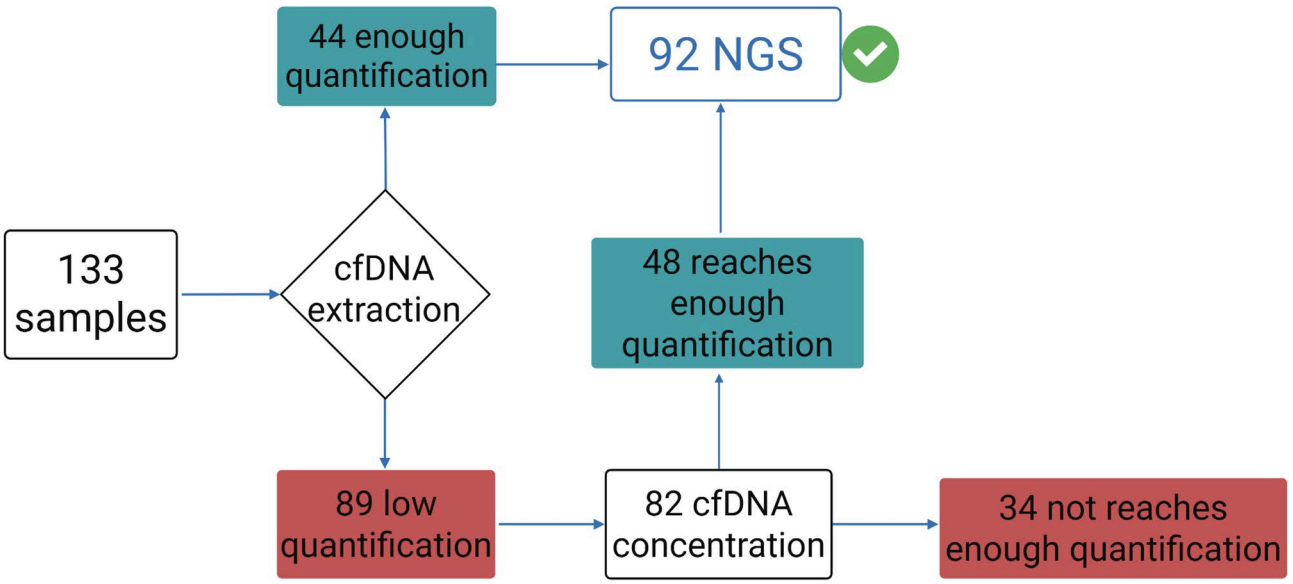

Fig. 3. Flowchart indicating the workflow utilized. Plasma samples used according to cfDNA extraction yield, enabling 44 samples to direct library construction, while of 89 remaining samples 82 were concentrated, with 48 reaching enough concentration to perform NGS and 34 did not, resulting in a final 92 samples submitted to cfDNA sequencing.

The cfDNA extracted was quantified by Bioanalyzer HS DNA analysis (Fig. 4), showing variation in the sample concentrations between 112 and 71,160 pg/µL, as depicted in Fig. 5A. Considering the proposed NGS methodology requires a minimum of 10 ng of cfDNA, a concentration threshold was set at 1.200 pg/µL (indicated by a horizontal red line – Fig. 5A). On that, 44 (33.1%) samples met this threshold for direct library construction. Subsequently, 82 of the remaining 89 cfDNA samples were concentrated, resulting in a notable increase in concentration ($p < 0{,}0001$) (Fig. 5B). Specifically, 73 of 82 samples (89.02%) showed an average

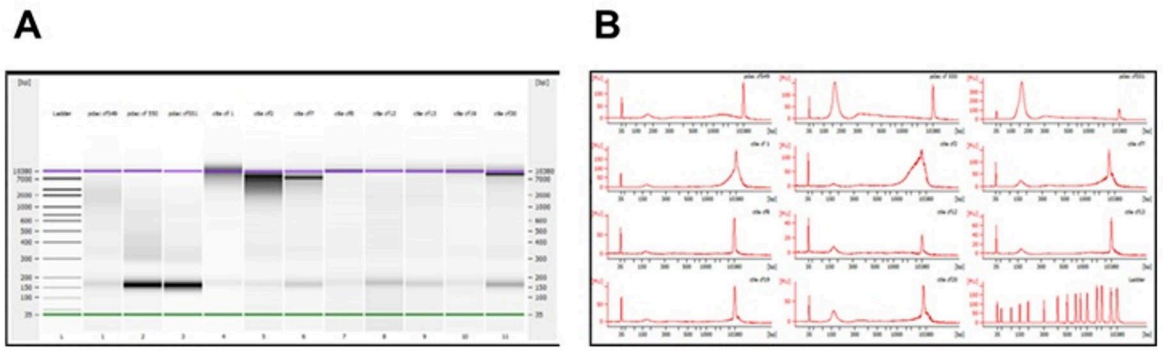

Fig. 4. Bioanalyzer visualization plots. (**A**) Bioanalyzer virtual gel, with green and purple lines indicating the lower and upper markers, respectively. The ladder corresponds to the first lane, and cell-free DNA (cfDNA) is represented by the band between 150–200 bp. (**B**) Bioanalyzer electropherograms, showing the left and right peaks at 35 and 10,380 bp correspond to lower and upper markers, respectively. cfDNA is represented by the peak between 150–200 bp.

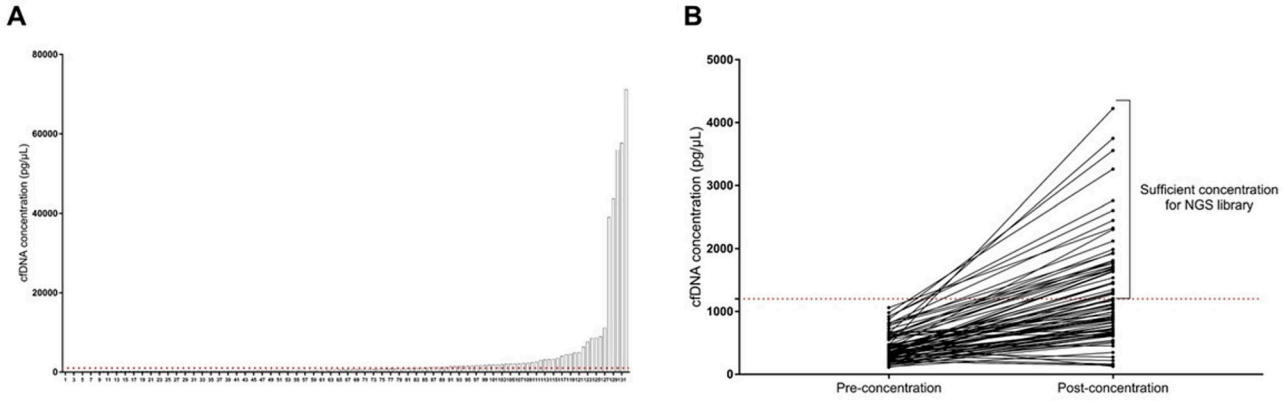

Fig. 5. cfDNA concentration after extraction. (**A**) cfDNA quantification for 133 samples, with the horizontal red line indicating the minimum concentration required for NGS library preparation. (**B**) cfDNA concentration before and after Speed-Vac concentration, showing a statistically significant increase of cfDNA from 426pg/µL± 226pg/µL before concentration to 1.248pg/µL ± 819pg/µL after concentration ($p < 0.0001$).

concentration increase of 3.35x. In contrast, four samples (4.88%) showed no concentration difference, while five samples (6.10%) had a reduction in concentration (> 80% of the initial level) (Supplementary Table 1). On that, 48 of the 82 lyophilized samples (58.53%) reached sufficient concentration for NGS library preparation. In the final dataset, 92 of the 133 samples (44 directly after cfDNA extraction and 48 following SpeedVac concentration), or 69.18%, achieved the required concentration for NGS library construction. Furthermore, no significant difference was observed in cfDNA concentrations between samples collected using K3 EDTA tubes and PAXgene ccfDNA tubes (Table 2).

Cell-free DNA liquid biopsy cohort The NGS was performed on 58 cfDNA samples from 28 PDAC patients. Among these, eleven patients contributed one plasma sample each, nine contributed two samples, three provided three samples, and five had four samples. For patients with multiple samples, the intervals between sample collections averaged 80.33 ± 20.60 days, ranging from 18 to 133 days (Supplementary Table 2). The limited number of samples resulted from technical constraints and optimizations to enhance NGS performance. This cohort description represents a significant bias, as factors such as stage at diagnosis, treatment regimen, CA19-9 levels, and clinical outcomes are critical for the analysis and interpretation of the results, as shown in Table 3.

In this cohort, sixteen patients had their first sample collected before treatment. Five patients underwent FOLFIRINOX standard chemotherapy, two received neoadjuvant chemotherapy followed by surgery, and five had previously undergone surgery. The survival of this cohort was evaluated by dividing patients into three groups: surgery, chemotherapy, and no chemotherapy, with no statistical difference between groups as shown in Fig. 6. Regarding patient outcomes, 17 of 28 patients (60.72%) died within an average of 229.05 ± 113.74 days (ranging from 52 to 511 days), while the surviving patients were followed for an average of 498.1 ± 158.05 days. Notably, three patients who were alive with no evidence of cancer had undergone surgery.

Sample	PAXgene (pg/µL)	K3 EDTA (pg/µL)
10,530 P5	696	767
11,601 P2	2.121	918
11,606 P2	625	580
11,611 P1	727	981
11,613 P1	1,747	1,419
11,614 P1	900	3,219
11,607 P2	741	280
11,616 P1	209	294
11,611 P2	179	1,523
11,617 P1	350	408
11,618 P1	364	588
11,619 P1	1,930	1,839
Mean SD	882 (±676)	1,068 (±842)

Table 2. cfDNA concentration in 12 PDAC samples comparing PAXgene cfDNA and K3 EDTA tubes, showing no significant differences between the two collection methods. $p = 0.5576$.

Patient	Treatment at first sample collection [1]	Sample number	Diagnosis to first sample collection (days)	Outcome	Follow-up (days)	CA19-9 quantification in each sample			
						1	2	3	4
10,521	Without	1	55	Death from cancer	102	> 10,000			
10,522	CH	2	160	Death from cancer	511	101	66.7		
10,523	Without	2	174	Alive with cancer	573	18.5	< 14		
10,524	SUR	2	283	Alive with cancer	785	86.3	NA		
10,525	Without	4	27	Death from cancer	361	622	147	1,180	1,440
10,527	CH + SUR	3	146	Alive with cancer	636	54.8	33.7	15.5	
10,529	Without	4	55	Death from cancer	316	1,330	327	> 10,000	> 10,000
10,530	CH + SUR	4	0	Alive with no cancer evidence	455	< 14	< 14	< 14	< 14
10,531	Without	2	154	Alive with cancer	525	47.4	30.9		
10,532	SUR	1	49	Death from cancer	179	1,920			
10,533	Without	2	160	Alive with cancer	536	< 14	24.2		
10,535	Without	1	98	Death from cancer	105	130			
10,536	SUR	4	75	Alive with no cancer evidence	491	< 14	19.5	21.8	< 14
10,537	Without	1	96	Death from cancer	160	> 10,000			
10,538	Without	2	54	Death from cancer	191	> 10,000	NA		
10,539	Without	2	38	Death from cancer	136	> 10,000	> 10,000		
10,540	CH + SUR	3	32	Death from cancer	212	22.6	36.9	8,860	
10,541	CH	3	60	Death from cancer	450	471	161	388	
10,542	SUR	4	100	Alive with no cancer evidence	531	130	42.1	75 > 6	48.3
10,543	Without	2	50	Alive with cancer	474	45.3	89.9		
10,545	Without	1	56	Death from cancer	113	321			
10,546	CH	1	41	Death from cancer	52	159			
10,549	CH	1	50	Death from cancer	411	> 10,000			
10,550	Without	2	44	Death from cancer	182	< 14	28.9		
10,553	CH	1	95	Death from cancer	242	153			
10,557	Without	1	103	Death from cancer	171	< 14			
11,602	Without	1	63	Alive with cancer	248	4,100			
11,603	Without	1	44	Alive with cancer	225	> 10,000			

Table 3. PDAC patients enrolled in the project, detailing treatment at the first sample collection, number of samples collected, days from diagnosis to first sample collection, outcome, follow-up duration, and CA19-9 values for each sample. [1] CH: Chemotherapy; SUR: Surgery; NA: Not available.

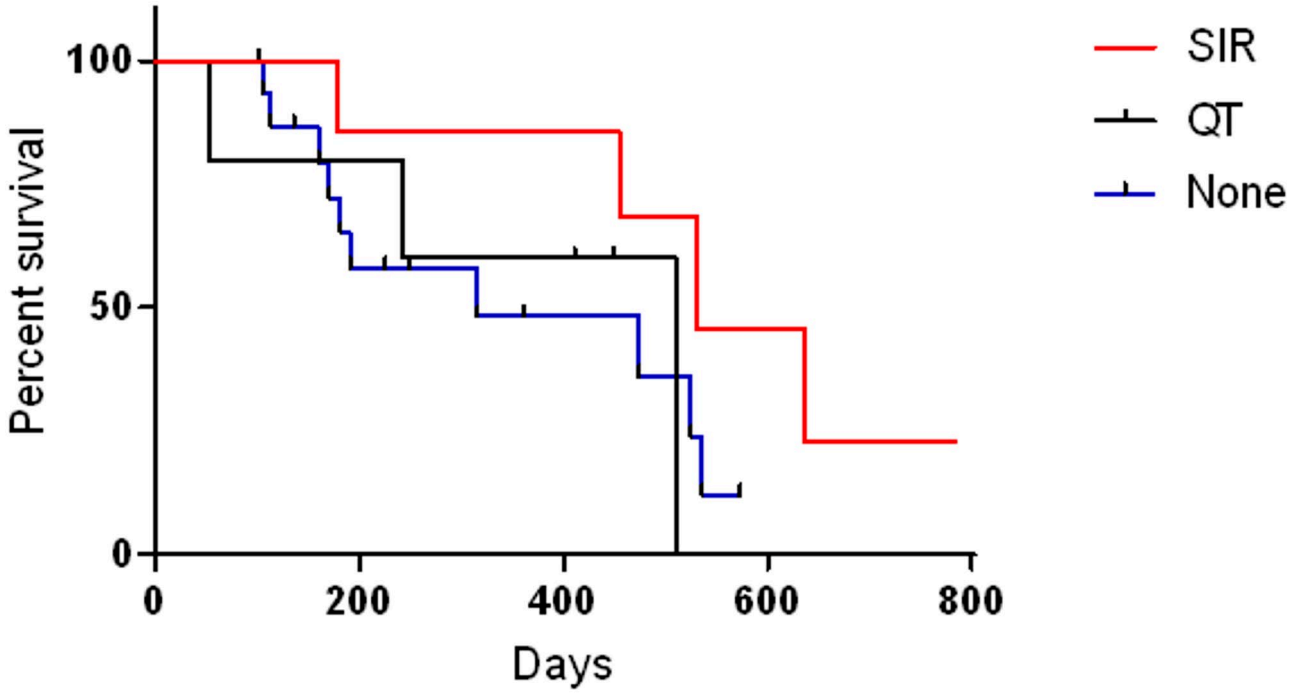

Fig. 6. Survival curve for PDAC patients based on treatment group. Despite not statistically significant, patients who underwent surgery had longer survival compared to those who received chemotherapy or no pharmacological treatment at the time of the first sample collection. QT: Chemotherapy; SIR: Surgery.

Pathogenic mutations in PDAC patients The NGS library for cfDNA was constructed as outlined in the methodology, which performs redundant sequencing for each base using different primers. This redundancy is crucial, allowing for a theoretical coverage of 20,000x due to the primer redundancy. Figure 7 depicts the NGS depth, the number of variants identified, and their genetic locations. Figure 7A shows the mean sequencing coverage for each sample, averaging 6,132x ± 2,383x, with a range from 1,577x to 10,698x. Variant analysis, performed using CLC software (Qiagen), is shown in Fig. 7B and Supplementary Table 3, categorizing variants by total count and those with a frequency below 50%. The genetic locations in *KRAS*, *CDKN2A*, *TP53*, and *SMAD4*, for both total variants and those with a frequency below 50%, are represented in Fig. 7C and D, respectively.

Given the large number of variants generated, manual analysis would have been highly labor-intensive. To address this, as a form of preliminary and limited assessment, 10 samples were analyzed for clinical significance using QIAGEN Clinical Insight Interpret software, which evaluates variants based on established medical and scientific evidence. The results, summarized in Table 4, identified five pathogenic or likely pathogenic variants, 12 variants of uncertain significance (VUS), and five benign or likely benign variants. Among the pathogenic variants, two were identified in *KRAS* (c.35G > T - G12V and c.35G > A - G12D), one in *TP53* (c.396G > C - K132N), and two in *SMAD4* (c.1173dup - K392* and c.1082G > A - R361H).

After that, mutations classified as pathogenic/likely pathogenic for *KRAS*, *TP53*, *CDKN2A* and *SMAD4* based on the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) and those pathogenic variants found after CLC analyzes were manually screened across all samples. As a result, 11 pathogenic variants were identified across 13 samples of 11 patients (Table 5). In nine patients, *KRAS* mutations were detected, including five cases of c.35G > A G12D, three of c.35G > T G12V, one of c.34G > C G12R, and one of c.183 A > T Q61H. For *TP53*, four pathogenic variants were identified, each present in one patient: c.396G > C p.K132N, c.476 C > T (p.Ala159Val), c.818G > T (p.Arg273Leu), and c.743G > A (p.Arg248Gln). Meanwhile, pathogenic and likely pathogenic variants in *SMAD4* were observed, with c.1082G > A (p.R361H) detected in two patients, c.1173dup (p.K392)* in one patient, and c.711G > A in another patient.

An important characteristic on this finding is regarding mutant *KRAS* detection. For those 9 patients with a mutant variant on this gene, 6 patients had just one sample and died closely after the sample collection (patients 521, 535, 537, 545, 546 and 553). While that, mutant *KRAS* was detected in last two samples of patient 529 (529P3 and 529P4), with an increasing frequency of 2.92% to 49.94%, followed by patient death. On the other hand, samples 525P3 and 539P1 showed mutant *KRAS* (frequency of 4.09% and 2.46%, respectively) with the following sample negative (525P4 and 539P2), however the patient's death by cancer. On these 2 patients, we highlight the NGS coverage on the mutant *KRAS* and the following sample, where patient 539P1 and 539P2 a mean read depth of 5.162 and 1.297, respectively, a greatly reduction in 539P2 in comparison with 539P1. On the other hand, 525P3 and 525P4 present a mean read depth of 1.120 and 1.489, respectively.

Since mutations in *KRAS* are widely recognized as the earliest genetic alterations in PDAC and occur with high incidence, a higher percentage of positive samples was anticipated. To evaluate these findings, dPCR was

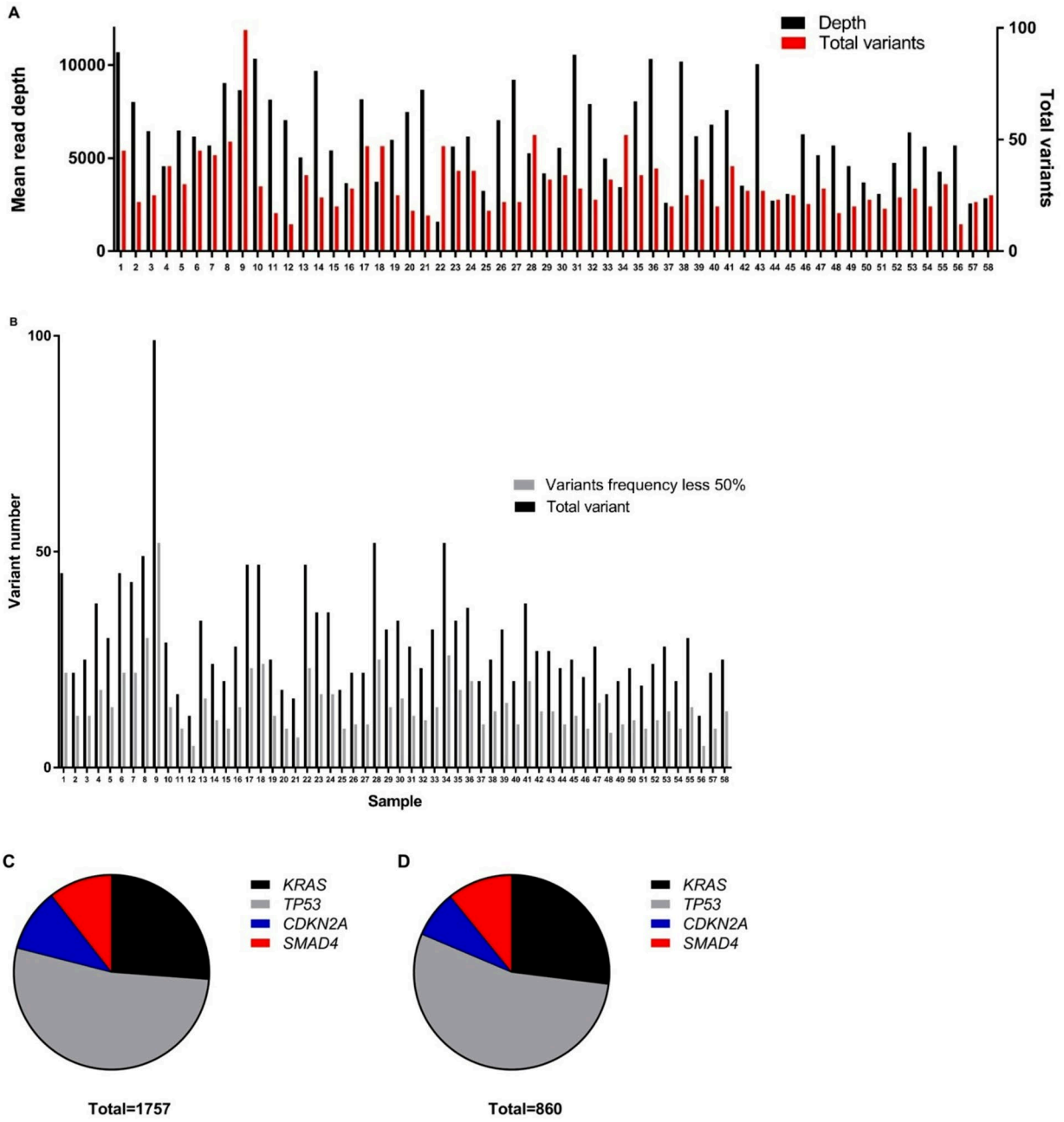

Fig. 7. NGS results. (**A**) NGS mean coverage depth (black bars) and number of total variants (red bars) for each cfDNA sample subjected to NGS sequencing; (**B**) Number of total variants (black bars) and variants with a frequency below 50% (gray bars) for each patient, to initially separate somatic and possible germline variant; (**C**) Genetic location of total variants; (**D**) variants with a frequency below 50%.

employed to detect mutated *KRAS* in 8 NGS-positive (2 *KRAS*-G12V, 5 *KRAS*-G12D and 1 *KRAS*-Q61H) and 11 NGS-negative samples. As demonstrated in Table 6, seven of the eight mutated *KRAS* patients detected by NGS were also detected by dPCR, highlighting that the dPCR undetected patient showed a mutation frequency of 0.67% in NGS. When NGS negative mutated *KRAS* samples were evaluated for G12D/V/C/R and Q61H mutation by dPCR it was observed one sample positive for G12D and another for Q61H, as demonstrated in Supplementary Table 4.

A 2×2 contingency table considering the 3 variants G12D, G12V and Q61H was constructed to perform concordance analysis between NGS and dPCR. dPCR showed an overall percent agreement (OPA) of 84.2% (CI 95% 0.604–0.966), a positive percent agreement (PPA) of 87.5% (CI 95% 0.473–0.997) and a negative percent agreement (NPA) of 81.8% (CI 95% 0.482–0.977) when compared with NGS. Cohen's kappa was 0.68, indicating good agreement beyond chance. McNemar's test ($p \approx 1.0$) indicated no significant systematic disagreement

Sample	Stage at diagnosis	KRAS	TP53	CDKN2A	SMAD4	CA19-9	Metastasis at diagnosis	Outcome	Follow-up (days)
521P1	IV	c.35G > T - G12V (P)	c.396G > C p.K132N (P) c.215 C > G p.P72R (V) c.994-1017del (B)	c.*29G > C (V)	c.1173dup p.K392* (P) c.1086T > C p.F362F (LB)	> 10,000	Liver	Death by cancer	102
523P2	II	c.141T > C p.D47D (V) c.451-9G > A (B) c.519T > C p.D173D (B)	None	c.*29G > C (V) c.*30G > A (V)	None	< 14	No	Alive with cancer	573
525P2	III	None	None	c.*29G > C (V)	None	147	Interaortocaval and retrocaval lymph nodes	Death by cancer	361
529P4	IV	c.35G > A p.G12D (P) c.451-9G > A (B)	None	None	c.1082G > A p.R361H (P)	> 10,000	Liver	Death by cancer	316
537P1	IV	c.519T > C p.D173D (B)	c.215 C > G p.P72R (V) c.1014 C > T p.H338H (LB) c.994-1017del (B)	c.*29G > C (V)	None	> 10,000	Peritoneum, liver	Death by cancer	160
538P1	III	c.112-7825T > C (V) c.112–3227 A > G (V)	None	None	c.1447 + 11 T > C (V) c.1102T > C p.S368P (V) c.134 A > G p.K45R (V) c.127T > C p.L43L (V) c.114 A > G p.R38R (V)	> 10,000	Liver, bile ducts, peritoneum	Death by cancer	191
539P1	III	c.35G > A p.G12D (P)	c.215 C > G p.P72R (V)	c.*29G > C (V)	None	> 10,000	No	Death by cancer	136
539P2	III	None	None	None	None	> 10,000	No	Death by cancer	136
541P1	II	None	c.215 C > G p.P72R (V)	c.*29G > C (V)	None	471	No	Death by cancer	450
549P1	IV	c.451-9G > A (B)	c.994-1017del (B)	c.*29G > C (V) c.457 + 1093 C > T (V)	None	> 10,000	Liver	Death by cancer	411

Table 4. Variants generated by QIAGEN Clinical Insight Interpret in 10 PDAC patient samples for *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*. The table also includes corresponding CA19-9 levels, presence of metastasis at diagnosis, clinical outcomes, and follow-up duration (days). (P): Pathogenic; (V): Variant of Uncertain Significance (VUS); (LB): Likely Benign; (B): Benign.

Sample	Gene	Variant	Mutation frequency (%)	Outcome
521P1	*TP53*	c.396G > C p.K132N	2.16	
	SMAD4	c.1173dup p.K392*	2.65	Death from cancer
	KRAS	c.35G > T G12V	4.61	
525P2	*SMAD4*	c.711G > A	0.86	Death from cancer
525P3	*KRAS*	c.35G > T G12V	4.09	
529P3	*SMAD4*	c.1082G > A p.R361H	1.28	Death from cancer
	KRAS	c.35G > A G12D	2.92	
529P4	*SMAD4*	c.1082G > A p.R361H	31.27	
	KRAS	c.35G > A G12D	49.94	
531P4	*TP53*	c.476 C > T (p.Ala159Val)	1.47	Alive with cancer
533P3	*TP53*	c.818G > T (p.Arg273Leu)	1.64	Alive with cancer
535P1	*KRAS*	c.35G > A G12D	0.67	Death from cancer
537P1	*KRAS*	c.35G > A G12D	1.75	Death from cancer
539P1	*KRAS*	c.35G > A G12D	2.46	Death from cancer
545P1	*KRAS*	c.35G > T G12V	12.08	Death from cancer
546P1	*KRAS*	c.183 A > T Q61H	2.75	Death from cancer
553P1	*KRAS*	c.34G > C G12R	9.01	Death from cancer
	TP53	c.743G > A (p.Arg248Gln)	4.98	

Table 5. Pathogenic and likely pathogenic variants identified in 13 of 58 PDAC samples.

	G12V		G12D		Q61H	
Samples	NGS Frequency	dPCR Frequency (CI)	NGS Frequency	dPCR Frequency (CI)	NGS Frequency	dPCR Frequency (CI)
521 P1	4.61%	10.9% (18.6%)				
545 P1	12.08%	12.85 (14.8%)				
529 P3			4.09%	2.86% (46.2%)		
529 P4			49.94%	50.3% (2.1%)		
535 P1			0.67%	Not detected		
537 P1			1.75%	1.36% (59.1%)		
539 P1			2.75%	36.9% (21.8%)		
546 P1					2.75%	49.8% (3.6%)

Table 6. dPCR result for NGS positive *KRAS* mutated samples.

Type	Variant	Gene	Number of samples
VUS	c.141T > C p.D47D	*KRAS*	2
	c.112-7825T > C	*KRAS*	1
	c.*29G > C	*CDKN2A*	53
	c.*30G > A	*CDKN2A*	2
	c.457 + 1093 C > T	*CDKN2A*	1
	c.215 C > G p.P72R	*TP53*	49
Benign, likely benign	c.519T > C p.D173D	*KRAS*	23
	c.451-9G > A	*KRAS*	9
	c.994-1017del	*TP53*	2
	c.1014 C > T p.H338H	*TP53*	8

Table 7. Number of samples with variants of uncertain significance (VUS) and benign/likely benign variants, detailing their genetic locations and highlighting the VUS c.112-7825T > C as a somatic variant.

between the methods. These results support that dPCR provides confirmatory value for KRAS mutation detection in plasma samples.

Upon critical analysis of the variants according to patient outcomes, none of the three patients who underwent surgery showed any evidence of cancer at the end of follow-up, harbored pathogenic variants. Similarly, four patients who underwent surgery and were alive but still had cancer also showed no pathogenic variants. This finding suggests that pathogenic variants were exclusively associated with patients whose cancer remained present and displayed aggressive behavior, as reflected by the high mortality rate. Six VUS were detected: two in *KRAS*, three in *CDKN2A*, and one in *TP53*. In this context, we highlight the *KRAS* variant c.112-7825T > C, which showed a frequency of 1.35%, strongly suggesting a somatic origin. In contrast, the remaining variants, with frequencies around 50% or 100%, are likely germline alterations, characterized as heterozygous or homozygous. Additionally, two benign and two likely benign variants were identified in both the *KRAS* and *TP53* genes (Table 7).

A deeper analysis was conducted to screen for uncharacterized variants with a frequency below 20%, which were therefore classified as somatic variants. Table 8 describes these variants, including their associated genes, the number of positive samples, NGS quality, and variant frequency. Supplementary Table 4 shows the total coverage and variant count.

In this analysis, 100% of patients who died and 90% of patients who were alive showed the presence of at least one unknown variant. Specifically, 32 samples from 18 deceased patients showed 85 uncharacterized variants, while 27 samples from 10 living patients displayed 40 uncharacterized variants. This indicates that patients who died had a higher number of uncharacterized variants compared to those who survived. Furthermore, four uncharacterized variants detected in the last sample from each patient were selected for survival rate analysis. The uncharacterized variant 8 showed statistically significant differences ($p < 0.05$ and HR = 4.14) in survival curves, present in five of 18 patients who died but absent in all 10 who survived (Fig. 8).

Discussion

In this study, we analyzed cfDNA from 58 samples of 28 patients with PDAC using an NGS liquid-biopsy panel and confirmation by dPCR was performed, since not at the same samples due to the fact of insufficient cfDNA volume to both approaches. We detected KRAS mutations in 17.2% of cases, by NGS, which is consistent with previous reports (12–23%). Moreover, 81.8% of patients with pathogenic variants were deceased, reinforcing the prognostic value of circulating mutations. These findings demonstrate that, even with low input material, cfDNA sequencing provides clinically relevant information.

PDAC has become one of the leading causes of cancer-related mortality, with early diagnosis representing a critical factor in reducing both mortality rates and improving patients' quality of life. A potential strategy to

Variant	Gene	Number of positive samples	NGS average quality (mean ± SD)	Frequency % (mean ± SD)
Uncharacterized 1	*KRAS*	1	51	0.85
Uncharacterized 2	*KRAS*	3	39.82 ± 2.09	2.00 ± 1.07
Uncharacterized 3	*KRAS*	2	42.22 ± 2.82	0.74 ± 0.23
Uncharacterized 4	*KRAS*	2	41.28 ± 4.79	1.82 ± 0.64
Uncharacterized 5	*KRAS*	2	43.38 ± 0.18	0.67 ± 0.23
Uncharacterized 6	*KRAS*	24	46.59 ± 5.58	1.18 ± 0.69
Uncharacterized 7	*KRAS*	4	45.31 ± 6.41	1.67 ± 0.63
Uncharacterized 8	*SMAD4*	12	43.05 ± 2.25	1.10 ± 0.38
Uncharacterized 9	*TP53*	8	38.53 ± 3.36	1.35 ± 0.52
Uncharacterized 10	*TP53*	6	42.06 ± 6.06	1.46 ± 0.60
Uncharacterized 11	*TP53*	18	39.17 ± 4.19	1.89 ± 0.79
Uncharacterized 12	*TP53*	19	39.21 ± 5.30	1.91 ± 0.63
Uncharacterized 13	*TP53*	29	38.02 ± 2.43	2.79 ± 1.19

Table 8. Uncharacterized variants identified with high NGS quality rates and frequencies below 20%, along with their genetic location and number of positive samples.

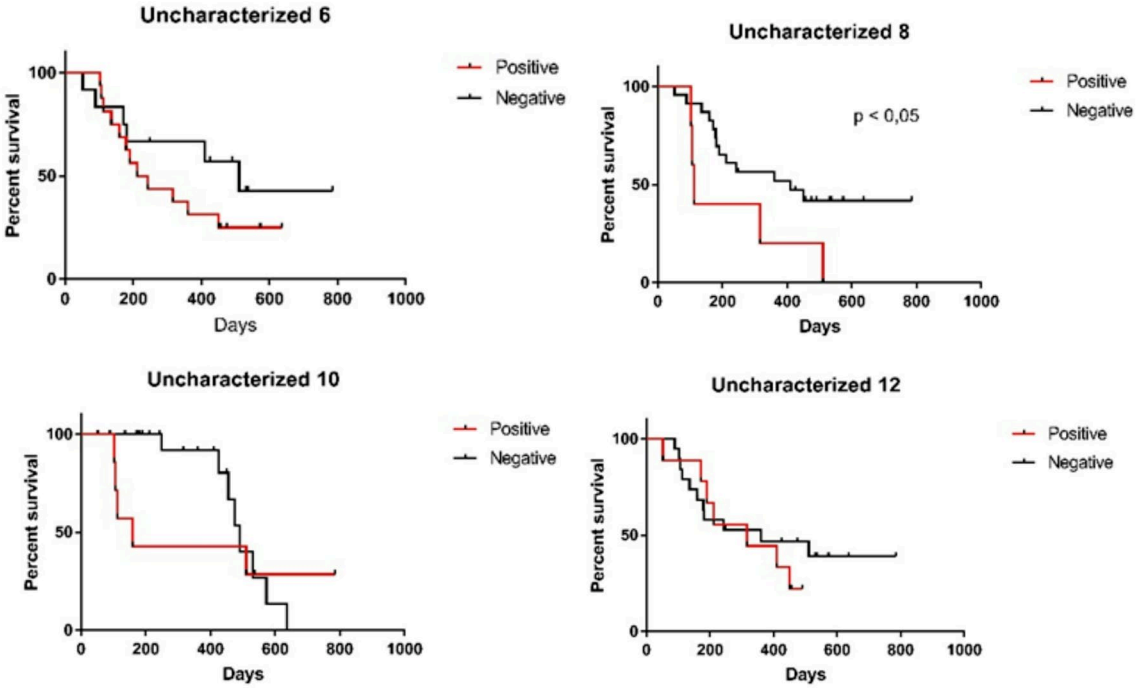

Fig. 8. Survival curve of uncharacterized variants 6 ($p = 0.23$, HR = 1.77), 8 ($p = 0.047$, HR = 4.14), 10 ($p = 0.645$, HR = 1.322), and 12 ($p = 0.518$, HR = 1.394), showing that the presence of uncharacterized variant 8 was associated with a reduced survival rate.

address early diagnosis in PDAC is screening in high-risk populations, including individuals with genetic and behavioral risk factors, such as cigarette smoking, heavy alcohol consumption[29–31], high body mass index[32,33] and chronic pancreatitis, which is also recognized as a risk factor, while the association with diabetes mellitus is complex and involves various other clinical variants[34–36].

In the germline genetic context, high-penetrance genetic predispositions account for approximately 10% of cases[37,38], with pathogenic germline alterations observed in genes such as *BRCA1*, *BRCA2*, *ATM*, *PALB2*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *CDKN2A*, and *TP53*[39]. Beyond monogenic predisposition, both familial and sporadic PDAC are increasingly recognized as polygenic diseases[40–43], and genome-wide association studies have identified multiple low-penetrance alleles contributing to disease risk[44–47].

The ESMO Clinical Practice Guideline for pancreatic cancer[48] recommends CT as the primary imaging modality, with MRI reserved for cases where CT is not feasible, inconclusive, or involves pancreatic cystic lesions. Although CA 19 − 9 is not recommended for screening purposes, it remains a significant prognostic marker. Regarding somatic genomic biomarkers, this guideline highlights that *KRAS*-wild type metastatic

PDAC represents a distinct molecular subset with potential therapeutic implications. Furthermore, multigene sequencing is emphasized as a valuable approach for identifying rare but actionable molecular alterations[49,50]. In this context, liquid biopsy, particularly through the detection of ctDNA, emerging as a promising alternative for early diagnosis of PDAC. One of the earliest studies on PDAC cancer and cfDNA identified the *KRAS* G12D mutation using allele-specific PCR, detecting its presence in 22 of 47 patients[51]. Subsequent research employed dPCR to investigate mutant *KRAS* in cfDNA of PDAC patients[52,53]. Moreover, Le Calvez-Kelm et al.[54] introduced the first approach using NGS for detecting mutant *KRAS* in blood-derived cfDNA. Liquid biopsy targeting cfDNA through NGS has since emerged as a novel and promising approach for PDAC, and in this study we present promising and complementary data to the associated discoveries and challenges.

CfDNA yields remain a central technical limitation of liquid biopsy for early diagnosis. Smaller tumors release less cfDNA into the plasma, even though they are the primary target for early detection using this methodology. In our study, using 5mL of plasma collected in EDTA tubes, only 33.1% of samples yielded cfDNA concentrations exceeding 1,200 pg/μL, with the remaining samples requiring concentration. Early-stage (asymptomatic) tumors are challenging to detect by liquid biopsy due to insufficient release of ctDNA to be detectable in a typical blood draw of 10 mL[55]. Pan et al. used 10mL of peripheral blood collected in Streck tubes from 139 patients with unresectable, locally advanced non-small cell lung cancer (NSCLC) and found that most samples had cfDNA concentrations below 1ng/μL[56]. Similarly, another study detected cfDNA in only 51% of pretreated NSCLC patients, including 24% of patients with stage I, 77% with stage II, and 87% with stage III of the disease[57]. Martin-Alonso et al. addressed the challenge of cfDNA degradation by developing two types of priming agents, which significantly enhanced the sensitivity of liquid biopsy techniques in mouse models, representing a promising alternative for clinical practice in oncology[58].

Regarding blood collection methods, we found no significant differences in cfDNA yield between the EDTA and PAXgene tubes when samples were immediately processed and frozen at −80 °C, however, such immediate processing may not always be feasible in routine clinical settings. Additionally, while increasing cfDNA concentration might improve the sensitivity of NGS-based methodologies, it limits methodological flexibility because it requires utilizing the entire cfDNA sample.

Our approach identified *KRAS* mutations in 10 of 58 patients (17.25%), a detection rate below the somatic alteration level reported for PDAC in the literature. However, similarly low detection rates of *KRAS* mutations in liquid biopsy for PDAC using NGS technology have been reported by other studies[59]. For example, Theparee et al. detected a 12.3% mutation rate in 81 patients using the Oncomine™ Lung cfDNA Assay on the Ion Torrent S5 sequencing platform[60]. Lee et al. found a comparable rate of 18.9% using the AVENIO ctDNA Expanded Kit and Illumina NextSeq 550[61], while Guo et al. reported a rate of 23.01% using the KAPA sequencing library kit and Illumina Hi-Seq 2500[62].

The detection of variant allele frequencies (VAFs) is directly influenced by NGS coverage, which is closely tied to cost, as greater coverage indicates a smaller number of patients per NGS and, consequently, higher costs. Reports also indicate reduced detection rates of mutant *KRAS* in cfDNA by dPCR[63,64]. For example, while *KRAS* mutations in codons 12 or 13 were identified in tissue samples from 45 of 47 PDAC patients, these mutations were detected in cfDNA from only 23 patients, predominantly in those with liver or lung metastases[65]. These findings highlight a limitation in detecting plasma-mutated *KRAS* in PDAC patients, emphasizing the need for improved extraction methods and technical advancements to achieve better detection outcomes.

Our study found 860 variants with VAF ranging from 50% to 0.5% in cfDNA, distributed across 11 pathogenic variants, six VUS, and four benign variants among 58 samples of 28 PDAC patients. Notably, 81.8% of patients with pathogenic variants were deceased. Among these, nine of 11 patients had *KRAS* mutations, four had *TP53* mutations, and three had *SMAD4* mutations. It is worth emphasizing that the quantity and mutant allele frequency (MAF) of mutated cfDNA are directly correlated with tumor stage[66,67]. For example, *KRAS* mutations in cfDNA were detected in 39% of treatment naïve patients and 31% of patients following neoadjuvant therapy[68]. Affolter et al. found a significant difference in the estimated median survival of PDAC patients based on the presence or absence of detectable ctDNA pre-operatively[69]. Additionally, the presence of mutated *KRAS* in cfDNA was associated with shorter survival[59]. A recent study analyzed cfDNA from 81 PDAC patients using NGS, showing that tumor stage did not significantly influence the detection of ctDNA mutations. However, the presence of *KRAS* or *TP53* mutations in cfDNA was associated with the survival outcomes, independent of tumor stage, age, and sex[60]. These findings further support that liquid biopsy for PDAC using NGS is a promising tool not only for diagnostic purposes but also for prognostic evaluation. Another important finding involves the identification of novel somatic variants potentially associated with PDAC onset and progression. Screening for such variants directly in cancer tissue often presents significant medical challenges. For instance, the identification of the uncharacterized variant 8 exemplifies genomic findings linked to prognosis that can be accessed through minimally invasive liquid biopsy.

Overall, our analysis discusses the major obstacles in applying cfDNA-based NGS for PDAC, including limited yields, low tumor-derived VAFs, and high analytical cost, factors that particularly restrict use in real-world settings in Brazil. Despite these constraints, complementary dPCR testing provided confirmatory evidence for NGS findings across both early and advanced disease samples, supporting its clinical utility for diagnosis, prognosis, and recurrence monitoring. While the study lacks paired tumor tissue sequencing and suffers from modest sequencing depth, it contributes valuable translational data from a low- to middle-income setting. As research and innovation advance, liquid biopsy is poised to evolve into a significant tool for oncological management[70,71], but its large-scale implementation will require technological accessibility, cost reduction, and methodological refinement.

Methods

Study design and participants

This prospective study was conducted between March 2023 and June 2024. The samples were collected from PDAC patients at the São Paulo Cancer Institute (ICESP), São Paulo, Brazil, following the ICESP Biobank protocol. The study received ethical approval from the National Research Ethics Committee (CONEP 023/2014) and the local ethics committee (CAAE 66587023.6.0000.5248), in accordance with Brazilian guidelines and regulations. The study was described in detail to all participants, who read, discussed, and signed an informed consent form before sample collection. Eligible participants were adults (18 years or older) with histologically confirmed PDAC (ICD 25.0, 25.1 and 25.2) and clinically approved conditions as determined by the medical team. No restrictions were imposed regarding tumor stage or treatment status at the time of enrollment. The study followed a sequential sampling design based on each patient's pathological and clinical status, following the TNM staging system established by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). For PDAC patients deemed operable, borderline cases indicated for neoadjuvant treatment, and those with local metastasis, blood samples were collected every two to three months. Patients with advanced, systemically metastatic PDAC had a single blood sample collection. Demographic and clinical data for each participant were also gathered, including date and place of birth, self-declared ethnicity, alcohol consumption, tobacco usage, diagnosis date, tumor stage, treatment, and vital status.

Blood collection, cfDNA extraction, and quantification

A total of 15 mL of peripheral blood was collected into K3 EDTA tubes by the ICESP Biobank team. The samples were centrifuged at 1,900 g for 10 min at 4 °C, after which the plasma was collected and transferred to another tube. A second centrifugation was performed at 16,000 g for 10 min at 4 °C, and the resulting supernatant was collected and immediately stored at −80 °C. For comparison, to a subset of 12 samples, additional blood samples were collected using PAXgene blood ccfDNA tubes (Qiagen, Hilden, Germany). These samples were centrifuged at room temperature for 15 min at 2,000 g, with the plasma transferred to another tube for a second centrifugation at 2,000 g for 10 min, also at room temperature. The supernatant was collected and immediately stored at −80 °C. The plasma samples were subsequently transferred to the Carlos Chagas Institute (Curitiba, Paraná, Brazil), thawed at room temperature, and cfDNA was extracted from 5 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The cfDNA was quantified by high-resolution automated electrophoresis on a 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, USA) using the High Sensitivity DNA kit (Agilent Technologies, Santa Clara, USA), following the manufacturer's instructions. The cfDNA quantifications of the samples were performed within the range of 100–300 bp.

Speed-vac concentration

Samples showing insufficient concentration were concentrated using an Alpha 2–4 LD plus Speed-Vac (Marin Christ, Osterode, Germany). The samples were dried completely and resuspended in 12 µL of nuclease-free water. Quantification was subsequently performed using the 2100 Bioanalyzer System with the High Sensitivity DNA kit, according to the manufacturer's instructions.

Next-generation sequencing

NGS was performed to sequence all exons of the four main mutated genes in PDAC patients: *KRAS*, *CDKN2A*, *TP53*, and *SMAD4*. The library was constructed using the Qiaseq Targeted DNA Pro Custom kit (Qiagen, Hilden, Germany), which included 195 primers designed to cover all target gene exons in duplicate (i.e., each exon was sequenced using two distinct primer sets). Samples were labeled with the Qiaseq Targeted DNA Pro UDI Set A (Qiagen, Hilden, Germany), and the library was prepared with a minimum of 10ng of cfDNA, following the manufacturer's instructions as outlined in the QIAseq Targeted DNA Pro Handbook. The workflow included fragmentation and end preparation, adapter ligation, target enrichment (Panel with 195 primers), and universal PCR (during which samples were indexed). The libraries were quantified using the Qubit™ High Sensitivity dsDNA Assay (ThermoFisher, Walham, USA), and the mean fragment size was determined using the 2100 Bioanalyzer System with the High Sensitivity DNA kit, targeting a fragment size of 300–500 bp, and also to identify primers dimers or over-clustering. The final library was normalized for NGS using the Qiaseq Universal Normalizer Kit (Qiagen, Hilden, Germany) to achieve the optimal concentration of 4nmol/L for the Illumina platform. The libraries were subsequently pooled and quantified by Qubit™ High Sensitivity dsDNA Assay, and the mean fragment sizes were determined using the 2100 Bioanalyzer System with the High Sensitivity DNA kit. These methods resulted in a pooled library concentration of approximately 4nmol/L. NGS was conducted on the MiSeq platform (Illumina, San Diego, USA) using the MiSeq Reagent kit V3–600 cycles (Illumina, San Diego, USA), with 25 samples per run and a theoretical coverage of 20,000x.

dPCR for mutated *KRAS*

dPCR was performed on the Qiacuity Platform (Qiagen, Hilden, Germany) using 10ng of cfDNA in Qiacuity Nanoplate 26k for the detection of mutated *KRAS* variants: G12V (c.35G > T), G12D (c.35G > A), G12R (c.34G > C), G12C (c.34G > T), and Q61H (c.183 A > T). The assay utilized QIAcuity Probe PCR Master Mix (Qiagen) and TaqMan Mutation Detection Digital PCR assay (ThermoFisher, Walham, USA) labeled with VIC/FAM.

Data analysis and statistics

NGS variants were analyzed using Qiagen CLC and QCI softwares (Qiagen), with customized target regions and primers and *Homo sapiens* hg38 as the reference sequence. Variants were filtered for base quality (QUAL > 25)

and variant quality (average > 35), excluding low-frequency SNPs and indels (< 0.5%) with an average quality < 30. For numerical comparison between two groups, the Student's t-test was used, and for the survival rate curve, the Log-rank (Mantel-Cox) test was employed Agreement between dPCR and NGS was perfomed by overall Percent Agreement (OPA), Positive Percent Agreement (PPA), and Negative Percent Agreement (NPA). For each estimate, 95% confidence intervals were calculated using the exact Clopper–Pearson method, with agreement beyond chance was assessed using Cohen's kappa coefficient, and the presence of asymmetry in the discordant classifications between methods was investigated using McNemar's test, obtained using RStudio and *irr* package. Statistical significance was set at $p < 0.05$. All analyses were performed using Prism GraphPad software.

Data availability

The raw datasets generated during the current study are available in the Sequence Read Archive (SRA) of the NIH with Submission ID: SUB15862039 and BioProject ID: PRJNA1403045.

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Author contributions

AMM and BET plan and execute experiments, analyze data, write the draft; DKW execute experiments, analyze data, statistical analyze; DDA, MU and MJFA recruited participants, organized clinical and demographic data, coordinate samples logistics, analyze data; RC plan experiments, analyze data, scientific discussion, write the draft; GNN recruited participants, scientific discussion, analyze data; DLZ, LB and FC analyze data, scientific discussion, write the draft; MNA coordinate the project, provides budget, plan experiments, analyze data, scientific discussion, write the draft. All authors read and approved of the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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