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POTENTIAL APPLICATION OF CIRCULATING TUMOR DNA ANALYSIS IN A REAL-WORLD COHORT OF PANCREATIC DUCTAL ADENOCARCINOMA PATIENTS: THE "PANTA REI" STUDY

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Abstract

Background. Several studies suggested the feasibility and potential clinical utility of circulating tumoral (ct) DNA analysis as a prognostic marker to identify pancreatic ductal adenocarcinoma (PDAC) patients most likely to benefit from intensification of treatment pre- and/or post-operatively, and to guide decision-making among the different combinations available to metastatic PDAC. The *KRAS* gene is mutated in over 90% of PDAC and the heterogeneity of *KRAS* mutations between primary tumor and metastasis in individual patients is rare, suggesting that circulating mutant *KRAS* is a good biomarker for detecting the presence of cancer cells. However, the clinical significance of *KRAS*-mutated ct-DNA in PDAC has been inconsistent with respect to decision-making because limited points of detection of circulating mutant *KRAS* genes have been used, without a longitudinal monitoring throughout the treatment. We aimed to explore the feasibility and clinical utility of ct-DNA analysis to inform decision-making focusing on the prognostic and predictive role of circulating mutant *KRAS* genes in the blood and urine of PDAC patients, at any stage and with a longitudinal evaluation at several time-points. We presented preliminary data of a case series of patients with early- and late-stage PDAC.

Material and Methods. Matched tumor, blood and urine samples were prospectively collected at diagnosis and at the following time-points: in patients with resectable PDAC (cohort 1), plasma and urine samples were collected at each CT imaging during the neoadjuvant treatment, at 4 weeks before surgery, at 4-8 weeks after surgery, at each CT evaluation during the adjuvant treatment and at each follow-up visit; in patients with advanced PDAC (cohort 2), samples were collected at each CT evaluation during the first-line treatment. Formalin-fixed paraffin-embedded specimens were evaluated for *KRAS* mutations in exons 2, 3, 4, using MassArray. Plasma and urine samples were

analyzed for mutations in *KRAS* exons 2, 3, 4 by droplet digital polymerase chain reaction (ddPCR). We employed QX200 ddPCR system (Bio-Rad, Berkeley, California). Semi-quantitative index of fractional abundance of mutated allele was used and correlated to clinical outcome performing the Pearson's test. Median PFS and OS were estimated by the Kaplan-Meier method.

Results. From January 2020 to May 2023, 152 patients were prospectively enrolled: 45 and 107 were included in cohort 1 and in cohort 2, respectively. In cohort 1, the median age resulted 58.4 years with 24 males, in cohort 2 the median age was 64.6 years with 64 males. Among all the recruited patients, 20 patients with *KRAS*-mutated PDAC presented definitive results from plasma-based ddPCR analysis and were included in the present case series: 11 (55%) men, with a median age of 65.9 years; 4 (20%) enrolled in cohort 1. In the case series, median PFS was 4.05 months and median OS from the therapy start was 11.18 months. The total sensitivity of *KRAS* mutation detection in plasma at baseline was 55%: in cohort 1 it resulted 25% and in cohort 2 62.5%. Correlation between the percentage of increment of fractional abundance and PFS was strongly negative performing the Pearson's test (correlation index: -0.85).

Conclusions This study was planned to demonstrate for the first time that ct-DNA detection in PDAC at any stage and its expression level dynamics by longitudinal evaluation could be used for prognosis determination and disease monitoring, respectively. The present case series casted light on the several challenges to be overcome in order to make ct-DNA a feasible and accurate biomarker on the management of PDAC patients. Our definitive results from the large study population could confirm the insights drawn from the present case series regarding the prognostic and predictive role of ct-DNA analysis.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) ranks as the fourth leading cause of cancer-related mortality worldwide[1]. Although it is not a common disease, ranked 11th in incidence in men and 9th in women[1], the high mortality rate of this disease leads PDAC-associated death to be a significant burden on society. This burden is constantly growing, mostly due to the growing obesity epidemic[2]. The high mortality rate of PDAC stems from late diagnosis and relapse risk despite surgery. Only 20% of PDAC patients are suitable for curative resection at initial presentation and relapse occurs in 85% of cases even after primary tumor resection[1]. Patients with metastatic disease have a 5-year overall survival (OS) rate of 2%[1]. Therefore, highly sensitive diagnostic methods for early detection and novel treatment strategies are a significant unmet need.

Although the prognosis of PDAC is still grim, several new treatment combinations have been approved in this decade[3], both in the adjuvant setting[4,5] and in the first-line chemotherapy for advanced disease[6,7]. This increasing number of therapeutic options for PDAC patients, both in localized and advanced stages, combined with their significant and different toxicities, raises the need for better non-invasive tools to drive treatment decision-making.

In addition, as the disease progresses, new genetic changes may occur, leading to more aggressive and less treatment-responsive cells, resulting in secondary treatment resistance[8]. The ability to detect these changes without using invasive techniques can potentially help to identify genetic targets with therapeutic implications[9]. For example,

patients with PDAC with homologous recombination deficiency have better OS and progression-free survival (PFS) when treated with platinum-based chemotherapy[10,11].

Minimally invasive tools to predict recurrences and prognosis, to personalize treatment, to monitor chemotherapy response and to switch between all the current therapeutic options are urgently needed. Even if carbohydrate antigen 19.9 (Ca 19.9) is the most validated marker, it presents several limitations[12]. Ca 19.9 assessment may lead to frequent false positive results in patients with cholestasis (frequently present in PDAC of the pancreatic head), diabetes mellitus (that can be induced by PDAC), cirrhosis, chronic pancreatitis, and other gastrointestinal cancers. False negatives can also represent a severe limitation with 5-10% of the Caucasian population of the Lewis-null blood phenotype[13].

In this context, translational research aims to study and to develop plasma alternatives to traditional blood-based protein biomarkers, also known as "liquid biopsy", such as circulating tumor cells, cell-free circulating tumor DNA (ct-DNA) and circulating microRNAs. Management and therapies of several epithelial cancer patients are already performed using some of these markers[14-16].

In 1983, Shapiro et al. first described high levels of peripheral blood circulating free DNA (cf-DNA) in pancreatic cancer[17]. Ct-DNA origin seems a complex phenomenon, some works sustain a passive origin (necrosis, apoptosis), others an active tumor secretion[18-20].

Ct-DNA has been shown to carry tumor-specific genetic or epigenetic alterations, such as point mutations, copy number variations, chromosomal rearrangements, and DNA methylation patterns[21-24]. Ct-DNA is highly tumor specific and can accurately detect the presence of metastatic and minimal residual disease at a threshold ratio of 10000:1 normal background DNA to mutant allele ct-DNA[25,26]. This level of sensitivity, ease of access from peripheral blood and the short half-life of ct-DNA (≈2 hours) make this liquid biopsy

a suitable biomarker for short-term dynamics of tumor burden that can be monitored throughout treatment[25,26].

Identification of ct-DNA may be difficult because of its potential dilution among total cfree (f) -DNA. Especially in early tumor stages as resectable patients, ct-DNA may represent <1% of total cf-DNA, and its detection may require highly sensitive methods[27]. Among them, the two most common approaches are polymerase chain reaction (PCR)-based also known as digital PCR, and next generation sequencing (NGS) techniques. Digital PCR methods are highly sensitive but can only examine a single or a few mutations of interest at any one time. NGS approaches have the ability to look at a larger number of genes simultaneously, but they are usually limited by the need for high sensitivity and specificity and cost associated with sequencing. Thus, in comparison to NGS, the digital PCR seems to be more applicable in daily clinical practice because it is faster, much cheaper, and sensitive (0.001% vs 1%) and does not require complex bioinformatic analysis.

Whatever the technique, the main target for ct-DNA identification in PDAC patients is the *KRAS*^{mut}, which represents the initiating event in 90% of pancreatic cancer[28]. This single-nucleotide mutation replacement concerns mainly the codon 12 (exon 2) (G12D, G12V or G12R) and can occur on codons 11, 13 or 61, although less frequently. In combination with all these characteristics, the rare heterogeneity of *KRAS* mutations between tumor center and invasion front and between primary tumor and metastasis in individual patients with PDAC[29] suggests that circulating mutant *KRAS* ct-DNA in the blood is an excellent candidate biomarker for detecting the presence of cancer cells.

Detection and analysis of ct-DNA for somatic driver *KRAS* mutations is an evolving field in the management of patients with PDAC but it is not yet part of the day-by-day clinical practice.

An increasing number of studies have suggested the feasibility and potential clinical utility of ct-DNA analysis as a prognostic marker to identify PDAC patients most likely to benefit from intensification of treatment pre- and/or post-operatively[30-32], and also to guide therapy decision-making among the different combinations currently available to metastatic PDAC[33-35]. However, the clinical significance of *KRAS*-mutated ct-DNA in PDAC has been inconsistent with respect to decision-making. This inconsistency could be induced by low sample size and/or limited points of detection of circulating mutant *KRAS* genes in the blood at a few time-points, without a longitudinal monitoring throughout the treatment follow-up at any stage of PDAC.

The aim of this project was to explore the feasibility and potential clinical utility of ct-DNA analysis to inform therapy decision-making focusing on the prognostic and predictive role of circulating mutant *RAS* genes in the blood and in the urine of PDAC patients at any stage and with a longitudinal evaluation at several time-points.

Materials and methods

Study design, participants, and data collection

The present work was conceived as an exploratory longitudinal cohort observational study.

Key eligibility criteria were the following: histological or cytological diagnosis of PDAC, borderline resectable disease eligible for neoadjuvant therapy or resected primary tumor eligible for adjuvant therapy (cohort 1), metastatic or locally advanced disease eligible for systemic chemotherapy (cohort 2), availability of tumor biopsy material, the planning of systemic treatment and the possibility of adequate clinical and radiological follow-up. Patient characteristics (age, sex, Eastern Cooperative Oncology Group (ECOG) performance status (PS), Ca 19.9, vascular involvement primary tumor, localization of metastases, type of neoadjuvant, adjuvant, first-line chemotherapy, recurrence-free survival (RFS), PFS, OS) were retrieved from medical records by a trained medical doctor.

Matched tumor, blood and urine samples were prospectively collected at diagnosis and at the following time-points: in cohort 1, plasma and urine samples were collected at each computed tomography (CT) imaging during the neoadjuvant treatment, at 4 weeks before surgery, at 4-8 weeks after surgery, at each CT-scan evaluation during the adjuvant treatment and at each follow-up visit; in cohort 2, plasma and urine samples were collected at each CT evaluation during the first-line treatment (Figure (Fig) 1).

Ca 19.9 measurements and CT imaging were evaluated as part of the patients' routine follow-up. All treatments were delivered as per standard of care and blinded to ct-DNA results.

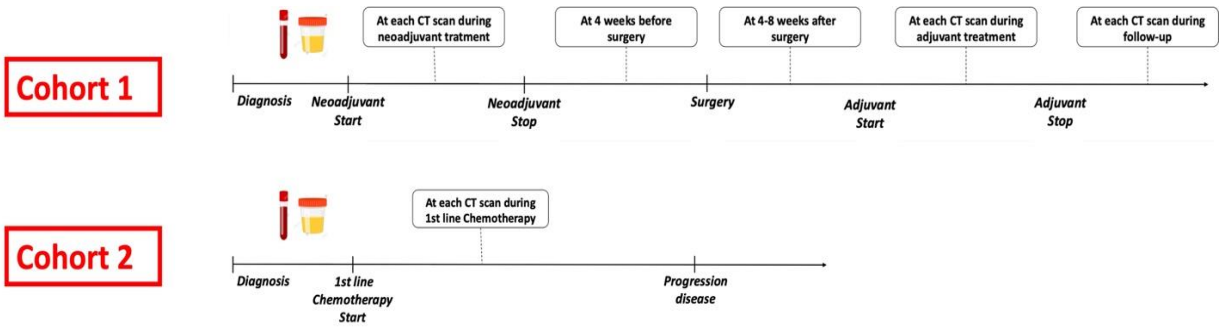


Fig. 1 Time-points and cohorts of the study

All these consecutive patients provided informed consent and the present study was conducted in accordance with the precepts of the Declaration of Helsinki and with approvation of the Veneto Institute of Oncology's Ethical Board (Prot. N. 0018920/21).

Tissue and plasma-based *RAS* mutation testing

Formalin-fixed paraffin-embedded (FFPE) specimens (either primary tumor or metastasis) were evaluated for *KRAS* and *NRAS* mutations in exons 2, 3, 4, using MassArray according to the procedures established in routine clinical use at our institution.

A single peripheral blood sample from each patient was collected in a K2 EDTA Vacutainer tube. Plasma samples were prepared from collected blood within 4h of phlebotomy with two subsequently centrifugation: the first one at 1600g for 10 minutes, then the isolated plasma was centrifuged at 3000g for 10 minutes and stored at -80°C. Ct-DNA was extracted from 4 ml of plasma samples using the QIAamp MinElute cf-DNA Mini Kit (Cat. ID 55284 Qiagen, Valencia, CA). The droplet (d) digital (d) PCR™ Screening Multiplex Kit (Bio-Rad, Hercules, CA) were used to assess the mutational

status of *KRAS* (codons 12 and 13), *NRAS* (codons 12,13 and Q16) genes. DdPCR analysis is based on the co-amplification of mutant or wild-type alleles (FAM- and HEX-labeled probes, respectively). QX200 droplet generator and Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystem, Waltham, Massachussetts) were used for the DNA amplification with the following protocol: 95°C for 10 minutes followed by 40 cycles of 94°C for 30 seconds and 55°C for 1 minute, then 98°C for 10 minutes. DdPCR allows for the enumeration of rare mutant variants in complex mixtures of DNA (wild type and mutant) based on an emulsion droplet technology and Poisson's distribution. Mutation specific amplification occurs in each individual droplet, and counting the positive droplets gives precise and absolute target quantification. As positive control we used DNA extracted by FFPE of PDAC *RAS*-mutated patients and as negative control DNA extracted by PDCA *RAS*-wild type patient. The results, obtained using a QX200 ddPCR system (Bio-Rad, Valencia, CA), are reported as copies per ml (copies/ml) of mutant DNA alleles. The fractional abundance quantifies the abundance of mutant DNA alleles in the wild-type background. Droplets were read in the QX200 droplet reader and analysed using the Quantasoft software version 1.0.596 (Bio-Rad, Berkeley, California). The sensitivity allowed the detection of very low allele frequencies down to < 0.02%.

Urine-based *RAS* mutation testing

Urine samples taken for ddPCR analysis were centrifuged to remove debris and contaminants. Urine sample containers were pre-filled with 10 ml EDTA (0.5 M, pH 8.0) and approximately 30 mL of urine was collected for each patient. Subsequently, urine samples were transferred and preserved into cf-DNA-BCT® tubes (Streck, Omaha, NE / USA). In this study, such contauners were used to ensure comparability between ct-DNA

extracted from plasma and urine samples, as the same ddPCR method was used for the analysis. The cell fraction was removed by centrifugation (20 minutes at 2000 g at +4°C) and the supernatant was carefully transferred into a 15 ml tube. A second centrifugation was performed at 16,000 g at +4°C to remove any cellular residues; 4 mL of processed urine was recovered for DNA extraction. The ct-DNA was extracted using the QIAamp® cf-DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Statistical analysis

Characteristics of patients with or without detectable ct-DNA were compared using a Student's *t*-test (normally distributed continuous variables), Mann-Whitney *U*-test (non-normally distributed continuous variables).

To evaluate the accuracy of the ct-DNA analysis we calculated the sensitivity and specificity of the ddPCR on our cohort. To define the false and true negatives or positives at the ct-DNA analysis we referred to the tissue-based RAS^{mut} analysis as the gold standard.

Results of liquid biopsy were considered both as a static parameter (presence versus absence of *RAS* mutation at each time-points) and as dynamic parameter (increase of fractional abundance from baseline and from nadir versus stable/decreasing value) and correlated with clinical outcomes. Delta 1 was defined as the difference between the fractional abundance at progression disease (PD) and that at the nadir value; delta 2 was defined as the difference between the fractional abundance at PD and that at the basal

value (i.e. T0, at the study start). Any change of fractional abundance was considered for statistical correlation.

To assess the potential clinical utility of ct-DNA analysis we investigated correlation of delta 1 and 2 with Ca 19.9, tumor burden, CT imaging, the oncological history as it emerged from the medical records. Correlation between delta 2 and PFS was statistically tested using Pearson's correlation test (normally distributed).

PFS was defined as the time from the beginning of the systemic treatment corresponding to T0 – the time of the baseline sample draw- to the first documentation of objective disease progression or death due to any cause, whichever occurred first. For alive patients free from progression at data cutoff, PFS was censored at the time of the last evaluable tumour assessment documenting absence of progressive disease.

OS was defined as the time from study enrolment to the date of death due to any cause. Patients still alive at the time of analysis were censored for OS on the last date they were known to be alive. Median PFS and OS were estimated using the Kaplan-Meier method and reported with 95% confidence interval (95%CI).

No formal sample size estimation and power calculation were made for this retrospective study. Statistical analyses were performed using the open-source statistical software package R 4.2.0.

Objectives

Primary objective of the PANTA REI study was to describe the feasibility, accuracy and potential utility of ct-DNA analysis in PDAC patients. In particular, we described all the several challenges existing about the integration of ct-DNA analysis in the clinical workflow.

Secondary objective was to investigate ct-DNA both as static and dynamic analyte according to the clinical data and the established biomarker Ca 19.9 in order to identify newer predictive or prognostic tools for survival in PDAC.

RESULTS

From January 2020, 178 consecutive patients diagnosed with PDAC were screened undergoing at least one plasma and urine sample collection at our center (UOC Oncology 1, Veneto Institute of Oncology, IOV-IRCCS, Padua).

To date, a total number of 152 patients was deemed eligible according to the above-mentioned inclusion criteria and having an enough number of collected time-points (Fig. 2). As detailed in our previous reports, data collection and ct-DNA analysis resulted conditioned by the study suspension during the 3-months SARS-Cov19 lockdown, but also by too long approval times for funding and materials supply. Therefore, the data lock was postponed to 2nd May 2023 and we are yet carrying out the ddPCR analyses on the whole study population.

We reported herein the preliminary results from a case series of 20 patients.

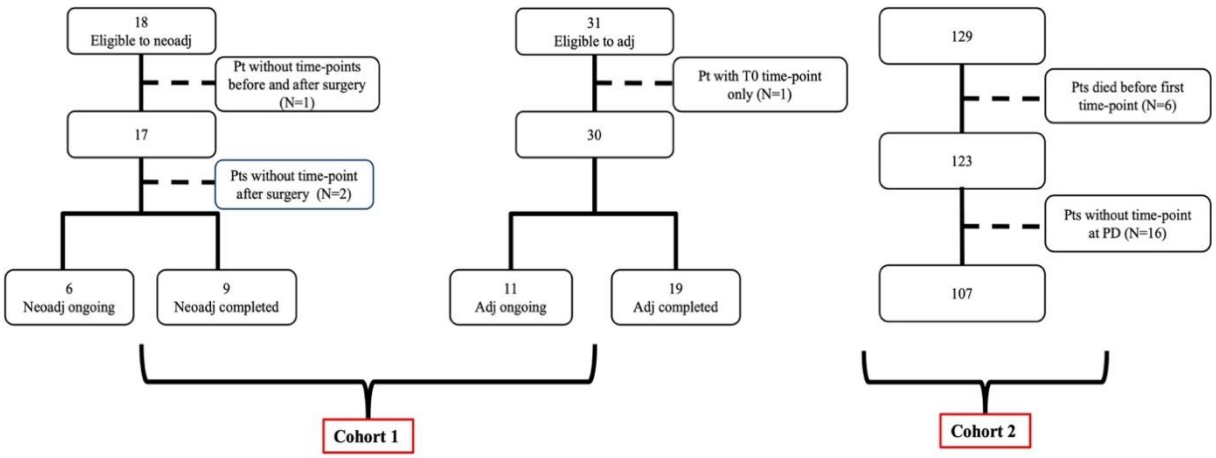


Fig. 2 Workflow diagram of the study

Clinical and pathological characteristics of all enrolled patients

Among the 152 enrolled patients, 45 and 107 were included in cohort 1 and in cohort 2, respectively. In cohort 1, the median age resulted 58.4 years with 24 (53.3%) males, in cohort 2 the median age was 64.6 years with 64 males (59.8%).

In cohort 1, a total number of 15 subjects underwent neoadjuvant therapy due to borderline resectable disease according to NCCN criteria 2.2022[36]. FOLFIRINOX was administered in 9 patients, PAXG in 4, Gemcitabine plus Nab-paclitaxel in 1 patient and FOLFOX in 1 case only. Among them, 2 patients have recently presented a disease progression and are just ready to molecular analysis. In patients receiving neoadjuvant chemotherapy, median Ca 19.9 was 830 kU/L at PDAC diagnosis.

Cohort 1 also included 30 patients who underwent curative and upfront pancreatectomy and referred to our center for adjuvant chemotherapy: 22/30 (73.3%) FOLFIRINOX, 6/30 (20%) Gemcitabine plus Capecitabine, 2/30 (6.7%) Capecitabine. Among them, 2 subjects have recently presented a disease progression and are just ready to ctDNA analysis. In patients receiving adjuvant chemotherapy, median Ca 19.9 was 230 kU/L at PDAC diagnosis.

In cohort 2, 34/107 (31.7%) patients presented a locally advanced (LA) disease, while PDAC resulted metastatic (m) at first diagnosis in 73 (68.3%) subjects. In the subset of patients with mPDAC liver was the first site of metastasis.

In cohort 2, the systemic chemotherapy regimens administered after the first visit at our center were the following: Gemcitabine plus Nab-paclitaxel 65/107 (60.8%), PAXG 13/107 (12.2%), FOLFIRINOX 13/107 (12.2%), Gemcitabine 7/107 (6.5%), FOLFOX 4/107 (3.7%), FOLFIRI 3/107 (2.8%), GEMOX 2/107 (1.8%). Among patients enrolled in cohort 2, a disease progression was recently detected in 29 patients who are ready to

ctDNA analysis on plasma and urine collected at various time-points. In cohort 2, median Ca 19.9 was 15.700 kU/L at diagnosis.

Clinical and pathological characteristics of the case series

Among the 20 patients with completed plasma-based ddPCR analysis and including in the present case series, 11 (55%) were men, with a median age of 65.9 years (range: 51-81). Table (tab) 1 summarized all the features of the case series.

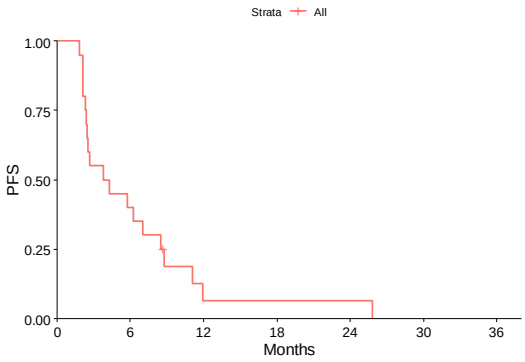
Out of these 20 PDAC patients, 4 (20%) were enrolled in cohort 1 having received neoadjuvant (N=2) or adjuvant only (N=2).

ID Number Patient	Age (y) at treatment start (T0)	Cohort	Primary tumor location	Primary tumor resected	Number of site of disease	Sites of disease	Biliary stenting before/during PANTAREI	Stage of PDAC at baseline	Baseline ECOG PS	Body Mass Index at T0	Albumin at T0 (g/dl)	Total Bilirubin at T0 (umol/L)	Ca 19.9 at T0 (ng/mL)	CA 19.9 at PD (ng/mL)	Therapy	Setting of Chemotherapy	Site of PD
2	72	1	head	Y (during study)	1	pancreas	N	IIA (BR)	0	20.4	42	25.7	9765	10000 (vs 435 at nadir)	Gem - Nab-paclitaxel	neoadj	Blood (Ca 19.9)
9	65	2	head	N	2	pancreas, liver	N	IV	1	31.2	24	8.8	758.2	312.1	Gem - Nab-paclitaxel	I line	liver
10	73	2	head	N	1	pancreas	N	III (LA)	0	20	32	11.6	3564	204 (vs 82 at nadir)	Gem - Nab-paclitaxel	I line	Blood (Ca 19.9)
7	72	2	tail	N	3	pancreas, liver, peritoneum	N	IV	1	24	missing	8.3	< 2.0	< 2.0	Gem - Nab-paclitaxel	I line	liver and peritoneum
13	77	2	head	N	2	pancreas, liver	N	IV	1	21.9	27	11.7	23160	24794 (vs 8163 at nadir)	Gemcitabine	I line	liver
6	66	2	head	Y (before study)	1	pancreas	N	IV	1	29	27	9,6	7,3	38	Gem - Nab-paclitaxel	I line	liver
15	74	1	head	Y (during study)	1	pancreas	N	IB (R)	1	22.6	31	5.3	191.2	missing (not available after surgery)	FOLFOX	neoadj	performance status
14	62	1	tail	Y (before study)	0	NA	N	pIIB	0	19.6	29	7	84.2	59.8	FOLFIRINOX	adj	peritoneum
17	52	2	body	Y (before study)	1	liver	N	IV	0	23.3	39	6.4	13710	12758	FOLFOX	II line	liver, lymphnodes, soft tissue
19	59	2	tail	N	1	pancreas	N	III (LA)	1	19.8	33	6.9	1160	2755	Gem - Nab-paclitaxel	I line	blood (Ca 19.9) and one single liver node
20	81	2	uncinate	N	1	pancreas	N	IIA (LA)	1	22.2	missing	4.1	93.5	122	Gemcitabine	I linea	local
21	51	2	head	Y (before study)	1	peritoneum	Y	IV	0	19.8	33	17.4	122.4	15.1	Gem - Nab-paclitaxel	I line	local
22	66	2	head	N	2	pancreas, liver	N	IV	1	19.8	36	5.7	3716	4779	Gem - Nab-paclitaxel	I line	liver
23	77	2	tail	N	2	pancreas, liver	N	IV	1	23.4	40	5.8	4564	39507	Gem - Nab-paclitaxel	I line	local and liver
24	63	2	tail	N	2	pancreas, liver	N	IV	0	24.7	missing	4.9	4482	23013	Gem - Nab-paclitaxel	I line	peritoneum and lymphnodes
25	52	2	body/tail	N	2	pancreas, liver	N	IV	0	20.9	38	5.0	3497	37698	PAXG	I line	local and liver
26	79	2	head	N	1	pancreas	N	III (LA)	0	24.9	41	9.3	2417	660 (vs 80 at nadir)	Gem - Nab-paclitaxel	I line	local
28	53	1	head	N	1	pancreas	Y	IB (BR)	0	22.7	24	27.4	30059	113000	FOLFIRINOX	neoadj	liver
29	52	2	head	Y (before study)	1	pancreas	N	IV	0	19	29	8.6	1620	2816	Gem - Nab-paclitaxel	I line	local
30	72	2	head	N	2	pancreas, liver, lung	Y	IV	1	24.1	missing	51.8	< 2.0	< 2.0	Gem - Nab-paclitaxel	I line	lung

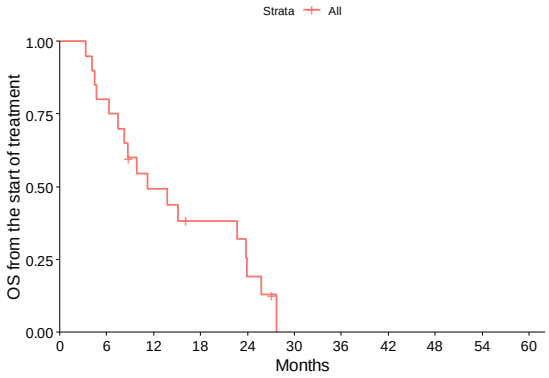
Tab.1 Characteristics of case series of 20 patients with PDAC

As shown in fig. 3, median PFS of the case series after 19 events was 4.05 months (95%CI: 2.47-8.75); after 17 events, median OS from diagnosis was 16.05 months

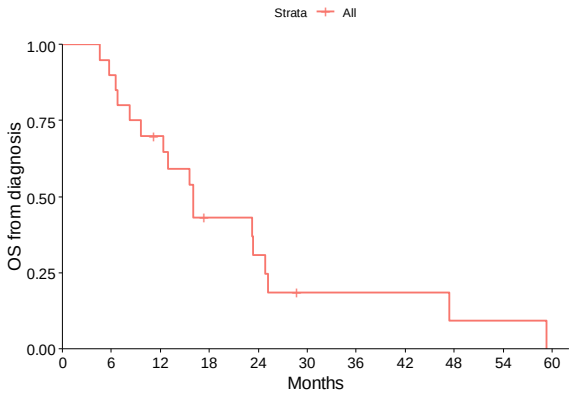
(95%CI: 12.37-NA), and median OS from the therapy start at T0 was 11.18 months (95%CI: 8.22-25.79).



Median PFS: 4.05 months
(95%CI: 2.47-8.75)
19 events



Median OS from T0: 11.2 months (95%CI: 8.22-25.79)
17 events



Median OS from diagnosis: 16.05 months
(95%CI: 12.4-NA)
17 events

Fig. 3 Median PFS and OS in the case series

Tissue-based *RAS* mutation testing of all enrolled patients

At this preliminary analysis, *RAS* mutational status was known for 24/45 (53.4%) and 86/107 (80.4%) patients of cohort 1 and 2, respectively. Tissue-based *RAS* mutation testing was performed only in 5 of 15 subjects receiving neoadjuvant chemotherapy because of unavailability of suitable cytological and or histological material at first diagnosis. To date, *RAS* mutations were detected in 20/24 (83.3%) patients in cohort 1 with *KRAS* G12D as the most common. In cohort 2, 72/86 (83.8%) patients harbored *KRAS* mutations with *KRAS* G12R as the most common. Final tissue-based results will be available when *RAS* mutation testing could be carried on resected tumor for patient's cohort 1 and on required tumor samples for all the patients (cohort 1 and 2) who do not have any archival tissue sample at our center.

Tissue-based *RAS* mutation testing of case series

All the 20 (100%) patients of the present case series harbored a *KRAS* mutation at the tissue-based analysis. On detail, the most common mutations resulted *KRAS* G12D (N=6, 30%), *KRAS* G12R (N=6, 30%), *KRAS* G12V (N=6, 30%); *KRAS* G12C mutation was detected in only one case (5%), as well *KRAS* Q61H (5%).

Plasma-based *RAS* mutation testing at T0 in the case series

All the 20 *KRAS*-mutated patients were tested in plasma on the same day of first administration of systemic treatment (baseline, T0). *KRAS* mutation was found in 11/20 cases by plasma-based ddPCR. The total sensitivity of *KRAS* mutation detection in plasma at baseline was 55%: in cohort 1 it resulted 25% and in cohort 2 62.5%.

In our series association was found between the presence of *KRAS* mutation in plasma and tumor burden, in terms of the presence of metastatic disease, the number of metastatic sites or the presence of liver localizations.

The detection of mutation in plasma at baseline was not confirmed as negative prognostic marker: the presence of *KRAS* mutation on plasma-based ct-DNA analysis was not statistically associated with PFS and OS.

Tab. 2 detailed concentrations of extracted ct-DNA, total amount of ct-DNA available for analysis, values of ct-DNA analyzed for each mutation, total number of events and fractional abundance at each time-point in our case series.

ID number patient	Cohort	RAS mutation on tissue-based	Date of plasma sample	Plasma volume utilized (mL)	Time Point	Conc. of cfDNA extracted (ng/uL)	Volume of basal cfDNA (uL)	Total ammount of cfDNA available (ng)	cfDNA analyzed per mutation (ng)	Well number used	Mut detected in ddPCR	Events number	Positive events per Mut.	Positive event wild-type	Fractional Abundance (%)	Delta Fractional Abundance PD vs Nadir	Delta Fractional Abundance PD vs Basal	Conc. Mut. (copies/µL)	Conc. wild-type (copies/µL)	PFS	S from diagnos	OS from T0
2	1	KRAS G	16/02/2021	4	2 T0	1,31	33	43,23	10	2	KRAS G12/13	22207	0	1158	0	0,00		0	63,01	8,75	25,23	23,85
			13/05/2021	4	2 T1R1	1,1	33	36,3	10	2	KRAS G12/13	22893	1	1126	0,09			0,05	59,34			
			15/07/2021	4	2 T2	2,13	33	70,29	10	2	KRAS G12/13	18871	0	1076	0			0	69,07			
			02/11/2021	4	2 PD	0,88	33	29,04	10	2	KRAS G12/13	19213	0	896	0			0	56,19			
9	2	KRAS G	05/03/2021	4	9 T0	5,24	38	199,12	20	4	KRAS G12/13	37574	2307	2748	45,64	-45,45	-45,45	74,44	89,085	3,82	6,51	6,28
			21/05/2021	4	9 T1R1	0,57	38	21,66	20	4	KRAS G12/13	24824	2	1058	0,19			0,085	65,03			
10	2	KRAS Q	06/08/2020	4	10 T0	0,71	33	23,43	20	4	KRAS Q61	37287	1	2687	0,04	0,01	0,01	0,025	87,83	8,52	24,84	23,88
			25/03/2021	4	10 PD	0,3	35	10,5	20	4	KRAS Q61	53543	1	2123	0,05			0,02	47,765			
7	2	KRAS G	27/01/2021	4	7 T0	1,09	38	41,42	10	2	KRAS G12/13	17491	5	1111	0,43		-0,27	0,34	77,21	11,09	23,22	22,70
			06/08/2021	4	7 T1R1	2,01	38	76,38	10	2	KRAS G12/13	17703	0	1030	0			0	70,52			
			08/10/2021	4	7 T1R2	0,76	38	28,88	10	2	KRAS G12/13	22719	1	1267	0,08			0,05	67,51			
			30/12/2021	4	7 PD	0,78	38	29,64	10	2	KRAS G12/13	24712	2	1244	0,16	0,16		0,1	60,77			
13	2	KRAS G	27/05/2021	4	13 T0	22,1	33	729,3	10	2	KRAS G12/13	22637	934	539	63,62		-5,60	49,57	28,35	5,79	8,19	7,43
			03/09/2021	4	13 T1R1	8,46	33	279,84	10	2	KRAS G12/13	24027	16	1138	1,35			0,78	57,08			
			26/11/2021	4	13 PD	4,79	33	158,07	10	2	KRAS G12/13	24163	1575	1150	58,02	56,67		79,3	57,37			
6	2	KRAS G	28/01/2021	4	6 T0	0,53	38	20,14	10	2	KRAS G12R	12474	2	642	0,3		-0,30	0,19	62,16	25,79	59,28	25,79
			06/05/2021	4	6 T1R1	3,04	38	115,52	10	2	KRAS G12R	19287	0	899	0			0	56,16			
			19/08/2021	4	6 T1R3	4,5	38	171	10	2	KRAS G12R	18598	0	1077	0			0	70,18			
			15/10/2021	4	6 T1R4	1,46	38	55,48	10	2	KRAS G12R	17702	0	923	0			0	63			
			16/12/2021	4	6 T1R5	1,43	38	54,34	10	2	KRAS G12R	22086	1	5377	0,02			0,05	328,23			
			17/02/2022	4	6 T1R6	1,15	38	43,7	10	2	KRAS G12R	19513	0	1058	0	0,00		0	65,58			
15	1	KRAS G	19/08/2022	4	15 T0	1,19	33	39,27	10	2	KRAS G12V	23926	1	1460	0,07		0,19	0,05	74,07	8,65	11,09	8,65
			28/10/2022	4	15 T1R1	20	33	660	10	2	KRAS G12V	24512	0	576	0			0	27,98			
			03/03/2023	4	15 T3	1,4	33	46,2	10	2	KRAS G12V	19038	3	1127	0,26	0,26		0,19	71,79			
14	1	KRAS G	06/04/2021	4	14 T4R1	0,42	38	15,96	10	2	KRAS G12V	20013	0	720	0		0,00	0	43,11	2,66	12,93	11,18
			08/07/2021	4	14 PD	1,6	38	60,8	10	2	KRAS G12V	23484	0	986	0	0,00		0	50,46			
17	2	KRAS G	04/06/2021	4	17 T0	5,6	33	184,8	10	2	KRAS G12V	21308	9	1309	0,66			0,5	74,41	2,53	16,05	4,67
			19/08/2021	4	17 PD	5,3	33	174,9	10	2	KRAS G12V	20917	117	1182	8,79	8,13	8,13	6,6	68,43			
19	2	KRAS G	30/09/2021	4	19 T0	0,72	35	25,2	10	2	KRAS G12R	18258	0	993	0			0	65,79	7,01	15,56	13,78
			27/10/2021	4	19 T1R1	0,53	35	18,55	10	2	KRAS G12R	17961	0	865	0			0	58,07			
			07/01/2022	4	19 T1R2	0,5	35	17,5	10	2	KRAS G12R	18393	1	927	0,11			0,06	60,84			
			03/03/2022	4	19 T1R3	0,61	35	21,35	10	2	KRAS G12R	18560	2	1026	0,19			0,13	66,9			
			26/05/2022	4	19 PD	1,3	35	45,5	10	2	KRAS G12R	21897	4	1303	0,3	0,30	0,30	0,21	72,18			
20	2	KRAS G	16/03/2021	4	20 T0	0,56	35	19,6	10	2	KRAS G12R	15717	0	830	0			0	63,83	2,37	16,05	15,10
			05/08/2021	4	20 PD	0,68	35	23,8	10	2	KRAS G12R	8911	0	373	0	0,00	0,00	0	50,31			
21	2	KRAS G	02/12/2020	4	21 T0	1,64	35	57,4	10	2	KRAS G12R	17673	0	839	0			0	57,22	2,11	47,37	27,73
			04/02/2021	4	21 PD	2,24	35	78,4	10	2	KRAS G12R	17394	0	1068	0	0,00	0,00	0	74,55			
22	2	KRAS G	30/09/2021	4	22 T0	0,38	36	13,68	10	3	KRAS G12D	24564	5	977	0,5			0,24	47,75	2,11	12,37	9,90
			27/10/2021	4	22 T1R1	1	36	36	10	2	KRAS G12D	18148	8	1127	0,68			0,52	75,43			
			05/01/2022	4	22 PD	0,56	36	20,16	10	2	KRAS G12D	20272	1	1097	0,09	-0,41	-0,41	0,06	65,45			
23	2	KRAS G	12/11/2021	4	23 T0	0,89	36	32,04	10	2	KRAS G12V	21376	5	1362	0,35			0,28	77,46	2,30	6,81	4,51
			07/02/2022	4	23 PD	2,26	36	81,36	10	2	KRAS G12V	18621	3	1318	0,22	-0,13	-0,13	0,19	86,36			
24	2	KRAS G	03/12/2021	4	24 T0	3,2	36	115,2	10	2	KRAS G12D	19365	101	1122	8,06			6,15	70,22	2,47	4,54	3,36
			18/02/2022	4	24 PD	10	36	360	10	2	KRAS G12D	24441	204	1184	14,44	6,38	6,38	9,86	58,42			
25	2	KRAS G	28/01/2022	4	25 T0	1,47	36	52,92	10	2	KRAS G12V	18728	66	1082	5,6		-1,52	4,13	70,01	4,28	17,30	16,05
			22/04/2022	4	25 T1R1	0,95	36	34,2	10	2	KRAS G12V	19524	4	965	0,4			0,24	59,63			
			15/06/2022	4	25 PD	0,72	36	25,92	10	2	KRAS G12V	15367	40	914	4,08	3,68		3,07	72,14			
26	2	KRAS G	05/02/2021	4	26 T0	0,46	35	16,1	9,66	3	KRAS G12R	40391	1	1453	0,07		0,04	0,03	43,1	11,94	28,62	26,97
			20/05/2021	4	26 T1R1	0,63	35	22,05	13,23	3	KRAS G12R	38395	2	1814	0,11			0,06	56,94			
			12/08/2021	4	26 T1R2	0,76	35	26,6	15,96	3	KRAS G12R	35214	2	2023	0,1			0,07	69,61			
			07/10/2021	4	26 T1R3	1	35	35	21	3	KRAS G12R	37448	2	2807	0,07			0,06	91,67			
			10/03/2022	4	26 PD	0,66	35	23,1	13,86	3	KRAS G12R	34970	2	1756	0,11	0,04		0,07	60,61			
28	1	KRAS G	20/01/2023	4	28 T0	0,6	35	21	12,6	3	KRAS G12D	30721	15	1362	1,07		-0,64	0,57	53,35	1,84	5,76	4,18
			31/03/2023	4	28 PD	2,45	35	85,75	51,45	3	KRAS G12D	31086	34	7004	0,43	-0,64		1,29	300,34			
29	2	KRAS G	28/01/2021	4	29 T0	0,75	35	26,25	15,75	3	KRAS G12D	30754	4	1809	0,21		0,44	0,15	71,32	6,22	23,39	8,65
			06/05/2021	4	29 T1R1	0,85	35	29,75	17,85	3	KRAS G12D	30488	2	1845	0,1			0,08	73,44			
			10/08/2021	4	29 T1R2	1	35	35	21	3	KRAS G12D	26077	10	2203	0,43			0,45	103,84			
			10/08/2021	4	29 PD	1	35	35	21	3	KRAS G12D	36163	20	2935	0,65	0,55		0,65	99,58			
30	2	KRAS G	06/05/2021	4	30 T0	5	35	175	105	3	KRAS G12V	17553	4	6695	0,13		-0,10	0,27	565,09	2,11	9,57	8,22
			02/07/2021	4	30 PD	2,41	35	84,35	50,61	3	KRAS G12V	28376	2	5803	0,03	-0,10		0,08	269,16			

Tab.2 Details of plasma-based *KRAS*^{mut} testing by ddPCR on the case series

Monitoring of plasma-based *RAS* mutation testing in the case series

With a mean of 3 time-points for each patient in the present case series, we explored the correlation of the results of ct-DNA analysis with each clinical outcome (i.e. PD) and data (i.e. Ca 19.9 trend, tumor burden, site of PD) considering liquid biopsy also a dynamic parameter (increase of fractional abundance versus stable/decreasing value).

At PD, we observed an increase of fractional abundance compared to its nadir value (delta 1) in 10/20 (50%) patients, while delta 2 (defined as the difference between the fractional abundance at PD and that at the basal value) resulted > 0 in 7/20 (35%) subjects. As reported in tab. 2, in 4 (20%) patients both delta 1 and delta 2 were equal to 0, because no ct-DNA was detected in the plasma by ddPCR analysis. These are 2 patients from cohort 1 (ID#2, ID#14) and 2 from cohort 2 (ID#20 and ID#21).

For patients experiencing increase in fractional abundance at PD, correlation between the percentage of increment of fractional abundance and the PFS value was negative performing Pearson's test. The correlation index between delta 1 and PFS was – 0.23 [-1, -1], while the other one between delta 2 and PFS resulted -0.85 [-1, -1].

Fig. 4 illustrated this strongly negative correlation between delta 2 and PFS.

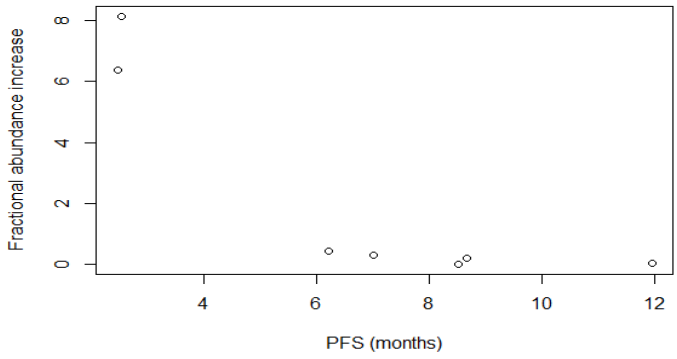


Fig. 4 Pearson's test of correlation between delta 2 and PFS in the case series.

In fig. 5 we reported results of ddPCR analysis for patient #29 at each time-points, in fig. 6 those for patient #17 at baseline and PD. Finally, fig. 7 showed the results of patient #9 with decrease of fractional abundance at PD versus baseline (delta 2 < 0).

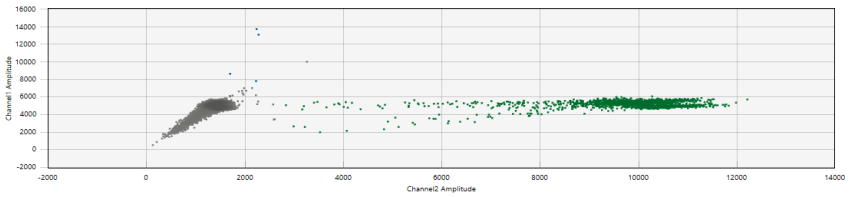


Fig. 5a Dot-plot of ddPCR at T0 in patient #29

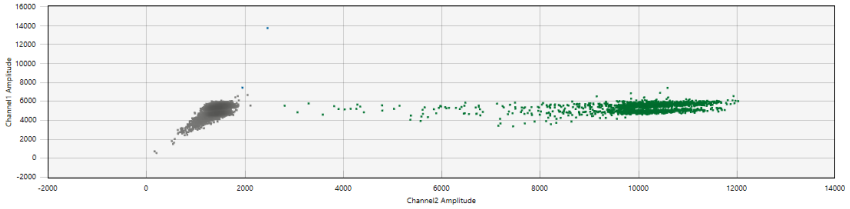


Fig. 5b Dot-plot of ddPCR at T1 in patient #29

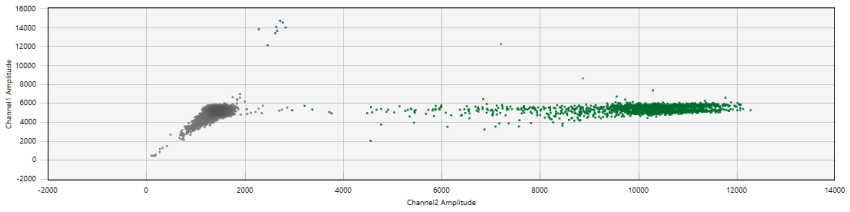


Fig. 5c Dot-plot of ddPCR at T2 in patient #29

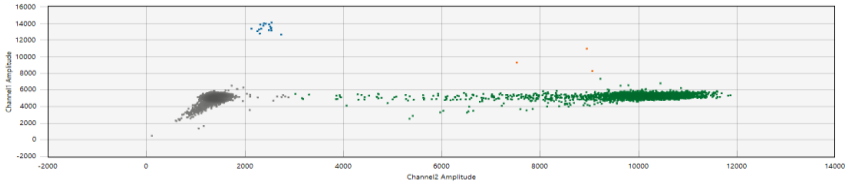


Fig. 5d Dot-plot of ddPCR at PD in patient #29

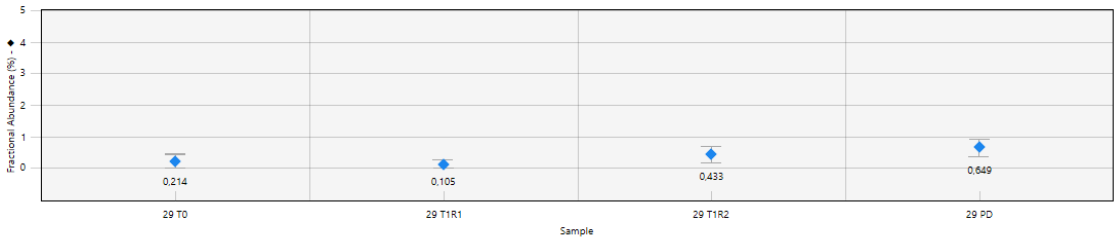


Fig. 5e Trend of fractional abundance at various time-points in patient #29

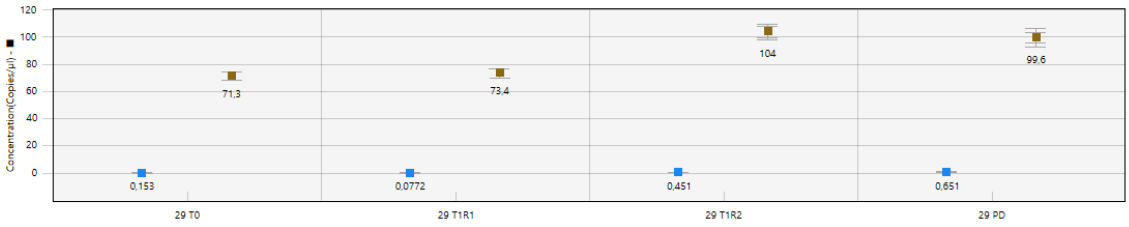


Fig. 5f Concentrations of extracted ct-DNA in patient #29

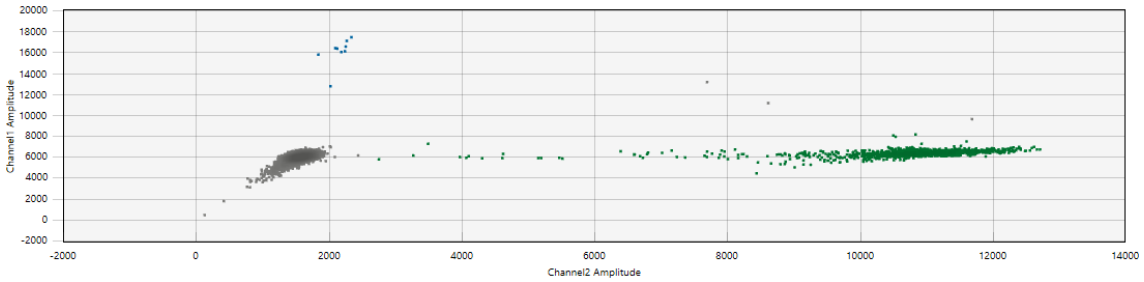


Fig. 6a Dot-plot of ddPCR at T0 in patient #17

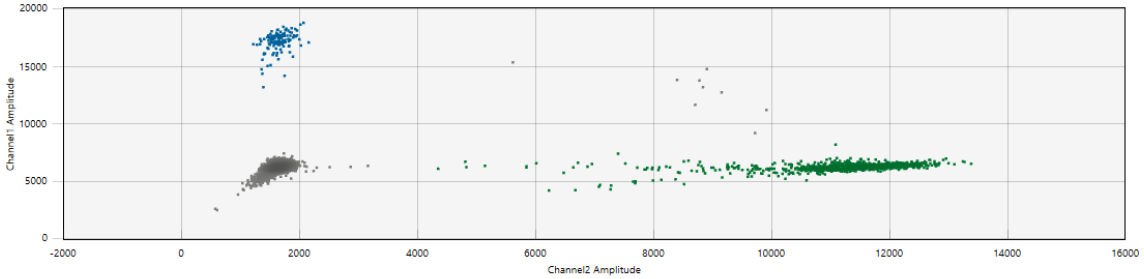


Fig. 6b Dot-plot of ddPCR at PD in patient #17

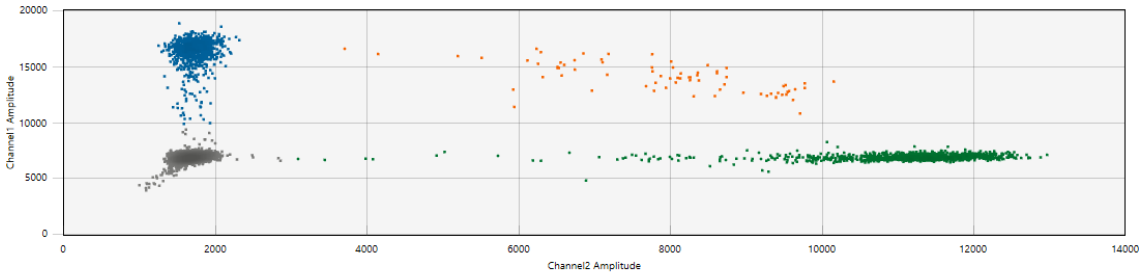


Fig. 7a Dot-plot of ddPCR at T0 in patient #9

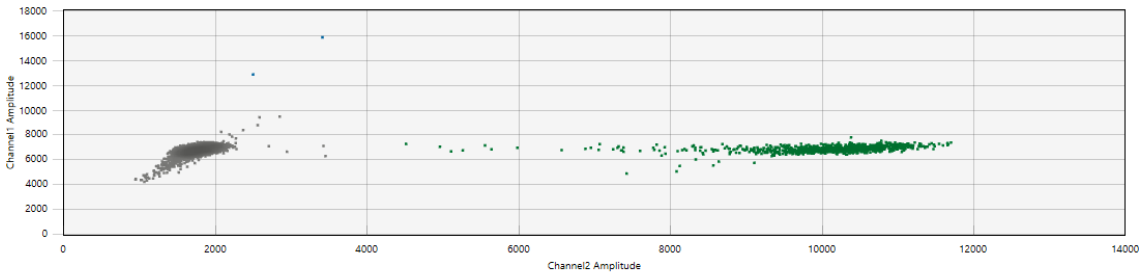


Fig. 7b Dot-plot of ddPCR in patient #9

With regard to the correlation of delta 2 with Ca 19.9 trend, we found correspondence in 9 (45%) cases: at PD, Ca 19.9 increased as the fractional abundance increased with respect to the baseline. In one case it was not possible to detect any correlation because Ca 19.9 was not dosed at the corresponding time-point (after surgery); in the remaining 10 (50%) cases instead there was no correlation between delta 2 and Ca 19.9 changes. Delta 1 and Ca 19.9 trend appeared to vary much more consensually.

Discussion

We reported prospective evaluation of ct-DNA analysis performed at several and predefined time-points leading to a serial monitoring of quantitative mutant *RAS* ct-DNA levels in early and advanced stages of PDAC. In the effort to develop an effective treatment follow-up marker, we investigated whether liquid biopsy could be a suitable tool to predict biological effects of anticancer treatments. To be integrated into daily management of patients with PDAC, this tool should meet the following requirements: it needs to be easy to use, with inexpensive and readily available method for clinical practice; it needs to be specific and sensitive, and it must closely correlate with disease response to treatment and progression[34].

Detection and analysis of ct-DNA for somatic driver mutations is an evolving field, clinically and academically. A search for term "ct-DNA" in PubMed showed a continuous rise in the number of publications, with 507, 721 and 988 articles published in 2016, 2019 and 2022, respectively. Moreover, ct-DNA analyses for specific mutations are now part of clinical routine for many tumors[14-16], but not for PDAC yet. Studies using ct-DNA have recently been gaining popularity also in PDAC because of several challenges facing tissue-based analyses, such as the lack of resected tumor tissue specimens, the low neoplastic cellularity of most of them and the poor general conditions of most PDAC patients that do not allow an invasive biopsy.

Feasibility of ct-DNA analysis

The primary objective of the PANTA REI study is to assess the feasibility of ct-DNA analysis in a real-world series of patients with PDAC, both in early and advanced stages. The many difficulties emerged during all the steps of our analyses demonstrated that

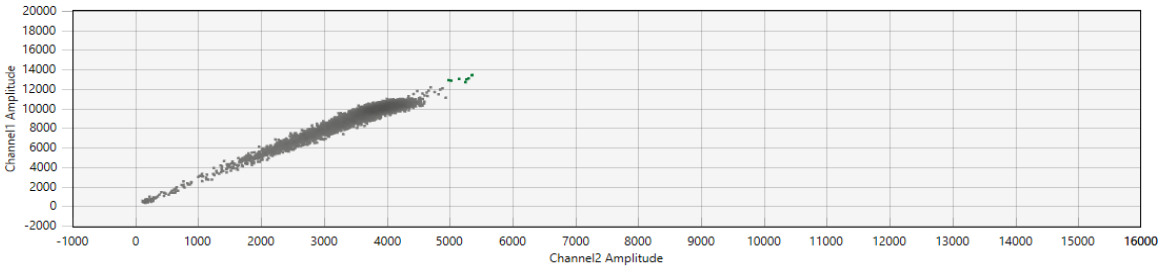
integrating the liquid biopsy into the clinical PDAC workflow requires a considerable investment of time, resources, and knowledge. State-of-the-art ddPCR, laboratory technicians and bioinformaticians for data analysis are part of the essential investments. Beyond the delays attributable to the SARS-Cov19 pandemic and to the too long times for approval and material supply, our analyses have also been slowed down by the training and education necessary for hospital personnel (nurses, physicians, and pathologists) in applying and interpreting the new biomarker. This has meant that we could only report the plasma-based (not yet urine) ddPCR results of 20 patients out of the 152 deemed eligible and we are yet carrying out the ddPCR analyses on the remaing study population. To demonstrate how much work still needs to be done to make the liquid biopsy feasible in daily practice, we reported some of the challenges we encountered. For example, the ct-DNA analysis started on plasma samples from the first 5 patients was repeated because results were deemed unreliable as the recorded droplets number suggested. As a matter of fact, an index that can be used to evaluate the methodological quality and data reliability is the droplets number that the QX200 droplet reader recognizes as adequate ("number of events"). The number of events must be equal to or greater than 10000 and can be influenced by several causes concerning both preanalytical and analytical steps:

i. We observed a lot of variability in the amount of extracted ctDNA in the first 5 patients analysed (range: 0.5–11.8 ng/uL). In cases in whom we recorded an insufficient number of droplets to proceed with analysis, the concentration of extracted ct-DNA resulted the lowest of those recorded in the entire population under preliminary investigation (range: 0.5-2.6 ng/uL). The low value of extracted ct-DNA could be due to the long interval (> 2 years) between plasma sampling

and cf-DNA extraction, as we assumed for patient #10 in which ctDNA may be degraded over all this time. In the other remaining patients who presented a number of events lower than 10000, the low amount of extracted ct-DNA could be due to a really low disease burden and therefore to a low ct-DNA shedding.

ii. The ctDNA analysis by ddPCR presented some preanalytical challenges also in the sample's preparation, as we found in the present preliminary results. In patient #5, the number of events was inadequate (< 10000) due to an operator-dependent error in the step of samples preparing for PCR: the formation of some air bubbles did not allow an adequate sample mixing.

In fig. 9, the dot-plot of the "elongated cloud" demonstrated the inadequate mixing between reaction mix and ct-DNA in patients for whom the PCR analysis had to be repeated given the lower number of events.



Observing the tab. 2, we could note that the number of events in the repeated and definitive analyzes of our case series resulted above 10000 at each time-point for each patient and that the mean number of events is even equal to 23758,8 (range:12470-53543). In addition, repeating the analyses, we decided to extract the maximum amount of ct-DNA for each time-point to reduce the variability of the number of events as much as possible.

All these issues demonstrated the good quality of the present case series results but also confirmed a necessary learning curve for technicians approaching PCR for the first time, especially if this is not completely automated as in our case. Similarly, the physician who received the assay report needed the training to read it, interpret the results and act accordingly. Education in new technologies and new approaches to care should also be taught in the university curriculum in the future, given the important development of personalised medicine in oncology.

Accuracy of ct-DNA analysis as a static and dynamic marker

In our case series with 20 *KRAS*-mutated patients, *KRAS* mutation was found in 11/20 cases by plasma-based ddPCR at T0 (as a "static biomarker") leading to a sensitivity of 55% consistent with ranges reported in literature[33,37]. Perets et al.[34] concluded for a slightly lower percentage of *KRAS* positive ct-DNA because his cohort included some *KRAS* wild-type PDAC unlike our case series.

The ct-DNA detection rate resulted 25% in cohort 1 and in cohort 2 62.5%, as expected considering that in cohort 1 PDAC were at early stages and therefore with a lower disease burden than those in cohort 2 including LA and mPDAC. This finding confirmed the demonstrated association[35] between the presence of *KRAS* mutation in plasma at baseline and tumor burden, in terms of the presence of metastatic disease, the number of metastatic sites or the presence of liver localizations.

Monitoring plasma-based *RAS* mutation testing in the present case series, we assessed liquid biopsy as a "dynamic biomarker" according to disease progression. For patients experiencing increase in fractional abundance at PD, we observed a strongly negative correlation between the magnitude of fractional abundance increase at PD and PFS value:

patients with the greatest value of *KRAS* mutants on ddPCR analysis had the shortest PFS, as demonstrated by Pearson's test. This finding suggested that ct-DNA could correlate with disease progression or activity. Similar to our results, previous studies have also highlighted the association between the presence and changes of *KRAS* mutations in plasma and poor survival in patients with resectable and advanced PDAC[34,38,39].

Interestingly, the 4 patients with undetectable ct-DNA at any time-point confirmed the correlation between ct-DNA analysis and disease load because these patients were at early stage (ID#2 and ID#14 in cohort 1) or with only a local recurrence (ID#20 and ID#21 in cohort 2). No wonder patient #21 registered the highest OS in our case series (OS from PDAC diagnosis was 47.4 months, OS from treatment start was 27.7 months): he had only disease recurrence at his duodenal prosthesis without any other metastatic localization. Instead, the surprising fact was that in this patient Ca 19.9 was significantly reduced at PD (-87.6% versus baseline), despite also the multiple cholangitis occurred.

Once again, these observations indirectly could confirm the prognostic value of liquid biopsy and the need to integrate all the information deriving from established (Ca 19.9, imaging-based tumor response) and emergent biomarkers (ct-DNA) to predict outcomes in PDAC patients. The too small sample size of the present series cannot allow to draw firm conclusions on impact of ct-DNA analyte on PFS and OS.

Case-by-case evaluation of patients with decrease of *KRAS*^{mut} on plasma-based ddPCR analysis performed at PD and compared to baseline.

In 9 of 20 PDAC patients we registered a negative value of delta 2 that implied a decrease in ct-DNA at the time of disease progression. Other studies[40,41] reported that the ct-DNA fractional abundance did not parallel changes in tumor volume in a minority part of

patients. Therefore, we proposed to analyze this incongruence between ct-DNA (delta 2) and tumor response in detail, by evaluating all these patients with a case-by-case approach:

- In patient #6, #25 and #30, we observed only minimal radiological PD (≥ 20%) but there was a significant increase of Ca 19.9 (>50%).
- In patient #7 and #13, both radiological and biochemical progression resulted minimal, but we registered a relevant increase of $KRAS^{mut}$ at PD in comparison to its nadir value (i.e. delta 1).
- In patient #23, PD was only biochemical, with Ca 19.9 increase > 50%.
- In patient #22, imaging-based evaluation showed mixed response on target lesions and Ca 19.9 increased slightly (+28%), but the PD was decreed due to the poor tolerance to administered chemotherapy.
- In patient #28, we constated that the number of positive events at PD was six times higher than at baseline. We supposed that standardizing the ct-DNA concentration between these two time-points could modify results in this case.
- In patient #9, PD was diagnosed on the basis of a radiological comparison with a baseline CT-scan performed more than one month before the treatment start. Similarly to the decrease of fractional abundance, Ca 19.9 resulted significantly reduced (> 50%).

Conclusions and future directions

The reported preliminary results highlighted some challenges of ct-DNA analysis, both in analytical and pre-analytical phases. Regardless of known delays due to SARS-Cov19 pandemic and of unexpected ones for funding and material supply, it took a long time to improve the manual skills of the technicians, to standardize the various steps of ddPCR analysis and to teach clinicians to read ddPCR results also according to clinical history of each patient. Much more challenges were faced in ddPCR analysis on samples of urine due to the lower ct-DNA levels than those reported in plasma ones; for these reasons it took even more time for urine-based results which will be available shortly.

Thanks to more reliable results in terms of number of events and thanks also to much more data with all collected time-points, we will be able to verify the feasibility and real clinical utility of the fractional abundance *delta* (i.e. the percentage change of the fractional abundance between subsequent time-points) in the entire study population.

As partially suggested by the presented first results, we expect different findings between the two patients' cohorts due to their different disease burden (early stages in cohort 1, advanced stages in cohort 2). To improve the ddPCR sensitivity in cohort 1, it may be necessary to increase the amount of extracted DNA in blood and urine samples so that minimal residual disease could be detected more easily.

As far as we know, it is the first study with the ambitious aim of monitoring ct-DNA as a static and dynamic biomarker, with so many time-points, performing ddPCR both on plasma and urine samples of patients with early- and late-stage PDAC.

When we will finish examining the longitudinal levels of circulating mutant *KRAS* and *NRAS* genes according to clinic-pathologic, treatment and outcome data of the 152

enrolled patients, we could reinforce insights emerged from the present case series. Even with the evident limits due to small sample size, we could draw some insights from these preliminary results: the correlation between ct-DNA analysis and clinical outcomes (disease progression) or data (Ca 19.9, tumor burden) may suggest the feasibility and utility of combining liquid biopsy with other established biomarkers into daily clinical practice to guide adjuvant and first-line therapy decisions in early- and late-stage PDAC, respectively. An interesting illustration of this promising approach in the diagnostic setting is the CancerSEEK test, which used a combination of ct-DNA and established serum markers to detect early-stage cancers, showing high sensitivity for the detection of five cancers, including PDAC[42].

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