

## ORIGINAL ARTICLE

# Circulating tumor DNA as a potential marker of adjuvant chemotherapy benefit following surgery for localized pancreatic cancer

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**Background:** In early-stage pancreatic cancer, there are currently no biomarkers to guide selection of therapeutic options. This prospective biomarker trial evaluated the feasibility and potential clinical utility of circulating tumor DNA (ctDNA) analysis to inform adjuvant therapy decision making.

**Materials and methods:** Patients considered by the multidisciplinary team to have resectable pancreatic adenocarcinoma were enrolled. Pre- and post-operative samples for ctDNA analysis were collected. PCR-based-SafeSeqS assays were used to identify mutations at codon 12, 13 and 61 of *KRAS* in the primary pancreatic tumor and to detect ctDNA. Results of ctDNA analysis were correlated with CA19-9, recurrence-free and overall survival (OS). Patient management was per standard of care, blinded to ctDNA data.

**Results:** Of 112 patients consented pre-operatively, 81 (72%) underwent resection. *KRAS* mutations were identified in 91% (38/42) of available tumor samples. Of available plasma samples ( $N = 42$ ), *KRAS* mutated ctDNA was detected in 62% (23/37) pre-operative and 37% (13/35) post-operative cases. At a median follow-up of 38.4 months, ctDNA detection in the pre-operative setting was associated with inferior recurrence-free survival (RFS) [hazard ratio (HR) 4.1;  $P = 0.002$ ] and OS (HR 4.1;  $P = 0.015$ ). Detectable ctDNA following curative intent resection was associated with inferior RFS (HR 5.4;  $P < 0.0001$ ) and OS (HR 4.0;  $P = 0.003$ ). Recurrence occurred in 13/13 (100%) patients with detectable ctDNA post-operatively, including in seven that received gemcitabine-based adjuvant chemotherapy.

**Conclusion:** ctDNA studies in localized pancreatic cancer are challenging, with a substantial number of patients not able to undergo resection, not having sufficient tumor tissue for analysis or not completing per protocol sample collection. ctDNA analysis, pre- and/or post-surgery, is a promising prognostic marker. Studies of ctDNA guided therapy are justified, including of treatment intensification strategies for patients with detectable ctDNA post-operatively who appear at very high risk of recurrence despite gemcitabine-based adjuvant therapy.

**Key words:** circulating tumor DNA, pancreatic ductal adenocarcinoma, pancreatic cancer, liquid biopsy, biomarkers, adjuvant therapy

## Introduction

Only 20% of pancreatic ductal adenocarcinoma (PDAC) patients are suitable for curative intent resection at initial presentation. Relapse occurs in 80%–85% of cases despite ‘curative’ resection [1]. The use of adjuvant gemcitabine with capecitabine or FOLFIRINOX both improve overall survival (OS) compared with gemcitabine alone [2, 3]. Preliminary results from the APACT trial, demonstrate a nominal survival benefit from the combination of gemcitabine with Abraxane compared with gemcitabine alone, with no improvement in recurrence-free survival (RFS) [4].

Circulating tumor DNA (ctDNA), arises from somatic tumor DNA fragments released into the blood circulation during cell death [5]. ctDNA is highly tumor specific and can accurately detect the presence of metastatic [6, 7] and minimal residual disease across many solid tumors [7, 8] at a threshold ratio of 10 000 : 1 normal background DNA to mutant allele ctDNA [5, 9]. This level of sensitivity, ease of access from peripheral blood and the short half-life of ctDNA ( $\approx 2$  h) [10] make ctDNA an ideal dynamic biomarker of tumor burden that can be monitored throughout treatment [7].

The *KRAS* gene is mutated in over 90% of PDAC tumors [11]. The use of the noninvasive blood-based biomarker ctDNA focusing on mutations in the *KRAS* gene [11], could be used to identify PDAC patients most likely to benefit from intensification of treatment pre- and/or post-operatively.

There are multiple challenges to undertaking biomarker studies in early PDAC, including the relatively small number of suitable participants undergoing surgery compared with more prevalent malignancies, a higher proportion of patients with inoperable disease identified at the time of surgery, and difficulties obtaining adequate PDAC tissue for analyses particularly if prior neoadjuvant therapy was undertaken. In this explorative biomarker study, we examined the feasibility of a blood-based biomarker study in localized pancreas cancer, including pre- and post-operative samples. We also examined the potential clinical utility of ctDNA as a prognostic marker to guide adjuvant therapy decisions in early-stage PDAC.

## Patients and methods

This prospective multicenter biomarker trial recruited patients with early-stage operable pancreatic adenocarcinoma at centers in Australia, New Zealand and Singapore (Australian New Zealand Clinical Trials registry number ACTRN12612000763842) from January 2015 till June 2017. Matched tumor and blood samples were prospectively collected at diagnosis and at 4–8 weeks after surgery (before adjuvant chemotherapy), alongside clinico-pathologic, treatment and outcome data. All tumor tissue and blood analyses were conducted at Johns Hopkins University. All treatments were delivered as per standard of care and blinded to ctDNA results. Follow-up included 3-monthly clinical review for 2 years. The study was approved by the institutional review boards for human research at each institution. All participants provided informed written consent for the collection of specimens and data analysis. This report was written according to the Reporting recommendations for tumor MARKer prognostic studies (REMARK) [12].

## Identification of somatic mutations in tumor tissue

Formalin fixed paraffin embedded tumor tissue were macro-dissected to ensure a neoplastic cellularity of  $>30\%$ . The ctDNA was purified from 3.5 ml plasma using a QIASymphony DP DNA Midi Kit (cat. no. 937255) Hilden, Germany.

## ctDNA analysis

A specifically designed PCR-based assay was used to identify somatic mutations at codon 12, 13 and 61 of the *KRAS* gene and in surrounding codons in the primary tumor [6]. The identified mutation was quantified in matching plasma using the Safe-Sequencing System (SafeSeqS) [9], which utilizes uniquely labeled molecular barcodes, for each template molecule. This minimizes replication errors that frequently occur in massively parallel sequencing. This technique can detect 1 mutant template amongst 10 000 normal templates, allowing detection and accurate quantification even at mutant-DNA levels as low as  $<0.1\%$  of the total cell free DNA. Analysis for ctDNA was blinded to clinical data.

## Algorithm for classifying ctDNA status

ctDNA was categorized as detectable (ctDNA-positive) or undetectable (ctDNA-negative) based on a permutation test (R package perm software version 3.2.3) comparing the mutational frequency in the sample of interest with the mutational frequency in healthy disease control individuals [7]. A *P*-value of 0.1 was chosen as the threshold to classify a sample of interest as ctDNA-positive ( $P < 0.1$ ) or ctDNA-negative ( $P > 0.1$ ). A specificity of at least 0.90 was considered desirable, and a *P* value of equal to 0.1 yielded 0.90 specificity when performing cross validation on the controls.

## Statistical analysis

The primary outcome measure was RFS evaluated by standard RECIST criteria [13]. RFS was defined from date of surgery to first radiologic recurrence or death due to PDAC. OS was the secondary end point and defined from the date of diagnosis till death. Univariate and multivariate analyses were carried out with backward stepwise Cox regression modeling. Chi-squared or Fisher’s exact test and the Mann–Whitney (rank-sum) test were carried out. The Kaplan–Meier method was used to estimate recurrence and survival over time. Statistical analysis was carried out using GraphPad Prism version 7.0 (GraphPad Software Inc.) and R 3.4.2, where *P* values  $<0.05$  were considered significant.

## Results

### Patient characteristics

Of the 112 patients with PDAC consented pre-operatively, 81 (72%) proceeded to ‘curative’ resection, the remaining 31 (28%) cases having unresectable disease that was identified intra-operatively. Baseline patient characteristics are summarized in Table 1. Ultimately, 142 blood samples from 42 resected early-stage PDAC cases were analyzed in triplicate for ctDNA (Figure 1). The presence of a *KRAS* gene mutation was identified in 90% (38/42) of the tissue specimens. ctDNA analysis was carried out pre-operatively in 37 cases and post-operatively in 35, with insufficient tumor tissue available in 1 case, and insufficient plasma volume collected post-operatively in 3 cases. When detected, there was 100% concordance between the specific *KRAS* mutation codon identified in the tumor tissue and in the plasma *KRAS* mutant-ctDNA from the same patient.

Table 1. Patient's and tumor characteristics according to ctDNA status

Clinico-pathologic features	Pre-op ctDNA-negative N = 14 (%)	Pre-op ctDNA-positive N = 23 (%)	P value	Post-op ctDNA-negative N = 22 (%)	Post-op ctDNA-positive N = 13 (%)	P value
Median age (years)	66	65		66	65	
Age range	48–79	48–81		47–79	48–77	
Sex						
Male	10 (71)	13 (57)	0.49	12 (55)	9 (69)	0.49
Female	4 (29)	10 (43)		10 (45)	4 (31)	
CA-19-9 < 36 U/ml	6 (43)	4 (17)				
CA-19-9 > 36 U/ml	8 (57)	19 (83)	0.13			
Tumor location						
Pancreas head	10 (71)	20 (87)	0.64	16 (73)	12 (92)	0.37
Pancreas body	1 (7)	3 (13)		3 (14)	1 (8)	
Pancreas tail	2 (15)	0 (0)		2 (9)	0 (0)	
Not reported	1 (7)	0 (0)		1 (4)	0 (0)	
T stage						
T1	0 (0)	1 (4)	0.43	0 (0)	0 (0)	>0.99
T2	2 (15)	1 (4)		2 (9)	1 (8)	
T3	12 (85)	21 (91)		20 (91)	12 (92)	
N status						
N0	4 (29)	2 (9)	0.17	5 (23)	1 (8)	0.37
N1	10 (71)	21 (91)		17 (77)	12 (92)	
R status						
R0	5 (36)	11 (48)	0.12	12 (55)	5 (38)	0.15
R1	9 (64)	7 (30)		10 (45)	6 (46)	
Rx	0 (0)	3 (13)		0 (0)	2 (16)	
Lymphovascular invasion						
Absent	4 (29)	6 (26)	0.53	5 (23)	3 (23)	0.53
Present	10 (71)	15 (65)		16 (73)	8 (61)	
Not reported	0 (0)	2 (9)		1 (4)	2 (16)	
Perineural invasion						
Absent	2 (15)	6 (26)	0.32	5 (23)	2 (16)	0.83
Present	12 (85)	15 (65)		16 (73)	10 (76)	
Not reported	0 (0)	2 (9)		1 (4)	1 (8)	
Adjuvant chemotherapy						
Yes	13 (93)	16 (70)		21 (96)	7 (54)	
No	1 (7)	7 (30)		1 (4)	6 (46)	

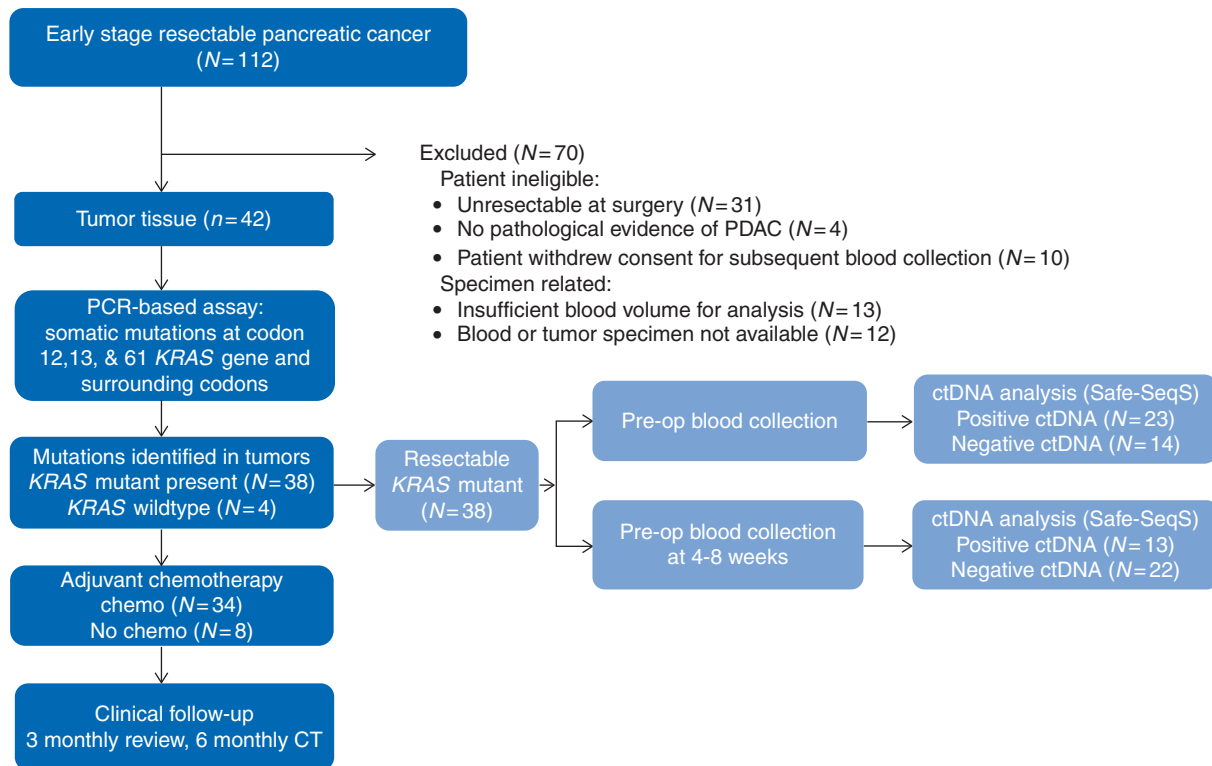
Adjuvant therapy was administered following standard institutional protocols and blinded to ctDNA analysis. Eighty-one percent (34/42) of cases received adjuvant therapy. Nineteen patients were treated with single agent gemcitabine, nine received gemcitabine plus capecitabine and six received gemcitabine plus nab-paclitaxel. At a median follow-up of 38.4 months recurrence has occurred in 27 (65%) patients and 20 patients (48%) have died.

**Prognostic value of pre-operative ctDNA.** *KRAS* mutant ctDNA was detected in 62% (23/37) of patient samples pre-operatively, with 4 tumors being *KRAS* wildtype. Pre-operative detectable (positive) ctDNA when compared with the undetectable (negative) ctDNA cohort was associated with an increased risk of recurrence with a median RFS of 10.3 months versus RFS not reached [hazard ratio (HR) 4.1; 95% confidence interval (CI), 1.8–9.0;  $P=0.002$ ] (Figure 2A); and an inferior median OS of 13.6 months versus OS not reached (HR 4.1; 95% CI 1.6 to 10.5;

$P=0.015$ ) (Figure 2B). On multivariate and univariate analysis (Table 2), the presence of ctDNA in the pre-operative setting was the only variable that had a statistically significant association with recurrence (HR 4.1; 95% CI 1.4–12.1;  $P=0.018$ ) and with OS (HR 4.1; 95% CI 1.0–16.6;  $P=0.049$ ).

**Prognostic value of post-operative ctDNA.** Post-operative ctDNA plasma specimens were collected at a median of 6.3 (range 4–8) weeks. The presence of detectable ctDNA following curative resection predicted for recurrence, with a 100% positive predictive value at a median follow-up of 38.4 months, with a specificity and sensitivity of 100% and 57%, respectively.

Univariate analysis of six independent variables was carried out. Post-operative ctDNA status demonstrated a significant prognostic impact on RFS (HR 5.4; 95% CI 1.9–15.2;  $P<0.0001$ ) (Table 2). HR's for other prognostic factors (Table 2) all trended in the expected direction but did not reach statistical significance, likely because of the sample size. On multivariate analysis, ctDNA



**Figure 1.** Consort diagram of patient enrolment, specimen collection and clinical management.

detection remained a significant predictor of recurrence (HR 6.3; 95% CI 2.4–16.2;  $P \leq 0.0001$ ) and death (HR 7.5; 95% CI 2.1–27.7;  $P = 0.002$ ).

Detectable (positive) ctDNA compared with undetectable ctDNA (negative) following curative resection was associated with a shorter time to recurrence of 5.4 versus 17.1 months (HR 5.4, CI 1.9–15.2;  $P < 0.001$ ) (Figure 2C), and worse median OS of 10.6 months versus OS not reached (HR 4.0, CI 1.2–13.6;  $P = 0.003$ ) (Figure 2D).

Of the 22 patients with undetectable ctDNA post-operatively, 10 patients have relapsed at a median follow-up of 38.4 months. Within the ctDNA-positive cohort, all 13 patients relapsed. In the post-operative ctDNA-positive cohort, the use of doublet chemotherapy showed a trend toward improved RFS with median RFS of 10.1 months compared with 5.1 months (HR 0.36, CI 0.1–1.6;  $P = 0.15$ ) favoring doublet chemotherapy over gemcitabine alone (supplementary Figure S1, available at *Annals of Oncology* online).

**Pre-operative versus post-operative results.** Analyses of matched pre- and post-surgery plasma collected from the same patients, revealed that of the 23 of 42 (55%) patients that had detectable ctDNA before surgery there were 11 that still had detectable ctDNA post-surgery. Two patients with undetectable ctDNA levels before surgery, were found to have detectable ctDNA following surgery, bringing the total number of patients with detectable ctDNA following surgery to 13 (31%) of 42 patients. This corresponds to a 24% decrease in the proportion of patients with detectable ctDNA following surgery.

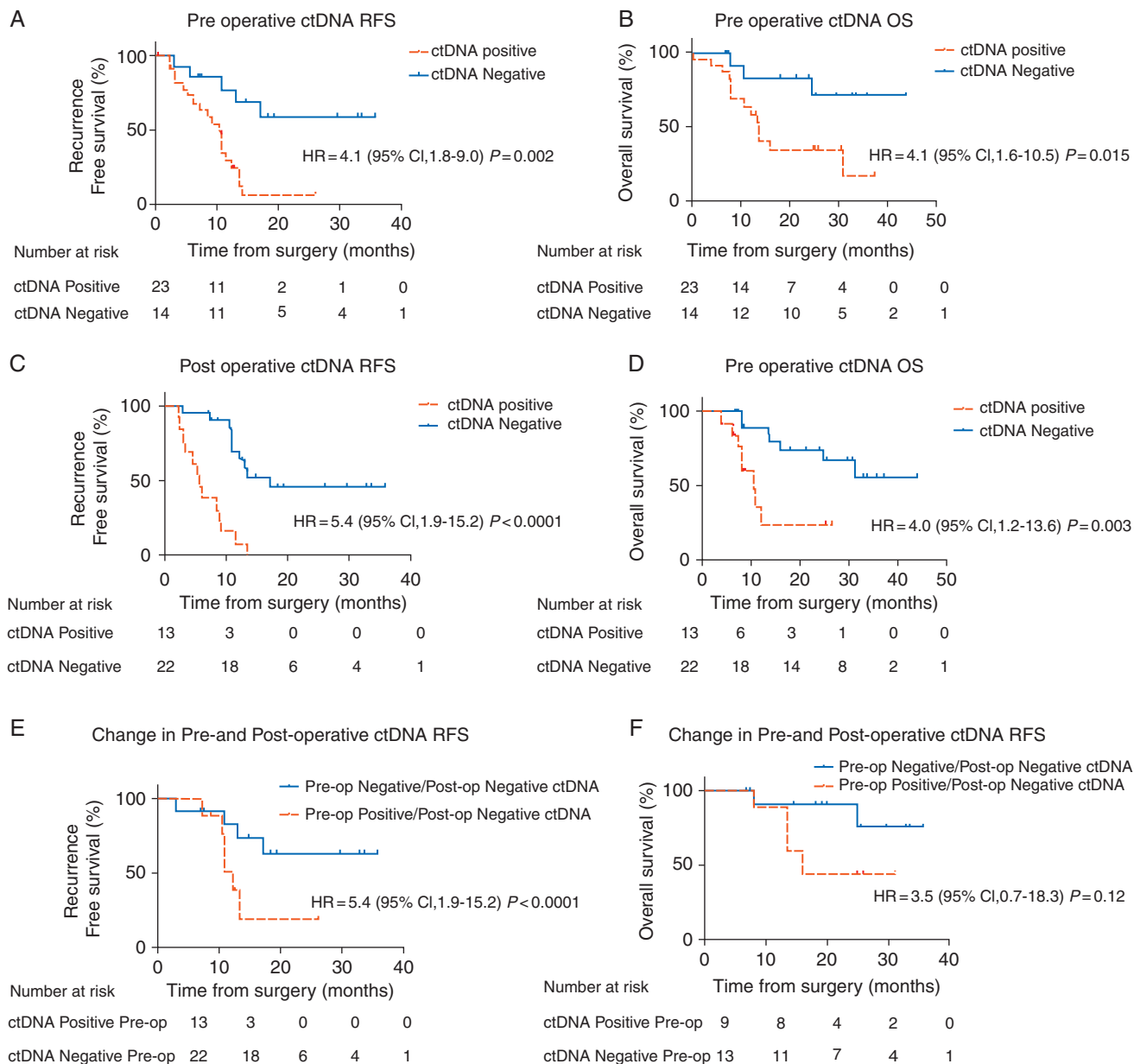
In patients with detectable ctDNA pre-surgery who then had undetectable ctDNA following surgery, the median RFS was

12.2 months. In the cohort with undetectable ctDNA both pre- and post-surgery median RFS was not reached (HR 3.2, CI 0.8–12.0;  $P = 0.05$  versus detectable ctDNA pre-surgery) (Figure 2E). Where patient status went from detectable ctDNA pre-surgery to undetectable post-surgery median the OS was 15.9 months (HR 3.5, CI 0.7–18.3;  $P = 0.12$ ) (Figure 2F).

## Discussion

This is the first study in early-stage pancreatic cancer patients undergoing surgery with curative intent that was designed to collect and report on both pre- and post-operative ctDNA analyses. This study highlights the challenges faced in the conduct of such a study and the treatment of PDAC. Issues include managing resectable versus unresectable ‘early’ stage disease, access to sufficient tumor tissue for testing, and collecting blood samples according to strict protocols. Despite these challenges we demonstrated the validity of ctDNA as a liquid biopsy and tool to evaluate disease response and potentially guide therapy. We also demonstrated that ctDNA was detectable at diagnosis in 62% of early-stage *KRAS* mutated PDAC. This is consistent with other studies in the pre-operative setting that have reported detection rates of 31%–55% [6, 14–16].

Our data suggest a potential role for ctDNA as a prognostic marker in the pre-operative setting. Of the 23 patients with detectable ctDNA at diagnosis, 19 (83%) have recurred at a median follow-up of 38.4 months despite standard therapy being administered in 81%. These higher risk patients might be a population where neoadjuvant therapy strategies could be explored, rather than proceeding straight to surgery.



**Figure 2.** (A) Kaplan–Meier estimates of recurrence-free survival (RFS) for all assessable patients undergoing curative intent surgery for early pancreatic cancer, stratified by pre-operative ctDNA status: detectable (positive) versus undetectable (negative). (B) Kaplan–Meier estimate for overall survival (OS) for matched patients, stratified by pre-operative ctDNA status. (C) Kaplan–Meier estimates for RFS, stratified by post-operative ctDNA status. (D) Kaplan–Meier estimates for OS, stratified by post-operative ctDNA status. (E, F) Kaplan–Meier estimates for RFS (E) and OS (F) for patients’ whose ctDNA status changes from detectable (positive) pre-operatively to undetectable (negative) post-operatively compared with patients whose ctDNA status remains undetectable (negative) both pre- and post-operatively.

In the post-operative setting, our finding that ctDNA is detectable in 31% of patients following curative intent surgery is consistent with other small series. These reported a post-operative detection rate of 44% in 4/9 patients [17] and 45% post-operative detection rates in a sub-group analysis [15, 18] with a median time to recurrence of 9.9 months in 10 patients with detectable post-operative ctDNA ( $P = 0.019$ ) [15]. In our series, the presence of detectable ctDNA was associated with an increased risk of relapse, with a median RFS of 10.3 months for pre-op ctDNA detection ( $P = 0.005$ ) and 5.4 months for post-op ctDNA

detection ( $P < 0.0001$ ); and significantly worse OS compared with patients with undetectable ctDNA ( $P = 0.04$  and  $P = 0.001$ , respectively).

Post-operative analysis of ctDNA appears most informative. Our preliminary data indicate that where ctDNA is detectable at diagnosis but becomes undetectable post-operatively that this is associated with a reduction in relapse risk compared with those where ctDNA remains detectable, but further adjuvant therapy measures should still be considered given the high recurrence risk. We would suggest that even with larger patient numbers and

**Table 2. Recurrence-free survival and overall survival analysis by clinico-pathologic variables and pre- and post-operative ctDNA status**

Pre-operative variable and RFS						
Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
CA19-9 at diagnosis, <36 U/ml versus >36 U/ml	1.6	0.7–3.5	0.31			
Tumor stage, T3/4 versus T1/2	4.4	0.6–32.9	0.145	6.4	0.7–57.5	0.100
Lymph node status, N1 versus N0	1.7	0.6–5.0	0.318	0.8	0.2–3.2	0.731
Resection margin status, R1 versus R0	1.3	0.6–3.0	0.488	2.1	0.5–8.7	0.325
Lymphovascular invasion status, present versus absent	2.3	0.8–6.8	0.130	1.1	0.3–4.0	0.887
Perineural invasion status, present versus absent	0.8	0.3–2.1	0.726	0.3	0.1–1.2	0.083
ctDNA status, positive versus negative	3.9	1.4–10.8	0.008	4.1	1.4–12.1	0.018
Pre-operative variable and OS						
Tumor stage, T3/4 versus T1/2	2.9	0.4–22.0	0.300			
Lymph node status, N1 versus N0	1.0	0.3–3.0	0.960	0.7	0.1–3.6	0.653
Resection margin status, R1 versus R0	1.4	0.5–4.0	0.533	2.0	0.6–7.1	0.290
Lymphovascular invasion status, present versus absent	1.3	0.4–4.2	0.616			
Perineural invasion status, present versus absent	0.8	0.3–2.4	0.715			
ctDNA status, positive versus negative	3.6	1.0–13.1	0.051	4.1	1.0–16.6	0.049
Post-operative variable & RFS						
Tumor stage, T3/4 versus T1/2	4.4	0.6–32.9	0.145			
Lymph node status, N1 versus N0	1.7	0.6–5.0	0.318	0.2	0.0–1.1	0.068
Resection margin status, R1 versus R0	1.3	0.6–3.0	0.488	2.4	0.6–10.1	0.221
Lymphovascular invasion status, present versus absent	2.3	0.8–6.8	0.130	59.6	3.0–1178.8	0.007
Perineural invasion status, present versus absent	0.8	0.3–2.1	0.726	0.1	0.0–0.5	0.009
ctDNA status, positive versus negative	5.4	1.9–15.2	<0.0001	6.3	2.4–16.2	<0.0001
Post-operative variable & OS						
Tumor stage, T3/4 versus T1/2	2.9	0.4–22.0	0.300			
Lymph node status, N1 versus N0	1.0	0.3–3.0	0.960	1.3	0.3–7.3	0.733
Resection margin status, R1 versus R0	1.4	0.5–4.0	0.533	0.6	0.2–2.3	0.494
Lymphovascular invasion status, present versus absent	1.3	0.4–4.2	0.616			
Perineural invasion status, present versus absent	0.8	0.3–2.4	0.715			
ctDNA status, positive versus negative	5.5	1–17.4	0.004	7.5	2.1–27.7	0.002

a prospective randomized trial it is unlikely that a ctDNA-negative group at such low risk of recurrence could be identified such that adjuvant therapy could be avoided.

Ours is the first series to examine the impact of adjuvant chemotherapy according to ctDNA status. Of the 13 patients with detectable ctDNA post-operatively, 5 received adjuvant gemcitabine alone and 2 received gemcitabine and capecitabine, the latter emerging as the standard of care following the ESPAC4 trial results that demonstrated improved median OS of 28 versus 25.5 months ( $P=0.032$ ) with gemcitabine with capecitabine compared with gemcitabine alone, and an improved median RFS of 13.9 versus 13.1 months ( $P=0.082$ ) [3]. Despite receiving adjuvant therapy all patients subsequently relapsed, providing preliminary evidence that current standard adjuvant therapy may not be of benefit in this very high-risk subset, and alternative adjuvant strategies need to be explored. Longitudinal monitoring using liquid biopsy samples through exoDNA and ctDNA provides both predictive and prognostic information that could be used for therapeutic stratification [19].

Results from the PRODIGE and APACT trials, evaluating the use of adjuvant FOLFIRINOX and adjuvant gemcitabine with

nab-paclitaxel (Abraxane), respectively, against gemcitabine alone, (which did not stratify patients according to ctDNA status) reported that the use of adjuvant FOLFIRINOX compared with single agent gemcitabine significantly improved RFS (21.6 versus 12.8 months,  $P<0.001$ ) [2] whilst the use of gemcitabine with nab-paclitaxel compared with single agent gemcitabine did not [4]. However, a nominal improvement in OS was observed in the gemcitabine with nab-paclitaxel cohort [4]. Whether a subset of patients at higher risk of recurrence can be identified with a biomarker such as ctDNA post-operatively and then managed with a risk-adapted treatment strategy remains to be seen.

We have initiated a larger prospective clinical trial exploring a ctDNA informed approach to adjuvant treatment (CTRA U1111-1209-6200). Patients are randomized to standard of care treatment blinded to ctDNA analysis or to ctDNA informed treatment. This study will prospectively collect plasma and data to validate the findings of the current study regarding the impact of standard of care treatments in the ctDNA-positive and -negative patients. As suggested in the ASCO-CAP joint review on ctDNA analysis in patients with cancer, the most reliable method to demonstrate the clinical utility of ctDNA is through

prospective clinical trials as a stand-alone diagnostic test, that include a patient cohort that resembles the intended-use population with defined entry criteria [20].

This present study had several important limitations: only a modest number of patients could be included in the major analysis, relatively small numbers in subsets of interest, the explorative study design and a missing validation cohort. The attrition rate partly reflects the nature of pancreatic cancer, where cases that initially appear to be resectable have inoperable disease at surgery. Our experience confirms that large biomarker studies in the setting of resectable pancreatic cancer can be challenging to complete, with cases being excluded due to a wide range of reasons. Future studies enrolling patients preoperatively should allow for a high proportion of patients not completing study related treatment and biomarker collection. Ensuring a minimum of 30 ml of whole blood is collected, can also prevent issues of insufficient sample for analysis.

In conclusion, we have shown the potential clinical utility of ctDNA analysis, before and after curative intent surgery, as a prognostic marker in patients with pancreatic cancer. These findings raise interesting possibilities around potential adjuvant and neo-adjuvant treatment strategies in ctDNA defined subsets, but these approaches need to be validated in prospective randomized clinical trials. It is crucial that studies involving ctDNA analysis should explore how to guide patient treatment rather than simply collecting plasma from patients in routine care, as demonstrating clinical utility and patient benefit from biomarker informed treatment is the end goal.

## Acknowledgements

We thank all participating hospitals (Royal Melbourne Hospital, Western Health, Cabrini Health, Prince of Wales Hospital, Royal Brisbane Hospital, Eastern Health, Austin Health, Monash Medical Centre, Alfred Hospital, Auckland City Hospital, New Zealand, National Cancer Centre, Singapore) for patient enrolment and sample collection; M. Chapman, J. Nolan for administrative assistance; and the Victorian Cancer Biobank for sample processing.

## Funding

This study was supported by National Institutes of Health (NIH) grants (CA57345, R37-CA43460, U01-CA152753 and P30-CA006973) and by the Virginia and DK Ludwig Fund for Cancer Research, the Conrad N. Hilton Foundation, the Sol Goldman Sequencing Facility at Johns Hopkins, Victorian Cancer Agency Clinical Research Fellowship (to JT), Walter & Eliza Hall Institute Hemstrich Centenary Fellowship (to BL). There are no applicable grant numbers for this funding.

## Disclosure

The authors have declared no conflicts of interest.

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