Intratumoural expression of dihydropyrimidine dehydrogenase is an independent prognostic factor in resected pancreatic ductal adenocarcinoma treated with adjuvant gemcitabine

HAKON BLOMSTRAND[1,2], MALIN BODARVE[2], FREDRIK GROTH[2], PETER NAREDI[3],
MALIN SUND[4,5], CAROLINE VILHAV[3], HENRIK GREEN[6,7], BERGTHOR BJÖRNSSON[1,8],
DANIEL ÖHLUND[9], STINA LINDBLAD[9], OSKAR FRANKLIN[4,10] and NILS O. ELANDER[1,11]

[1]Division of Surgery, Orthopaedics and Oncology, Department of Biomedical and Clinical Sciences, Linköping University, SE-58185 Linköping, Sweden; [2]Department of Clinical Pathology, Linköping University Hospital, SE-58185 Linköping, Sweden; [3]Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, SE-40530 Gothenburg, Sweden; [4]Department of Diagnostics and Intervention, Umeå University, SE-90187 Umeå, Sweden; [5]Department of Surgery, University of Helsinki and Helsinki University Hospital, 00014 Helsinki, Finland; [6]Division of Clinical Chemistry and Pharmacology, Department of Biomedical and Clinical Sciences, Linköping University, SE-58185 Linköping, Sweden; [7]Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Linköping University, SE-58185 Linköping, Sweden; [8]Department of Surgery, Linköping University Hospital, SE-58185 Linköping, Sweden; [9]Department of Diagnostics and Intervention, and Wallenberg Centre for Molecular Medicine, Umeå University, SE-90187 Umeå, Sweden; [10]Division of Surgical Oncology, Department of Surgery, University of Colorado School of Medicine, Aurora, CO 80045, USA; [11]Clatterbridge Cancer Centre NHS Foundation Trust, Liverpool L7 8YA, United Kingdom

Abstract. Pancreatic ductal adenocarcinoma (PDAC) is associated with a poor prognosis, and biomarkers to guide treatment decisions in PDAC are generally lacking. Intratumoural expression of dihydropyrimidine dehydrogenase (DPD) is a potential prognostic parameter in patients with PDAC undergoing surgical resection and postoperative chemotherapy. In the present study, DPD was analysed by immunohistochemistry of a tissue microarray platform including a real-world cohort of 495 patients with PDAC who had undergone resection with curative intent at any of three tertiary centres, including Northern, Western and Southeastern regions of Sweden, between 1993 and 2019. DPD level (high/low) was analysed and overall survival associations were assessed in treatment subgroups using a multivariate Cox regression model accounting for potential confounders. In patients who had not received adjuvant chemotherapy (n=182), the median overall survival time was 11.6 months (95% CI 9.6-13.5), compared with 28.8 months (25.0-32.6) among those who had (n=313; log-rank P<0.001). The most common type of chemotherapy was gemcitabine single agent (Gem, n=239) followed by gemcitabine plus capecitabine (GemCape, n=39). Tumour-Node-Metastasis (TNM) stage and DPD expression were statistically significant prognostic parameters in the Gem group (HR 1.19, 95% CI 1.01-1.41, P=0.036), with high expression of DPD linked with worse survival. In addition, tumour grade and TNM stage were statistically significant prognostic factors in the group that did not receive any chemotherapy (P≤0.001). No statistically significant parameters were identified in the GemCape group. Taken together, intratumoural expression of DPD may be considered a prognostic marker for patients with PDAC treated with adjuvant gemcitabine following surgical resection, with low expression levels predicting better survival. Further studies in larger cohorts of patients receiving multi-drug or non-gemcitabine based regimens are warranted.

Correspondence to: Dr Nils O. Elander, Division of Surgery, Orthopaedics and Oncology, Department of Biomedical and Clinical Sciences, Linköping University, 7 Sandbäcksgatan, SE-58185 Linköping, Sweden
E-mail: nils.elander@liu.se

Introduction

Pancreatic ductal adenocarcinoma (PDAC) represents a challenging type of cancer with increasing mortality counts, projected to replace breast cancer as the third highest ranked cause of cancer-related deaths in Europe by 2025 (1). Historically, the outcome for patients with PDAC has been poor with almost no long-time survivors, yet recent data indicate a positive trend with 10-year survival (all stages combined) now climbing towards 10% (2).

One key factor for improved prognosis has been the introduction of adjuvant chemotherapy following curative intent resection in patients with disease limited to the locoregional area and who are in reasonably good performance status. Initially, 5-fluorouracil (5-FU) and folinic acid was mainstay treatment (3) however over the past 10-15 years protocols including gemcitabine single agent (Gem) (4), gemcitabine plus the 5-FU prodrug capecitabine (GemCape) (5), and 5-FU, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) (6) have largely replaced this standard. In addition, a combination of the 5-FU oral prodrug tegafur in combination with enzyme inhibitors gimeracil and oteracil (S-1) has evolved as a viable option mainly in Asian populations (7). Irrespective of the treatment regimen administered, benefit from chemotherapy is highly variable between patients and finding prognostic and treatment predictive markers to better guide therapeutic strategy has proven to be a goal yet to be accomplished.

Dihydropyrimidine dehydrogenase (DPD) is an enzyme encoded by the *DPYD* gene that is involved in the catabolism of thymine and uracil and is expressed in various tissues and cells of the body, including the liver, bone marrow and mononuclear cells of the blood, as well as in tumour tissue (8). In addition to its physiological function, DPD is also a key enzyme in the conversion of 5-FU into its pharmacologically inactive form dihydrofluorouracil (9). High expression of DPD mRNA and protein has been reported in several types of adenocarcinoma including those of the stomach, head and neck, and pancreas (10).

Germline variations of the *DPYD* gene are closely linked to severe toxicity to 5-FU and other pyrimidine analogues, and there is now a general recommendation to check all patients for such variants prior to starting treatment (11). Beside this, additional reports indicate that intratumoural expression of DPD (either due to germline or somatic mutations, epigenetic alterations, or post-transcriptional upregulation) is a marker for poor prognosis in various types of cancer as well as poor response to chemotherapeutic anti-metabolic drugs including 5-FU, capecitabine and S-1 when given alone or in combination with gemcitabine (12-17). In addition, *in vitro* and *in vivo* studies in urinary bladder cancer have implied DPD expression levels to interfere with sensitivity and resistance to gemcitabine (18).

The current study aimed to evaluate the potential prognostic impact of intratumoural DPD expression in a large real-world multi-centre cohort of patients with resected PDAC treated with adjuvant chemotherapy.

Materials and methods

TMA construction. A tissue microarray (TMA) with multiple biopsies of tumour tissue representing PDAC patients who underwent surgical resection between 1993-2019 in any of the Northern, Western, and Southeastern health care regions of Sweden, was constructed. The cohort from the Southeast region included all resected cases between 2009-2019 and has been described in detail in a previous publication (19).

All available slides were reviewed for each case and paraffin blocks corresponding to slides with the highest proportion of tumour cells were selected for the TMA. The TMA was manufactured with the previous ESPAC3 material-based TMA as a template (15). Two 1 mm in diameter micro core biopsies were taken from tumour cell rich areas in two blocks from the primary tumour and one 1 mm in diameter core

biopsy from a block with lymph node metastasis (if present), in total 4-5 cores per case with an automated TMA Master or TMA Grandmaster (3DHistech Kft., Budapest, Hungary). In a few cases where poor core quality was readily detected during biopsy transferral (e.g., due to half or broken biopsies), additional blocks were retrieved (if available) to reach the total micro biopsy number of 4-5. In 86 other cases tumour tissue was embedded in new paraffin blocks as tumour was only found in xl-blocks in the original case (not compatible with the TMA machine). The biopsies were arranged in a grid pattern in receiving paraffin blocks with micro biopsies containing control tissue (alternating benign liver, pancreatic, colonic, and renal tissue) arranged in a fence-like manner in the perimeter of the grid (Fig. 1). Receiving blocks were then mildly heated to melt the cores with surrounding paraffin and subsequently 3.5 μm sections were taken with a HM355S microtome (Thermo Fisher Scientific, Waltham MA, USA) and mounted on Cut frosted microscope slides (Epredia, Kalamazoo MI, USA). One section from each block was stained with haematoxylin-eosin (HE) for reference and validation of tumour cell content in the micro biopsies.

Immunohistochemical staining. One additional slide, sectioned at a thickness of 3.5 μm, was retrieved from all TMA blocks and baked for 1 h at 60°C. Deparaffinization and staining were performed in BOND III stainers (Leica) using heat-induced epitope retrieval (HIER) with Bond Epitope Retrieval Solution 2, effective heating time of 20 min at 100°C, and Bond Polymer Refine Detection Kit (all reagents supplied by Leica). The primary antibody (rabbit anti-DPD, Abcam ab 134922, Abcam, Cambridge, UK) was used at a dilution of 1:2,000, with EnVision FLEX Antibody Diluent (Dako) and incubated for 15 min at room temperature. The staining procedures were designed according to a previously validated and optimised protocol for immunohistochemical analysis of intratumoural DPD expression in paraffin embedded TMA biopsies of PDAC (15).

DPD staining intensity assessment. Staining intensity was evaluated and scored by HB and NE in an individual and blinded manner in four tiers 0-3, replicating the methods used in the previous work utilizing the ESPAC3 tissue material (15). If heterogeneous staining intensity was present, the predominant pattern was chosen. When the raters scored the same core differently, the case was discussed, and a consensus score was established.

Representative stained slides are displayed in Fig. 1. Following completion of scoring, the cores were deciphered and cases with less than two evaluable tumour cores (e.g., no tumour in the core, section lost during preparation, no tissue left in the TMA block etc.) were excluded from further analysis. For included cases a mean score was calculated based on all cores from the same case rounded to the nearest integer. Cases were then dichotomized into low (0-1) or high (2-3) expression.

Statistical analysis. All cases fulfilling the inclusion criteria were included in the statistical analyses that were performed with SPSS v29 (IBM, Armonk, USA) and R Statistical Software (v4.3.1; R Foundation for Statistical Computing, Vienna, Austria). P<0.05 was considered to indicate a
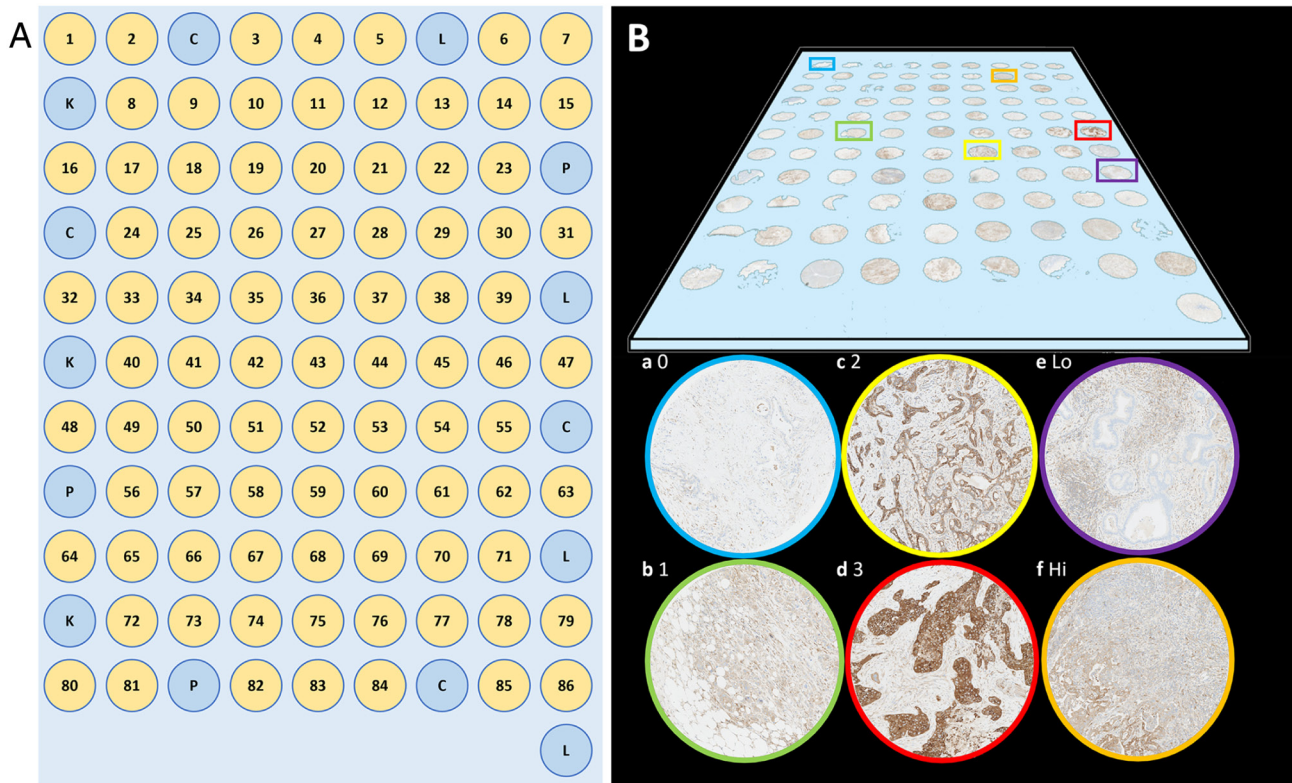
Figure 1. (A) Schematic of a TMA paraffin block. Blue circles: control tissue. Orange circles: tumour tissue. C: colonic mucosa. L: liver parenchyma. K: kidney tissue. P: pancreatic parenchyma. (B) Example of a TMA slide with representative tissue cores stained for DPD (magnification, x20). (Ba) Negative (0), (Bb) weak (1), (Bc) moderate (2) and (Bd) strong (3) staining. (Be) Metastatic lymph node with negative staining (0). (Bf) Metastatic lymph node with moderate staining (2). DPD, dihydropyrimidine dehydrogenase; TMA, tissue microarray.

statistically significant difference. If not else stated, descriptive statistics were reported as median and interquartile range for continuous variables and as frequencies and percentages for categorical values. Comparisons were made with Mann Whitney U or χ^2-test and unpaired Student's t-test for categorical and continuous parameters, respectively. Cohen's κ was used to assess interrater variability concerning IHC scoring. Primary outcome was median overall survival (OS), defined as time from the date of surgery until death or censoring, whatever occurred first. Kaplan-Meier survival analysis was used to estimate survival times and the log-rank test was utilized to detecting significant differences between subgroups. Assuming proportional hazards, univariate Cox regression analysis was used to identify potentially prognostic factors. Spearman's rank correlation was used to determine covariation between selected variables. A subsequent multivariate Cox regression model, including factors with P<0.10 in the univariate analysis, was used to determine independent prognostic factors. To calculate median follow-up time, the reverse Kaplan-Meier method was used (20).

Results

A total of 2,323 tumour cores were transferred to the TMA blocks, representing the total cohort of 552 included cases of resected PDAC. Fifty-seven cases were excluded due to less than two evaluable tumour containing micro biopsies being available, leaving a total of 495 cases in the final cohort available for analysis (Fig. 2). DPD staining was performed, and
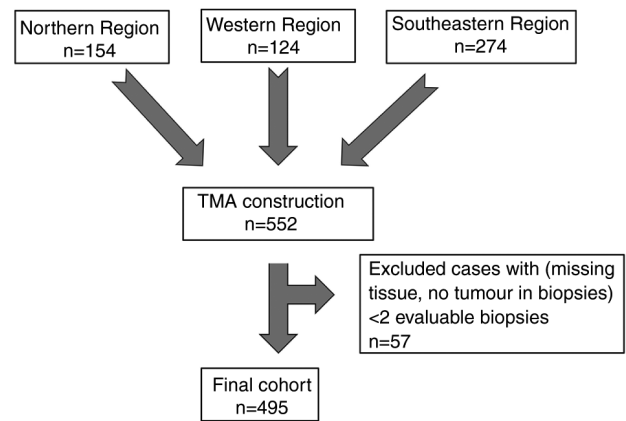

Figure 2. Flowchart for inclusion of cases in the study. TMA, tissue microarray.

staining intensity was scored by two independent assessors. The staining pattern was generally homogenous and clear, with excellent inter-rater concordance (Cohen's κ=0.81).

Descriptives. Seventeen patients were diagnosed with PDAC of a specified histological subtype, whilst 478 cases were diagnosed with PDAC not otherwise specified (NOS). Descriptive patient characteristics are shown for the full cohort and stratified as per DPD intensity level in Table I. DPD levels were significantly associated with tumour differentiation grade and overall survival, with low levels generally being linked with

Table I. Patient characteristics for the total cohort and when divided into subgroups per DPD staining intensity level.

Characteristic	Total (n=495)	DPD low (n=383)	DPD high (n=112)	P-value[a]
Age, years[b]	69 (63-75)	68 (9)	69 (10)	0.196
Tumour size, mm[b]	30 (24-39)	30 (23-39)	30 (25-40)	0.230[c]
Sex, female	269 (54)	201 (52.5)	68 (60.7)	0.152
Year of surgery				0.181
1993-2005	23 (4.9)	19 (6.3)	4 (3.9)	
2006-2010	101 (21.7)	79 (21.7)	22 (21.5)	
2011-2015	207 (44.4)	169 (46.4)	38 (37.3)	
2016-2019	135 (29.0)	97 (26.6)	38 (37.3)	
Differentiation grade				<0.001
High	48 (9.9)	42 (11.2)	6 (5.5)	
Medium	237 (49.0)	203 (54.1)	34 (31.2)	
Low	199 (41.1)	130 (34.7)	69 (63.3)	
Margin status				0.568
R0	274 (56.1)	217 (57.1)	57 (52.8)	
R1	205 (42.0)	157 (41.3)	48 (44.4)	
R2	9 (1.8)	6 (1.6)	3 (2.8)	
Sampled nodes[b]	15 (10-21)	15 (9-20)	17 (11-23)	0.051[d]
Positive nodes[b]	2 (0-5)	2 (0-5)	2 (0.8-5.3)	0.583[d]
TNM 7th ed Stage				0.151
I	50 (10.5)	41 (11.1)	9 (8.3)	
II	353 (78.2)	292 (79.2)	81 (75.0)	
III	14 (2.9)	8 (2.2)	6 (5.6)	
IV	40 (8.4)	28 (7.6)	12 (11.1)	
TNM 8th ed Stage				0.360
I	70 (19.5)	56 (20.8)	14 (15.7)	
II	144 (40.1)	112 (41.5)	32 (35.9)	
III	105 (29.2)	74 (27.4)	31 (34.8)	
IV	40 (11.1)	28 (10.4)	12 (13.5)	
Neoadjuvant treatment	25 (5.1)	21 (5.5)	4 (3.6)	0.567
Adjuvant treatment				0.971
None	182 (37.1)	140 (36.7)	42 (38.2)	
5FU	10 (2.0)	9 (2.4)	1 (0.9)	
Gem	239 (48.7)	184 (48.3)	55 (50.0)	
GemCape	39 (7.9)	31 (8.1)	8 (7.3)	
Gem/NabP	4 (0.8)	3 (0.8)	1 (0.9)	
FOLFIRINOX	6 (1.2)	5 (1.3)	1 (0.9)	
Other	11 (2.2)	9 (2.4)	2 (1.8)	
Relapse				0.528
None	68 (21.6)	50 (20.9)	18 (23.7)	
Local	51 (16.2)	35 (14.6)	16 (21.1)	
Distant	114 (36.2)	90 (37.7)	24 (31.6)	
Local and distant	73 (23.2)	58 (24.3)	15 (19.7)	
M1 at surgery	9 (2.9)	6 (2.5)	3 (3.9)	
OS[e]	19.6 (17.4-23.8)	22.5 (18.6-26.1)	16.2 (13.1-21.5)	0.005[f]

Data are presented as number (%) unless otherwise stated. [a]χ^2 test unless otherwise specified; [b]Median (IQR); [c]Student's T-test; [d]Mann-Whitney U test, [e]months (95% CI); [f]log-rank test. 5FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; FOLFIRINOX, 5-FU, leucovorin, irinotecan and oxaliplatin combination chemotherapy; Gem, gemcitabine monotherapy; GemCape, gemcitabine capecitabine combination chemotherapy; Gem/NabP, gemcitabine/nab-paclitaxel; OS, median overall survival.

better prognostic features (high tumour differentiation grade and longer overall survival).

Overall survival. Median overall survival in the total cohort, including all patients who had and who had not received any adjuvant chemotherapy, was 19.6 months (95% CI 17.4-23.8, Table I).

Patients who did not receive adjuvant chemotherapy had a worse outcome than those who did, with a median overall survival of 11.6 months (95% CI 9.6-13.5) vs. 28.8 months (95% CI 25.0-32.6), P<0.001, log-rank test).

Further subgrouping of patients, according to the type of chemotherapy received, revealed overall survival estimates of 28.1 months (95% CI 24.1-32.0) and 28.1 months (95% CI 15.0-41.2) for the most commonly utilized protocols Gem (n=239) and GemCape (n=39), respectively. The other subgroups, including FOLFIRINOX, gemcitabine with nab-paclitaxel (Gem/nab-P), and 5-FU single agent, were too small (n<11 for each individual regimen) to perform separate subanalyses on.

When categorising the patients according to the intra-tumoural DPD expression levels, high expression was associated with shorter survival in the total cohort of patients (log-rank P=0.0053). This impact was most prominent in the Gem treated population, with median OS of 30.3 months (95% CI 26.3-34.5) and 21.5 months (95% CI 17.4-27.7) in the DPD low and high expression subgroups, respectively (P=0.005). A similar yet not statistically significant trend was evident in the group of patients who did not receive any postoperative chemotherapy (P=0.056), whereas no difference was observed in the GemCape treated subgroup (P=0.960) (Fig. 3).

Univariate analyses of overall survival. Given the heterogenous nature of the main groups of patients (no chemotherapy, Gem, and GemCape), these were separately assessed with Cox regression analysis in terms of potential prognostic factors and overall survival.

In the untreated group of patients, TNM stage, R-status, year of surgery and tumour differentiation grade were all statistically significant prognostic parameters (Table II).

In the Gem treated group, DPD level, sex, R-status, and TNM stage were prognostic whereas no factors showed significant prognostic value in the GemCape group (Table III). When combining all patients treated with any type of chemotherapy, DPD-level, sex, R-status, and TNM stage were prognostic in terms of overall survival (Table II).

Multivariate analyses of overall survival. All factors that returned P<0.10 in the univariate regression analyses were included in the subsequent multivariate Cox regression analyses (Kaplan-Meier curves for these factors are seen in Fig. S1). As Spearman's rank correlation analysis revealed no association between the year of surgery variable and the dependent variable DPD expression level (rho 0.109, negligible correlation; data not shown), the year of surgery variable was excluded in the multivariate analysis.

In the group of patients who had not received any chemotherapy, differentiation grade and TNM stage were both statistically significant independent prognostic factors with regards to overall survival (Table II).

In the Gem treated group of patients, TNM stage and DPD expression levels were both independent parameters for survival (with P=0.018 and P=0.036, respectively), whereas sex was borderline significant (P=0.050, Table III). Amongst GemCape treated patients, neither DPD-expression nor any other factors were found statistically significant (Table III). Upon grouping all types of chemotherapy together, only sex was a statistically significant factor in terms of survival (Table II).

Discussion

Over the past 20 years, adjuvant chemotherapy has evolved as mainstay treatment in patients who have undergone curative intent resection of pancreatic adenocarcinoma. Despite significant therapeutic improvement, the prognosis remains poor, and there are still patient groups that do not benefit from the chemotherapy given. There are currently few, if any, treatment predictive molecular biomarkers that tell us who will be at high vs. low risk for recurrent disease and who will have good outcomes following adjuvant treatment.

With newer and more intense multi-drug regimens at hand, the need for prognostic profiling of the tumour, that may indicate what type of patient that will need more intense treatments and follow up, has become imminent.

Previous studies on DPD expression in various types of cancer including colorectal and pancreatic adenocarcinomas have indicated a potential prognostic and/or predictive value in patients treated with 5-FU or other fluoropyrimidines alone or in combination with Gem (12-17). In addition, DPD has been implied as a molecular marker for response to Gem in urinary bladder cancer cell lines and tumours (18).

The present study focused on the potential value of intratumoural DPD expression levels in a large cohort of patients with PDAC who underwent curative intent surgery over a period of 26 years (1993-2019) and covering three major catchment areas of Sweden.

As expected, outcomes were very poor amongst patients who did not receive any type of adjuvant chemotherapy. This was not surprising and is at least partly likely to be explained by selection bias in terms of patients with poor performance status and/or severe complications following the surgical resection being less likely to be fit for chemotherapy and, notably, the survival in this group was closely mirroring the observation arm of the ESPAC-1 trial (3). In addition, early relapses, preceding the window of starting adjuvant chemotherapy, may have contributed to the dismal outcome in this type of patients. Independent prognostic factors (following multivariate regression analysis) in patients who did not receive adjuvant chemotherapy were differentiation grade and TNM stage.

Amongst patients who did receive adjuvant chemotherapy, median overall survival was 28.8 months, which is in line with outcomes reported in the literature (3-6). There was no numerical difference between the two most common chemotherapy regimens utilised (Gem and GemCape) as median overall survival was 28.1 months in both groups. It should however be noted that the Gem group made up the vast majority of the population treated with chemotherapy (n=239, 75% of those who received any type of chemotherapy) whereas just

Table II. Univariate and multivariate Cox regression analyses on all patients combined, and in the subgroups who received and who did not receive adjuvant chemotherapy.

Variable	All patients		No adjuvant chemotherapy (n=182)				Any adjuvant chemotherapy (n=309)			
	HR Univariate	P-value	HR Univariate	P-value	HR Multivariate	P-value	HR Univariate	P-value	HR Multivariate	P-value
Adjuvant										
None	1	<0.001	-	-	-	-	-	-	-	-
5-FU	0.18 (0.07-0.43)	<0.001	-	-	-	-	-	-	-	-
Gem	0.47 (0.38-0.58)	<0.001	-	-	-	-	-	-	-	-
GemCape	0.45 (0.31-0.68)	<0.001	-	-	-	-	-	-	-	-
Gem/NabP	0.82 (0.26-2.57)	0.734	-	-	-	-	-	-	-	-
FOLFIRINOX	0.56 (0.21-1.50)	0.248	-	-	-	-	-	-	-	-
Age										
<70 years	1		1		-		1		-	
≥70 years	1.19 (0.98-1.44)	0.078	0.79 (0.57-1.07)	0.129	-	-	1.17 (0.91-1.51)	0.211	-	-
DPD-level										
Low	1		1		1		1		1	
High	1.38 (1.10-1.73)	0.005	1.19 (0.99-1.42)	0.058	1.06 (0.70-1.59)	0.787	1.17 (1.01-1.36)	0.038	0.76 (0.56-1.02)	0.070
Sex										
Male	1		1		-		1		1	
Female	0.85 (0.71-1.03)	0.104	1.12 (0.82-1.51)	0.488	-	-	0.85 (0.75-0.96)	0.011	1.36 (1.06-1.74)	0.016
Grade										
Low	1		1	<0.001	1	<0.001	1	0.333	-	
Medium	0.75 (0.61-0.91)	0.005	0.54 (0.38-0.76)	<0.001	0.60 (0.42-0.87)	0.006	0.85 (0.66-1.10)	0.217	-	-
High	0.54 (0.36-0.77)	<0.001	0.26 (0.15-0.45)	<0.001	0.33 (0.19-0.58)	<0.001	0.76 (0.48-1.20)	0.242	-	-
Neoadjuvant treatment										
No	1		1		-		1		-	
Yes	0.83 (0.52-1.31)	0.424	1.04 (0.53-2.04)	0.912	-	-	0.70 (0.37-1.31)	0.263	-	-
Margin status										
R0	1		1		1		1		1	
R1-2	1.34 (1.10-1.63)	0.004	1.41 (1.03-1.95)	0.035	1.01 (0.72-1.41)	0.981	1.43 (1.11-1.84)	0.005	1.29 (1.00-1.68)	0.054
Year of surgery										
1993-2006	1		1	0.008	-		1	0.604	-	
2007-2010	0.64 (0.42-0.96)	0.032	0.63 (0.36-1.08)	0.090	-	-	0.93 (0.46-1.90)	0.848	-	-
2011-2014	0.66 (0.45-0.97)	0.033	0.91 (0.55-1.49)	0.693	-	-	0.90 (0.46-1.79)	0.765	-	-
2015-2019	0.47 (0.32-0.70)	<0.001	0.48 (0.28-0.83)	0.008	-	-	0.77 (0.39-1.52)	0.448	-	-

Table II. Continued.

Variable	All patients		No adjuvant chemotherapy (n=182)				Any adjuvant chemotherapy (n=309)			
	HR Univariate	P-value	HR Univariate	P-value	HR Multivariate	P-value	HR Univariate	P-value	HR Multivariate	P-value
TNM 7th										
Stage I	1		1	<0.001	1	<0.001	1	0.024	1	0.097
Stage II	2.07 (1.46-2.94)	<0.001	3.56 (1.89-6.69)	<0.001	3.55 (1.83-6.89)	0.013	1.75 (1.14-2.70)	0.011	1.63 (1.04-2.55)	0.033
Stage III	4.45 (2.34-8.45)	<0.001	6.10 (2.30-16.14)	<0.001	4.10 (1.35-12.43)	<0.001	2.91 (1.10-7.68)	0.031	2.67 (1.00-7.16)	0.050
Stage IV	3.77 (2.38-5.99)	<0.001	5.92 (2.81-12.45)	<0.001	4.81 (2.21-10.46)	<0.001	2.33 (1.21-4.48)	0.011	1.92 (0.97-3.79)	0.062
TNM 8th										
Stage I	1		1	<0.001	-	-	1	<0.001	-	-
Stage II	1.57 (1.12-2.22)	0.010	0.42 (0.28-0.63)	<0.001	-	-	1.40 (0.90-2.16)	0.135	-	-
Stage III	2.66 (1.86-3.81)	<0.001	0.91 (0.68-1.22)	0.535	-	-	2.69 (1.64-4.10)	<0.001	-	-
Stage IV	3.66 (2.37-5.64)	<0.001	1.33 (0.98-1.80)	0.069	-	-	2.45 (1.30-4.59)	0.005	-	-

5FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; FOLFIRINOX, 5-FU, leucovorin, irinotecan and oxaliplatin combination chemotherapy; Gem, gemcitabine monotherapy; GemCape, gemcitabine capecitabine combination chemotherapy; Gem/NabP, gemcitabine/nab-paclitaxel; HR, hazard ratio.

39 patients (14%) received GemCape. Therefore, selection bias cannot be excluded and any inter-group comparisons should be made with greatest caution.

Independent prognostic factors remaining statistically significant following multivariate Cox regression analysis included TNM stage and DPD expression status in the Gem group, whereas no factors appeared statistically significant in the GemCape group of patients. Again, the relatively small number of subjects in the latter (meaning a low power to detect any significant findings) should prompt careful interpretation.

As other types of chemotherapy including 5-FU with folinic acid, FOLFIRINOX, and gemcitabine plus nab-paclitaxel were only sporadically given in this cohort (n<11 in each treatment group), no meaningful analyses of prognostic factors including DPD-high vs. low were possible to do within these subgroups.

The results of this study cannot be directly compared with previous results on intratumoural DPD expression in patients of the ESPAC3 randomised controlled trial (15), as this was a real world cohort where no strict inclusion or exclusion criteria nor any randomisation to various treatments were applied. In the ESPAC3 trial population, DPD appeared as an independent prognostic marker in the 5-FU treated arm of patients but not in the Gem arm (although a non-significant numerical difference was still evident). On the other hand, the Kondo study (16) on patients receiving a combination of S-1 and gemcitabine revealed that DPD was an independent prognostic marker, with high expression being linked with worse prognosis.

Whereas the present results indicate that DPD is an independent predictor of the outcome in PDAC patients treated with postoperative Gem, the cohort studied here cannot be used to answer whether guidance to any of the more intense multi-drug protocols with GemCape (5), Gemcitabine and Nab-paclitaxel (21), or FOLFIRINOX (6) would have been beneficial for patients with high expression of DPD. Although 39 patients in the present population were treated with GemCape, and no statistically significant factors were evident in the multivariate regression model, statistical power would not be sufficient to rule out any impact of DPD (or any of the other potentially prognostic markers) in this or any of the even smaller subgroups. In addition, it would be most relevant for a future prospective trial to explore whether the addition of a DPD inhibitor such as gimeracil, one of three active substances in the S-1 combination, might be able to override the negative impact of high levels of DPD in the tumour. In theory, such a Gem plus gimeracil combination might be particularly valuable in patients with high expression of DPD in their tumour.

The weaknesses of this study are mainly inherent to the retrospective study design, and as there was no randomisation to various treatment arms any inter-arm differences observed should be interpreted with caution. Selection of treatment is likely to have been affected by background patient factors and comorbidity status as well as postoperative recovery and occurrence of complications. During the studied period, the predominant adjuvant protocol was Gem, with a smaller proportion of patients being subjected to the more recent multi-drug regimens that are now available and generally recommended.

The main strengths include the long term and comprehensive multi-centre real-world approach, meaning that a large

Table III. Univariate and multivariate Cox regression analyses on patients treated with adjuvant Gem and GemCape.

Variable	Gem (n=239)				GemCape (n=37)	
	HR Univariate	P-value	HR Multivariate	P-value	HR Univariate	P-value
Age						
<70 years	1		-		1	
≥70 years	1.26 (0.95-1.67)	0.105	-		1.43 (0.64-3.18)	0.380
DPD-level						
Low	1		1		1	
High	1.25 (1.06-1.47)	0.007	1.19 (1.01-1.41)	0.036	1.02 (0.65-1.60)	0.944
Sex						
Male	1		1		1	
Female	0.84 (0.73-0-96)	0.010	0.87 (0.76-1.00)	0.050	0.92 (0.64-1.34)	0.664
Grade						
Low	1	0.47	-		1	
Medium	0.89 (0.67-1.21)	0.48	-		0.75 (0.35-1.61)	0.464
High	0.74 (0.45-1.21)	0.234	-		-	
Neoadjuvant treatment						
No	1		-		1	
Yes	0.73 (0.33-1.36)	0.457	-		1.02 (0.35-2.93)	0.976
Radicality						
R0	1		1		1	
R1-2	1.40 (1.05-1.86)	0.021	1.23 (0.91-1.64)	0.174	1.75 (0.74-4.13)	0.203
Surgery						
1993-2006	1	0.366	-		-	
2007-2010	0.62 (0.30-1.28)	0.200	-		1	0.735
2011-2014	0.67 (0.34-1.33)	0.254	-		0.69 (0.10-4.96)	0.713
2015-2019	0.56 (0.28-1.12)	0.102	-		0.57 (0.13-2.45)	0.448
TNM 7th						
Stage I	1	0.003	1	0.018	1	0.566
Stage II	1.92 (1.20-3.06)	0.006	0.48 (0.30-0.76)	0.002	1.93 (0.26-14.30)	0.520
Stage III	5.41 (1.82-16.07)	0.002	0.88 (0.63-1.22)	0.429	1.24 (0.08-19.83)	0.880
Stage IV	2.77 (1.32-5.82)	0.007	2.26 (1.05-4.87)	0.037	6.64 (0.39-112.6)	0.190
TNM 8th						
Stage I	1	<0.001	-		1	0.261
Stage II	1.40 (0.84-2.33)	0.199	-		2.16 (0.70-6.72)	0.183
Stage III	3.01 (1.76-5.15)	<0.001	-		2.21 (0.69-7.05)	0.181
Stage IV	2.88 (1.38-6.04)	0.005	-		8.77 (0.89-86.80)	0.063

Gem, gemcitabine; GemCape, gemcitabine with capecitabine.

number of patients with PDAC undergoing curative intent resection in three major health care regions were included. Detailed clinical information was available and follow up time was sufficient to yield robust data on overall survival. To our knowledge, this is the largest real-world cohort of patients with resected PDAC treated with gemcitabine where DPD has been explored as a prognostic marker.

Future studies should focus on exploring the value of intratumoural DPD expression levels in patients undergoing adjuvant chemotherapy with contemporary multi-drug regimens, as well as exploring other potential enzymes and transport proteins involved in the metabolism and turnover of nucleic acids hence playing a potential role for sensitivity to-anti pyrimidine chemotherapeutics. Such candidate biomarkers include (but would not be limited to) thymidylate synthase, orotate phosphoribosyl transferase, cytidine deaminase, human equilibrative nucleoside transporter-1, and intratumoural human antigen all known to be involved in the turnover of antimetabolic chemotherapeutics (15,16,22-25). In addition, preclinical research will be necessary to dissect the mechanisms by which DPD and other metabolic enzymes affect the sensitivity to 5-FU, gemcitabine and other compounds.
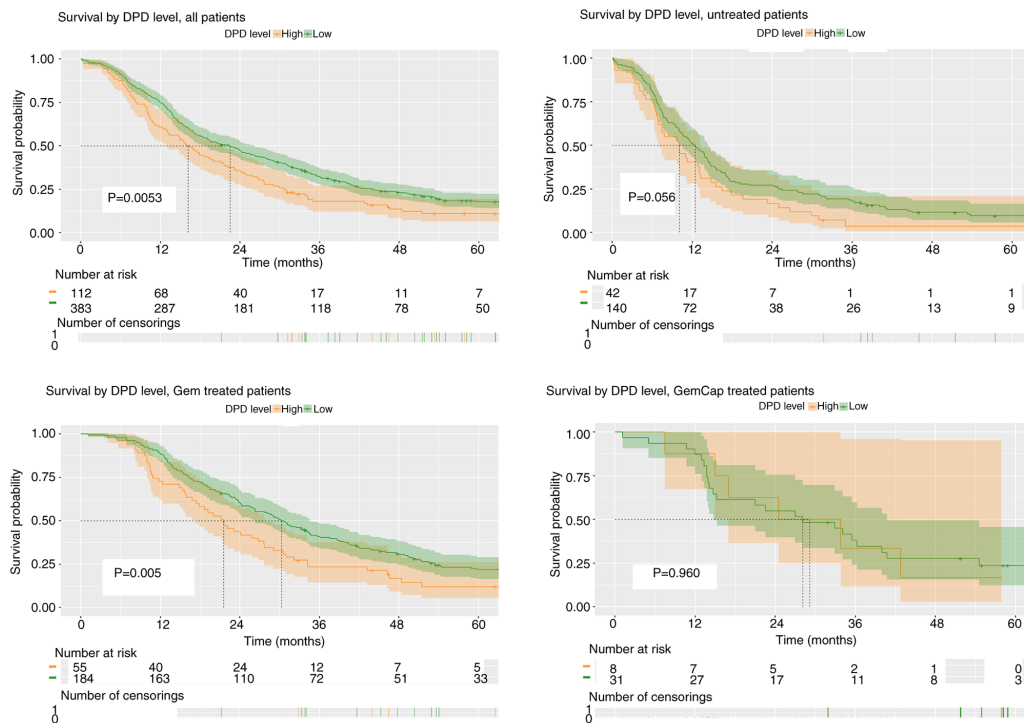
Figure 3. Kaplan-Meier curves estimating overall survival depending on DPD expression level in different subgroups according to adjuvant treatment. P-values for log-rank test are shown in each panel. DPD, dihydropyrimidine dehydrogenase; Gem, gemcitabine monotherapy; GemCap, gemcitabine capecitabine combination chemotherapy.

Ideally, individual or panels of predictive biomarkers should be identified and explored as treatment guiding tools in a prospective trial setting, to establish algorithms for optimal adjuvant treatment strategies and drug(s) of choice in patients with localised PDAC undergoing surgical resection.

In conclusion, intratumoural DPD expression is an independent prognostic factor in patients with PDAC undergoing surgical resection followed by adjuvant gemcitabine. Additional studies of DPD as a potential predictive biomarker in cohorts of PDAC patients treated with gemcitabine and non-gemcitabine based multi-drug chemotherapy protocols are warranted.

Acknowledgements

Funding

Availability of data and materials

The data generated in this study are available at reasonable request to the corresponding author.

Authors' contributions

Conceptualisation and design were performed by HB, PN, MS, CV, HG, BB, DÖ, SL, OF and NOE. HB, MB, FG, PN, MS, CV,

DÖ, SL and OF constructed the TMA. HB, MB and FG were responsible for tissue management and preparation. Staining was performed by MB and FG, scoring by HB and NOE. Data analysis was conducted by HB, HG, BB, DÖ, SL, OF and NE. HB and NOE drafted the manuscript. HB and NOE confirm the authenticity of all the raw data. All authors contributed to, and read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was performed according to The Declaration of Helsinki and was approved by the Swedish Ethics Review Board (approval number 2020-01511). Due to the retrospective non-interventional nature of the study, the review board waived the need for informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Cancerfonden: The Swedish Cancer Society Report. Cancerfonden, Stockholm, 2023.
3. Neoptolemos JP, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, Beger H, Fernandez-Cruz L, Dervenis C, Lacaine F, *et al*: A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. N Engl J Med 350: 1200-1210, 2004.
4. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, Padbury R, Moore MJ, Gallinger S, Mariette C, *et al*: Adjuvant chemotherapy with fluorouracil plus folinic acid vs. gemcitabine following pancreatic cancer resection: A randomized controlled trial. JAMA 304: 1073-1081, 2010.
5. Neoptolemos JP, Palmer DH, Ghaneh P, Psarelli EE, Valle JW, Halloran CM, Faluyi O, O'Reilly DA, Cunningham D, Wadsley J, *et al*: Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): A multicentre, open-label, randomised, phase 3 trial. Lancet 389: 1011-1024, 2017.
6. Conroy T, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, Choné L, Francois E, Artru P, Biagi JJ, *et al*: FOLFIRINOX or gemcitabine as adjuvant therapy for pancreatic cancer. N Engl J Med 379: 2395-2406, 2018.
7. Uesaka K, Boku DN, Fukutomi A, Okamura Y, Konishi M, Matsumoto I, Kaneoka Y, Shimizu Y, Nakamori S, Sakamoto H, *et al*: Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: A phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). Lancet 388: 248-257, 2016.
8. Padmanabhan S: Handbook of pharmacogenomics and stratified medicine. Academic Press, Cambridge, MA, USA, pp 341-364, 2014.
9. Longley DB, Harkin DP and Johnston PG: 5-fluorouracil: Mechanisms of action and clinical strategies. Nat Rev Cancer 3: 330-338, 2003.
10. Fukui Y, Oka T, Nagayama S, Danenberg PV, Danenberg KD and Fukushima M: Thymidylate synthase, dihydropyrimidine dehydrogenase, orotate phosphoribosyltransferase mRNA and protein expression levels in solid tumors in large scale population analysis. Int J Mol Med 22: 709-716, 2008.
11. European Medicines Agency: EMA recommendations on DPD testing prior to treatment with fluorouracil, capecitabine, tegafur and flucytosine. EMA, Press Release, Amsterdam, 2020.
12. Falvella FS, Cheli S, Martinetti A, Mazzali C, Iacovelli R, Maggi C, Gariboldi M, Pierotti MA, Di Bartolomeo M, Sottotetti E, *et al*: DPD and UGT1A1 deficiency in colorectal cancer patients receiving triplet chemotherapy with fluoropyrimidines, oxaliplatin and irinotecan. Br J Clin Pharmacol 80: 581-588, 2015.
13. Zhang YH, Shi WN, Wu SH, Miao RR, Sun SY, Luo DD, Wan SB, Guo ZK, Wang WY, Yu XF, *et al*: SphK2 confers 5-fluorouracil resistance to colorectal cancer via upregulating H3K56ac-mediated DPD expression. Oncogene 39: 5214-5227, 2020.
14. Vallböhmer D, Yang DY, Kuramochi H, Shimizu D, Danenberg KD, Lindebjerg J, Nielsen JN, Jakobsen A and Danenberg PV: DPD is a molecular determinant of capecitabine efficacy in colorectal cancer. Int J Oncol 31: 413-418, 2007.
15. Elander NO, Aughton K, Ghaneh P, Neoptolemos JP, Palmer DH, Cox TF, Campbell F, Costello E, Halloran CM, Mackey JR, *et al*: Expression of dihydropyrimidine dehydrogenase (DPD) and hENT1 predicts survival in pancreatic cancer. Br J Cancer 118: 947-954, 2018.
16. Kondo N, Murakami Y, Uemura K, Sudo T, Hashimoto Y, Nakashima A and Sueda T: Combined analysis of dihydropyrimidine dehydrogenase and human equilibrative nucleoside transporter 1 expression predicts survival of pancreatic carcinoma patients treated with adjuvant gemcitabine plus S-1 chemotherapy after surgical resection. Ann Surg Oncol 19 (Suppl 3): S646-S655, 2012.
17. Kuramochi H, Hayashi K, Uchida K, Nakajima G, Hatori T, Danenber KD, Danenberg PV and Yamamoto M: High intra-tumoral dihydropyrimidine dehydrogenase mRNA levels in pancreatic cancer associated with a high rate of response to S-1. Cancer Chemother Pharmacol 63: 85-89, 2008.
18. Tsukahara S, Shiota M, Takamatsu D, Nagakawa S, Matsumoto T, Kiyokoba R, Yagi M, Setoyama D, Noda N, Matsumoto S, *et al*: Cancer genomic profiling identified dihydropyrimidine dehydrogenase deficiency in bladder cancer promotes sensitivity to gemcitabine. Sci Rep 12: 8535, 2022.
19. Blomstrand H, Olsson H, Green H, Björnsson B and Elander NO: Impact of resection margins and para-aortic lymph node metastases on recurrence patterns and prognosis in resectable pancreatic cancer-a long-term population-based cohort study. HPB (Oxford) 25: 1531-1544, 2023.
20. Schemper M and Smith TL: A note on quantifying follow-up in studies of failure time. Control Clin Trials 17: 343-346, 1996.
21. Tempero M, Pelzer U, O'Reilly EM, Winter J, Oh DY, Li CP, Tortora G, Chang HM, Lopez CD, Bekaii-Saab T, *et al*: Adjuvant nab-paclitaxel + gemcitabine in resected pancreatic ductal adenocarcinoma: Results from a randomized, open-label, phase III trial. J Clin Oncol 41: 2007-2019, 2023.
22. Miyake K, Imura S, Yoshizumi T, Ikemoto T, Morine Y and Shimada M: Role of thymidine phosphorylase and orotate phosphoribosyltransferase mRNA expression and its ratio to dihydropyrimidine dehydrogenase in the prognosis and clinicopathological features of patients with pancreatic cancer. Int J Clin Oncol 12: 111-119, 2007.
23. Oba A, Ban D, Kirimura S, Akahoshi K, Mitsunori Y, Matsumura S, Ochiai T, Kudo A, Tanaka S and Minoru T: Clinical application of the biomarkers for the selection of adjuvant chemotherapy in pancreatic ductal adenocarcinoma. J Hepatobiliary Pancreat Sci 23: 480-488, 2016.
24. Aughton K, Elander NO, Evans A, Jackson R, Campbell F, Costello E, Halloran CM, Mackey JR, Scarfe AG, Valle JW, *et al*: hENT1 predicts benefit from gemcitabine in pancreatic cancer but only with low CDA mRNA. Cancers (Basel) 13: 5758, 2021.
25. Wei CH, Gorgan TR, Elashoff DA, Hines OJ, Farrell JJ and Donahue TR: A meta-analysis of gemcitabine biomarkers in patients with pancreaticobiliary cancers. Pancreas 42: 1303-1310, 2013.