Detecting drug resistance in pancreatic cancer organoids guides optimized chemotherapy treatment

Alexander Hennig1,2,3,4,5*, Franziska Baenke14†, Anna Klimova1,2,3,4,5,6, Stephan Drukewitz2,3,4,5,7, Beatrix Jahnke1, Sascha Brückmann1, Ramona Secci1,2,3,4,5,6, Christof Winter2,9,10, Tim Schmäche1,2,3,4,5,7, Therese Seidlitz1, Jean-Paul Bereuter1, Heike Polster1, Lisa Eckhardt1, Sidney A Schneider1, Stefan Brückner1, Renate Schmelz1,2, Jana Babatz1, Christoph Kahler1,2,3,4,5, Marius Distler1,2,3,4,5, Jochen Hampe12,13, Maximilian Reichert11,14, Sebastian Zeitläbig1,2,11,12, Gunnar Folprecht1, Jürgen Wetz1,2,3,4,5, Daniela Aust1, Thilo Welsch14 and Daniel E Stange1,2,3,4,5‡

1 Department of Visceral, Thoracic and Vascular Surgery, Medical Faculty and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany
2 National Center for Tumor Diseases (NCT), Dresden, Germany
3 German Cancer Research Center (DKFZ), Heidelberg, Germany
4 Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany
5 Helmholtz-Zentrum Dresden – Rossendorf (HZDR), Dresden, Germany
6 Institute for Medical Informatics and Biometry, Technical University Dresden, Dresden, Germany
7 Core Unit for Molecular Tumor Diagnostics (CMTD), Technical University Dresden, Dresden, Germany
8 Institute of Pathology and Tumor- and Normal Tissue Bank of the University Cancer Center (UCC), University Hospital Carl Gustav Carus, Medical Faculty, Technische Universität Dresden, Dresden, Germany
9 Institute of Clinical Chemistry and Pathobiochemistry, School of Medicine, Technical University of Munich, Munich, Germany
10 TranslaTUM, Center for Translational Cancer Research, Technical University of Munich, Munich, Germany
11 German Cancer Consortium (DKTK), Partner Site Munich, and German Cancer Research Center (DKFZ), Heidelberg, Germany
12 Department of Medicine I, University Hospital Carl Gustav Carus, Dresden, Germany
13 Center for Regenerative Therapies (CRTD), Technische Universität (TU) Dresden, Dresden, Germany
14 Translational Pancreatic Cancer Research Center, Medical Clinic and Polyclinic II, Klinikums rechts der Isar, Technical University of Munich, Munich, Germany
15 Center for Protein Assemblies (CPA), Technische Universität München, Munich, Germany

*Correspondence to: DE Stange, University Hospital Carl Gustav Carus, Department of Visceral, Thoracic and Vascular Surgery, Technische Universität Dresden, Fetscherstrasse 74, House 59, 01307 Dresden, Germany. E-mail: daniel.stange@uniklinikum-dresden.de
†Equal first authors.
‡Co-senior authors.

Abstract

Drug combination therapies for cancer treatment show high efficacy but often induce severe side effects, resulting in dose or cycle number reduction. We investigated the impact of neoadjuvant chemotherapy (neoCTx) adoptions on treatment outcome in 59 patients with pancreatic ductal adenocarcinoma (PDAC). Resections with tumor-free margins were significantly more frequent when full-dose neoCTx was applied. We determined if patient-derived organoids (PDOs) can be used to personalize poly-chemotherapy regimens by pharmacotyping of treatment-naïve and post-neoCTx PDAC PDOs. Five out of ten CTx-naïve PDO lines exhibited a differential response to either the FOLFIRINOX or the Gem/Pac regimen. NeoCTx PDOs showed a poor response to the neoadjuvant regimen that had been administered to the respective patient in 30% of cases. No significant difference in PDO response was noted when comparing modified treatments in which the least effective single drug was removed from the complete regimen. Drug testing of CTx-naïve PDAC PDOs and neoCTx PDOs may be useful to guide neoadjuvant and adjuvant regimen selection, respectively. Personalizing poly-chemotherapy regimens by omitting substances with low efficacy could potentially result in less severe side effects, thereby increasing the fraction of patients receiving a full course of neoadjuvant treatment.

Keywords: pancreatic cancer; patient-derived organoids; chemotherapy; personalized medicine; treatment response

Received 13 September 2021; Revised 11 March 2022; Accepted 30 March 2022

No conflicts of interest were declared.
Introduction

With an overall 5-year survival rate of about 5%, pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest malignancies with a steady increase in worldwide incidence [1]. Surgical resection within a multimodal treatment concept is to date the only potentially curative approach. However, 80% of patients present with unresectable locally advanced (LA) or metastasized disease [2]. The reasons for the ‘silent’ progression of PDAC are the lack of symptoms at early stage and the early tumor infiltration into major blood vessels, often precluding surgical resection [3].

Systemic neoadjuvant chemotherapy (neoCTx) may transform unresectable LA-PDACs into an operable stage in the event of a good response [2]. For many years, gemcitabine monotherapy was the favored chemotherapy in PDAC treatment [4]. The ACCORD11 study replaced gemcitabine with FOLFIRINOX, a combination of 5-fluorouracil (5-FU), leucovorin (folinic acid), irinotecan, and oxaliplatin, as the standard-of-care systemic chemotherapy [5]. Patients treated with FOLFIRINOX showed improved progression-free survival (PFS) and significantly enhanced overall survival (OS). In 2013, the IMPACT trial introduced the combination of gemcitabine and nab-paclitaxel (Gem/nab-Pac) as an additional regimen-driven improvement of PFS and significantly higher 2-year OS for neoadjuvant FOLFIRINOX and Gem/nab-Pac, opening up the question of which biomarker could guide treatment selection [7].

However, poly-component regimen-driven improvements were accompanied by significantly higher incidences of grade 3 or 4 toxicities compared with gemcitabine alone (FOLFIRINOX 46% versus gemcitabine 21%; Gem/nab-Pac 38% versus gemcitabine 27%) [5,6]. Recommendations for the choice of neoCTx vary between different guidelines (i.e. ESMO [8] and ASCO [9]). Important parameters are concomitant secondary diseases and Eastern Cooperative Oncology Group (ECOG) performance status [2]. Irrespective of treatment, many patients undergoing combination chemotherapy are unable to complete their treatment, due to adverse events, resulting in a lower relative dose intensity (RDI) administered [5,6,10]. However, chemotherapy with reduced RDI decreases PFS and OS, as shown for several tumor entities [9–13]. In the neoadjuvant setting, a favorable response increases the chance of surgical resection, which is associated with improved OS compared with patients not undergoing surgery [2]. Therefore, neoCTx is associated with improved patient survival, optimization of therapy protocols with the goal of lowering side effects while maintaining high efficacies is urgently needed [14]. Adjuvant chemotherapy may also improve PFS and OS but is administered only to patients with good performance status [15,16]. The choice of adjuvant regimen usually does not take into account a potentially developed resistance during neoadjuvant treatment.

Patient-derived organoids (PDOs) constitute a three-dimensional cell culture system that may serve as a model system to guide optimized therapy by directing tailored treatments. PDOs can be generated from surgical resection specimens and ultrasound-guided fine needle aspirations (EUS-FNAs) within weeks, with high efficiency. PDOs can be used to delineate drug response and resistance as they harbor most of the genetic alterations present in the original tumors [17–23]. The first study to correlate PDO response to treatment outcome was performed in gastrointestinal cancer, and other studies have provided additional evidence that patient response may be predicted by ex vivo testing [24,25].

Here, we retrospectively analyzed the impact of an adaptation of neoCTx treatment in patients with LA-PDAC on prognostic markers such as pathological response. We then pharmacotyped PDOs from chemotherapy-naïve and -pretreated patients using standard-of-care treatments and analyzed the efficiency of individualized treatment.

Materials and methods

Study approval

Sample collection, PDO generation, and cultivation were approved by the local ethics committee at Technische Universität Dresden, Germany (#EK451122014), and written informed consent was received from participants before study inclusion.

Patient cohort and PDAC tissue sampling

Data from patients presenting with LA-PDAC at the University Hospital Dresden between 2014 and 2021 were retrospectively analyzed to investigate the effect of dose or cycle reduction on therapy outcome. Inclusion criteria were the availability of information about the administered neoCTx and the pathological assessment of the tumor regression grade (TRG) according to Le Scodan et al. [26]. NeoCTx was considered ‘full course’ when FOLFIRINOX was administered for at least four 14-day cycles and Gem/nab-Pac was administered for at least three 28-day cycles without dose reduction. Patients who changed neoCTx after cycle 2 or who received systemic treatment other than FOLFIRINOX or Gem/nab-Pac were excluded. Board-certified pathologists diagnosed PDAC disease according to the WHO criteria [27] in all tissue samples from which PDOs were derived. An overview of the treatments that the patients of the 20 PDAC PDOs were administered can be found in supplementary material, Figures S1 (CTxnaive PDOs) and S2 (neoCTx PDOs).

Generation and cultivation of human PDAC organoids

PDAC organoid generation from surgical resection specimens was performed as described previously [17].
The protocol for processing FNA biopsies was modified by reducing the digestion time to 45 min and ACK lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) was used when erythrocytes were present. PDAC organoids were cultured in DMEM/F12 media supplemented with 1 × Glutamax (Invitrogen), 1 × HEPES (Invitrogen), and 1 × Pen/Strep (Invitrogen) supplemented with WNT3a-conditioned medium (10% v/v), RSPO1- and Noggin-conditioned medium (10% v/v), and gastrin (1 nM; Sigma-Aldrich, St Louis, MO, USA), primocin (1 mg/ml; InvivoGen, San Diego, CA, USA), N-acetyl-l-cysteine (1 mm, Sigma-Aldrich), nicotinamide (10 mm, Sigma-Aldrich), recombinant human fibroblast growth factor 10 (hFGF10, 100 ng/ml; Peprotech, Hamburg, Germany), recombinant murine epidermal growth factor (mEGF, 50 ng/ml; Invitrogen), and A-83-01 (0.5 μM; Tocris Bioscience, Bristol, UK). Successful PDAC line generation was determined when stable growth (> passage 10 to exclude normal organoids) and/or a KRAS mutation was confirmed.

DNA isolation and KRAS sequencing

The QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used for DNA isolation from PDAC PDOs. If required, Matrigel contamination was removed using cell recovery solution (Corning, New York, NY, USA). PCR was performed using the following primers for the KRAS gene: exon 2 forward 5'-AGCGTCGATGGAGGAGT TTG-3', exon 2 reverse 5'-TGTATCAAAGAATGGTC CTGCAC-3', exon 3 forward 5'-CCAGACTGTGTTTC TCCCTTC-3', exon 3 reverse 5'-TGCATGCGCATTAG CAAAGAC-3', and Phusion Taq polymerase (Thermo Fisher Scientific, 66.1 °C annealing temperature).

Panel sequencing

DNA was obtained from cryopreserved primary tissue by macrodissection. For targeted sequencing, 120 ng of total DNA per sample was used. Library preparation using the TruSight Oncology 500 Kit (Illumina, San Diego, CA, USA) was performed. The barcoded libraries were pooled and sequenced (2 × 150 bp paired-end) on an Illumina NextSeq 500 platform. Raw sequencing data were analyzed using the TSO500 pipeline (ruo-2.2.0.12) provided by Illumina. Resulting small variants were annotated using gnomAD exomes.r2.1.1 [28], ClinVar (last accessed 19 November 2021: https://www.ncbi.nlm.nih.gov/clinvar/), and COSMIC (last accessed 23 November 2021) [29], using SnpSift [30]; the effect of variants was predicted with SnpEff [31]. Before the comparison of organoid and tumor samples, all variants were quality-filtered according to the following criteria: (1) PDO samples: minimal variant coverage of 100X, minimal allele frequency of 5%; and (2) primary tumor samples: minimal variant coverage of 200X, minimal allele frequency of 1%. The tumor samples of DD394, DD439, DD578, DD412, and DD864 showed a reduced median target coverage. Consequently, variants were filtered with slightly adapted criteria: minimal variant coverage 40X, minimal allele frequency 5%. All results were visualized with Python using the Matplotlib library (https://www.computer.org/csdl/magazine/cs/2007/03/c03090/13RUwbJD0A) and CoMut [32].

Histology

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) for CK19 (ab20210, pH 6.0, 1:100; Abcam, Cambridge, UK) and TP53 (#2524, pH 6.0, 1:200; Cell Signaling Technology, Danvers, MA, USA) were conducted on paraffin-embedded material following standard protocols. Organoids were fixed in 4% paraformaldehyde for 30 min, dehydrated, and paraffin-embedded prior to sectioning and staining. Images were acquired using an EVOS FL Auto microscope (Life Technologies, Carlsbad, CA, USA).

Single- and combination-drug assays

PDO maintenance and experiments were carried out as previously described except that 75% Matrigel was used [17]. Chemotherapeutics were provided by the local pharmacy department at the University Hospital Dresden and used with the following dilution ranges: irinotecan 300 μM to 100 nM, oxaliplatin 500 μM to 500 nM, 5-FU 1000 μM to 10 nM, gemcitabine 390 nM to 1 nM, and paclitaxel 200 nM to 200 μM. Each dilution was assessed in duplicate. Normal growth medium alone and Matrigel covered with growth medium were used as negative controls. Mean IC_{50} values for each single substance from the first ten PDAC PDO lines were calculated and subsequently used as the drug concentration in combination: the dilution step n for FOLFIRINOX contained 10 μM irinotecan + 35 μM oxaliplatin + 35 μM 5-FU and n for Gem/Pac was 14 nm gemcitabine + 9 nm paclitaxel. Controls were analogous to those for single-drug assays. After 72 h, media were refreshed. Assay readout was carried out after 144 h by measuring cell viability using PrestoBlue (Invitrogen) in a VarioSCAN LUX plate reader (Thermo Fisher Scientific) as previously reported [17]. Drug assays were repeated at least three times within a period of five passages for each PDO line.

Modified drug combination assays

Modified drug combination assays were performed as described above. PDOs for FOLFIRINOX, leave-one-out modified assay (irinotecan + oxaliplatin, irinotecan + 5-FU, and oxaliplatin + 5-FU), and alternative treatment (Gem/Pac) were prepared from the same PDO pellet. Gem/Pac treatments were compared with single-agent assays already performed. Overlapping regions of Gem/Pac and single-agent assays were used to calculate the area under the curve (AUC) values.
Statistical analysis of drug assays

Analysis of the impact of full-course or adapted neoCTx on LA-PDAC patient treatment outcome was done via chi-squared testing in GraphPad Prism 8.4 (GraphPad Software, San Diego, CA, USA).

Pharmacotyping assays were analyzed as follows: relative viability was calculated by normalizing to the mean of the negative controls after blank subtraction. Plotting of dose-dependent relative viability curves and calculation of AUC was carried out using GraphPad Prism 8.4. Pharmacotyped PDO lines were grouped into CTx-naïve and FOLFIRINOX- or Gem/Pac-pretreated. Relative AUC (relAUC) was calculated by dividing the AUC by the maximum area for the individual drug dilution range. Single- and combination-drug assay relAUC values from the analyzed PDO lines were averaged in each group and values compared using Mann–Whitney tests.

AUC z-score normalization used the formula $z = (x - \mu)/\sigma$, where $x$ is the mean AUC from the PDO line tested in three individual experiments, $\mu$ is the mean AUC from all PDO lines analyzed, and $\sigma$ is the standard deviation from all PDO lines analyzed.

Analysis of modified FOLFIRINOX and Gem/Pac assays was as follows: Calculation of the viabilities for each drug dilution and AUC determination were carried out analogous to unmodified combination assays. Statistical analysis of the leave-one-out drug mixtures was performed using three-way ANOVA with multiple comparisons to the FOLFIRINOX triple drug data. For modified Gem/Pac, a one-way ANOVA test was used ($p < 0.05$, $**p < 0.01$, $***p < 0.001$).

To estimate whether drugs that are part of the Gem/Pac combination treatment act in additive, synergistic, or antagonistic ways, the drug responses of the single-agent treatment were fitted in a regression model with Gem, Pac, and their interaction Gem $\times$ Pac as explanatory variables.

Results

Adaptation of neoadjuvant treatment decreases the pathological response

First, we studied in our PDAC cohort if the adaptation of the neoCTx required by severe side effects affected outcome. We identified 59 patients with LA-PDAC who met the criteria for the retrospective analysis (supplementary material, Table S1). CTx was applied without adaptation (‘full course’) in 70.7% and 72.2% of patients using the FOLFIRINOX ($n = 41$) or the Gem/nab-Pac ($n = 18$) regimen, respectively (Figure 1A). An adaptation impacted the pathological regression grade: the distribution of regression grades shifted to a worse outcome in the FOLFIRINOX cohort; major (TRG3) and intermediate (TRG2) responses were less likely when a treatment needed to be modified/adapted ($p = 0.144$, chi-squared test) (Figure 1B). Similarly, no TRG3 response was observed in patients who received an adapted therapy in the Gem/nab-Pac cohort (Figure 1B). Since R and N status have been shown to be independent predictors for OS in PDAC, we further investigated how the adaption of CTx influenced these two parameters. An R0 resection was significantly more frequent when patients received full-course neoCTx (89.2% versus 62.5%, $p = 0.023$, chi-squared test) (Figure 1C). Accordingly, the percentage of patients with R0 resection paralleled the TRG score (TRG1 75%, TRG2 81.25%, TRG3 100% ($p = 0.25$, chi-squared test). N status was not significantly affected by adapted neoCTx ($p = 0.602$, chi-squared test; N0 35.7% versus 47.1%, N1 42.9% versus 41.2%, N2 21.4% versus 11.8% for full course versus adapted, respectively), but a TRG2/3 response increased the chance of lymph node negativity (N0 33.3%, 74%, and 54.5% in TRG1, TRG2, and TRG3, respectively) and resulted in a significantly improved lymph node status ($p = 0.02$, chi-squared test) (Figure 1D). In summary, adaptation of CTx was necessary in about 30% of patients and resulted in a worse outcome concerning TRG, R, and N status. This finding opens the question of whether an adaption of standard-of-care chemotherapy could be avoided by optimizing the regimen to prevent cytotoxic side effects without impacting efficacy.

Establishing a PDO biobank of chemotherapy-naïve and -pretreated PDAC

In order to evaluate the effect of neoCTx on tumor cells, we established a biobank of PDAC PDoS from surgical resections and EUS-FNA biopsies (supplementary material, Figure S3 A). A total of 54 PDAC PDO lines were cultured, of which 41 PDoS were derived from CTx-naïve patients (CTx-naïve PDoS, including 19 obtained by EUS-FNA) and 13 PDO lines were derived from patients who had received neoCTx (neoCTx PDoS). The efficiency of generating PDOs increased over time: continuous redefining of the culture conditions increased the take rate from 20% for the first ten samples to 80% and 40% for the last ten surgical specimens from CTx-naïve and neoCTx patients, respectively (supplementary material, Figure S3 A). Notably, failure of organoid outgrowth was observed whenever the patient showed a major response to neoCTx (take rate for TRG1: 56%; for TRG2: 57%; and for TRG3: 0%). Of note, the success rate of the last ten EUS-FNA biopsies from CTx-naïve patients was 90%. Continuous and robust growth of the majority of the PDO cultures was achieved within 1 month after culture initiation (Figure 2A), which allows pharmacotyping within this time frame (supplementary material, Figure S1B). Some PDO outliers required longer periods of culture. PDO morphologies ranged from cystic or (thin- or thick-walled) dense with no lumen to grape-like clusters (Figure 2C). We confirmed the epithelial origin of PDAC PDOs by IHC staining of CK19 (supplementary material, Figure S4A). As cystic PDAC PDOs cannot be distinguished from normal pancreatic organoids based on morphology, sequencing of the frequently mutated Kirsten rat sarcoma virus (KRAS) gene
Figure 1. Neoadjuvant chemotherapy (neoCTx) dose adaptation alters tumor regression grade as well as R and N status in LA-PDAC patients. (A) Frequency of patients receiving full-course versus adapted neoCTx with either FOLFIRINOX or gemcitabine + nab-paclitaxel (Gem/nab-Pac). (B) Distribution of minor, intermediate, and major pathological response rates (tumor regression grade; TRG1–3, respectively) in patients administered full-course or adapted neoCTx with FOLFIRINOX or Gem/nab-Pac. (C) Impact of full-course or adapted neoCTx dose on tumor-free (R0) or microscopic tumor-infiltrated (R1) resection margins and distribution of R0/R1 according to TRG. (D) Impact of full-course or adapted neoCTx dose on the number of tumor-infiltrated lymph nodes (N0 = 0, N1 < 4, N2 ≥ 4) according to TRG.
of exon 2 and, if required, exon 3 was performed. Commonly known mutations in codon 12 (G12V 35.19%, G12R 16.67%, G12D 25.93%) were detected, as well as rare single nucleotide changes in codons 14 (V14A) and 61 (Q61H) (supplementary material, Figure S4B).

Figure 2. Generation of a PDO (patient-derived organoid) PDAC (pancreatic ductal adenocarcinoma) biobank from treatment-naïve and neoadjuvant chemotherapy-pretreated patients. (A) A box plot depicts the time in days of the PDO growth dynamics from tissue processing until the earliest possible cryopreservation (six dense wells) of PDAC PDOs of the last ten tumor specimens processed from either surgical resection (CTX-naïve or neoCTX-pretreated) or CTX-naïve fine needle aspirations (EUS-guided FNA). (B) Representative bright-field images from passage 0 of PDO cultures to time point of first cryopreservation from one EUS-FNA (DD882) and two surgical resection specimen (DD909 CTX-naïve and DD996 neoCTX)-derived PDO lines. Scale bar: 1000 μm. (C) Representative bright-field images displaying the four main morphology types of PDAC PDOs. Scale bar: 400 μm. (D) Oncoplot depicting prevalent genetic alterations in PDAC tumor tissue and PDO lines.
Figure 3  Legend on next page.
established PDOs recapitulates the primary cancer, targeted sequencing was performed of cryopreserved tissue by macrodissection and of corresponding PDO lines (ten CTx-naïve and ten neoCTx PDAC PDOs, respectively) (Figure 2D). Of note, four out of 20 primary cancers (DD372, DD429, DD577, and DD996) could not be analyzed due to no, or very low, tumor content in the available cryosections (supplementary material, Table S2). The analyses revealed that previously described most prevalent PDAC mutations (e.g. in KRAS, TP53, SMAD4, and CDKN2A) were detected in 95% of the analyzed PDOs and matched primary cancers. Only PDO DD394 did not match the primary cancer in the four most prevalent genes; nevertheless, a common mutation in the GNAS gene was detected.

NeoCTx shaped the drug response profiles of PDOs

To assess the impact of neoCTx on drug sensitivity, we compared ten neoCTx PDOs with the ten CTx-naïve PDOs (supplementary material, Tables S2 and S3). Firstly, the 20 PDOs were analyzed for proliferative capacity using EdU-positive cells to rule out this potential confounder. The proliferation rate was not significantly different between the CTx-naïve and neoCTx PDOs (p = 0.2172, two-tailed t-test; supplementary material, Figure S4C). Next, the PDO lines were exposed to the FOLFIRINOX and Gem/Pac regimen (Figure 3A,C). Individual PDO lines showed a diverging response pattern, with some PDOs responding to low doses of chemotherapy, while for others the viability reduced only at higher doses. The AUC was calculated and PDOs were grouped according to the neoCTx administered to the corresponding patient (Figure 3B,D). For both the FOLFIRINOX- and the Gem/nab-Pac-pretreated patient cohort, a fraction of the PDOs responded poorly (defined as an AUC outside of 1 standard deviation of naïve PDOs) to the combinatorial regime administered neoadjuvantly: 33% of FOLFIRINOX- and 25% of Gem/Pac-pretreated PDOs, respectively. Thus, while no overall statistical significance was reached, a first indication that the neoCTx might impact the sensitivity of residual tumor cells was found.

Next, we sought to determine the response of the neoCTx PDOs to the standard alternative-of-care CTx regimen (i.e. the one not administered to the corresponding patient). NeoCTx PDOs exposed to the alternative CTx displayed similar (p = 0.3524 for FOLFIRINOX and p = 0.5905 for Gem/Pac) AUC values to those of neoCTx PDOs treated with the CTx regimen that the corresponding patient had received neoadjuvantly (supplementary material, Figure S5A,B). A switch to the alternative regimen for the adjuvant therapy might therefore not necessarily result in a higher treatment effect for some patients, and the choice of the adjuvant therapy regimen potentially needs to be tested for each individual patient.

PDOs might help to guide selection of the neoCTx regimen

Recent clinical trial data showed comparable efficacy for the FOLFIRINOX and Gem/nab-Pac regimens [7]. A biomarker for treatment selection does not exist. We therefore examined if drug testing in PDOs might be useful in selecting the more efficient regimen. To this end, the FOLFIRINOX and Gem/Pac treatment z-scores were calculated from the relative AUC values of the CTx-naïve PDO lines (Figure 3E). Six out of ten CTx-naïve PDO lines exhibited similar response patterns to both multi-drug treatments (DD372, DD376, DD385, DD429, DD439, DD442), while for the remaining four PDO lines a decision for one therapy with better efficacy could be taken. In addition, Pearson correlation analysis of the whole cohort showed that PDO sensitivities to both regimens were not linked (R^2 = 0.4377, p = 0.206) (Figure 3F). Taken together, drug testing of PDAC PDOs might guide the selection of the neoadjuvant treatment regimen.

Combination regimens can be adapted without compromising in vitro efficacy

Severe therapy-associated side effects necessitated an adaption of the dosing or number of applied neoCTx cycles in a substantial number of patients (Figure 1A) [33]. We asked whether an optimization of these regimens would be possible by modifying the multi-drug therapy composition. Single-drug assays were performed for ten CTx-naïve and ten neoCTx PDO lines to analyze the response to the FOLFIRINOX single substances irinotecan, oxaliplatin, and 5-FU, as well as the Gem/Pac components gemcitabine and paclitaxel (supplementary material, Figure S5C–E). A wide variation of treatment responses could be observed for each drug. We then calculated the z-scores of relative AUCs to assess common patterns between single- and combination-drug assays (Figure 3G). A good response to FOLFIRINOX was observed, with one exception...
Figure 4  Legend on next page.
(DD776), in PDOs that showed in general not more than one resistance to the single drugs irinotecan, oxaliplatin, and 5-FU, while FOLFIRINOX non-response was associated with poor single-drug efficacy in at least two single drugs (exception: DD385). Similar observations were seen for the Gem/Pac \( z \)-score analyses: a good response to Gem/Pac was achieved when at least one drug showed a good response in single-drug assays, except for DD577, DD412, and DD864. Thus, for both the FOLFIRINOX and the Gem/Pac therapies, several PDOs showed good sensitivity to treatment even if the response was poor for one or two single FOLFIRINOX drugs (e.g. DD376, DD776, DD439, DD578) or one Gem/Pac drug (DD376, DD372, DD442). We therefore hypothesized that at least in vitro, drug combination efficacy might not be driven by all compounds equally. Linear regression analyses of \( z \)-scores revealed that the most efficient single drug for each PDO correlated well with the complete combination therapy (FOLFIRINOX \( R^2 = 0.6018, p < 0.0001 \); Gem/Pac \( R^2 = 0.4718, p = 0.0008 \); supplementary material, Figure S5F,G).

To reduce side effects, one possibility could be to omit chemotherapeutics for which resistance is observed in PDO pretesting. To evaluate this strategy, the PDO lines DD385, DD439, and DD564 were chosen, as each of these lines showed resistance to at least one compound (irinotecan, oxaliplatin or 5-FU) (Figure 3E and supplementary material, Figure S6B). We compared cytotoxicity by the triple combination FOLFIRINOX with the combination of only two drugs. For the irinotecan-resistant PDO line DD385, no reduction in drug response was observed after removing irinotecan from the complete combination treatment (Oxa + 5FU versus FOLFIRINOX, \( p = 0.653 \); three-way ANOVA) (Figure 4A,B). The lack of 5-FU led to a significant reduction in efficacy (Iri + Oxa versus FOLFIRINOX; \( p < 0.001 \); three-way ANOVA), while the presence of 5-FU showed a response comparable to that seen for FOLFIRINOX (Iri + 5FU versus FOLFIRINOX; \( p = 0.345 \); three-way ANOVA). This suggested that 5-FU was the driving mediator of cytotoxicity for DD385. The same pattern was present in the irinotecan-resistant PDO line DD439 (Iri + Oxa or Iri + 5-FU or Oxa + 5-FU versus FOLFIRINOX: \( p = 0.007, p = 0.949, \) and \( p = 0.929, \) respectively; three-way ANOVA). Analysis of DD564, an irinotecan-sensitive but oxaliplatin- and 5-FU-resistant PDO line, revealed that treatment efficacy was only negatively altered when irinotecan was not applied (Iri + Oxa or Iri + 5-FU or Oxa + 5-FU versus FOLFIRINOX: \( p = 0.995, p = 0.929, \) and \( p = 0.043, \) respectively; three-way ANOVA). Taken together, for all three PDOs, the combination treatment could be reduced from three to two drugs without affecting efficacy.

Performing the same leave-one-out analyses for gemcitabine or paclitaxel compared with the Gem/Pac combination treatment resulted in similar patterns (Figure 4C,D). In the PDO line DD372 (resistant to gemcitabine but sensitive to paclitaxel), removing gemcitabine from the mixture did not change cytotoxicity, while removing paclitaxel resulted in a significantly reduced response (Gem versus Gem/Pac or Pac versus Gem/Pac; \( p = 0.020 \) and \( p = 0.824, \) respectively; one-way ANOVA). DD376 and DD442, both showing a poor response to paclitaxel, responded significantly in the opposite way (DD376 Gem versus Gem/Pac or Pac versus Gem/Pac; \( p = 0.171 \) and \( p < 0.001, \) respectively; DD442 Gem or Pac versus Gem/Pac, \( p = 0.542 \) and \( p = 0.24, \) respectively). Of note, statistical synergy analyses using a regression model revealed no consistent pattern of the interaction effect between Gem and Pac: while synergy was estimated for DD372, additive effects were seen for DD376 and DD442 (supplementary material, Figure S6A). Thus, for each PDO treatment, the effects differed and needed to be evaluated on an individual basis.

Another possibility to limit the toxicity of the triple chemotherapy regimen FOLFIRINOX is to combine drugs with a good response from both the FOLFIRINOX and the Gem/Pac regimens, e.g. irinotecan and gemcitabine (GemIri). Three PDO lines (DD376, DD429, and DD564) showing a good irinotecan and gemcitabine response were chosen to test the efficacy of GemIri (supplementary material, Figure S6B). GemIri-treated PDOs showed a similar, statistically not different response curve compared with FOLFIRINOX. Thus, a combination of effective drugs from both CTx regimens could replace the standard-of-care multi-drug treatments (supplementary material, Figure S6C).

**Discussion**

We used our PDAC PDO biobank from both chemotherapy-naïve and -pretreated patients to explore novel treatment possibilities for this devastating disease. The success rate of organoid generation from pretreated patients correlated with TRG, which can be explained
by the low number of viable tumor cells in specimens from patients with a major response (TRG3). Despite a limited sample number, a tendency towards drug resistance in neoCTX PDOs according to the combination therapy administered prior to the respective patients could be observed. The development of resistance or the enrichment of already existing resistant clones within the tumor under systemic chemotherapy might explain our results. Interestingly, not all neoCTX PDO lines showed resistance to the respective regimen, nor were they all more susceptible to the alternative regimen. Consequently, PDOs generated after treatment might play a role when selecting the most efficient adjuvant treatment in the future.

Thus, selecting the right neoCTX is currently hampered by the lack of biomarkers indicating a better efficiency of the FOLFIRINOX or Gem/nab-Pac regimen for the individual patient. The latest findings from the SWOG-S1505 trial showed similar efficacy for both regimens in the neoadjuvant setting. We showed that in ~50% of PDOs, a differential response for FOLFIRINOX versus Gem/nab-Pac could be documented. Therefore, PDOs might act as biomarkers to support clinicians by choosing the most promising regimen for neoadjuvant treatment of LA-PDAC. The cohort of PDAC patients for which pharmacotyping might play a role will potentially increase in the near future, since it has been shown that even patients with primarily resectable PDAC benefit from neoCTX [34]

Combining cytotoxic agents represents a common strategy to increase treatment efficiency in advanced cancers, and promising results have been achieved in the past [5,6,35–37]. Nevertheless, it has been shown that much of the benefit of combination therapy can be explained by independent drug action [38]. Indeed, in our analysis of interaction between the drugs of the combination regimen Gem/Pac, in two out of three PDOs additive effects were estimated, whilst the drugs displayed synergistic effects in only one PDO line. Administering more than one drug increases the chance of bypassing drug resistance frequently present in genetically heterogeneous tumors. The downside of poly-drug approaches is higher incidences of toxicities that require drug-dosing adjustments, a change of therapy, or termination of chemotherapy. In line with published studies in non-small-cell lung, breast, and liver cancers [12,39,40] as well as PDAC [41], we also observed in our PDAC cohort an inferior response to neoCTX after adapting the standard treatment due to side effects. TRG distribution differed between patients who received full-course first-line therapy and patients who did not. In turn, a good response to neoCTX increased the chance of R0 resection and significantly reduced the number of tumor-infiltrated lymph nodes.

PDOs can be used as a preclinical model to deliver in vitro data on drug sensitivities for individual patients. Successful generation and biobanking of PDAC organoids is feasible and has resulted in a better understanding of the disease [17–21]. In our hands, the generation of stably growing PDOs was feasible within 1 month on average, allowing pharmacotyping within a clinically reasonable time frame. In addition, tumors might evolve under the selective pressure of drug treatment, and therefore longitudinal biopsies and PDO generation to (re)assess drug sensitivities could reveal evolutionary trajectories leading to adaptations of the treatment.

The starting material from EUS-FNA samples was much smaller compared with surgical resection samples, yet the efficiency of PDO generation was higher. Rapid processing of EUS-FNA samples within minutes after biopsy retrieval might explain the success rate. This could be of clinical relevance, since histological confirmation of PDAC disease is required before the start of neoCTX, and frequently repeated EUS-FNAs need to be taken due to insufficient material yield before a pathological confirmation can be achieved. While expanding PDO lines, cfDNA from the supernatant can be used to confirm the origin of PDAC by droplet digital PCR and next-generation sequencing of frequently mutated genes in PDAC [21]. Combining EUS-FNA-guided PDO generation with mutation calling from cfDNA has the potential to significantly shorten the time to diagnosis.

In our retrospective analysis, 29% (17/59) of all the patients received an adapted therapy, regardless of which neoCTX they received. As we excluded patients from the analysis who did not undergo surgical resection after neoCTX or who had switched from FOLFIRINOX and Gem/Pac to the respective other regimen, the number of patients with an adaption of treatment would be expected to be even higher. Further treatment improvements could be achieved by identifying the compounds of poly-drug chemotherapy protocols that actually drive cytotoxicity. Using PDOs as an avatar of the patient’s tumor, we have shown that removal of inefficient drugs from a poly-drug regimen results in similar dose response curves. This finding was true for both clinically relevant combination regimens (FOLFIRINOX and Gem/Pac). Omitting one drug from a combination regimen could result in it being less drug-specific but also there being fewer general side effects associated with chemotherapy. Optimizing CTX to achieve a higher fraction of patients that receive full-course neoCTX could increase the rate of intermediate and good pathological response, potentially leading to a better OS of LA-PDAC patients.

Only 50% of patients can undergo adjuvant therapy due to a reduced ECOG status after surgery [2]. Yet survival is improved when PDAC patients manage to receive adjuvant CTX, regardless of nodal and resection margin status [15,16]. The same concept of optimizing neoadjuvant poly-drug CTX by administering fewer, but only effective, drugs could thus also be applied to patients in the adjuvant setting. In addition, administering fewer different drugs might allow slightly higher dosing of the used drugs, potentially even increasing the response rate.

In summary, PDAC PDOs constitute a promising in vitro system with the potential to optimize both the neoadjuvant and the adjuvant treatment by identifying the most efficient drug combinations. Increasing the
fraction of patients that receive full-course neoCTx could increase the rate of pathological major response, potentially resulting in a clinically relevant prolongation of overall survival. Prospective clinical trials are now needed to prove the translation of in vitro data to the in vivo situation in line with similar approaches in colorectal cancer [24,42,43].

Acknowledgements

We thank the patients and their families for supporting our research. We thank the Core Unit for Molecular Tumor Diagnostics (CMTD) of the National Center for Tumor Diseases (NCT) of the German Cancer Research Center (DKFZ), especially Dr Sandra Oster, for their excellent support. AH, TSch, TW, and DES were funded by a grant from the National Center for Tumor Diseases (NCT) within the Proof of Concept program. MR is supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, Project-ID 329628492 and DFG-RE 3723/4-1) and by the German Cancer Aid Foundation (Deutsche Krebshilfe #111273 and #70114328). DES is supported by the German Cancer Aid Foundation (Deutsche Krebshilfe #70113745).

Author contributions statement

AH generated and analyzed data and prepared the manuscript and figures. FB generated and interpreted data and contributed to figure preparation, discussion, and manuscript revision. AK performed statistical analyses. SD and LE generated data, analyzed panel sequencing data, and revised the manuscript. BJ generated data and stained tissue and organoids. SaB assessed pathological and graded pathological regression. RS generated data, performed ddPCR, and revised the manuscript. CW generated and interpreted data and performed ddPCR. TiS and ThS interpreted data and contributed to the discussion. JP generated data and revised the manuscript. HP was responsible for sample/tissue procurement from patients and patient follow-up. SAS generated data. StB, RS, JB, JH, and SZ performed endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNA). CK and MK performed surgical resections and revised the manuscript. MR and GF interpreted data and contributed to the discussion. JW contributed to manuscript revision. DA confirmed PDAC, assessed the pathology, and graded pathological regression. TW discussed data and contributed to revision of the manuscript. DES designed the project, supervised experiments, discussed data, and contributed to preparation of the manuscript and figures and finalization of the manuscript.

References

1. GBD 2017 Pancreatic Cancer Collaborators. The global, regional, and national burden of pancreatic cancer and its attributable risk factors in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Gastroenterol Hepatol 2019; 4: 934–947.
2. Müller PC, Frey MC, Ruzza CM, et al. Neoadjuvant chemotherapy in pancreatic cancer: an appraisal of the current high-level evidence. Pharmacology 2021; 106: 143–153.
3. Vincent A, Herman J, Schulick R, et al. Pancreatic cancer. Lancet 2011; 378: 607–620.
4. Burreis HA 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997; 15: 2403–2413.
5. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 2011; 364: 1817–1825.
6. Von Hoff DD, Ervin T, Arena FP, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 2013; 369: 1691–1703.
7. Sohal D, Duong MT, Ahmad SA, et al. SWOG S1505: results of perioperative chemotherapy (peri-op CTx) with moflib Roxin versus gemcitabine/nab-paclitaxel (Gem/nabP) for resectable pancreatic ductal adenocarcinoma (PDA). J Clin Oncol 2020; 38: 4504.
8. Ducrêux M, Cuhn A, Caramella C, et al. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2015; 26: v56–v68.
9. Balaban EP, Mangu PB, Yee NS. Locally advanced unresectable pancreatic cancer: American Society of Clinical Oncology clinical practice guideline summary. J Oncol Pract 2017; 13: 265–269.
10. Okusaka T, Ikeda M, Fukutomia A, et al. Phase II study of FOLFIRINOX for chemotherapy-naive Japanese patients with metastatic pancreatic cancer. Cancer Sci 2014; 105: 1321–1326.
11. Lütkauksiene S, Grizas S, Jureniene K, et al. Retrospective analysis of the impact of anthracycline dose reduction and chemotherapy delays on the outcomes of early breast cancer molecular subtypes. BMC Cancer 2018; 18: 453.
12. Crawford J, Divulis N, Patt D, et al. Phase II study of FOLFIRINOX for chemotherapy-naive Japanese patients with metastatic pancreatic cancer. J Clin Oncol 2019: 37: 8276–8284.
13. Blazevic I, Vaillant W, Basso M, et al. Survival and relative dose intensity of 5-fluorouracil, oxaliplatin and irinotecan in real-life treatment of metastatic colorectal cancer. Contemp Oncol (Pozn) 2020; 24: 150–156.
14. Prapatpi PW, Walter K, Gout J, et al. Pancreatic cancer-derived organoids – a disease modeling tool to predict drug response. United European Gastroenterol J 2020; 8: 594–606.
15. Flaum N, Hubner RA, Valle JW, et al. Adjuvant chemotherapy and outcomes in patients with nodal and resection margin-negative pancreatic ductal adenocarcinoma: a systematic review and meta-analysis. J Surg Oncol 2019; 119: 932–940.
16. DePeralta DK, Ogami T, Zhou JM, et al. Completion of adjuvant therapy in patients with resected pancreatic cancer. HPB (Oxford) 2020; 22: 241–248.
17. Hennig A, Wolf L, Jahnke B, et al. CFTR expression analysis for subtyping of human pancreatic cancer organoids. Stem Cells Int 2019; 2019: 1024614.
18. Tiriac H, Belleau P, Engle DD, et al. Organoid profiling identifies common responders to chemotherapy in pancreatic cancer. Cancer Discov 2018; 8: 1112–1129.
19. Seino T, Kawasaki S, Shimokawa M, et al. Human pancreatic tumor organoids reveal loss of stem cell niche factor dependence during disease progression. Cell Stem Cell 2018; 22: 454–467.e6.
20. Driehuis E, van Hoeck A, Moore K, et al. Pancreatic cancer organoids recapitulate disease and allow personalized drug screening. Proc Natl Acad Sci U S A 2019; 116: 26580–26590.
21. Dantes Z, Yen HY, Pfarr N, et al. Implementing cell-free DNA of pancreatic cancer patient-derived organoids for personalized oncology. JCI Insight 2020; 5: e137809.
22. Huang L, Bockorny B, Paul I, et al. PDX-derived organoids model in vivo drug response and secrete biomarkers. JCI Insight 2020; 5: e135544.
23. Hirt CK, Booij TH, Grob L, et al. Drug screening and genome editing in human pancreatic cancer organoids identifies drug–gene interactions and candidates for off-label treatment. Cell Genom 2022; 2: 100095.
24. Vlachogiannis G, Hedayat S, Vatsiou A, et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. Science 2018; 359: 920–926.
25. Wensink GE, Elias SG, Mullenders J, et al. Patient-derived organoids as a predictive biomarker for treatment response in cancer patients. NPJ Precis Oncol 2021; 5: 30.
26. Le Scodan R, Mornex F, Partensky C, et al. Histopathological response to preoperative chemoradiation for resectable pancreatic adenocarcinoma: the French Phase II FFCD 9704-SFRO trial. Am J Clin Oncol 2008; 31: 545–552.
27. Hruban RH, Boffetta P, Hiraoka N, et al. Ductal adenocarcinoma of the pancreas. In WHO Classification of Tumours of the Digestive System, WHO Classification of Tumours (4th edn), Bosman FTJ, Lakhani SR, Ohgaki H (eds). International Agency for Research on Cancer: Lyon, 2010; 281–291.
28. Karczewski KJ, Franciosi LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature 2020; 581: 434–443.
29. Tate JG, Bamford S, Jubb HC, et al. COSMIC: the catalogue of somatic mutations in cancer. Nucleic Acids Res 2019; 47: D941–D947.
30. Cingolani P, Patel VM, Coon M, et al. Using Drosophila melanogaster as a model for genotoxic chemical mutational studies with a new program, SnpSift. Front Genet 2012; 3: 35.
31. Cingolani P, Platts A, Wang LL, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 2012; 6: 80–92.
32. Crowdis J, He MX, Reardon B, et al. CoMut: visualizing integrated molecular information with comutation plots. Bioinformatics 2020; 36: 4348–4349.
33. Suker M, Beumer BR, Sadot E, et al. FOLFIRINOX for locally advanced pancreatic cancer: a systematic review and patient-level meta-analysis. Lancet Oncol 2016; 17: 801–810.
34. Janssen QP, O’Reilly EM, van Eijk JH, et al. Neoadjuvant treatment in patients with resectable and borderline resectable pancreatic cancer. Front Oncol 2020; 10: 41.
35. Bayat Mokhtari B, Homayouni TS, Baluch N, et al. Combination therapy in combating cancer. Oncotarget 2017; 8: 38022–38043.
36. Al-Batran SE, Homann N, Pauligk C, et al. Perioperative chemotherapy with fluorouracil plus leucovorin, oxaliplatim, and docetaxel versus fluorouracil or capetitabine plus cisplatin and epirubicin for locally advanced, resectable gastric or gastro-oesophageal junction adenocarcinoma (FLOT4): a randomised, phase 2/3 trial. Lancet 2019; 393: 1948–1957.
37. de Grammont A, Figer A, Seymour M, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 2000; 18: 2938–2947.
38. Palmer AC, Sorger PK. Combination cancer therapy can confer benefit via patient-to-patient variability without drug additivity or synergy. Cell 2017; 171: 1678–1691.e13.
39. Qi W, Wang X, Gan L, et al. The effect of reduced RDI of chemotherapy on efficacy of FOLFIRINOX in patients with advanced pancreatic cancer. J Natl Cancer Inst 2011; 103: 13421.
40. Kirino S, Tsuchiya K, Kurosaki M, et al. Relative dose intensity over the first four weeks of livaftinib therapy is a factor of favorable response and overall survival in patients with unresectable hepatocellular carcinoma. PLoS One 2020; 15: e0231828.
41. Kobayashi S, Ueno M, Omae K, et al. Influence of initial dose intensity on efficacy of FOLFIRINOX in patients with advanced pancreatic cancer. Oncotarget 2019; 10: 1775–1784.
42. Oodd SN, Weeber F, Dijkstra KK, et al. Patient-derived organoids can predict response to chemotherapy in metastatic colorectal cancer patients. Sci Transl Med 2019; 11: eaay2574.
43. Yao Y, Xu X, Yang L, et al. Patient-derived organoids predict chemoradiation responses of locally advanced rectal cancer. Cell Stem Cell 2020; 26: 17–26.e6.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods
Figure S1. Patient follow-up data summarized for the ten CTx-naive PDO lines
Figure S2. Patient follow-up data summarized for the ten neoCTx PDO lines
Figure S3. Growth dynamics of individual PDO lines
Figure S4. Histological and molecular characterization of pancreatic tumor PDOs
Figure S5. Multi- and single-drug pharmacotyping of pancreatic tumor PDOs
Figure S6. Tailored drug combination testing of pancreatic tumor PDO
Table S1. Neoadjuvant chemotherapy – PDAC patient data
Table S2. PDAC patient and patient-derived organoid data
Table S3. Follow-up data of PDAC patients