Neoadjuvant Chemotherapy or Combined Chemo-Radiation Therapy of Pancreatic Ductal Adenocarcinoma Differentially Shift Immune Activation, ECM Composition, Energy Metabolism, and Ribosomal Proteins of the Residual Tumor Mass

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Abstract

Background:

Pancreatic ductal adenocarcinoma (PDAC) belongs to the deadliest malignancies worldwide. Curative therapeutic options are limited as the majority of patients are diagnosed at advanced stages that disqualify them for surgical resection. Currently, neoadjuvant therapy regimens are being discussed as they may offer improved tumor resectability. Although features of the tumor biology (e.g. molecular markers) may potentially guide adjuvant therapy, little is known about the biological tumor alterations after neoadjuvant therapy.

Methods:

We performed mass spectrometry-based proteomics to characterize the proteomes of 67 PDAC resection specimens of patients who received either neoadjuvant chemo or chemo-radiation therapy. We employed data-independent acquisition (DIA), yielding a proteome coverage in excess of 3,500 proteins. Further, we combined our dataset with the proteomic PDAC CPTAC dataset to compare treatment-naïve and neoadjuvant treated residual PDAC.

Results:

The two neoadjuvant therapies yielded highly distinguishable proteome profiles of the residual tumor mass. Components of the immune system (e.g., complement cascade and immunoglobulins) and the extracellular matrix are increased in the chemo-radiation group, ribosomal proteins, the protein biosynthesis, degradation via the proteasome, and metabolic turnover in the chemotherapy group. Further analyses on the correlation of protein expressions and patient overall survival identified several proteins of the immune system as potential prognostic candidate biomarkers in both groups. The comparison of treatment-naïve and neoadjuvant treated tumors revealed an increase of ribosomal translation and the aldehyde dehydrogenase family.

Conclusion:

We present the first proteomic characterization of the residual PDAC tumor mass after neoadjuvant chemo and combined chemo-radiation therapy as well as potential candidates of protein markers associated with survival. We conclude that residual PDAC exhibits fundamentally different proteome profiles between both neoadjuvant therapies with immune system components correlating with overall survival. Notably, ribosomal translation and aldehyde dehydrogenases are suggested to be activated upon neoadjuvant treatment.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most prevalent tumor of the pancreas, accounting for more than 90% of all pancreatic malignancies. PDAC is expected to emerge as the second leading cause of cancer-related deaths in the US by 2030 [1]. The worldwide 5-year survival rate is below 10% [2], ranging between 2 and 9% in European countries and the USA [3]. The poor prognosis results from the advanced tumor stage (52% stage IV) at the time of diagnosis [4] as early stages are usually clinically silent [5]. Surgical resection is the exclusive curative treatment modality for PDAC [6, 7], however merely 15–20% of the patients present with initially resectable tumors [8]. Following resection, adjuvant chemotherapy was shown to be beneficial and
life-prolonging [9, 10] while the addition of radiation remained controversial in the literature [5, 11, 12]. Thus, it emerged that in Europe patients with PDAC typically receive adjuvant chemotherapy, whereas in the USA chemo-radiation is more common. Even in a resectable tumor stage (stage I - III) the long genetic evolution [13] with the subsequent inter- [14–16] and intra-tumor heterogeneity, the aggressive disperse growth pattern [17], and the development of resistances against chemotherapeutical drugs (e.g. Gemcitabine, 5FU, FOLFIRINOX, nab-Paclitaxel) are leading to high local recurrence rates up to 80% after surgical resection [18].

Recently, neoadjuvant chemo- or chemo-radiation therapy regimens have been gaining increased attention in order to improve the nodal and resection margin status [19, 20]. The benefit of chemo-radiation over chemotherapy alone is under debate as patients’ overall survival time does not improve significantly [21–25]. A comparison of neoadjuvant treatment to “surgery first” treatment suggests that the overall survival time and incidence of resections are similar [24–26]. Nevertheless, it has been observed that neoadjuvant treatments have the capacity for tumor downstaging, facilitating the surgery conditions and/or shortening surgery time [24, 26, 27].

Poor responders to neoadjuvant treatments still face early disease recurrence and shortened overall survival [28], highlighting the need for further research on the tumor biology of residual PDAC following neoadjuvant treatment. Two recent studies used mass spectrometry-based proteomics to determine predictive biomarkers identifying poor responders for neoadjuvant-treated patients [10, 29]. These studies found that among good responders, acute phase signaling and macrophage activation were increased, which indicated elevated immune responses in the tumor. Another mass spectrometry-based proteomic study, that focused on the comparison of PDAC tissue after neoadjuvant chemotherapy and treatment-naïve PDAC tissues, showed a significantly lower expression of metabolic proteins in the neoadjuvant-treated samples and a higher potential for stem cell-like properties of surviving cancer cells in the residual tumors after neoadjuvant therapy [30]. Neoadjuvant chemotherapy (Folrinox-based) treated tumors showed an enrichment of cytotoxic T cells and an anti-tumorigenic immune microenvironment [31], which was predominantly located in the stromal regions. Recently, heterogeneity of the tumor microenvironment was described to shape the immune phenotypes of PDAC [32].

Here, we present the first proteomic characterization of residual PDAC after neoadjuvant chemo- or combined chemo-radiation therapy. We use data independent acquisition (DIA) [33] mass spectrometry (MS) to characterize and compare the proteomes of the residual tumors after both neoadjuvant therapies. In DIA, the mass spectrometer acquires MS/MS spectra independent of MS1 ion intensities [33, 34]. The protein coverage and quantification of DIA outperforms the standard data-dependent acquisition [35–38].

We analyzed formalin-fixed and paraffin-embedded (FFPE) tissue specimens. FFPE tissues are a valuable resource for histopathological, proteomic, and genomic analysis as this fixation procedure preserves the cellular morphology and prevents tissue degradation for decades. For each neoadjuvant treatment (viz. chemo and chemo-radiation), we correlate the proteome expression data with the survival time to identify potential prognostic candidate markers.

**Methods**
Patient cohort and tissue fixation

67 PDAC resections were collected at the Memorial Sloan Kettering Cancer Center, NY, USA, between 2012 and 2018. Patients received neoadjuvant treatment as described previously [39, 40] and underwent either a chemotherapy or a combined chemo-radiation therapy before tumor resection. Resected tumor specimens were formalin-fixed and paraffin-embedded (FFPE) according to routine protocols. From the FFPE tissue blocks, 10 µm thick slices were cut and mounted on glass slides for macrodissection. An overview of the experimental procedure is demonstrated in Fig. 1.

LC-MS/MS Sample Preparation

10 µm thin tissue specimens were deparaffinized and rehydrated using a decreasing xylol and ethanol series. Specimens were stained with hemalaun for 5 seconds and macro-dissected by a pathologist (S. T.-B.). Aiming at a sample size between 1 and 3 mm³, two slides were pooled when necessary. Heat-induced antigen retrieval and protein extraction were performed as described earlier [41]. In short, proteins were extracted in an acid labile buffer (0.1% RapiGest in 1 M HEPES, pH 8.0) with the aid of sonication (Bioruptor®, Diagenode), reduced with f. c. 5 mM DTT and alkylated with f. c. 15 mM iodacetamide. Proteins were double-digested with trypsin (Sequencing Grade Modified Trypsin, V5111, Promega) and Lys-C (Lysyl Endopeptidase®, Mass Spectrometry Grade (Lys-C), FUJIFILM Wako Pure Chemical Corporation) at 50°C for 2 hours and afterwards overnight at 37°C. Peptides were acidified with trifluoroacetic acid (TFA) to pH 2.0 and desalted using PreOmics cartridges (iST Kit, PreOmics). Peptides, bound to cartridges, were washed first with 1% TFA in isopropanol, and then with 0.2% TFA in ddH₂O. Peptides were eluted in 2% trimethylamine in 80% acetonitrile. Peptide concentration was determined using the bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, #23225, ThermoFisher) according to the manufacturer's instructions. Peptides were vacuum dried and frozen at -80°C until further use.

**LC-MS/MS Data-independent Acquisition (DIA)**

All liquid chromatography-tandem mass spectrometry (LC-MS/MS) data were acquired on a Q-Exactive Plus mass spectrometer (Thermo Scientific™) coupled to an EASY-nLC™ 1000 UHPLC system (Thermo Scientific™). The analytical column was self-packed with C18 silica beads (Reprosil Pur C18-AQ, d = 3 Å; Dr. Maisch HPLC GmbH) and coupled to a PepMap™ precolumn (C18, 75 µm diameter, 20 mm length; Thermo Scientific™). A two-step linear gradient increasing buffer B (0.1% formic acid in 80% acetonitrile, Fluka) from 8–43% over 90 min and from 43–65% over 20 min was applied to separate peptides on the analytical column. For each measurement, 800 ng peptides were injected. For DIA, MS1 scans were performed at 70,000 resolution, an AGC target of 3e6, and a maximal injection time of 50 ms. The survey scans were conducted on a scan range between 385 and 1015 m/z. The MS2 isolation window size was set to 24 m/z, and 25 consecutive MS2 scans were performed in a stepwise overlapping manner. The resolution was set to 17,500, the AGC target to 1e6, the maximal injection time to 80 ms, and the stepped NCE to 25 and 30.

Spectral Library Generation

To generate a cohort-specific spectral library, 2 pooled reference samples were repeatedly measured using gas phase fractionation (GPF) [34, 42, 43]. Each reference sample consisted of the same peptide amount from 8
samples of the patient cohort in consideration of the clinicopathological features. 6 measurements, injecting 800 ng per measurement, were conducted on the Q-Exactive Plus mass spectrometer. A fractionation window range from 395 m/z to 1005 m/z was covered in 100 m/z wide steps (hence 6 measurements). The MS2 isolation window size was set to 4 m/z. MS2 scans were performed at 17,500 resolution, an AGC target of 1e6, and a maximal injection time of 80 ms, using a stepped NCE of 25 and 30. MS1 survey scans were performed at 70,000 resolution, an AGC target of 1e6, and a maximal injection time of 50 ms. DIA-NN 1.8 [44] was used to generate the spectral library from the gas phase fractionated measurements. Data files were converted from the vendor-specific .raw file format to the software-specific .dia file format and analyzed against a proteome fasta file, downloaded from Uniprot on the 14th June 2021 (https://www.uniprot.org/), which contained 25,853 proteins. For library generation, the precursor FDR was set to 10%, deep learning-based spectra, RTs and IMs prediction was enabled. The peptide length was set between 7 and 30 amino acids, the precursor charge range between 1 and 4, the precursor m/z range between 400 and 1000, and the fragment ion m/z range between 200 and 1800. N-terminal methionine excision and cysteine carboxamidomethylation were enabled. Trypsin was set as the digestion protease, and 2 missed cleavages were allowed. The final, GPF refined library comprised 12,348 proteins, 15,335 protein groups, and 79,058 precursors.

DIA Sample Analysis

Samples were analyzed using DIA-NN 1.8. Data files were converted from the vendor-specific .raw file format to the software-specific .dia file format and analyzed against the cohort-specific spectral library generated beforehand. For sample analysis, the precursor FDR was set to 1%, the peptide length was set between 7 and 30 amino acids, the precursor charge range was between 1 and 4, the precursor m/z range was between 400 and 1000, and the fragment ion m/z range was set between 200 and 1800. N-terminal methionine excision, cysteine carboxamidomethylation, and match between runs (MBR) were enabled. Trypsin was set as the digestion protease, and 2 missed cleavages were allowed. 1 sample was removed from the analysis after an initial analysis due to a low protein content (less than 3% of the average of all samples).

Differential Protein Expression Analysis

The statistical analysis was performed using R in the RStudio environment [45, 46]. The protein group intensity matrix was inspected for missing values and filtered such that protein groups that have at most 30% missing values per treatment subgroup (viz. chemotherapy or chemo-radiation therapy) were retained. As the protein digestion has been performed in multiple batches, the resulting batch effect in the intensities was corrected using the ComBat algorithm [47] and median normalization. Missing values were imputed via the ImpSecRob algorithm [48, 49], which was suggested by DIMAR [50] as the best-performing imputation algorithm for this specific data set. The partial-least squares discriminant analysis (PLS-DA) and principal component analysis (PCA) were performed using the mixomics package [51], and the linear model analysis using the linear model for microarrays data (limma) package [52]. The subsequent pathway enrichment analysis was performed using the topGO approach and the Fisher's exact test [53].

Survival Analysis

For the survival analysis, the lubridate [54], survival [55], survminer [56], cmprsk [57] and CoxBoost [58] packages were used. The survival time was calculated from the day of diagnosis. To test whether there is a
difference in the incidence of death by PDAC between both treatments a competing risk analysis was performed (cmprsk package). A multivariate cox proportional hazards regression model was fitted (survival package) to identify clinical parameters that affect the overall survival time. In order to identify potential prognostic candidate markers the CoxBoost algorithm was applied to the clinical and expression data. The penalty value and optimal number of boosting steps were determined using package-specific cross validation-based functions. To visualize the effect of a prognostic candidate marker on overall survival, patients were divided into groups of high and low protein expression [59]. For each protein, the cut point value based on the distribution of expression values was calculated using the survminer package. Patients were separated according to a given expression value: if they showed a value below/above the given cut point they were assigned to the “low expression”/“high expression” group. Results were visualized using intrinsic functions from the packages, or the following packages: EnhancedVolcano [60], ggplot2 [61], cowplot [62], or forestmodel [63].

Proteogenomic analysis

The proteogenomic analysis was performed as described previously [64]. In short, the Galaxy environment [65] was used to analyze paired-end RNA-Seq data from the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra). The RNA-Seq reads (Supplementary Table 2) were mapped to the human reference genome GRCh38. The resulting proteoform database (subFASTA) containing single amino acid variants (SAAVs) was processed locally with Python scripts to generate tryptic variant peptides (3,596 entries) that were appended to the abovementioned human proteome reference database. This combined database was then used for the SAAV peptide search in DIA-NN 1.8. First, a spectral library was predicted (1,837,002 precursor and 7,769 proteins) and then the LC-MS/MS samples were analyzed against this library.

Results And Discussion

Overview of Patient Cohort

66 patients were included in the following analyses as one patient was initially removed due to many missing data points. All patients had been diagnosed with PDAC and received either neoadjuvant chemotherapy or a combined neoadjuvant chemo-radiation therapy before surgical resection. They were treated at the Memorial Sloan Kettering Cancer Center, New York, USA, and were between 33 and 85 years old (median 66.5 years, mean 65.5 years). 42 patients presented with tumor progression after the neoadjuvant therapy. The median survival was 2.034 years. Further clinicopathological details are summarized in Table 1 (and Supplementary Table 1).
Table 1
Clinicopathological parameters for individuals included in the study group.

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<th>Age</th>
<th>Differentiation</th>
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AWD: alive with disease, DOC: died of other cause, DOD: died of disease, DUC: died of unknown cause, NED: no evidence of disease; * Survival calculated starting from the day of diagnosis.
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AWD: alive with disease, DOC: died of other cause, DOD: died of disease, DUC: died of unknown cause, NED: no evidence of disease; * Survival calculated starting from the day of diagnosis.
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AWD: alive with disease, DOC: died of other cause, DOD: died of disease, DUC: died of unknown cause, NED: no evidence of disease; * Survival calculated starting from the day of diagnosis.

Table 1: Clinicopathological parameters for individuals included in the study group.

**Vascular Invasion Increases the Hazard Ratio to Dying by PDAC**

We aimed to analyze whether clinical parameters affected the overall survival of the patients included in this study. Therefore, we fitted a multivariate Cox proportional hazards model with the clinical patient data: treatment, differentiation status, perineural invasion status, vascular invasion status, occurrence of recurrence, age, and sex. We observed that vascular invasion was the only parameter to significantly affect the overall survival time of all patients (Fig. 2a). Vascular invasions have already been reported to accompany dismal overall survival [66]. Here, individuals with an evident vascular invasion in the initial tumor (n = 35) present a significantly increased hazard ratio of 3.24 (p = 0.004) compared to patients without a confirmed vascular invasion (n = 28).

**Cumulative Incidence of Death by PDAC Decreases in Patients who Received Neoadjuvant Chemo-Radiation Therapy**

We next compared the cumulative incidence of death by PDAC between both therapy subgroups using a competing risk analysis since patients in this study group died of either PDAC (DOD, n = 46), another cause (DOC, n = 2), an unknown cause (DUC, n = 1), or were alive with disease (AWD, n = 7), or with no evidence of the disease (NED, n = 10) at the end of the sampling time. For our cohort, we observed that patients who underwent the combined chemo-radiation therapy had a significantly lower cumulative incidence of death from PDAC than the chemotherapy subgroup (p = 0.038; Fig. 2b). In both therapy subgroups, patients received either folfinorox, folfox, gemcitabine or capecitabine-based chemotherapy regimens (Supplementary Table 1).

To date, the benefit of neoadjuvant treatment regimens for patients suffering from PDAC is under debate [12, 19–28]. There are theoretical benefits such as tumor shrinkage, downstaging of the lymph node status and increase of the chance for tumor resection [19, 20], but so far, there is no clear evidence for prolonged progression-free or overall survival [12, 26, 67, 68]. In accordance, there is no consensus which neoadjuvant treatment modality, chemotherapy alone or combined chemo-radiation therapy is beneficial for patients with PDAC. In a recent study from 2021, Chopra and colleagues showed that patients who underwent neoadjuvant chemo-radiation therapy demonstrated a longer disease-free survival than patients receiving neoadjuvant chemotherapy, however the overall survival was similar [24]. Patients included in their study received either gemcitabine-based or 5-fluorouracil-based (folfox, folfinorox) chemotherapy. Breslin and colleagues showed that the survival of patients with resectable tumors could be maximized by the aid of neoadjuvant chemo-radiation therapy [69], but they did not compare the survival to a group of patients receiving neoadjuvant...
Our results suggest a longer overall survival for patients who received neoadjuvant chemoradiation therapy compared to neoadjuvant chemotherapy.

**Overview of Proteomic Coverage, Batch Effect Removal and Imputation**

In order to study the PDAC proteome biology of the residual PDAC after neoadjuvant treatment, we performed explorative, mass spectrometry-based proteome profiling of FFPE resection specimens of patients with PDAC. The macro-dissected tissue volumes ranged between 0.08 mm$^3$ and 3.02 mm$^3$. After heat-induced antigen retrieval and protein extraction, proteins were enzymatically digested by trypsin and LysC to perform a prototypical "bottom-up" proteomic approach. In a recent benchmarking study, we have shown that DIA-type proteomics is a powerful approach for characterizing the proteome biology of distinct tissues, even in the presence of inter-individual heterogeneity [70]. Hence, we have used DIA-type proteomics for the present work.

We identified 3592 proteins. Interestingly, considerably more proteins were identified in the residual tumor mass that received neoadjuvant chemotherapy compared to the combined chemo-radiation therapy (Fig. 3a-b). Calculating a Pearson correlation between the number of identified proteins and the percentage of stromal tissue does not reveal coherence of both (Figure S1a-b). The proteome coverage remains below the recently published CPTAC (Clinical Proteomic Tumor Analysis Consortium) study on PDAC proteomics [71], but is substantially above the proteome coverage reported by non-fractionated DDA-type proteomics of PDAC [72].

Proteins that were present in at least 70% of the samples were included in the statistical analyses. We corrected for batch effects arising from the sample preparation procedure using the ComBat [47] algorithm (Fig. 3c). Following a median normalization (Fig. 3d) [73], missing protein expression values were imputed using the impSecRob algorithm [48, 49]. Using DIMAR [50], we evaluated which imputation algorithm performs best on our dataset. Finally, 2040 proteins were included for further analyses.

**Neoadjuvant Chemotherapy and Combined Chemo-Radiation Yield Distinct Residual PDAC Proteomes**

Supervised Partial Least-Squares Discriminant Analysis (PLS-DA) clearly separated both treatment groups based on their protein expression profiles (Fig. 4a). Patients that received neoadjuvant chemo-radiation formed one cluster, while the chemo treated patients separated into a second cluster. Two samples from the chemo-radiation subgroup cross the separation border and locate closely to the chemo subgroup (CR19 and CR26). In an unsupervised hierarchical clustering analysis (Euclidean distance and complete-linkage clustering), we, however, did not detect any clustering according to the treatment groups or within one treatment subgroup (Figure S2).

**Patient-Specific Factors/Variables Impact the Distinct Proteome Biology in the Residual PDAC Mass**

To identify differentially regulated proteins between the subgroups, we performed a linear model analysis via the limma package [52]. In the following, we describe proteins as significantly regulated, if their false discovery rate (FDR) is < 0.05. If in addition, the fold change (log2(chemo-radiation/chemo) is above 1.5, we
call these proteins significantly upregulated or below − 1.5 significantly downregulated, respectively. In order to examine, whether the clinical (age and sex) or histopathological (perineural invasion, vascular invasion, and differentiation status before the surgery) parameters affect the differential protein expression, we included those parameters as covariates to the linear model. Since under the null hypothesis (no impact) the p-value distribution from the linear model analysis of all proteins is expected to be uniform, the observed shift of the distribution towards zero as well as the substantial proportion of p-values below 0.05 suggest a strong global impact of those variables on the proteome (Fig. 4b-f).

**Combined Neoadjuvant Chemo-Radiation Therapy Yields Enrichment of Extracellular Matrix Proteins and Immune System Activation**

Using the linear model analysis and the above-described criteria, we identified substantial differences between the proteomes of the residual tumors after neoadjuvant combined chemo-radiation therapy and neoadjuvant chemotherapy (Fig. 5a). Overall, 134 proteins were significantly upregulated in the chemo-radiation subgroup, whereas 242 proteins were significantly upregulated in the chemotherapy subgroup.

Proteins that are significantly upregulated in the chemo-radiation subgroup present a strong fingerprint for apolipoproteins, extracellular matrix (ECM) proteins, the complement system, and immunoglobulins. They include six apolipoproteins (Apolipoprotein A1, A4, C2, C3, ApoD, and ApoL-II), 11 collagens (COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, COL6A1, COL6A2, COL8A1, COL11A1, COL12A1, and COL14A1), the procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1) that is essential for the assembly and cross-linking of collagen fibrils and is thereby promoting cancer proliferation, invasion and migration [72,73,74], and the matrix related proteins matrix metalloprotease-14 (MMP14), biglycan (PGS1), and matrix-remodeling-associated protein 5 (MXRA5), which shows anti-inflammatory and anti-fibrotic properties by decreasing the induction of chemokines and collagen expression [77].

A fibrotic ECM rich in collagens, the so-called desmoplastic reaction is a hallmark of PDAC, known to promote tumor progression and chemoresistance [78–80]. Especially the interstitial fibrillary collagens types I and III contribute to the desmoplastic reaction [81, 82]. Besides progression and resistance, tumors exploit collagen fibers for migration and to facilitate metastasis [83]. MMP14 contributes to matrix remodeling by exhibiting endopeptidase activity through which it cleaves components of the ECM such as collagens [84, 85]. MMP14 has already previously been found to be overexpressed in PDAC [85–89]. Though described as a hallmark of PDAC progression and potentially favoring the development of metastasis, we did not interpret this upregulation in the chemo-radiation subgroup as unfavorable for the patients outcome as only 6 of 28 chemo-radiation patients showed metastatic spread in lymph nodes, and the chemo-radiation subgroup showed a lower cumulative incidence of death.

A collagen-rich residual tumor mass may also arise from radiation therapy. Radiation induces tissue damage and subsequent wound healing. In the course of the long enduring repair process, ECM that surrounds tumor cells may undergo increased proteolysis (e.g. by MMPs) and enhanced activity of matrix proteins resulting in ECM remodeling [90].
For the present study, we were unable to include "healthy", normal pancreas, treatment-naïve PDAC, or radiation-only treated PDAC; hence, it remains beyond the scope of the study to interpret this observation as unfavorable or favorable. However, we observed an overall resemblance of the neoadjuvant treated tissues with treatment-naïve PDAC tissues from a large proteomic PDAC study by the Clinical Proteomic Tumor Analysis Consortium (CPTAC) (see section “Neoadjuvant treated residual PDAC mass groups with treatment-naïve PDAC from the CPTAC”).

Furthermore, we detected a large upregulation of immune related proteins in the chemo-radiation subgroup. Immunoglobulins overrepresented in the chemo-radiation subgroup included immunoglobulin heavy constants alpha 1, gamma 1–4, mu, heavy variables, the J chain, kappa constants and variables as well as a lambda variable and the lambda-like polypeptide 5. Upregulated complement proteins comprise four complement C1 subcomponents (C1QA, C1QC, C1R, and C1S), C2, C4-B, C6, C8-B, and C9, complement factor H, and complement factor H-related protein 2, the complement decay-accelerating factor (CD55), as well as C4b-binding protein alpha chain (C4BPA) [91, 92] that drive and control the complement pathway. C4BPA also interacts with Serum amyloid P-component (SAP) [93] and Vitamin K-dependent protein S [94], two proteins, which are significantly overexpressed in the chemo-radiation subgroup as well. CD55 suppresses the C3 convertase of the classical complement pathway [95, 96], whereas complement factor H [97–99], and complement factor H-related protein 2 [100] are both inhibitors of the alternative complement C3 convertase.

Recently, Dias Costa and colleagues (2022) reported an enrichment of T cells in neoadjuvant chemotherapy treated PDAC with subsequent additional radiotherapy only having a marginal effect on the so-called immune microenvironment [31]. In our study, we observed a strong impact on the expression of the complement system and of immunoglobulins in response to combined chemo-radiation therapy. Although, prototypical T cell markers such as CD3, CD4, CD8 or FOXP3 escaped proteomic detection, our data suggests an impact of radiotherapy on the PDAC immune microenvironment as compared to neoadjuvant chemotherapy alone.

The classical complement pathway is activated by the Fc fragment of immunoglobulins. Upon binding to the Fc fragment, the C1 complex (C1q, C1r and C1s subcomponents), C2 and C4 are activated to form the C3 convertase of the classical pathway [101–103]. C6, C8 and C9, together with C5b and C7 (latter two are not significantly upregulated: C5 fold change = 0.49, \( p_{\text{adjusted}} < 0.001 \), C7 fold change = 0.22, \( p_{\text{adjusted}} = 0.32 \)), build the membrane attack complex (MAC), which is the final event of the cascade leading to the cytolysis of the attacked cell. Thus, we see a strong expression of immunoglobulins along with an overexpression of the classical complement pathway.

There is an ongoing debate regarding the function and effect of the classical complement pathway in tumor progression and invasion [104–107]. There appears to be an increasing consensus that the implication of the complement cascade on tumor progression is dependent on the tumor context (e.g. cancer entity, localization, TME, cell type, tumor progression, and stage) [108, 109]. Evidence for implications of the complement system in cancer ranges from anti-tumor defense to tumor promotion [108]. The proteins C1-4, which initiate the complement cascade, are expressed in most tumor entities [108]. With the exception of cholangiocarcinoma, which is a cancer of the bile duct and very close to the pancreas, C6, C8 and C9 show only very low expression levels in most cancers [108]. In the chemo-radiation subgroup, we observed an upregulation of the complete complement cascade and its inhibitors.
Neoadjuvant Chemotherapy Yields Upregulation of Protein Biosynthesis and Energy Metabolism

Proteins overrepresented in the chemotherapy subgroup include proteins executing and controlling the protein biosynthesis and mRNA processing, ribosomal proteins, proteasome components, and proteins contributing to the metabolic energy turnover of a cell.

We detected the upregulation of 12 60S ribosomal proteins (RPL3, RPL8, RPL10A, RPL11, RPL17, RPL18, RPL19, RPL21, RPL27, RPL27A, RPL34, and RPL36) and the 40S ribosomal protein S5 (RPS5) that form and stabilize the ribosome [110]. Nuclear protein 56 (NOP56), a protein involved in the biogenesis of the 60S ribosome subunit, is also upregulated in the chemo subgroup. The eukaryotic translation initiation factor 3 subunit H (EIF3H) and factor 4 gamma 1 (EIF4G), which initiate translation [111], as well as the heterogeneous nuclear ribonucleoproteins F (HNRNPF) and H (HNRNPH1) are also upregulated in the chemo subgroup. Heterogeneous nuclear ribonucleoproteins regulate mRNA splicing and stabilize the mRNA during the transport to the translation site [112]. Methionine-tRNA ligase (MARS1), which catalyzes the attachment of amino acids to tRNAs [113] is also upregulated in the chemo subgroup. Interestingly, we also detected an increased expression of three signal recognition particles (SRP14, SRP68, and SR72) which bind to the ribosome upon recognition of proteins, that are in the process of being translated, and are targeted for the endoplasmic reticulum [114]. The upregulation of these proteins in the chemo subgroup suggests a dysregulation of the protein translation.

Proteasome subunits upregulated in the chemo subgroup include four 20S core components (PSMB4, PSMA5, PSMB5, and PSMB6), and the 26S complex chaperone PSMD9. We also found the proteasome adaptor and scaffold protein ECM29 to be significantly upregulated. In accordance with this, the ubiquitin-like modifier-activating enzyme 1 (UBA1), ubiquitin-like modifier-activating enzyme 6 (UBA6), E3 ubiquitin-protein ligase HECTD3, E3 ubiquitin/ISG15 ligase TRIM25, transcription intermediary factor 1-beta (TRIM28), and phospholipase A-2-activating protein (PLAA), which promote ubiquitination of proteins that are then targeted for proteasome degradation, are significantly upregulated in the chemo subgroup.

The upregulation of the proteasome subunits suggests an increased proteasome activity in the chemotherapy subgroup. The proteasome modulates the proteome by degrading regulatory, damaged, or misfolded proteins [115]. Together with the dysregulated protein translation, these processes represent a sign of proteome reorganization suggesting a stronger protein turn-over in the residual tumor mass after neoadjuvant chemotherapy.

Furthermore, four proteins, which execute the TCA cycle, are upregulated in the chemotherapy subgroup: isocitrate dehydrogenase (IDH2), 2-oxoglutarate dehydrogenase (OGDH), aconitate hydratase (ACO2) and succinate-CoA ligase (SUCLG2). Key components of the fatty acid oxidation such as medium-chain specific acyl-CoA dehydrogenase (ACADM), acetyl-CoA acetyltransferase (ACAT1), carnitine O-palmitoyltransferase 1 (CPT1A), and hydroxyacyl-coenzyme A dehydrogenase (HADH) are also co-regulated with the TCA cycle proteins. Directly coupled to the fatty acid oxidation and TCA cycle are the mitochondrial respiratory chain and the oxidative phosphorylation, which eventually produce ATP from the substrates delivered by the preceding processes. We detected increased expressions of proteins, which form the complexes I, III, and IV of
the respiratory chain, and of the ATP synthase subunit g (ATP5MG), which phosphorylates ATP from ADP in the process of oxidative phosphorylation. The proteins upregulated in complex I of the respiratory chain are the NADH dehydrogenases NDUFA2, NDUFA5, and NDUFB11, in complex II the cytochrome b-c1 complex subunit Rieske (UQCRFS1), and in complex IV the cytochrome c oxidase subunits 5B (COX5B) and 6B1 (COX6B1). Further metabolism-related proteins, which are upregulated in the chemotherapy subgroup, include the aldehyde dehydrogenases ALDH1B1, ALDH4A1, ALDH6A, and ALDH18A1. There have been numerous reports about upregulated aldehyde dehydrogenases in cancer [116–118]. ALDH1A1 is a family member that was shown to be overexpressed in PDAC after neoadjuvant chemotherapy [30], as well as after adjuvant chemotherapy and to provide chemoresistance [72]. Interestingly, Amrutkar et al. [30] observed a decrease of 46 metabolic proteins after neoadjuvant chemotherapy compared to treatment-naive PDAC.

A well-known energy metabolism-related process occurring in cancer progression is the Warburg effect [119]: most tumor cells undergo a dramatic shift in the energy supply using the so-called “aerobic glycolysis” in which pyruvate is reduced to lactate to produce ATP though oxygen is available [120, 121]. We conclude that the residual tumor mass of the chemotherapy subgroup likely shows a shift back to the TCA cycle and oxidative phosphorylation using the respiratory chain as it increases the expression of these proteins.

**Enrichment Analysis Enhances the Identification of Dysregulated Pathways**

In order to identify commonly dysregulated pathways between the chemo-radiation and chemotherapy subgroup, we performed a pathway enrichment analysis using the topGO approach. Only proteins, which presented an FDR < 0.05 in the linear model analysis, were included in the pathway enrichment analysis.

The enrichment analysis confirmed the overrepresentation of proteins involved in collagen fibril organization, complement activation, innate immune response, and humoral immune response mediated by circulating immunoglobulins in the chemo-radiation subgroup compared to the chemotherapy subgroup (Fig. 5b). Additionally, the enrichment analysis identified the upregulation of negative regulation of hydrolase activation, high-density lipoprotein particle remodeling, acute-phase response, reverse cholesterol transport, phospholipid efflux, and cholesterol efflux in the chemo-radiation subgroup. These pathways are mainly comprised of apolipoproteins and serpin family members.

In the chemotherapy subgroup, the enrichment analysis identified an overrepresentation of the cytoplasmic translation, and tRNA aminoacylation. This complements the above-described upregulation of protein translation and turnover as these pathways are dominated by the presence of eukaryotic translation initiation factors, ribosomal proteins, and tRNA synthethases (Fig. 5c). In addition, we again detected an overrepresentation of the TCA cycle, mitochondrial transport and the fatty acid oxidation in the chemo subgroup, supporting the majority of significantly dysregulated proteins identified via the linear model.

Interestingly, the enrichment analysis identified the telomere maintenance via telomere lengthening, and regulation of telomerase activity as upregulated in the chemotherapy subgroup. Telomeres are of special interest in the field of cancer studies, as they regulate the proliferation capacities of a cell. Telomere shortening can induce senescence and apoptosis, which is why cancer cells try to reduce the shortening by activating telomerases and maintaining telomere length [122, 123].
Prognostic Proteins in the Residual PDAC Mass After Neoadjuvant Combined Chemo-Radiation Therapy

We used the CoxBoost algorithm to identify prognostically relevant proteins. The clinical parameters (age, sex, differentiation, vascular invasion and perineural invasion status) were included to the model as covariates. Since both subgroups showed huge differences in their proteome profiles and a differing cumulative incidence of death, we decided to analyze the prognostic power of the proteome profiles in both subgroups independently.

In the chemo-radiation subgroup, we found four proteins that affected the survival time of these patients (Table 2, Fig. 6a). Proteasome subunit beta type-8 (PSMB8 or PSMB5i; P28062), C-terminal-binding protein 1 (CTBP1; Q13363), and Ubiquitin-associated protein 2-like (UBAP2L; Q14157) show estimated CoxBoost coefficients > 0, which is indicative of proteins where a high expression level is accompanied with a dismal overall survival (Fig. 7). In contrast, Afamin (AFM; P43652), a carrier protein essential for the solubility of Wnt family members [124], presented a coefficient < 0, meaning that a high protein expression is accompanied with a favorable overall survival.

Except for PSMB8, all putative prognostic proteins have already been described to affect disease outcomes of other cancer entities than PDAC. High AFM expressions have been identified as indicative of a favorable outcome for ovarian cancer patients [125]. In gastric cancer, low AFM serum levels have been even proposed as a predictive early disease marker [126].

UBAP2L is a poorly characterized protein, which is suggested to interact with protein aggregates after ubiquitin proteasome inhibition [127]. High UBAP2L expressions are reported to negatively affect glioma [128], breast [129], and prostate cancer [130]. CTBP1 is a transcriptional co-regulator facilitating epithelial to mesenchymal transition [131, 132]. It is upregulated and associated with poorer survival in breast (76–77 in Blevins), prostate [133, 134] and colon cancer [135, 136], melanomas [137], and leukemia [138].

PSMB8 is one of the three inducible subunits of the immunoproteasome. It is also called inducible PSMB5 (PSMB5i) as it replaces the catalytic beta 5 subunit of the conventional proteasome [139, 140]. The immunoproteasome degrades ubiquitin-tagged proteins, thereby generating peptides for MHC1 class antigen presentation. Interestingly, it was recently shown that in the context of acute pancreatitis PSMB5i deletion results in persistent pancreatic damage [141].

Prognostic Proteins in the Residual PDAC Mass After Neoadjuvant Chemotherapy

In the chemotherapy subgroup, we identified six proteins that affected overall survival (Table 2, Fig. 6b). Unconventional myosin-VI (MYO6; Q9UM54), Myeloperoxidase (MPO; P05164), Tetratricopeptide repeat protein 38 (TTC38; Q5R3I4), and the Polymeric immunoglobulin receptor (PIGR; P01833) show estimated CoxBoost coefficients < 0 (Fig. 8). Rho guanine nucleotide exchange factor 1 (ARHGEF1, Q92888), and Complement factor D (CFD; P00746) present CoxBoost coefficients > 0. Though detected to affect survival, we
excluded TTC38 from further analysis as only 4 patients showed a high protein expression, and one of these patients was lost to follow-up.

MYO6 is the only unconventional myosin that walks along actin filaments towards the minus end [142] and thus, is suggested to play a role in endocytosis [143]. MYO6 upregulation was observed in prostate [144] and ovarian cancer [145], however, it was not described to affect survival so far.

High MPO and PIGR expression levels have already been proposed to favor patient survival. MPO is part of the innate immune system [146]. Tumor infiltration of MPO-expressing cells is associated with a favorable prognosis and suggested as a prognostic marker for improved overall survival in breast and colorectal cancer [147–150]. PIGR, which transports immunoglobulins (IgA and IgM) within the lamina epitheliales mucosae, was already shown to predict survival in a similar way as observed in this study in patients suffering adenocarcinomas of the upper gastrointestinal [151] and pancreatico-biliary tract [152], or ovarian [153] cancer.

In contrast, high expressions of ARHGEF1 and CFD were associated with an unfavorable patient outcome in the chemotherapy subgroup. ARHGEF1 plays a pivotal role in the activation of Rho [154]. CFD is the only serine protease in blood that can catalyze the formation of the C3bBb complex, the C3-convertase in the alternative complement pathway [155, 156]. This is the key step in the activation of the alternative complement pathway. Increased CFD expressions were detected in cSCC tumors [157], and in early onset colorectal cancer, where a high expression was also associated with a worse progression-free survival [158].

Using our proteome data, we successfully determined prognostic candidate markers in both therapeutic subgroups, respectively. Interestingly, neither ECM proteins, which showed to be a hallmark in the chemoradiation subgroup, nor DNA damage proteins, which would have been expected due to radiation therapy, were candidates of prognostic markers. Though significantly upregulated, we did not detect ribosomal proteins or energy metabolism related proteins as prognostic candidate markers in the chemotherapy subgroup. Instead, proteins with immune-biological functions dominate, ranging from MPO to components of the immunoproteasome and the complement system.
Table 2
Prognostic Candidate Marker Proteins after Neoadjuvant Treatment

<table>
<thead>
<tr>
<th>Neoadjuvant treatment</th>
<th>CoxBoost coefficient</th>
<th>Uniprot ID</th>
<th>Protein name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemo-radiation</td>
<td>-0.1851</td>
<td>P43652</td>
<td>Afamin</td>
</tr>
<tr>
<td></td>
<td>0.1096</td>
<td>P28062</td>
<td>Proteasome subunit beta type-8 (PSMB5i)</td>
</tr>
<tr>
<td></td>
<td>0.1640</td>
<td>Q13363</td>
<td>C-terminal-binding protein1</td>
</tr>
<tr>
<td></td>
<td>0.2187</td>
<td>Q14157</td>
<td>Ubiquitin-associated protein 2-like</td>
</tr>
<tr>
<td>Chemo</td>
<td>-0.0901</td>
<td>Q9UM54</td>
<td>Unconventional myosin-VI</td>
</tr>
<tr>
<td></td>
<td>-0.0796</td>
<td>P05164</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td></td>
<td>-0.0660</td>
<td>Q5R3I4</td>
<td>Tetratricopeptide repeat protein 38</td>
</tr>
<tr>
<td></td>
<td>-0.0390</td>
<td>P01833</td>
<td>Polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td></td>
<td>0.0085</td>
<td>Q92888</td>
<td>Rho guanine nucleotide exchange factor 1</td>
</tr>
<tr>
<td></td>
<td>0.0441</td>
<td>P00746</td>
<td>Complement factor D</td>
</tr>
</tbody>
</table>

Coefficient < 0 = high protein expression is favorable; Coefficient > 0 = high protein expression is unfavorable

Proteogenomic analysis and identification of Single Amino Acid Variants

Proteogenomic analyses combine proteomic and genomic and/or transcriptomic data to identify potential sequence variants, such as single amino acid variants (SAAVs), or copy number variations [159]. The proteogenomic landscape of PDAC has been recently published as part of the Clinical Proteomic Tumor Analysis Consortium (CPTAC; https://proteomics.cancer.gov/programs/cptac) [71]. In our study, we used publicly accessible transcriptomic PDAC data (Supplementary Table 2) in order to identify potential SAAVs. The transcriptomic data were used to search for mutations that lead to SAAVs in peptide sequences. These sequences that contain SAAVs were appended to the human proteome reference database and a new search against the patient LC-MS/MS data was performed. In total, we identified 319 SAAVs among all patient samples. 264 SAAVs were identified in the chemo subgroup (median SAAVs per sample = 37), 184 SAAVs in the chemo-radiation subgroup (median SAAVs per sample = 29) (Fig. 9a). Since we identified a higher number of peptides in the chemo subgroup, we assessed the abundance of SAAVs in relation to the number of peptides per sample from the same search (Fig. 9b). In this comparison, we did not detect a significant difference in the abundance of SAAVs (Welch two sample t-test: p-value = 0.3775). Off note, we want to mention that the SAAVs we identified, represent potential benign mutations but also naturally occurring allele variants. Among the 13 most frequent SAAVs (present in at least 50% of samples; Table 3), 6 were already annotated as benign SAAVs in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/). However, to identify the mutational burden for each patient further experiments would be required which is beyond the scope of this study.
Table 3
List of the 13 most frequent SAAVs, which are present in at least 50% of all proteomic samples.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein Name</th>
<th>SAAV</th>
<th>ClinVar: Variation Annotation</th>
<th>ClinVar: Clinical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>O60504</td>
<td>Vinexin</td>
<td>I5556T</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P01619</td>
<td>Immunoglobulin kappa variable 3–20</td>
<td>S74N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P02751</td>
<td>Fibronectin</td>
<td>V2114I</td>
<td>NM_212482.4(FN1):c.6781G &gt; A (p.Val2261Ile)</td>
<td>Benign (Dec 18, 2021)</td>
</tr>
<tr>
<td>P06727</td>
<td>Apolipoprotein A-IV</td>
<td>S147N</td>
<td>NM_000482.4(APOA4):c.440G &gt; A (p.Ser147Asn)</td>
<td>Benign (Dec 18, 2021)</td>
</tr>
<tr>
<td>P07942</td>
<td>Laminin subunit beta-1</td>
<td>Q1022R</td>
<td>NM_002291.3(LAMB1):c.3065A &gt; G (p.Gln1022Arg)</td>
<td>Benign (Dec 18, 2021)</td>
</tr>
<tr>
<td>P30084</td>
<td>Enoyl-CoA hydratase, mitochondrial</td>
<td>T75I</td>
<td>NM_004092.4(ECHS1):c.224C &gt; T (p.Thr75Ile)</td>
<td>Benign (Dec 19, 2021)</td>
</tr>
<tr>
<td>P32455</td>
<td>Guanylate-binding protein 1</td>
<td>T349S</td>
<td>Protein not found on ClinVar.</td>
<td></td>
</tr>
<tr>
<td>Q01518</td>
<td>Adenyl cyclase-associated protein 1</td>
<td>S256A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Q16666</td>
<td>Gamma-interferon-inducible protein 16</td>
<td>R409S_</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Q5JTV8</td>
<td>Torsin-1A-interacting protein 1</td>
<td>M146T</td>
<td>NM_015602.4(TOR1AIP1):c.437T &gt; C (p.Met146Thr)</td>
<td>Benign (Sep 10, 2021)</td>
</tr>
<tr>
<td>Q969G5</td>
<td>Caveolae-associated protein 3</td>
<td>R8P</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Q96HC4</td>
<td>PDZ and LIM domain protein 5</td>
<td>S383N</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- SAAV not found in ClinVar database

Neoadjuvant treated residual PDAC mass groups with treatment-naïve PDAC from the CPTAC

We compared our data with the proteomic PDAC data of the Clinical Proteomic Tumor Analysis Consortium (CPTAC; https://proteomics.cancer.gov/programs/cptac). CPTAC aims to collect and integrate proteomics and genomics data to comprehensively study the molecular bases of cancer [160]. The CPTAC PDAC study
included tumor and normal adjacent tissues of 140 patients (135 PDAC and 5 pancreatic adenosquamous carcinoma patients, 67 paired normal adjacent tissues plus 9 normal non-tumor tissues) into their database [71] (expression data downloaded from LinkedOmics: http://www.linkedomics.org/data_download/CPTAC-PDAC/). Using a labor-intensive workflow, the authors were able to identify more than 11,000 proteins. We observed a huge overlap of the proteins identified in our study (called MSKCC dataset) and the CPTAC study (called CPTAC dataset), with 3,478 proteins being identified in both (Fig. 10a). To perform a correlation analysis, we calculated for each protein and each dataset the mean expression over all samples. The unprocessed data show a Pearson correlation of 0.67 (Fig. 10b). In order to directly compare the expression profiles, we corrected the expression values using the ComBat algorithm, as we observed separation of the MSKCC and CPTAC datasets arising from the different platforms (e.g., sample collection, storage, and preparation, mass spectrometer used, data acquisition and analysis; Fig. 10c-d), and performed a median normalization (Fig. 10e). After the processing, the correlation is close to 1.00 (Fig. 10f). Unsupervised Principal Component Analysis (PCA) demonstrated a grouping of the tumor tissues from both studies (Fig. 10g). The neoadjuvant treated tumor samples group together with the treatment-naïve tumor tissues of the CPTAC study. The CPTAC normal adjacent tissues and tumor tissues separate primarily according to their pathology, though some samples overlap and prevent a clear separation of tumor and normal tissues. This might partly be explained by the fact that the normal adjacent tissues stem from the PDAC patients and that the tissue might have already been affected by the tumor. This comparison shows that it is possible to collect and match data from different resources and platforms to comprehensively study molecular disease profiles. This approach is especially interesting, when studying rare disease types with a limited number of available patient tissue.

**Neoadjuvant therapy enriches for aldehyde dehydrogenases and ribosomal proteins**

In order to study the impact of neoadjuvant treatment, we performed a limma analysis comparing the entire MSKCC dataset and the tumor samples of the CPTAC dataset. We removed proteins, which presented more than 20% missingness, and refrained from imputation as both datasets stem from different mass spectrometry platforms. Hence, 1,499 proteins were included in the limma analysis. 58 proteins were upregulated and 15 proteins were downregulated in the neoadjuvant PDAC dataset as compared to the treatment-naïve PDAC dataset (Fig. 11a, Supplementary Spreadsheet 1). We found 15 ribosomal proteins upregulated after neoadjuvant treatment (Fig. 11b) and three members of the aldehyde dehydrogenase family. ALDH1A1, ALDH1L2 and ALDH6A1 showed the highest expression levels after neoadjuvant chemotherapy (Fig. 11c). In a previous study, we have linked ALDH1A1 to chemo-radiation resistance of PDAC [72]. The present results demonstrate its upregulation in response to neoadjuvant treatment. Mechanistically, ALDH1A1 is thought to contribute to detoxification in chemotherapy as a route to eventual therapy resistance [161].

We further investigated the abundance of the immune cell markers presented by Dias Costa and colleagues (2022) [31]. We identified the macrophage polarization marker CD163 in our combined proteomic dataset. It showed a decreased (p_adj = 0.008, fold change = -0.33) level after neoadjuvant treatment (lowest expression after neoadjuvant chemotherapy; Fig. 11d). The fold change is below the threshold that we
consider as significantly downregulated, however the adjusted p-value reaches significance. A decrease of CD163 may suggest a shift of the macrophage polarization to the M1 state, which is considered to be detrimental and to inhibit cell proliferation. Dias Costa and colleagues (2022) showed such a shift to the M1 state after neoadjuvant chemotherapy.

**Conclusion**

In the present study, we investigated the proteome biology of the residual PDAC tumor mass after neoadjuvant chemo or combined chemo-radiation therapy. We conclude that the proteome profiles differ fundamentally. Extracellular matrix, immunoglobulin and complement proteins are upregulated prominently in the residual tumors after chemo-radiation therapy. Whereas after chemotherapy, proteins associated with energy metabolism (TCA cycle, fatty acid oxidation, mitochondrial respiratory chain, and oxidative phosphorylation), the proteasome, and protein translation are increased. Interestingly, the immune system seems to be a pathological hallmark of PDAC. We observed a strong dysregulation of immunoglobulins and the complement cascade between both neoadjuvant treatments, and identified several prognostic candidate markers in the form of proteins involved in the immune system. The immune system related proteins PSMB8 (=PSMB5i) in the chemo-radiation subgroup, as well as MPO, PIGR, and CFD in the chemo subgroup were identified as prognostic candidate markers to predict survival outcome, indicating that the immune system is relevant in both therapy groups.

We did not have access to treatment-naïve PDAC and healthy pancreas tissue, and, hence, could not directly compare our proteome profiles to these tissues. However, we combined our data with the proteomic PDAC data of the CPTAC showing a large overlap of the identified proteins and a close relationship of the PDAC tissues. We identified several differentially regulated proteins between the treatment-naive CPTAC tumor samples and the neoadjuvant treated MSKCC dataset, though the final number of proteins that were consistently expressed in both datasets was limited to 1,499 proteins. In future, it might be possible to combine proteomic data from different resources to gain larger insights into different disease conditions. Yet, our results encourage more intense studies on the biology of neoadjuvant treatment modalities in PDAC.

**Abbreviations**

PDAC = pancreatic ductal adenocarcinoma  
DIA = data-independent acquisition  
GPF = gas phase fractionation  
FFPE = formalin fixed paraffin embedded  
LC-MS/MS = liquid chromatography tandem mass spectrometry  
FDR = False discovery rate  
PCA = Principal component analysis
CH = chemotherapy
CR = chemo-radiation therapy
CPTAC = Clinical Proteomic Tumor Analysis Consortium
SAAV = single amino acid variant
MSKCC = Memorial Sloan Kettering Cancer Center

Declarations

Funding and Acknowledgement

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Competing Interests

The authors have no conflict of interests to declare.

Authors Contributions

All authors contributed to the study design, conception and revised the manuscript. Experimental sample preparation and analyses, data curation, statistical analyses, visualization and manuscript preparation were performed by Maren Nicole Stillger. Laura Tang prepared the sample material, ethics approval and consent of patients. Sylvia Timme-Bronsert performed the tumor macro-dissection and the tumor/stroma quantification. Eva Brombacher and Clemens Kreutz supported the statistical analyses. Oliver Schilling provided funding. All authors read and approved the final manuscript.

Data Availability

The LC-MS/MS raw data, library files and analysis output files are available under restricted access due to medical data protection at the European Genome-phenome Archive (https://ega-archive.org) under the accession code: EGAD00010002388 (Dataset: “PDAC Proteomics After Neoadjuvant Therapy”). Access can be obtained via a data access agreement that corresponds to the ‘harmonised Data Access Agreement (hDAA) for Controlled Access Data’ as brought forward by the ‘European standardization framework for data integration and data-driven in silico models for personalized medicine - EUSTANDS4PM’. For data access, please contact the corresponding author under the Dac ID: EGAC000001002949 (Prof Dr Oliver Schilling).

Supplementary Data
The results of the limma analysis comparing the CPTAC and MSKCC datasets are reported in the excel spreadsheet named "Supplementary_Spreadsheet_1.xlsx". The detailed clinicopathological annotation of the neoadjuvant PDAC dataset can be found in "Supplementary_Table_1.docx". The SRA files used for the proteogenomic analysis are listed in “Supplementary_Table_2.docx”.

**Ethics Approval**

This study was performed in line with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the Memorial Sloan Kettering Cancer Center, New York, USA.

**References**


40. Katz MHG, Shi Q, Meyers J, Herman JM, Chuong M, Wolpin BM, et al. Efficacy of Preoperative mFOLFIRINOX vs mFOLFIRINOX Plus Hypofractionated Radiotherapy for Borderline Resectable Adenocarcinoma of the Pancreas: The A021501 Phase 2 Randomized Clinical Trial. JAMA Oncol. 2022 Sep 1;8(9):1263–70.


Figures

![Figure 1](image.png)

Figure 1

Schematic overview of the experimental procedure.
Figure 2

Cox proportional hazards and overall survival of PDAC patients (n=66). a) The hazard to decease for patients included in this study is increased by vascular invasion (p=0.004; multivariate cox proportional hazards model). b) Overall survival of patients who deceased of PDAC differs by the applied neoadjuvant treatment (chemotherapy or chemo-radiation therapy) assessed with a competing risk analysis (p=0.038).
Figure 3

Protein identification, intensity correction and normalization. a) Number of identified and quantified proteins per neoadjuvant treatment. b) Venn diagram showing the overlap of the identified proteins between neoadjuvant chemotherapy and chemo-radiation therapy. c) Principal Component Analysis (PCA) of unprocessed input (left) and batch corrected (right) data using the ComBat algorithm. d) Boxplot showing the median normalized log2 intensity values of all proteins quantified per sample. CH = neoadjuvant chemotherapy (blue), CR = neoadjuvant chemo-radiation therapy (orange).
Figure 4

Proteomic comparison between neoadjuvant chemo and combined chemo-radiation therapy and influence of clinical and histopathological co-variables. a) Partial least-squares discriminant analysis (PLS-DA) indicates separation of the proteome profiles after neoadjuvant chemo (blue, n=38) or combined chemo-radiation (orange, n=28) therapy. b-f) Histograms presenting the distribution of p values from proteins affected by clinical and histopathological factors. All variables show a large number of proteins that present p-values below 0.05 in the statistical analysis using limma.
Figure 5

Differentially regulated proteins and enriched pathways between neoadjuvant chemo (n=38) and chemo-radiation (n=28) therapy. a) Volcano plot showing the log2 fold change and −log10 of the adjusted p values of all 2040 proteins included in the limma model. Differentially regulated proteins: 134 proteins upregulated in chemo-radiation (orange) and 242 proteins upregulated in chemo (blue). b-c) Pathway enrichment analysis using the topGO approach. b) Pathways enriched in the chemo-radiation group and c) pathways enriched in the chemo group.
Figure 6

Prognostic proteins that affect patient survival time. CoxBoost coefficients deviating from zero indicate prognostic proteins. The earlier the coefficients deviate from zero, the higher their impact. Coefficients < 0 positively affect survival, coefficients > 0 have a negative impact. a) Prognostic proteins in the chemoradiation group (n=28). b) Prognostic proteins in the chemo group (n=38).
Figure 7

Kaplan-Meier curves showing the survival probability for the four prognostic proteins that affect patient survival in the chemo-radiation group divided into groups of high and low expression. Patients with a high Afamin protein expression present a higher survival probability than patients with low Afamin expression. High expressions of Proteasome subunit beta type-8, C-terminal-binding protein1 and Ubiquitin-associated protein 2-like are unfavorable.
Figure 8

Kaplan-Meier Curves showing the survival probability for the five prognostic proteins that affect patient survival in the chemotherapy group divided into groups of high and low expression. Low Complement factor D and Rho guanine nucleotide exchange factor 1 protein expression level are favorable, whereas low expressions of Myeloperoxidase, Polymeric immunoglobulin receptor and Unconventional myosin VI are unfavorable.
Figure 10

Combination and comparison of neoadjuvant treated PDAC proteomes (referred to as MSKCC data; Chemoradiation = 28 and chemo = 38) and PDAC proteomic data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC; Tumor = 140 and paired normal adjacent tissue (NAT) = 67). a) Overlap of the identified and quantified proteins between both studies. b) Correlation plot showing the Pearson correlation of the unprocessed MSKCC and CPTAC data. c-d) PCA of unprocessed input (c) and ComBat batch corrected (d) data. For batch correction, we considered the MSKCC dataset as one batch and the CPTAC dataset as the
second batch. e) Boxplot showing the mean of the median normalized intensity values per group. f) Correlation plot showing the Pearson correlation of the MSKCC and CPTAC datasets after batch correction and intensity median normalization. g) PCA of the CPTAC NAT and tumor, and MSKCC neoadjuvant treated PDAC samples. The CPTAC tumor and neoadjuvant treated tumor samples form one group based on their proteome profiles.
Differentially regulated proteins after neoadjuvant therapy. a) Comparison of treatment-naïve PDAC (tumor samples from CPTAC dataset) and neoadjuvant treated residual PDAC (MSKCC dataset). Differentially regulated proteins: 58 proteins upregulated (orange triangles) and 15 proteins downregulated (blue triangles) after neoadjuvant treatment. b) Expression levels of three selected ribosomal proteins, that showed the highest expression fold change. c) Expression levels of the three aldehyde dehydrogenases upregulated after neoadjuvant therapy. d) Expression level of CD163, a macrophage polarization (M2) marker, is decreased after neoadjuvant treatment.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1AB.pdf
- FigureS2.pdf
- SupplementarySpreadsheet1.xlsx
- SupplementaryTable1.docx
- SupplementaryTable2.docx