Aus der Klinik für Allgemein-, Viszeral- und Gefäßchirurgie

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DISSERTATION

Expression of phosphorylated estrogen receptor beta is an independent negative prognostic factor in pancreatic ductal adenocarcinoma

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von

Ioannis Pozios

aus Athen

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Abstract (German)

Einleitung: Die Rolle der Expression vom Östrogenrezeptor beta (ER-β) beim duktalen Pankreasadenokarzinom (PDAC) ist weitgehend unbekannt. Präklinische Daten deuten zusätzlich zur klassischen ligandenabhängigen nuklearen Aktivität auf eine östrogenunabhängige Aktivierung des ER durch andere Signalwege hin. In dieser Studie untersuchten wir den Effekt der Expression von ER-β, phosphoryliertem ER-beta (pER-β), STAT3, phosphoryliertem STAT3 (pSTAT3) und IL-6 auf das Gesamtüberleben und das rezidivfreie Überleben bei Patienten mit reseziertem PDAC.

Methodik: 175 Patienten, bei denen im Zeitraum zwischen 2003 und 2010 ein duktales Adenokarzinom des Pankreas reseziert wurde, wurden identifiziert. Aus dem paraffin-eingebetteten Tumormaterial wurden Tissue Microarrays (TMA) konstruiert, die mit spezifischen Antikörpern für die oben genannten Moleküle gefärbt wurden. Die Expression von ER-β und pER-β wurde standardisiert mit Hilfe des immunoreaktiven Scores nach Remmele (IRS) ausgewertet. Die Expression der Marker wurde dann mit klinischen und pathologischen Parametern korreliert und anschließend wurde eine univariate sowie multivariate Überlebensanalyse (Kaplan-Meier bzw. Cox-Regression) durchgeführt.

Ergebnisse: Alle fünf Marker wurden in der Mehrheit der Tumoren (>50%) exprimiert. Die univariate Analyse der Überlebensdaten ergab, dass ein höheres UICC Stadium, ein niedrigerer Tumordifferenzierungsgrad, das Vorhandensein von Residualtumor (R1) und die Expression von pER-β jeweils mit einer signifikant kürzeren gesamten und rezidivfreien Überlebenszeit einhergingen. Für die anderen Marker ergab sich keine signifikante Korrelation mit dem Überleben. Die multivariate Analyse bestätigte die pER-β-Expression als unabhängigen prognostischen Faktor. Die pER-β-Expression korrelierte mit einem kürzeren gesamten (hazard ratio 1.9; P=0.021) und tumorfreien Überleben (hazard ratio 1.9; P=0.033).

Schlussfolgerung: Die Expression von pER-β korreliert mit einer ungünstigen Prognose und stellt damit einen unabhängigen negativen prognostischen Faktor für das PDAC dar. Die zugrundeliegenden molekularen Mechanismen sind nicht ausreichend charakterisiert und bedürfen weiterer Untersuchung. Anhand dieser Daten könnte ein Kollektiv von Patienten identifiziert werden, die neben einer adjuvanten zytotoxischen Therapie von einer Therapie mit SERMs profitieren könnten.

Abstract (English)

Background: The role of estrogen receptor beta (ER-β) expression in ductal pancreatic adenocarcinoma (PDAC) is largely unknown. Ligand-independent phosphorylation and activation of ER-β may play a relevant role in the IL-6/STAT3 signaling pathway and, as a result, in tumor progression. Here, we examined the effect of ER-β, phosphorylated ER-β (pER-β), STAT3, phosphorylated STAT3 (pSTAT3) and IL-6 expression on the overall and recurrence-free survival in a cohort of patients with resected PDAC.

Methods: We identified 175 patients who underwent pancreatic resection for PDAC. Tissue microarrays were constructed from archival tumor specimens. These were stained with specific antibodies for the above molecules. The expression of ER-β and pER-β was evaluated using the immunoreactive score (IRS) by Remmele. The expression of the markers was then correlated with clinicopathological parameters and survival analysis was performed.

Results: More than half of the tumor samples showed high expression of all the five markers. Univariate survival analysis showed that higher UICC stage, tumor grade, residual tumor (R1) and expression of pER-β were correlated to shorter overall and disease-free survival. All the other markers investigated showed no prognostic relevance. Cox multivariate analysis revealed that pER-β expression was an independent factor correlating with a shorter overall survival (hazard ratio 1.9; P= 0.021) and disease-free survival (hazard ratio 1.9; P= 0.033).

Conclusions: Expression of pER-β constitutes an independent prognostic marker for PDAC and is correlated with poor prognosis. The underlying molecular mechanisms require further investigation. These data may help in identifying patients who could benefit from additional therapeutic regimens, including selective estrogen receptor modulators.

1 Introduction

1.1 Pancreatic cancer

1.1.1 Incidence

Malignancies of the pancreas account in about three percent of all cancers, but remain the fourth most common cause of cancer-related death in both sexes in the western world and the sixth worldwide.(1) Due to its typical late presentation and its refractory nature, PDAC has the worst survival rate of all cancers, with a 5-year survival rate of <5%. The disease is rare before the age of 45, but the incidence rises sharply thereafter. According to the German Centre for Cancer Registry Data of the Robert-Koch-Institut, the average age for men is 71 and for women 75 years in Germany. The incidence of pancreatic cancer varies by sex and race and is greater in younger men than in younger women, but decreases with increasing age (male-to-female ratio 1.3:1).(2) Disease rates are also greater in African Americans than in Caucasians.(3)

1.1.2 Risk factors

Acquired risk factors for pancreatic cancer are tobacco smoking, Type 2 diabetes mellitus, nonhereditary chronic pancreatitis, obesity and lack of physical activity.(4) There are also some studies concerning diet,(5–12) coffee, alcohol consumption,(13–15) Aspirin and NSAID use,(16–19) Helicobacter pylori and hepatitis B virus(20) as risk factors for pancreatic cancer, but the results are inconsistent. 5 to 10 percent of patients with exocrine pancreatic cancer have a first-degree relative with the disease.(21–23) This suggests a role for familial aggregation and/or genetic factors in pancreatic cancer.(24) These patients present with the disease at an earlier age than those with noninherited disease.(25,26) Between three and 16 percent of the patients are estimated to have a known genetic syndrome or a strong family history that predisposes them to the disease.(22,23)

1.1.3 Molecular pathogenesis

In pancreatic cancer, key signaling pathways are dysregulated contributing to pancreatic tumorigenesis. Multiple combinations of somatic mutations are commonly found in exocrine pancreas carcinoma.(27) Inherited and acquired mutations in specific cancer-associated genes lead to developing of pancreatic adenocarcinomas,(28–30) including mutational activation of oncogenes (KRAS), inactivation of tumor suppressor genes (TP53, p16/CDKN2A, SMAD4) and inactivation of genome maintenance genes (hMLH1 and MSH2). Apart from these, there are also

many other genetic aberrations in patients with a familial predisposition to pancreatic cancer.(31) A KRAS gene mutation was reported in more than 90 percent of pancreatic carcinoma.(28,32,33) KRAS mutations are also present at precancerous lesions of invasive pancreatic cancer, and the prevalence of mutations increases with increasing degrees of dysplasia in these lesions.(34–38) The progression of dysplasia to adenocarcinoma is biologically characterized by the accumulation of a variety of genetic aberrations. Furthermore, other molecular mechanisms such as methylation, mitochondrial mutations and micro-RNA expression have been described as possible factors in pancreatic tumorigenesis.

1.1.4 Pathology

The majority of pancreatic neoplasms -about 85 percent- are ductal adenocarcinomas, caused by malignant transformation of cells of the exocrine pancreas from the ductal epithelium. Precancerous lesions of invasive pancreatic cancer are mucinous cystic neoplasm (MCN), intraductal papillary mucinous neoplasm (IPMN) and pancreatic intraepithelial neoplasm (PanIN).(39) Histologic grading is based upon the degree of differentiation and the prevalence of mitotic cells. A three-tiered grading system is typically used (grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated), although highly anaplastic tumors are sometimes designated grade 4.(40) Because of the proximity to the adjacent structures (portal vein, superior mesenteric artery or vein, aorta) a negative resection margin can be difficult to achieve, resulting very often in microscopically positive resection margins (R1 resection). In published studies, the rate of R1 resections varies widely, ranging from 16% to >75% due to insufficient standardization of histopathological examination, concerning especially the circumferential resection margin (CRM).(41–43) Regional peripancreatic lymph nodes are frequently positive, while perineural invasion both within and beyond the pancreas also occurs in these tumors.

1.1.5 Localization and clinical symptoms

Characteristic early symptoms are missing. The localization of the cancer determines the symptoms. Approximately 65 percent of tumors arise in the pancreatic head, 15 percent in the pancreatic body and 10 percent in pancreatic tail. The anatomical boundary between the pancreatic head and body is the left edge of the superior mesenteric vein and between pancreatic body and tail, the left edge of the aorta. The main symptoms of pancreatic head carcinoma are pain, typically radiating to the back, weight loss and obstructive jaundice. Pain and weight loss are also symptoms of carcinoma of pancreatic body or tail. Other symptoms are diarrhea and

steatorrhea, malabsorption, glucose intolerance, and paraneoplastic syndromes such as thrombophilia inclusive thrombophlebitis migrans and Panniculitis nodularis (Pfeifer-Weber-Christian syndrome). Other non-specific symptoms of pancreatic cancer are asthenia and anorexia including nausea and vomiting, which are often caused by gastric outlet obstruction secondary to duodenal tumor invasion.

1.1.6 Diagnosis

Apart from a detailed history and a physical examination, the diagnostic evaluation of a patient with suspected pancreatic cancer includes serologic evaluation and abdominal imaging. Several serum markers for pancreatic cancer have been evaluated, the most useful of which is carbohydrate antigen 19-9 (also called cancer-associated antigen 19-9, CA 19-9).(44–48) The next step in the patient's evaluation is abdominal imaging, though the choice of test varies depending upon the patient's presenting symptoms. Moreover, following the initial evaluation, a biopsy-proven diagnosis of pancreatic cancer is dispensable before curative surgery, but obligatory prior palliative therapy. Important prognostic factors at the time of diagnosis are the general condition of the patient (ECOG), weight loss, pain and tumor markers (CA19-9).

1.1.7 Staging and Classification

The key goal of staging workup of a patient with pancreatic cancer is to assess the extent of disease spread and to evaluate the resectability of the pancreatic tumor. Computed tomography (CT) is the preferred method of staging pancreatic cancer. Other studies include transabdominal or endoscopic ultrasound, magnetic resonance imaging and positron emission tomography scanning. Staging laparoscopy is used for patients with clinically suspected peritoneal carcinomatosis to avoid a futile laparotomy. Infiltration of adjacent structures and presence of distant metastases define the unresectability of pancreatic tumors. Local unresectability is usually due to vascular invasion. The classification system for pancreatic cancer is based on the tumor-node-metastasis (TNM) staging system of the combined American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) as described in table 1.(40)

1.1.8 Therapy and Prognosis

At the time of initial diagnosis, only 15-20% of patients have a potentially curable disease. With tumor resection and adjuvant systemic therapy a median survival up to two years can be achieved. However, at the time of diagnosis approximately 15-20% of pancreatic cancer patients

have a non-resectable, non-metastatic tumor (Locally Advanced Pancreatic Cancer, LAPC), while the majority of patients (60-70%) already suffer from synchronous metastatic disease.

Table 1: Classification according to TNM staging system (40)

Stage	Primary tumor (T)	Regional lymph nodes (N)	Distant metastasis (M)
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1-3	N1	M0
III	T4	Any N	M0
IV	Any T	Any N	M1

Primary tumor (T)
TX Primary tumor cannot be assessed
T0 No evidence of primary tumor
Tis Carcinoma in situ*
T1 Tumor limited to the pancreas, 2 cm or less in greatest dimension
T2 Tumor limited to the pancreas, more than 2 cm in greatest dimension
T3 Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery
T4 Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor)
Regional lymph nodes (N)
NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Regional lymph node metastasis
Distant metastasis (M)
M0 No distant metastasis
M1 Distant metastasis
Note: cTNM is the clinical classification, pTNM is the pathologic classification.
* This includes lesions classified as PanInIII classification.

1.1.8.1 Operation

The only potentially curative option for patients with pancreatic cancer is the radical surgical resection.(49,50) Criteria for surgery are the tumor resectability based on the preoperative diagnostic and the comorbidity of the patients.(4) Even after a complete resection and adjuvant therapy, only 10 to 25 percent of these patients are alive after 5 years and median survival remains between 10 and 20 months.(51–53) The surgical procedure depends on the localization of the carcinoma. The standard procedures for cancers in the head of the pancreas are the classic Whipple procedure including partial gastrectomy and partial pancreaticoduodenectomy (Whipple) and the pylorus-preserving pancreaticoduodenectomy (PPPD or pp-Whipple). As far as the oncological result is concerned, the two procedures are equivalent.(54) Total pancreaticoduodenectomy or distal pancreatectomy are performed for carcinoma in the body or tail of the pancreas. Preoperative biliary drainage is indicated only in patients with cholangitis or when the surgery is delayed.(55)

1.1.8.2 Adjuvant therapy

After a R0 resection of the primary tumor, adjuvant therapy with Gemcitabine or 5-fluorouracil/folinic acid is indicated. It prolongs the disease-free and overall survival. Contraindications are poor general condition or severe comorbidities. These two therapeutic agents have comparable efficacy.(56–58) However, due to the slightly better tolerability and the administration form, gemcitabine is preferred. Neoadjuvant or adjuvant radiation therapy alone or in combination with chemotherapy is not indicated apart from clinical trials.

1.1.8.3 Locally Advanced Pancreatic Cancer (LAPC)

Approximately 15-20% of pancreatic cancer patients have at the time of diagnosis a non-resectable, non-metastatic tumor. The optimal treatment of these patients is controversial.(59) Patient selection is essential and the main treatment goal should be the downsizing of the tumor in order to render it resectable. These patients have a median survival of 9 to 11 months.(60) First of all, induction chemotherapy should be started in these patients. In patients who did not develop distant metastasis in the course of induction therapy, radiotherapy could be added to intensify the loco-regional treatment. After each treatment step, the resectability of the tumor should be reassessed.(61)

1.1.8.4 Palliative therapy

Treatment in advanced stages is palliative. In studies, patients with primary metastatic disease have a very limited median survival: between 4–6 months and approximate 5-year survival rates of 1–2%.(39) Nevertheless, chemotherapy leads to a prolongation of survival and improves the quality of life for patients with good performance status.(62,63) Palliative therapy also involves the treatment of symptoms and should be interdisciplinary. The first-line standard treatment until early 2000s was gemcitabine.(64) Recently, other chemotherapeutic agents were tested in combination with gemcitabine, and erlotinib is approved as a combination therapy with gemcitabine as the first-line therapy.(65) New studies also suggested two alternative first-line treatments: the combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin, known as FOLFIRINOX and the combination of gemcitabine/nab-paclitaxel.(66,67) Good general condition of the patient and the patient's will are important factors in deciding about the use of a second-line treatment. This includes 5-fluorouracil/folinic acid alone or plus oxaliplatin,(68) capecitabine,(69) docetaxel, irinotecan and platinum derivatives.(63)

1.2 Estrogen receptors

The estrogen receptor exists in two isoforms: estrogen receptor alpha (ERα, ESR1, NR3A) and estrogen receptor beta (ERβ, ESR2, NR3b). These two proteins bind estrogens with high affinity and specificity and are members of the superfamily of nuclear receptors (NRs). Nuclear hormone receptors are ligand-modulated transcription factors that regulate gene expression. This group constitutes receptors that bind steroids, thyroid hormone, and retinoids, and include also peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR), and liver X receptor (LXR) that mediate metabolic processes(70) and other receptors for which their ligands are still unknown.

1.2.1 Structure and signal transmission

The structure of both estrogen receptors is similar to the other nuclear receptors. ERs are composed of six functional domains (named A-F).(71) The important components are the C or DNA-binding domain (DBD), which binds with high affinity and specificity to DNA sequences - termed estrogen response elements (EREs) - to regulate transcription rates of target genes, and the E or ligand-binding domain (LBD), which binds estrogens and estrogen analogues. The ERs also contain two regions, known as activation functions (AF-1 and AF-2). AF-1 is located toward the amino-terminal end of the receptor and is ligand-independent, whereas AF-2 is located in the LBD and is ligand-dependent.(72,73) In spite of their homology, the two isoforms have important structural differences with implications on the regulation of gene expression. As described in Figure 1, in the DNA-binding C domain (DBD), there is a sequence identity of 97 percent, in comparison with only 59 percent identity in the ligand-binding E domain (LBD).(74)

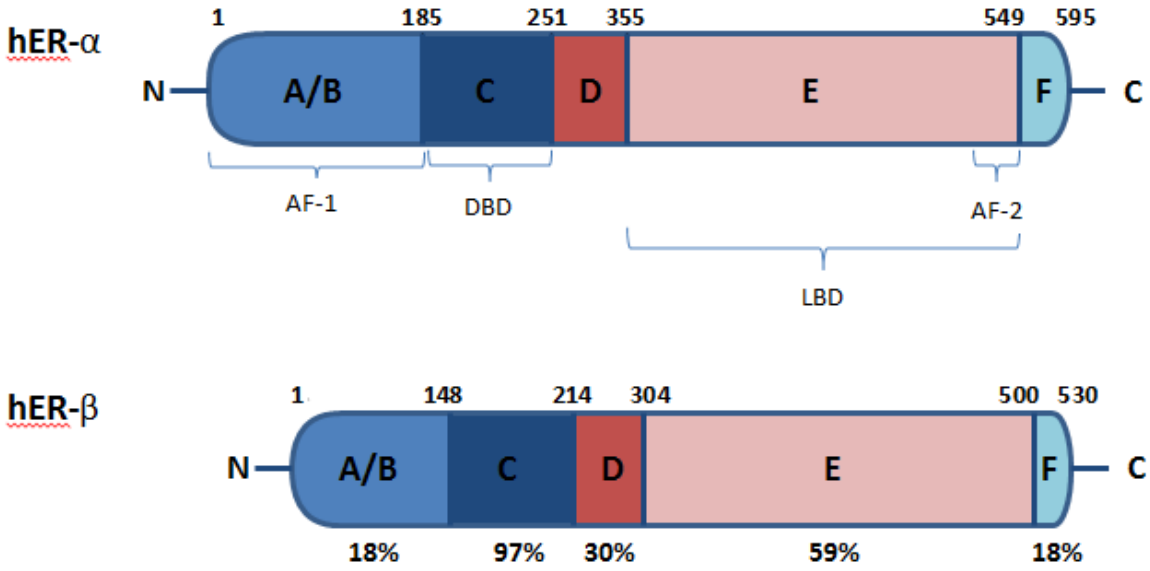


Figure 1: Schematic representation of the two human estrogen receptor isoforms (hER-α and hER-β). Full-length human ER-α is 595 amino acids long, while the hER-β isoform is 530 amino acids long. Both receptors consist of six functional domains, including the DNA-binding domain (DBD), the ligand-binding domain (LBD) and two transcriptional activation functions (AF), the ligand-independent AF-1 and the ligand-dependent AF-2 as indicated in hER-α. Percent sequence identity between the two isoforms is indicated in hER-β. (74,75)

ERs are generally classified as ligand-dependent transcription factors. After associating with their specific ligands, they bind specific genomic sequences (EREs) and interact with co-regulators to regulate the gene expression. However, in several studies, estrogen effects were also described, which occur after ligand activation of plasma membrane proteins, including ER-isoforms termed membrane-bound ERs (mER), complex of ER with other plasma membrane proteins and G protein-coupled receptor 30 (GPR30). This ligand-binding leads to activation of other signaling cascades via second messengers without genomic modulation and is termed "non-genomic".(76–80)

In addition to the classical ligand-induced activation of ERs and their ability to modulate the activity of selected promoters directly, recent studies reported that ERs can also be transcriptionally activated in the absence of a ligand. The unliganded activated ERs then interact with other signaling molecules in the nucleus or in the cytoplasm regulating the activity of other

major signaling cascades, including growth factor signaling.(75,81–88) In the absence of ligand the cascade of signaling events is different and either activation or repression may occur. A ligand-independent signaling pathway is thought to activate the ERs in cancerous tissues contributing to hormone-independent tumor growth.(75,89,90)

ERs have a major role in several systems including reproductive, cardiovascular, skeletal, immune and nervous systems. Thus, the complex tasks of ERs affect the entire organism. The two isoforms are found in different concentrations in every tissue. Moreover, the interactions between ERs and other molecules are complex, so that ERs and their ligands show completely different effects in different organs and organ systems. Considering the widespread expression of ERs and the variety of interactions with extracellular and intracellular signaling molecules, ERs may help to adjust single cell functions to the body homeostasis. Furthermore, estrogen receptor signaling pathways regulate important physiological processes such as cell growth and apoptosis.(83)

1.2.2 Estrogen receptors in breast cancer

Normal mammary gland maturation and development require the existence of ERa in breast tissue. ERs are overexpressed in malignant breast tissue and two-thirds of breast cancers express the ERa. Estrogen and its receptors play an essential role for growth, survival, and progression in ER-positive breast cancer. These insights into estrogen receptor biology led to the development of better chemotherapeutic agents for breast cancer treatment which interact with the receptor in order to block ER function and signaling. These agents can have either antagonist or agonist actions on the ER in different tissues. Three classes of these endocrine therapy drugs, including selective ER modulators (SERMs), selective ER downregulators (SERDs) and SERM/SERD hybrid agents (SSH), are in use in the treatment and prevention of ER-positive breast cancer.(91)

1.2.3 Estrogen receptors in pancreatic cancer

The incidence of pancreatic cancer varies by sex and is greater in younger men than in younger women.(2) In western countries and Japan, the male-to-female sex ratio is approximately 1.25:1 and 1.75:1, respectively, but it decreases with increasing age. This has raised interest in sex hormones and their receptors in the development of pancreatic cancer.(92,93) Since 1981, when Greenway and colleagues first reported the presence of estrogen receptors (ERs) in pancreatic cancer tissue,(94) diverse studies with controversial results have investigated the presence and role ERs in pancreatic cancer as well as the role of selective estrogen receptor modulators (SERMs) in its therapy.(95–100)

As mentioned above, in addition to the classical hormone-induced ER nuclear actions, newer studies demonstrated that ERs interact with cell membranes and signal transduction proteins in the absence of ligand activating diverse intracellular pathways.(87) An intricate cross-talk between ERs and growth factor signaling pathways observed in breast and ovarian cancer cell lines is also active in pancreatic tumors,(101) suggesting similar cross-talk between ERs and growth factors in pancreatic cancer.(100,102,103)

1.2.4 SERMs and IL-6-Inhibition in bone tissue

SERMs are competitive inhibitors of estrogen binding to estrogen receptors (ERs) and have a mixed antagonist/agonist effect on ERs, depending on the target tissue. SERMs increase the bone density providing partial protection against menopausal bone loss. Raloxifene is the SERM of choice to prevent osteoporosis in postmenopausal women. It inhibits bone resorption and reduces the risk of vertebral fracture, while reducing the risk of breast cancer. The molecular mechanism of its effect on bone tissue is not fully understood, but the cytocine interleukin-6 (IL-6) plays a key role. IL-6 mediates the increase in bone resorption that occurs following estrogen deficiency in rats. In vitro data showed also that raloxifene suppresses IL-6 and inhibits mammalian osteoclast differentiation and bone resorption activity only in the presence of IL-6.(104,105) Estrogen deficiency also leads to an IL-6-mediated stimulation of osteoclastogenesis, suggesting a mechanism for the increased bone resorption in postmenopausal osteoporosis.(105)

The aforementioned effect of raloxifene on bone tissue is transmitted through the ERs, suggesting a possible interaction between ER and IL-6.(104)

1.3 IL-6/STAT3 Pathway

1.3.1 Function and signal transmission

Interleukin 6 (IL-6) is a pleiotropic cytokine with biological effects on a wide variety of cells regulating many cellular functions, including cell proliferation, cell differentiation, immune defense mechanisms, and hematopoiesis. Signal transducer and activator of transcription-3 (STAT3) is a transcription factor and a member of the STAT protein family. It is encoded by the STAT3 gene, an oncogene that is expressed in several human cancers including pancreatic, having a well-established role in tumorigenesis.

IL-6 mediates part of its functions through the IL-6-receptor complex. The IL-6-receptor is a cell-surface type I cytokine receptor complex consisting of the ligand-binding IL-6-receptor-subunit (chain α) and the signal transducer glycoprotein 130 (gp130) (chain β). The binding of IL-6 to IL-6-receptor complex activates the STAT3 signal transduction cascade via tyrosine-phosphorylation of STAT3 (tyrosine 705) by the Janus kinase (JAK). Phosphorylated STAT3 (pSTAT3) then, forms homo- or heterodimers, which translocate to the cell nucleus.(106–110) Here, pSTAT3 regulates the transcription of target genes involved in proliferation, survival, cell cycle progression, angiogenesis and immunosuppression, playing a key role in many cellular processes.(111)

1.3.2 Signaling interactions

Activation of STAT3 also occurs via phosphorylation of tyrosine 705 in response to other ligands such as epidermal growth factor (EGF) and Interleukin 5 as well as via phosphorylation at serine 727, for example by mitogen-activated protein kinases (MAPK). This activation may occur directly through interaction with the ligand or indirectly mediated by JAKs.(112) Yamamoto et al. reported that active ER directly associates with, and acts as a transcriptional co-factor for, STAT3, which is induced by IL-6 in breast cancer cells. Furthermore, it was shown that 17beta-estradiol (E2) suppresses IL-6-induced activation of STAT3 activity and STAT3-mediated gene expression. E2-mediated inhibition of STAT3 activation was reversed by tamoxifen, which belongs to SERMs. Moreover, direct physical interactions between STAT3 and ER were also reported, which represent a novel form of cross-talk between STAT3 and ER signaling pathways and open up novel therapeutic prospects.(110)

1.3.3 IL-6/STAT3 in pancreatic cancer

IL-6 plays a major role in malignant transformation and progression of several tumors, including pancreatic cancer.(108,113–117) Recent studies demonstrated that stimulation with IL-6 activates phosphorylation of STAT3 in pancreatic cell lines.(113,118,119) The JAK/STAT pathway also stimulates cell proliferation and malignant transformation and inhibits apoptosis in the pancreas.(120) Additionally, elevated IL-6 levels are reported in pancreatic cancer and correlated with poor prognosis(121,122) as well as with weight loss and cachexia, which are negative prognostic factors for patients with pancreatic cancer.(123,124)

2 Objective

The underlying molecular mechanisms involved in pancreatic carcinogenesis require further investigation in order to identify novel targets for therapeutic intervention. In this study, we hypothesized that phosphorylation of ER-β and activation of IL-6/STAT3 signaling cascade contribute to tumor progression in PDAC. The goal of this study was to examine the following objectives:

- The expression of ER-β, phosphorylated ER-β (pER-β), IL-6, STAT3 and phosphorylated at tyrosine 705 form of STAT3 (pSTAT3) in a cohort of patients with resected PDAC.
- The prognostic relevance of the expression of these molecules for overall and recurrence-free survival in a cohort of patients with resected PDAC.
- The effect of clinicopathological parameters on the overall and disease-free survival in these patients.

3 Materials and Methods

3.1 Patients

In total, 211 patients who underwent surgical therapy of PDAC between 2003 and 2010 were considered for this study. Exclusion criteria were perioperative mortality (patients dying within 30 days after curative resection), the presence of macroscopic residual disease after resection and periampullary tumors other than PDAC, e.g. ampullary, distal cholangiocarcinomas, duodenal adenocarcinomas. As thirty-six patients were excluded from this study, 175 patients were finally considered for this study.

Data on clinical parameters and follow-up information were extracted from the tumor registry and the clinical records. Clinical Data were pseudonymized. The study was approved by the local ethics committee.

Overall survival was defined as the time interval between the date of resection and the date of death from any cause, or censoring based on the date of last contact. Pathological findings (tumor location, tumor invasion, lymph node status, grading) were obtained from the pathologists' original reports. The Tumor-Node-Metastasis (TNM) staging criteria of the International Union Against Cancer (UICC) were used for histologic classification.(125)

3.2 Tissue Microarrays

Tissue microarrays (TMAs) allow for the simultaneous histological analysis of several hundred separate tissue samples under the same conditions in a short time. They consist of paraffin blocks in which hundred tissue cores are assembled in array fashion to allow for multiplex analysis. This method requires a very limited amount of antibodies and reagents.

Tissue micro-arrays (TMAs) containing surgical tumor specimens (paraffin tissues) were constructed according to standard procedures.(126,127). The area of interest to be sampled was identified and marked on hematoxylin-eosin-stained tissue slides. After the preparation of wells in the empty paraffin block, one tissue core biopsy 0.6-mm in diameter was taken from a representative area of the tumor and then inserted into a recipient TMA block using a manual arrayer (Beecher Instruments, Sun Prairie, WI). A distance of 2.5 mm was defined between the samples of the individual patients. Each case was represented by two core biopsies from different parts of the pancreatic carcinoma. Two TMAs containing 422 samples from 211 patients were constructed. The blocks were then incubated for one hour at 37 °C to ensure an optimal fusion of the samples with the paraffin block. Finally, slices of 2µm were prepared with a slider microtome, mounted on a Superfrost Plus specimen slide (Menzel) and dried overnight at 50 ° C.

These sections of the TMA were then available for immunohistochemical staining. In total, 2110 specimens of pancreatic tissue including normal mucosa were evaluated.

3.3 Immunohistochemistry

Commercial antibodies employed were: ER-β (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK); pER-β^{Ser105} (Abcam, Cambridge, UK); STAT3 (Abcam, Cambridge, UK); pSTAT3^{Tyr705} (Cell Signaling Technology, Danvers, MA, USA) and IL-6 (Abcam, Cambridge, UK) (Table 3). Immunohistological staining of TMAs was performed according to standard procedures. The TMA slides were pretreated and then incubated with the antibodies, followed by antibody detection via biotinylated anti-mouse secondary antibody and a biotin-streptavidin-amplified detection system (Biogenex, San Ramon, CA, USA). Staining was visualized using a Fastred chromogen system (DAKO, Hamburg, Germany). The TMA-slides were evaluated by a pathologist blinded for the clinical data. The immunostaining of the cells concerning the expression of ER-β and pER-β was evaluated and scored according to the immunoreactive score of Remmele and Stegner (IRS) with a range between 0 and 12 (Table 3). IRS is calculated by multiplying the number of positively labeled cells (4 percentage groups) by the intensity of the staining reaction (3 grades).(128) For statistical evaluation, scores of 0 and 1 were considered as low expression, whereas scores of 2 or higher were considered as high expression. The immunohistochemical staining of the other three molecules (STAT3, pSTAT3 and IL-6) was scored semiquantitatively by a four-tier scale (0, negative; 1, weak; 2, moderate; 3, strongly positive) according to standard procedures.(127) This was reduced also to a two-tier system (0, negative; 1-3, positive) for the independently performed statistical analysis of single protein and its correlation with clinicopathological parameters including survival.

Intensity of Staining / Percentage of stained cells	0 = no color reaction	1 = mild reaction	2 = moderate reaction	3 = intense reaction
0 = no positive cells	IRS = 0	IRS = 0	IRS = 0	IRS = 0
1 = < 10% positive cells	IRS = 0	IRS = 1	IRS = 2	IRS = 3
2 = 10-50% positive cells	IRS = 0	IRS = 2	IRS = 4	IRS = 6
3 = 51-80% positive cells	IRS = 0	IRS = 3	IRS = 6	IRS = 9
4 = > 80% positive cells	IRS = 0	IRS = 4	IRS = 8	IRS = 12

Table 2: IRS-classification scoring system. Immunoreactive score of Remmele and Stegner (IRS) with a range between 0 and 12.(128) For statistical evaluation, scores of 2 or higher were considered as 'high' expression.

Antibodies	Company	Cat. No.
ER-β	Novocastra Laboratories Ltd (Newcastle upon Tyne, UK)	NCL-ER-beta
pER-β ^{Ser105}	Abcam (Cambridge, UK)	ab62257
STAT3	Abcam (Cambridge, UK)	ab119352
pSTAT3 ^{Tyr705}	Cell Signaling Technology (Danvers, MA, USA)	9145
IL-6	Abcam (Cambridge, UK)	ab154367

Table 3: Commercial antibodies

3.4 Statistical analysis

Data were analyzed with SPSS software, version 20.0 (IBM Corp., Armonk, NY, USA). p-values of <0.05 were considered statistically significant. The association between expression of the investigated parameters and clinicopathological characteristics was tested with a chi-square test. Kaplan-Meier curves and univariate survival analysis were performed for each investigated parameter. Survival curves were compared and assessed using the log-rank test. Multivariate survival analysis was performed using a proportional hazard model (Cox regression). Apart from age and sex, only parameters with p-values <0.05 in univariate survival analysis were included. As UICC stage summarizes the parameters of tumor size, lymph node status and the presence or absence of metastasis (TNM), these factors were not included separately in the Cox proportional risk model.(125) A stepwise procedure, including both backward elimination and forward selection, was used to analyze the independent prognostic factors.

4 Results

4.1 Clinicopathological parameters

The study population consisted of 94 males and 81 females ranging from 32 to 88 years (median, 68.4 years). The majority of patients were older than 60 years (76%) and underwent partial pancreatoduodenectomy (PD: Whipple procedure, 34.9%) or pylorus-preserving partial pancreatoduodenectomy (PPPD: pp-Whipple, 44.6%) for tumors in the head of the pancreas. As shown in Table 4, most of tumor samples showed advanced tumor infiltration (pT3 = 84.6%) and lymph node involvement (pN1= 64%), whereas 8.6% of the patients had already developed distant metastases. The median number of lymph nodes analyzed was 13 (range 0-41). The histopathological examination showed high-grade tumors (G2 and G3) in the great majority (96.5%) of tissue samples and microscopic residual disease after resection in 42.3% of the tumors. Most patients underwent perioperative chemotherapy (33.2%) or a combination of radio- and chemotherapy (45.1%) whereas 21.7% of the patients had no additional therapy. The characteristics of the study subjects are summarized in Table 4.

	Number of cases n=175	*%*
Age		
≤60 years	42	24.0
>60 years	133	76.0
Sex		
male	94	53.7
female	81	46.3
Operation		
PD	61	34.9
PPPD	78	44.6
DP	26	14.9
TP	10	5.7
pT status (UICC 2010)		
pT1	3	1.7
pT2	14	8.0
pT3	148	84.6
pT4	10	5.7
pN status (UICC 2010)		
pN0	63	36.0
pN1	112	64.0
cM status		
cM0	160	91.4
cM1	15	8.6
Stage (UICC 2010)		
I	9	5.2
IIa	45	25.7
IIb	96	54.9
III	10	5.7
IV	15	8.6
Residual tumor		
R0	97	55.4
R1	74	42.3
Grade		
G1	6	3.5
G2	52	29.7
G3	117	66.9
Perioperative Therapy		
No therapy	38	21.7
Chemotherapy	58	33.2
Radiochemotherapy	79	45.1

Table 4: Clinicopathological parameters of 175 patients after resection of PDAC (PD: pancreaticoduodenectomy or Whipple procedure; PPPD: pylorus-preserving pancreaticoduodenectomy or pp-Whipple procedure; DP: distal pancreatectomy; TP: total pancreatectomy).

4.2 Immunohistochemical analysis

High nuclear expression of ER-β was found in 61.7% and pER-β in 80.6% of the tumor samples. 54.3% of the tumors expressed STAT3 and 68% pSTAT3. Expression of IL-6 was observed in 76.6% of the specimens (Table 5). Expression of the molecules was also observed in the cytoplasmic cellular compartments. Representative examples of immunohistochemical staining of PDAC tissue microarrays for ER-β and IL-6/STAT3 pathway proteins are shown in Figure 2. No significant correlation of clinicopathological parameters with the expression of the molecules was found (Table 6).

Antibody	n	%	n	%
	low		*high*	
ER-β	60	34.3	108	61.7
pER-β	25	14.3	141	80.6
	negative		*positive*	
STAT3	71	40.6	95	54.3
pSTAT3	49	28.0	119	68.0
IL-6	37	21.1	134	76.6

Table 5: Expression of different antibodies
'Low' Expression: Scores 0 or 1 of Immunoreactive Remmele Score (IRS)*
'High' Expression: Scores 2 or higher of IRS
'Negative': score 0 by semi-quantitative immunostaining scale scoring system
'Positive': scores 1 (weak), 2 (moderate) or 3 (strongly positive)

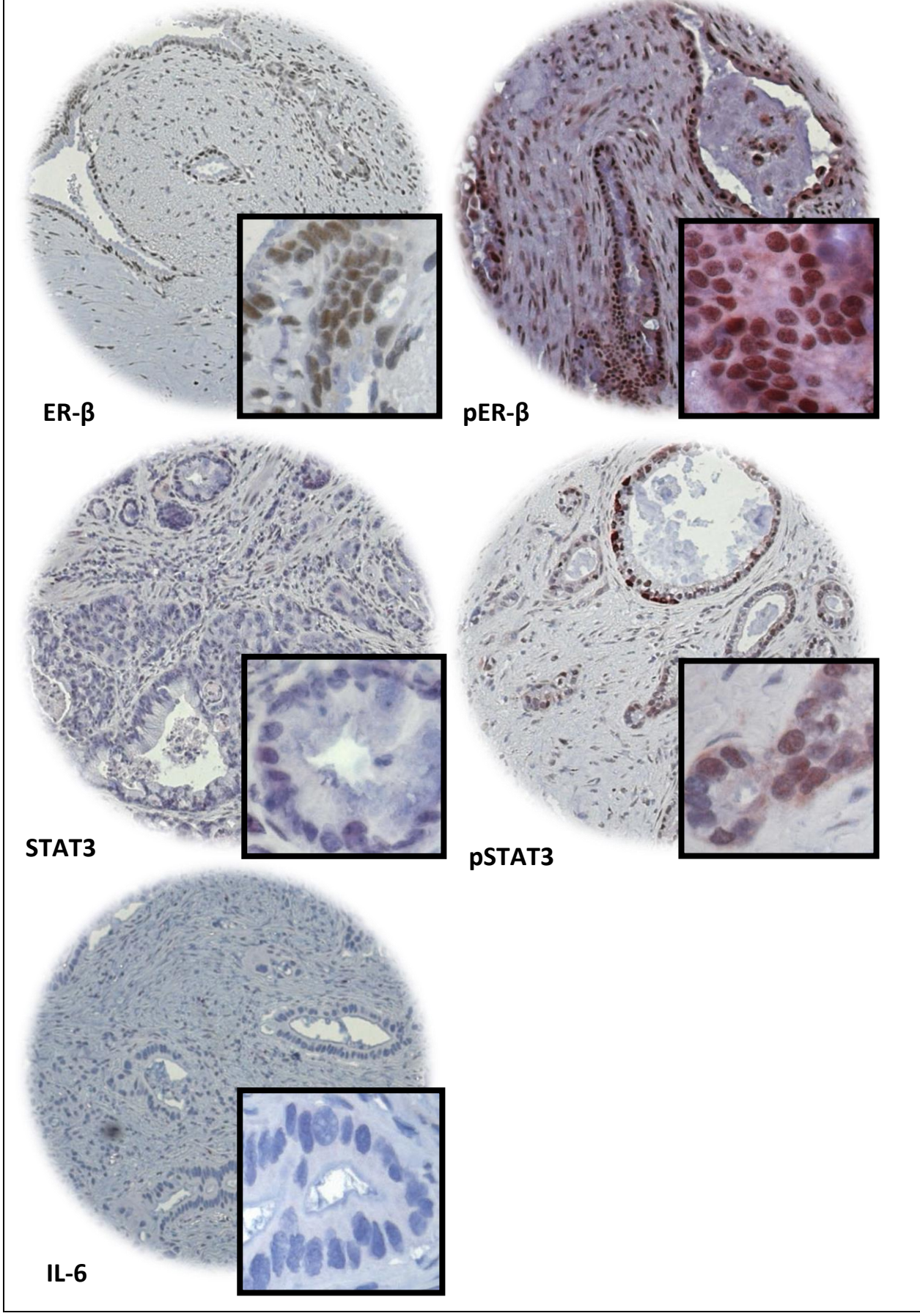

ER-β
pER-β
STAT3
pSTAT3
IL-6

Figure 2: Immunohistochemical staining of PDAC tissue microarrays for ER-β and IL-6/STAT3 pathway proteins. The first two panels for nuclear ER-β and pER-β show representative examples of biopsies scored as "high expression" according to Remmele immunoreactive score (IRS*). The other three panels concerning STAT3, pSTAT3 and IL-6 show representative examples of biopsies scored as "positive" according to the following score system: 0 = negative; 1-3 = positive [staining intensity 1 (weak), 2 (moderate) and 3 (strong)].

Characteristics		***n***	**pER-β expression [%]**	***p***
	Total	175	80.6	
Age	≤60 years	42	84.2	0.886
	>60 years	133	85.2	
Sex	Male	94	83.1	0.487
	Female	81	87.0	
Tumor size	T1-2	17	75.0	0.242
	T3-4	158	86.0	
Lymph node status	N0	63	85.0	0.987
	N1	112	84.9	
Metastasis	M0	160	84.1	0.341
	M1	15	93.3	
Tumor stage (UICC 2010)	0-IIa	54	84.3	0.881
	IIb-IV	121	85.2	
Grading	G1-2	58	86.3	0.749
	G3	117	84.3	
Residual Tumor	R0	97	81.9	0.096
	R1	74	91.2	
Chemotherapy	No	38	86.5	0.765
	CTx	137	84.5	
Radio-chemotherapy	No	96	84.9	0.998
	RCTx	79	84.9	

Table 6: Correlation of pER-β expression with clinicopathological parameters

4.3 Univariate survival analysis

Survival analysis was conducted to correlate overall and disease-free survival with the immunohistochemistry results. The median overall survival was 16.3 months and the mean overall survival 32.9 months (confidence interval (CI) 95% 27.2-38.6). The median disease-free survival was 33.9 months and the mean disease-free survival 15.5 months (CI 95% 27.0-40.7). At the end of follow-up, 32 patients (18.3%) were alive.

4.3.1 Correlation of clinicopathological parameters with patient survival

Overall survival was significantly related to tumor stage (stage I-IIa vs. stage IIa-IV, p=0.031), metastasis (M0 vs. M1, p<0.001), grading (low vs. high, p=0.002) and residual tumor (status R0 vs. R1, p=0.022) (Figure 3). Age, sex, tumor size, lymph node status and perioperative radiochemotherapy were not related to the overall survival rates (Table 7). Disease-free survival was correlated with tumor stage (stage I-IIa vs. stage IIa-IV, p=0.018), lymph node status (pN0 vs. pN1, p=0.037), metastasis (M0 vs. M1, p=0.025), grading (low vs. high, p=0.031) and residual tumor (status R0 vs. R1, p=0.005) (Figure 4). Age, sex, tumor size and perioperative radiochemotherapy were not significantly associated with disease-free survival (Table 8).

Characteristics		**n**	**Mean OS [Months]**	**95% CI**	**Median OS [Months]**	***p***
		175	32.889	27.203-38.575	16.300	
Age	≤60 years	42	34.080	23.952-44.209	18.533	0.550
	>60 years	133	32.672	25.911-39.434	16.000	
Sex	Male	94	31.784	24.868-38.701	18.533	0.733
	Female	81	33.207	24.429-41.985	14.800	
Tumor infiltration	T1-2	17	46.062	27.420-64.704	29.100	0.111
	T3-4	158	31.226	25.405-37.047	16.000	
Lymph node status	N0	63	37.054	27.949-46.160	21.433	0.103
	N1	112	29.721	22.819-36.622	15.033	
Metastasis	M0	160	34.686	28.633-40.740	17.367	0.000
	M1	15	10.867	6.561-15.173	6.933	
Tumor stage (UICC 2010)	0-IIa	54	39.611	29.632-49.590	23.400	0.031
	IIb-IV	121	29.050	22.512-35.587	15.000	
Grading	G1-2	58	42.988	32.728-53.248	28.033	0.002
	G3	117	27.390	21.143-33.636	14.167	
Residual Tumor	R0	97	38.508	30.082-46.933	21.433	0.022
	R1	74	25.195	18.519-31.871	14.167	
Chemotherapy	No	38	27.295	16.027-38.564	10.700	0.149
	CTx	137	34.214	27.781-40.647	18.533	
Radio-chemotherapy	No	96	32.583	25.076-40.091	15.567	0.853
	RCTx	79	31.750	23.938-39.562	16.300	
pER-β expression	low	25	47.184	29.332-65.036	28.967	0.016
	high	141	26.748	21.694-31.801	15.067	

Table 7: Univariate analysis of prognostic factors for overall survival in resected pancreatic ductal adenocarcinoma. CI: confidence interval; OS: overall survival.

Characteristics		**n**	**Mean DFS [Months]**	**95% CI**	**Median DFS [Months]**	***p***
		175	33.873	27.007-40.739	15.533	
Age	≤60 years	42	31.898	20.681-43.115	16.300	0.801
	>60 years	133	34.907	26.594-43.220	15.000	
Sex	Male	94	32.450	23.654-41.245	16.333	0.637
	Female	81	33.266	23.461-43.071	14.200	
Tumor infiltration	T1-2	17	44.645	22.320-66.970	16.333	0.187
	T3-4	158	32.143	25.111-39.174	15.000	
Lymph node status	N0	63	40.448	28.987-51.908	20.033	0.037
	N1	112	28.831	20.935-36.726	14.167	
Metastasis	M0	160	35.158	27.956-42.360	15.833	0.025
	M1	15	11.310	6.662-15.958	12.433	
Tumor stage (UICC 2010)	0-IIa	54	42.996	30.349-55.642	21.033	0.018
	IIb-IV	121	28.042	20.624-35.460	14.200	
Grading	G1-2	58	40.500	28.892-52.108	20.233	0.031
	G3	117	29.619	21.624-37.613	14.167	
Residual Tumor	R0	97	41.881	31.857-51.904	18.433	0.005
	R1	74	22.566	14.910-30.223	14.167	
Chemotherapy	No	38	34.482	19.271-49.693	12.433	0.932
	CTx	137	33.403	25.920-40.885	15.533	
Radio-chemotherapy	No	96	37.505	28.021-46.989	17.000	0.090
	RCTx	79	28.329	19.565-37.093	14.367	
pER-β expression	low	25	46.650	27.499-65.800	25.033	0.042
	high	141	29.160	22.496-35.824	14.200	

Table 8: Univariate analysis of prognostic factors for disease-free survival in resected pancreatic ductal adenocarcinoma. CI: confidence interval; DFS: disease free survival.

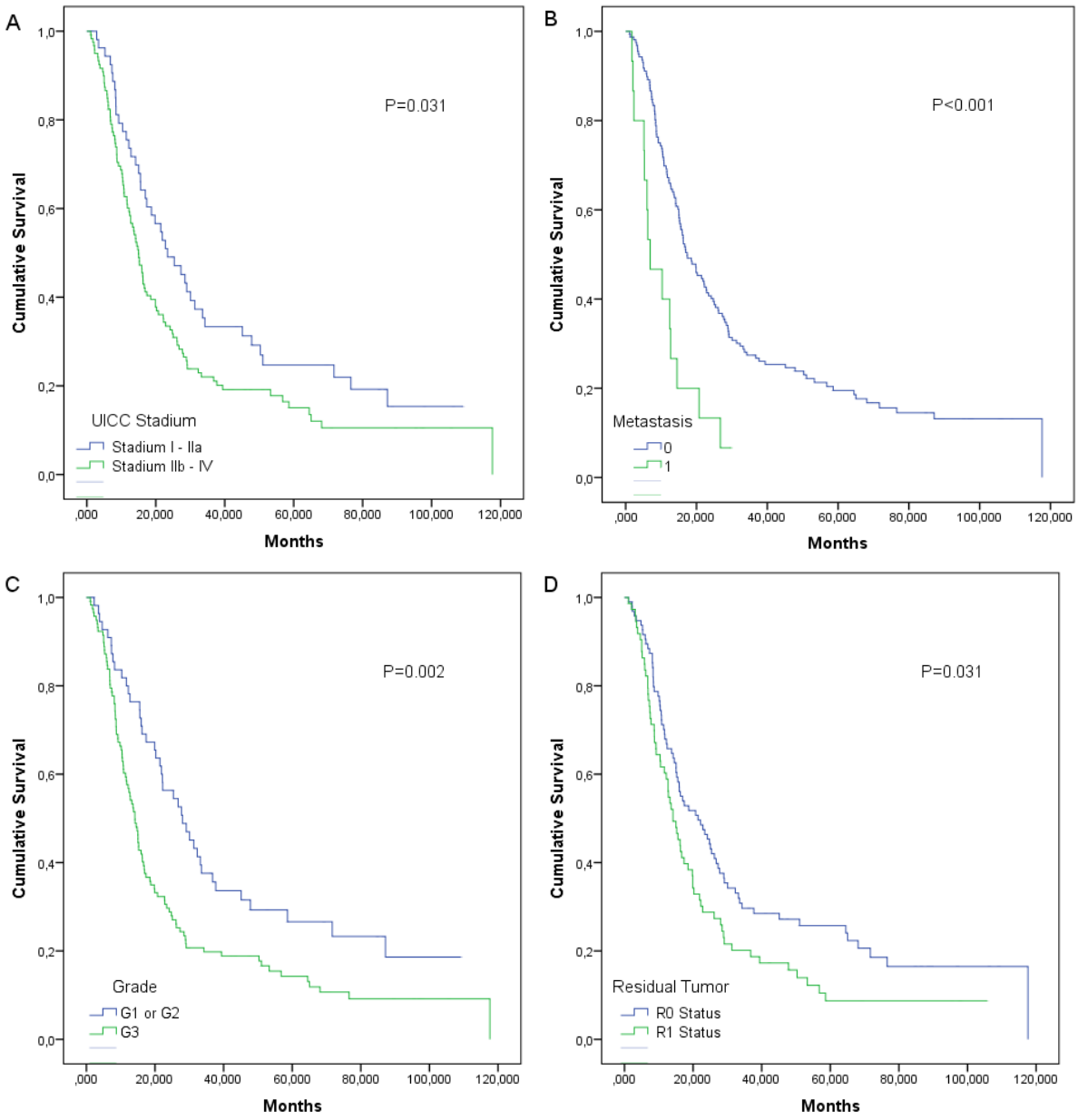
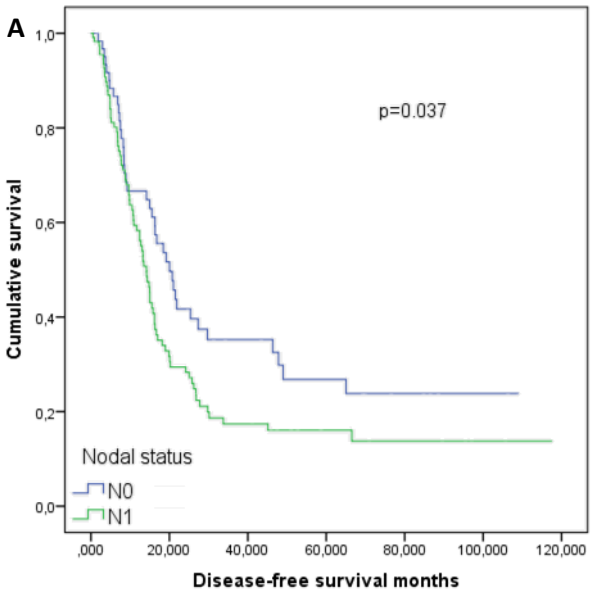
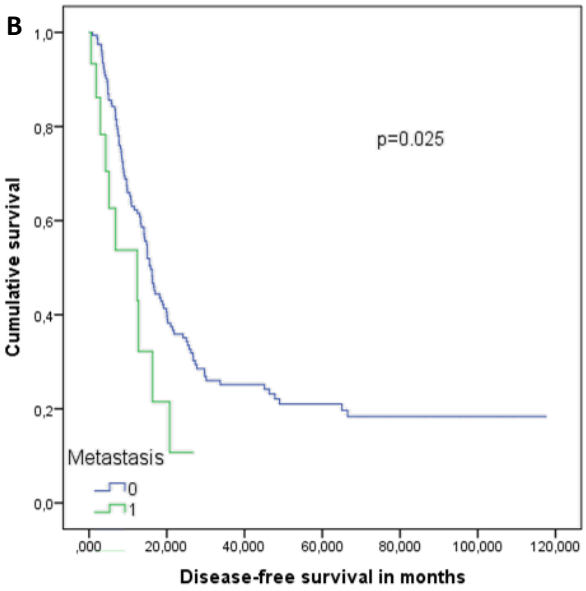
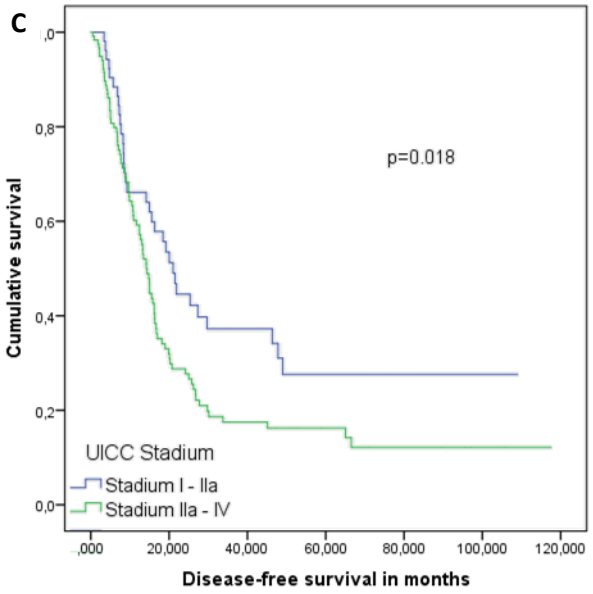
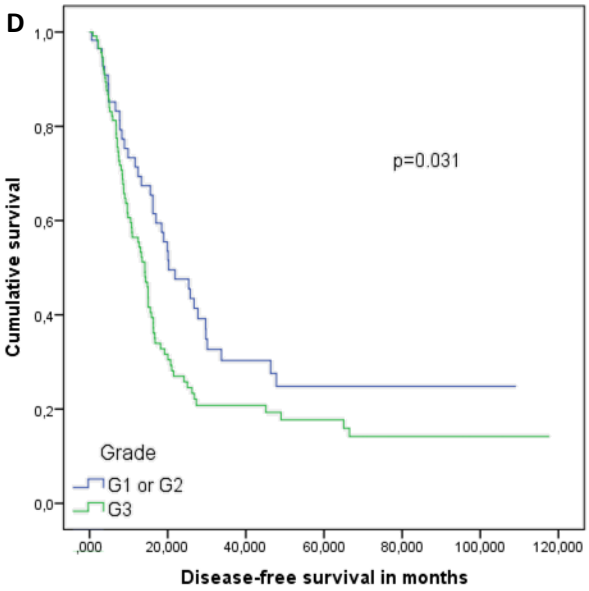


Figure 3: Univariate analysis of overall survival in correlation with clinicopathological parameters. Overall survival related to (A) tumor stage (stage I-IIa vs. stage IIa-IV, p=0.031), (B) metastasis (M0 vs. M1, p<0.001), (C) grading (low vs. high, p=0.002) and (D) residual tumor (status R0 vs. R1, p=0.031)


A
p=0.037
Cumulative survival
Nodal status
N0
N1
Disease-free survival months


B
p=0.025
Cumulative survival
Metastasis
0
1
Disease-free survival in months


C
p=0.018
Cumulative survival
UICC Stadium
Stadium I - IIa
Stadium IIa - IV
Disease-free survival in months


D
p=0.031
Cumulative survival
Grade
G1 or G2
G3
Disease-free survival in months

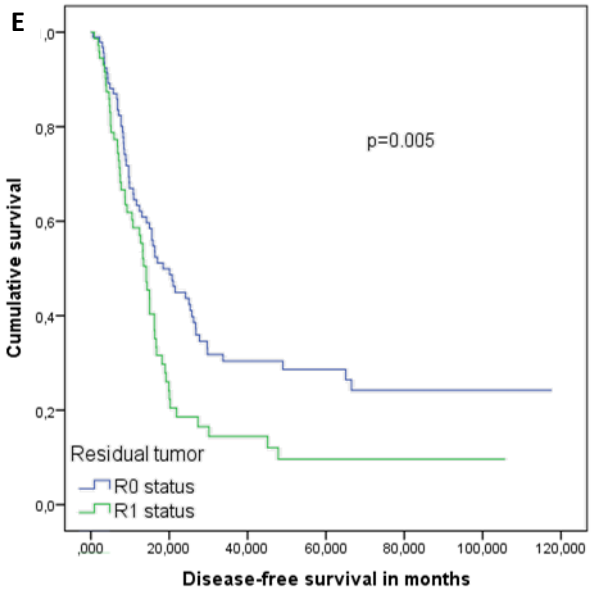

E
p=0.005
Cumulative survival
Residual tumor
R0 status
R1 status
Disease-free survival in months

Figure 4: Univariate analysis of disease-free survival in correlation with clinicopathological parameters. Disease-free survival related to (A) nodal status (N0 vs. N1, p=0.037), (B) metastasis (M0 vs. M1, p=0.025), (C) tumor stage (stage I-IIa vs. stage IIa-IV, p=0.018), (D) grading (low vs. high, p=0.031) and (E) residual tumor (status R0 vs. R1, p=0.005).

4.3.2 Correlation of expression of ER-β and STAT3/IL-6 pathway proteins in PDAC tissue with patient survival

The median overall survival for patients with low pER-β expression was 29 months, whereas for patients with high pER-β expression it was 15.1 months (p=0.016). The median disease-free survival for patients with low and high pER-β expression was 16.7 and 14.8 months, respectively, (p=0.042). The median overall survival of patients with low pER-β expression was at least 14 months longer in comparison with patients with high pER-β expression. All other investigated molecules showed no significant prognostic relevance (p>0.05). The corresponding survival curves according to the antibodies investigated (ER-β, pER-β, STAT3, pSTAT3 and IL-6 expression) are shown in figures 5 and 6.

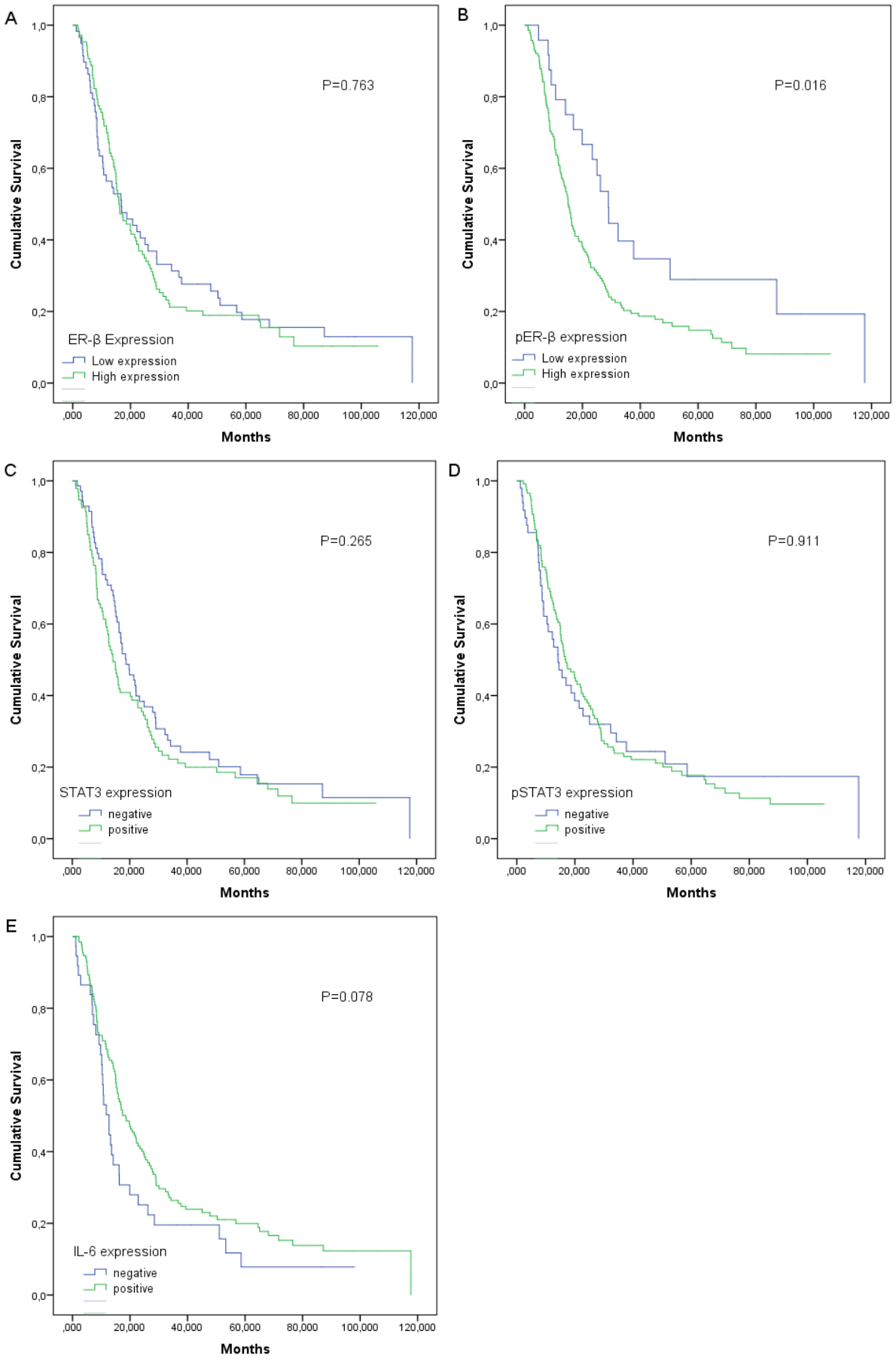

A
P=0.763
ER-β Expression
Low expression
High expression
Cumulative Survival
Months
B
P=0.016
pER-β expression
Low expression
High expression
Cumulative Survival
Months
C
P=0.265
STAT3 expression
negative
positive
Cumulative Survival
Months
D
P=0.911
pSTAT3 expression
negative
positive
Cumulative Survival
Months
E
P=0.078
IL-6 expression
negative
positive
Cumulative Survival
Months

Figure 5: Separate univariate analysis of overall patients' survival in correlation to expression of ER-β, pER-β, STAT3, pSTAT3 and IL-6 in PDAC TMAs. Patients' overall survival related to expression of (A) ER-β (B) pER-β, (C) STAT3 (D) pSTAT3, and (E) IL-6. Expression of pER-β was correlated to shorter overall survival ($p=0.016$), whereas all other molecules investigated showed no significant prognostic relevance ($p>0.05$).

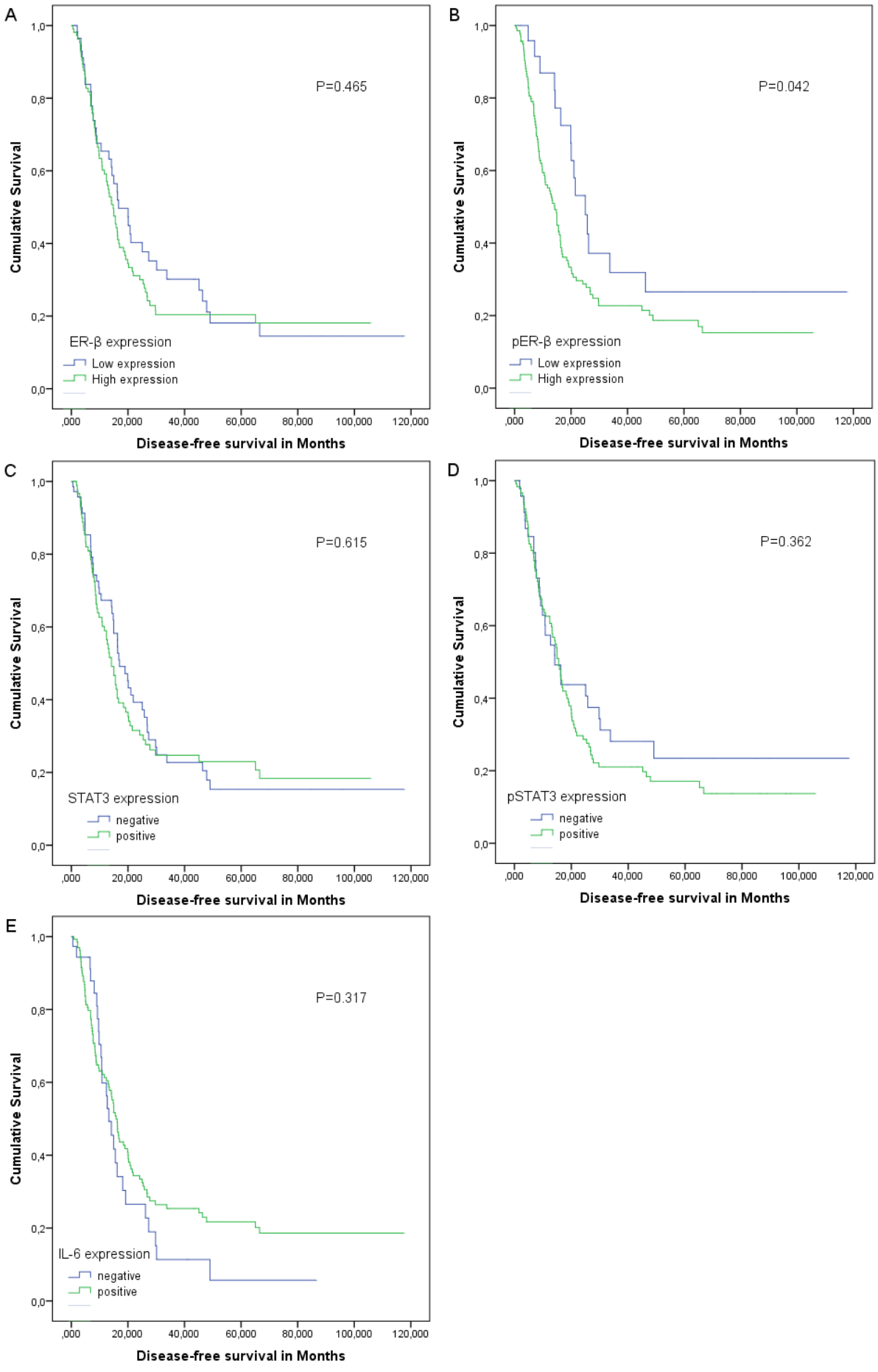

A
P=0.465
ER-β expression
Low expression
High expression
Cumulative Survival
Disease-free survival in Months
B
P=0.042
pER-β expression
Low expression
High expression
Cumulative Survival
Disease-free survival in Months
C
P=0.615
STAT3 expression
negative
positive
Cumulative Survival
Disease-free survival in Months
D
P=0.362
pSTAT3 expression
negative
positive
Cumulative Survival
Disease-free survival in Months
E
P=0.317
IL-6 expression
negative
positive
Cumulative Survival
Disease-free survival in Months

Figure 6: Separate univariate analysis of disease-free patients' survival in correlation to expression of ER-β, pER-β, STAT3, pSTAT3 and IL-6 in PDAC TMAs. Patients' disease-free survival related to expression of (A) ER-β (B) pER-β, (C) STAT3 (D) pSTAT3 and (E) IL-6. Expression of pER-β was correlated to shorter disease-free survival ($p=0.042$), whereas all other molecules investigated showed no significant prognostic relevance ($p>0.05$).

4.4 Multivariate survival analysis

For multivariate analysis, the following variables were taken into account: age, sex, tumor stage, grading, residual tumor, pER-β expression (Tables 9 and 10). High expression of pER-β, high tumor grading (G2 and G3) and presence of microscopic residual tumor proved to be independent predictors of overall survival in patients with PDAC correlating with a bad prognosis. Patients with high pER-β expression had a shorter overall survival with a hazard ratio of 1.9 (95% CI: 1.1-3.3; P=0.013).

The Cox proportional hazard model for disease-free survival revealed similar results as shown in Table 10. Multivariate analysis revealed high expression of pER-β, UICC stadium, high tumor grading and presence of microscopic residual tumor as independent predictors of disease-free survival associated with a bad prognosis. Patients with high pER-β expression were almost twice as likely to have a recurrence compared with patients with low pER-β expression (hazard ratio 1.9; 95% CI: 1.1-3.4; *P*=0.029).

Multivariate Analysis					
Characteristics		**n**	**HR**	**95% CI**	***p***
		175			
Age	≤60 years >60 years	42 133	1.384	0.908 – 2.110	0.130
Sex	Male Female	94 81	0.939	0.662 – 1.333	0.725
Tumor stage (UICC 2010)	0-IIa IIb-IV	54 121	1.260	0.863 – 1.841	0.232
Grading	G1-2 G3	58 117	1.732	1.163 – 2.578	0.007
Residual Tumor	R0 R1	97 74	1.516	1.068 – 2.150	0.020
pER-β expression	low high	25 141	1.993	1.153 – 3.443	0.013

Table 9: Multivariate analysis of overall survival with the following variables included: pER-β, UICC stage, grading, residual tumor, age and sex.

Multivariate Analysis					
Characteristics		**n**	**HR**	**95% CI**	***p***
		175			
Age	≤60 years >60 years	42 133	1.284	0.814 – 2.027	0.283
Sex	Male Female	94 81	0.892	0.606 – 1.311	0.560
Tumor stage (UICC 2010)	0-IIa IIb-IV	54 121	1.431	0.934 – 2.193	0.100
Grading	G1-2 G3	58 117	1.510	0.983 – 2.321	0.060
Residual Tumor	R0 R1	97 74	1.657	1.121 – 2.450	0.011
pER-β expression	low high	25 141	1.932	1.070 – 3.492	0.029

Table 10: Multivariate analysis of disease free survival with the following variables included: pER-β, UICC stage, grading, residual tumor, age and sex.

5 Discussion

Estrogen receptor-related pathways are implicated in the pathogenesis of pancreatic cancer, representing a suitable target for its treatment.(81) Although several studies about anti-hormone treatment with SERMs (e.g. Tamoxifen) in PDAC showed controversial results,(100,129,130) ligand-independent activation of ERs (e.g. phosphorylation) and therapeutic perspectives of this pathway remained unexplored in pancreatic cancer.(81)

Previous studies showed that raloxifene suppresses IL-6 and inhibits mammalian osteoclast differentiation and bone resorption activity only in the presence of IL-6, suggesting a possible interaction between ER and IL-6.(104,105) Importantly, Yamamoto et al. reported that active ER directly associates with, and acts as a transcriptional co-factor for, STAT3 induced by IL-6 in breast cancer cells. Moreover, direct physical interactions between STAT3 and ER were also reported, which represent a novel form of cross-talk between STAT3 and ER signaling pathways and open up novel therapeutic prospects.(110)

Based on the data above, this study focused on the ER-β and its phosphorylated form pER-β regarding their expression on PDAC tissue microarrays and their effect on the survival of patients with PDAC. Furthermore, we also investigated three other molecules (STAT3, pSTAT3 and IL-6), which are part of an important signaling cascade in tumor progression. We hypothesized that phosphorylation of ER-β and activation of several signaling cascades, including IL-6/STAT3, contribute to tumor progression in PDAC specifically affecting the survival of these patients.

5.1 ER-β/pER-β expression and prognostic relevance

ER-β and pER-β were highly expressed in the majority of tumors (61.7% and 80.6% respectively). pER-β expression was related to survival rates. Nuclear expression of pER-β indicated a poor clinical prognosis for overall and disease-free survival. Univariate and multivariate analysis revealed high expression of pER-β as an independent predictor of both overall and disease-free survival associated with a bad prognosis for these patients.

In 1981, Greenway et al. reported for the first time the presence of estrogen receptor (ER) in the carcinoma of the human exocrine pancreas.(94) Since then, there has been a sustained interest in the role of estrogens, including estrogen receptors and selective estrogen receptor modulators (SERMs) in pancreatic cancer. Diverse studies have been published investigating the presence of ERs in pancreatic tumors, but the results are inconsistent. Some studies reported the presence of ERs, although others failed to detect ERs at all.(95–99) Even the expression of the two ER isoforms, ER-α and ER-β, in pancreatic tumors remains controversial. Satake et al. reported that more than 90 percent of all published studies used antibodies that specifically recognized only the ER-α isoform. The expression pattern of ER-β in pancreatic cancer remained unclear to date.(100) Moreover, there are data showing that ER-β may play a more important role than ER-α in pancreatic cancer.(99) A recent study investigating in vitro pancreatic cell proliferation showed that ERs are frequently expressed in pancreatic cancer cell lines and especially ER-β expression usually outweighs ER-α expression.(130)

Our study is in agreement with these data showing that the majority of pancreatic tumors express strongly ER-β and pER-β. Furthermore, our data showed that pER-β was notably identified as an independent predictor of disease outcome for PDAC correlating with poor prognosis. This result provides additional strong evidence for ER-β in particular having an important role in PDAC. The fact that some tumors express strongly only the phosphorylated form of ER-β could be explained on the grounds that the phosphorylation of the ER-β reduces the percentage of not phosphorylated ER-β in the pancreatic cancer cell. ER-β was also present in the majority of the rest tumors, but not strongly expressed, so that they were rated “1” and categorized as „low expression“. According to immunoreactive score of Remmele and Stegner (IRS), Score “1” means less than 10 percent stained cells with mild reaction.

The interest in the role of ER-β has increased significantly since ER-β was discovered in 1996.(131) While the prognostic value of ER-β has already been evaluated in previous studies in

many tumors, there is no previous data in the existing literature about the prognostic relevance of ER-β and its phosphorylated form in pancreatic cancer. As mentioned above, the majority of all published studies about ER in PDAC used antibodies that specifically recognized only the ER-α isoform.(100) In contrast, the present study demonstrates the prognostic role of ER-β in PDAC: high pER-β expression associated with a higher mortality and recurrence rate representing a poor independent predictor of overall and disease-free survival.

Nevertheless, several studies investigating the role of ER-β in breast cancer have reported that ER-β might serve as a favorable prognostic factor, although the data are not entirely consistent.(132) The expression of ER-β is a protective factor of colorectal cancer.(133) As far as prostate cancer is concerned, the loss of ER-β expression is associated with progression from normal prostate epithelium to cancer, while those cancers that retained ER-β expression were associated with a higher recurrence rate.(134) ER-β is a prognostic marker of a favorable course of non-small cell lung cancer. Apart from the tumors mentioned above, there are also some studies regarding non-small cell and small cell lung cancer, esophageal, ovarian and brain tumors.(135,136) While they provide inconsistent results demonstrating the complex role of ER-β in cancer, ER-β expression seems predominantly to have a tumor-suppressive role in the tumors mentioned above. Nevertheless, our findings suggest that ER-β may have a tumor-promoting effect on pancreatic cancer, illustrating that several molecular mechanisms underlying the differential influence of ER-β in tumors, as for example ligand affinity, gene transcription, interactions with co-factors, heterogeneous dimerizations or splice variants of receptors.

Moreover, the rate of ER-α and ER-β has been described to be important in the hormone-dependent tumor progression in breast, ovary, colon and prostate cancer.(132) However, the expression of ER-α was not investigated in this study. Thus, the role of the balance between ER-α and ER-β still remains unclear in PDAC. Further investigation is needed to identify the prognostic role of ER-β expression, ER-α/ER-β rate and their effect on above tumors as well as in PDAC.

The present human PDAC cohort demonstrated that while the expression of the phosphorylated Ser105 active form of ER-β correlates significantly with poor overall and disease-free survival, ER-β showed no association with survival. This suggests that the phosphorylation of ER-β at serine 105 in the pancreatic cell may be an important component of pancreatic tumorigenesis resulting in poor prognosis.

ER activity is regulated at multiple levels including phosphorylation, one of the most important posttranslational modifications. It occurs in response to hormone and ligand-independent signals modulating ER transcriptional competence and mediating genomic and non-genomic action of the receptor. Specific phosphorylation sites were identified in the activation function-1 (AF-1), which is located in the N-terminus of the ER-α. Phosphorylation of the ER-α at serine sites has been reported to alter protein-protein interaction, subcellular localization, transactivation and the stability of the human ER-α.(137,138) Moreover, phosphorylation of ER-α at various serine sites is currently being evaluated for the classification of breast cancer,(139) as modulation of cancer cell proliferation due to phosphorylation of a specific serine in the ER-α has already been described.(140,141) Multiple phosphorylation sites on the human ER-α were shown to influence breast cancer carcinogenesis.

In contrast, our knowledge of human ER-β phosphorylation was derived primarily through studies of the mouse ER-β.(142) Three serines (Ser75, Ser87, and Ser105) in the N-terminus of human ER-β have recently been reported as phosphorylation targets of ERK1/2 and p38 kinases.(143) However, further information on the influence of ER-β phosphorylation on carcinogenesis is still not available and requires further investigation.

Immunohistochemical staining in our cohort of patients demonstrated that ER-β and mainly the phosphorylated active form pER-β are found not only in the nuclear but also in the cytoplasmic cellular compartments. This observation suggests that ER-β is not only active in the nucleus but it also has non-genomic or indirect genomic activity in the cytoplasm, where it could interact with other signaling molecules.

Similar to other NRs, the two ER isoforms are generally classified as ligand-dependent transcription factors. After the association with their specific ligands, they bind specific genomic sequences (EREs) and interact with co-regulators to regulate gene expression. In addition to the classical ligand-induced activation of ERs, recent studies described that ERs can be also transcriptionally activated in the absence of ligand by undergoing selected post-translational modifications that modify their stability, cellular localization and activity (e.g. phosphorylation). The unliganded activated ERs then interact with other signaling molecules in the nucleus or in the cytoplasm regulating the activity of other major signaling cascades.(75,81–84,86–88)

As mentioned above, activation of ERs are ligand-induced, and ERs are capable of modulating the activity of selected promoters directly. In addition to this classical way, recent studies reported that ERs can be also transcriptionally activated in the absence of ligand after activating

posttranslational modifications (e.g. phosphorylation) or through other signaling pathways, like growth factor (GF) signaling. In this mechanism (Figure 7), activated kinases activate the ER via phosphorylation, which then, after dimerization, translocates in the nucleus for gene regulation.(75,81–88) A previous study described the role of nuclear receptor phosphorylation showing the ligand-independent activation of ERβ via the MAPK pathway.(142) In the absence of ligands, the cascade of signaling events is different, and either activation or repression may occur. A ligand-independent signaling pathway is thought to activate the ERs in cancerous tissues contributing to hormone-independent tumor growth.(75,89,90)

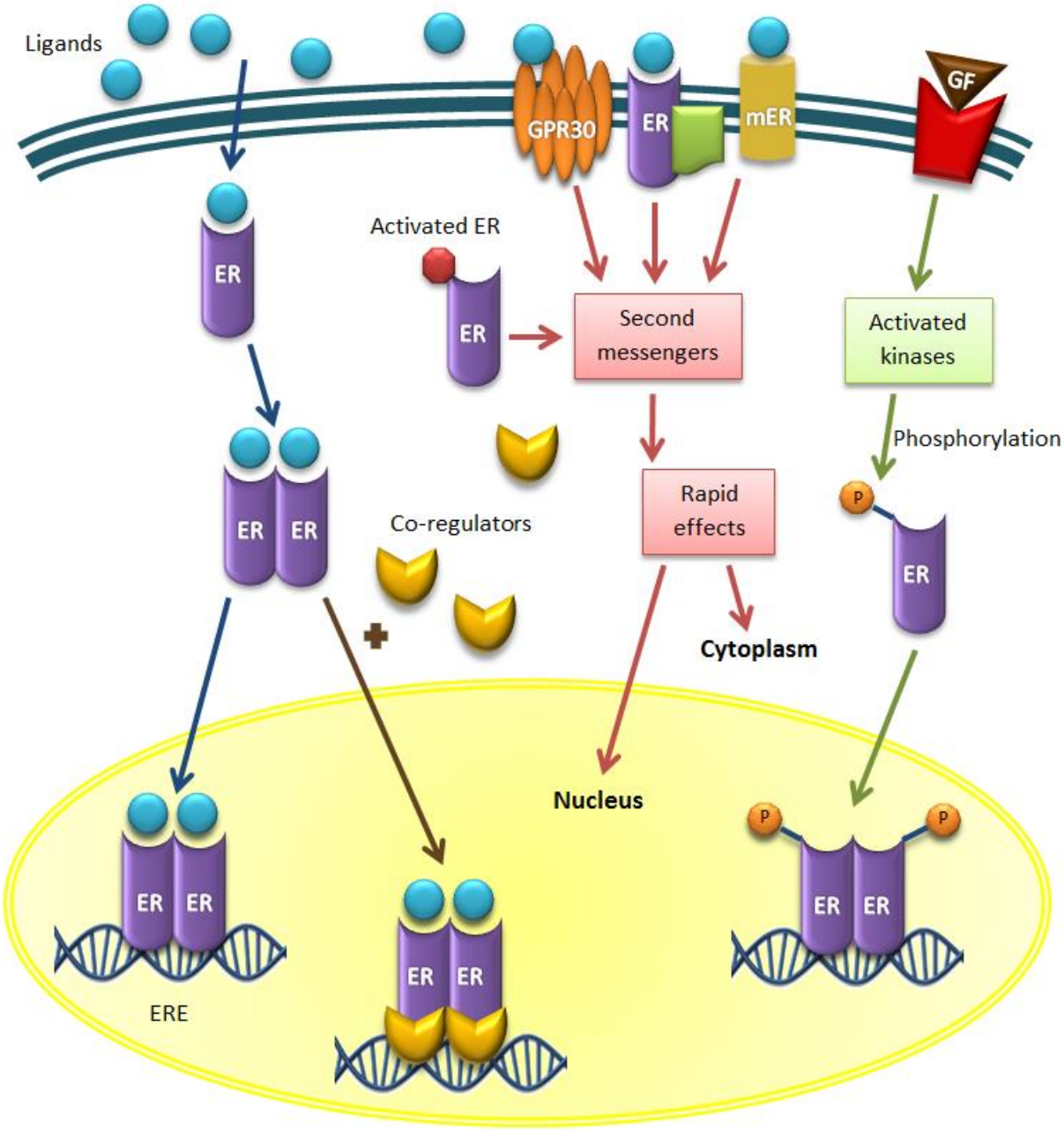


Figure 7: Schematic illustration of a model representing multiple molecular pathways of ER actions; ligand-dependent versus ligand-independent and genomic versus non-genomic. ERs were initially known only as ligand-dependent transcription factors with genomic functions. In the classical ligand-dependent genomic pathway (blue arrows), the ERs bind with their specific ligands and translocate into the nucleus, where they bind specific genomic sequences (EREs) affecting the transcription of these genes. This pathway also includes binding of the ligand-ER complex with other transcription factors (co-regulators), which modify the gene expression (brown arrow). In several studies, estrogen rapid effects were also described, which occur after ligand activation of plasma membrane proteins, including ER-isoforms termed membrane-bound ERs (mER), complex of ER with other plasma membrane proteins and the G protein-coupled

receptor 30 (GPR30). This ligand-binding leads to the activation of other signaling cascades via second messengers (SM) without genomic modulation, which are termed “non-genomic” (red arrows).(76–80) In addition to the classical ligand-induced activation of ERs and their capability to modulate the activity of selected promoters directly, recent studies reported that ERs can also be transcriptionally activated in the absence of ligands after activation via posttranslational modifications or other signaling pathways, like MAPK pathway or growth factor (GF) signaling. In this mechanism (green arrows), activated kinases activate the ER via phosphorylation, which then, after dimerization, translocates into the nucleus for gene regulation.(75,81–88)

5.2 IL-6/STAT3/pSTAT3 expression and prognostic relevance

This study on PDAC tissue microarrays showed that STAT3, pSTAT3 and IL-6 were expressed in more than half of the pancreatic tumors examined. Nevertheless, STAT3, pSTAT3 and IL-6 expression was not related to survival rates.

The IL-6/STAT3 pathway constitutes one of the essential signaling cascades in pancreatic cancer initiation and progression.(114) Recent studies demonstrated that stimulation with IL-6 activates phosphorylation of STAT3 in pancreatic cell lines(113,118,119) and that the JAK/STAT pathway also stimulates cell proliferation and malignant transformation and inhibits apoptosis in the pancreas.(120) IL-6 plays a major role in malignant transformation and progression of several tumors, including pancreatic cancer.(108,113–117) IL-6 acts either by affecting the tumor cells directly or modulating the tumor microenvironment. A study in KRAS-mutated mice showed the major role of IL-6 in PDAC reporting that IL-6 activates STAT3 pathway in order for the early PanIN lesions to be developed to PDAC.(121) Moreover, elevated IL-6 levels are described in pancreatic cancer and correlated with poor prognosis(121,122) as well as with weight loss and cachexia, which are negative prognostic factors for patients with pancreatic cancer.(123,124) Furthermore, IL-6 promotes angiogenesis in tumors.(144)

Immunohistochemical staining in our cohort of patients confirms the data above, as the majority of the examined pancreatic tumors express all three of the investigated components of IL-6/STAT3 pathway, supporting the importance of this cascade in pancreatic cancer. However, our survival analysis demonstrated no prognostic relevance of IL-6/STAT3 pathway proteins. Denley et al. reported that expression of IL-6R, JAK, STAT3 and pSTAT3^{Ser727} is not associated with the survival in a tissue microarray-based cohort of PDAC from 86 patients undergoing pancreaticoduodenectomy, confirming our results about STAT3 and IL-6 expression. In contrast, high pSTAT3^{Tyr705} expression was associated with reduced overall survival in univariate and multivariate analysis. Furthermore, Denley et al. reported high phosphorylated JAK (pJAK) expression as an independent adverse prognostic factor, and patients with a combination of high expression of pJAK and pSTAT3^{Tyr705} had an especially poor prognosis.(60) As far as prognostic relevance of pSTAT3^{Tyr705} is concerned, there has been only limited assessment of its prognostic utility in pancreatic cancer. Comprising 175 patients, our cohort represents to date the largest study investigating the expression of the IL-6/JAK/STAT3 signaling pathway. However, despite diverse studies trying to determine the clinicopathological impact of this inflammatory pathway

in resectable PDAC, IL-6/STAT3 pathway proteins in pancreatic tumorigenesis and tumor progression is not fully understood.

5.3 Other prognostic factors and study limitations

Overall and disease-free survival were significantly related to tumor stage, metastasis, grading and residual tumor. Lymph node status was significantly related to disease-free survival rate, but not to overall survival rate. Age, sex, tumor size and perioperative radiochemotherapy were not related to overall and disease-free survival rates (Table 6 and 7). For multivariate analysis, the following variables were taken into account: age, sex, tumor stage, grading, residual tumor and pER-β expression (Tables 9 and 10). Nodal status was not taken into account separately because it is included into UICC tumor stage.

While high expression of pER-β, high tumor grading and presence of microscopic residual tumor proved to be independent predictors of both overall and disease-free survival in our patients with PDAC correlating with a bad prognosis, UICC stadium was found to be an independent prognostic factor only of recurrence-free survival associated with a poor prognosis. The relatively small sample size of our cohort in comparison with the cohort below is likely to contribute to the lack of significance regarding overall survival. Furthermore, the high-risk estimate associated with high tumor grading and presence of microscopic residual tumor may explain why tumor stage did not achieve significance. Nevertheless, UICC stage was significantly related to both overall and disease-free survival rates in univariate analysis.

Tumor stage is the most important prognostic factor in pancreatic cancer. An analysis of the National Cancer Database from USA comprising 21,512 patients undergoing pancreatectomy for pancreatic adenocarcinoma illustrated the essential influence of tumor stage on survival.(145) For patients who underwent pancreatectomy, tumor size, nodal status, and distant metastases affect survival.(145)

In the present human PDAC cohort, while tumor size had no prognostic relevance, the presence of positive nodes was significantly associated with poor disease-free survival but not with overall survival. As far as metastasis is concerned, it was significantly related to both disease-free and overall survival rate. For the multivariate analysis, tumor size, nodal status, and distant metastases were not taken into account separately because they are all included in UICC tumor stage. The fact that tumor size did not affect significantly the prognosis of these patients in the univariate analysis may be explained by the relatively small sample size in our cohort. Furthermore, nodal status affected only disease-free survival but not the likelihood of death. While the prognostic role of lymph node status was well established in previous studies,(145)

there is recent evidence demonstrating that nodal status is not an independent prognostic factor.(146–148) These previous studies, which show the nodal status as an independent prognostic factor for the survival in patients with PDAC, were done mostly at a time when adjuvant therapy was not typically administered.(145) But adjuvant chemotherapy prolongs survival even for a short time, reducing consequently the direct prognostic effect of nodal status. This may explain this inconsistency in the results above.

In addition to tumor stage, other important prognostic factors for PDAC are tumor size, tumor grade, the presence of residual tumor, the width of the surgical margin, the presence of lymphatic invasion within the tumor, as well as preoperative and postoperative serum CA 19-9 levels.(51,52,146–149) As far as residual tumor and tumor grade are concerned, our study confirms the data above. The width of the surgical margin, tumor size, the presence of lymphatic invasion within the tumor and preoperative and postoperative serum CA 19-9 levels were not included in our analysis, constituting a limitation of our study.

While the majority of patients in this study were treated with adjuvant therapy, it should also be mentioned that the lack of a standardized adjuvant therapy protocol is a drawback. While many patients were treated only with chemotherapy, other patients were treated with a combination of adjuvant radiochemotherapy. Further evaluation of the molecules expressed and their prognostic relevance is required in a cohort with a standardized adjuvant therapy protocol, as adjuvant therapy could affect survival rates. Nevertheless, in the present study, the expression of the investigated molecules was not statistically associated with the type of adjuvant therapy.

5.4 Conclusion

In this study, we investigated the prognostic role of ER-β-phosphorylation and the IL-6/STAT3-cascade activation in PDAC. We showed that all five investigated molecules (ER-β, pER-β, STAT3, pSTAT3 and IL-6) were expressed in more than half of the examined pancreatic tumors. Especially, pER-β was strongly expressed in the majority of tumors and its expression constitutes an independent prognostic marker for PDAC, demonstrating its important role in pancreatic cancer. Our study showed that patients with a high pER-β expression have a poor prognosis. However, the underlying molecular mechanisms and the exact role of ER-β/pER-β and IL-6/STAT3 pathways in the cellular cascades in PDAC need further investigation.

The understanding of the molecular mechanisms of pancreatic carcinogenesis could potentially identify prognostic subtypes of PDAC, predict clinical and therapeutic outcomes accurately and define novel therapeutic targets. Furthermore, the lack of detailed studies evaluating the therapy with SERMs only to patients expressing ERs probably contributes to the inconsistency of published results concerning therapy with SERMs in pancreatic cancer. The present study suggests a thorough re-examination of the potential role of SERMs in pancreatic neoplasms with high pER-β expression. These data may help in identifying patients who could benefit from additional therapeutic regimens, including selective estrogen receptor modulators.

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List of abbreviations

AF-1 and AF-2	activation function 1 and 2
AJCC	American Joint Committee on Cancer
CA 19-9	carbohydrate antigen 19-9 or cancer-associated antigen 19-9)
CI	confidence interval
CRM	circumferential resection margin
CT	computed tomography
cTNM	clinical Tumor-Node-Metastasis
DBD	DNA-binding domain
DFS	disease free survival
DNA	deoxyribonucleic acid
DP	distal pancreatectomy
E2	17beta-estradiol
ECOG	Eastern Cooperative Oncology Group
EGF	epidermal growth factor
ER	estrogen receptor
ER-α	estrogen receptor alpha
ER-β	estrogen receptor beta
ERE	estrogen response elements
ERK1/2	extracellular signal-regulated kinases 1 and 2
FOLFIRINOX	folinic acid – fluorouracil - irinotecan - oxaliplatin
GF	growth factor

gp130	glycoprotein 130
GPR30	G protein-coupled receptor 30
hER-α and hER-β	human estrogen receptor alpha and beta
hMLH1	human homolog of the Escherichia coli DNA mismatch repair gene, mutL
HR	hazard ratio
IL-6	interleukin 6
IPMN	intraductal papillary mucinous neoplasm
IRS	immunoreactive score by Remmele
JAK	Janus kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAPC	locally advanced pancreatic cancer
LBD	ligand-binding domain
LXR	liver X receptor
MAPK	mitogen-activated protein kinases
MCN	mucinous cystic neoplasm
mER	membrane-bound ERs
MSH2	DNA mismatch repair MutS protein homolog 2
Nab-paclitaxel	nanoparticle albumin–bound paclitaxel
NR	nuclear receptors
NSAID	nonsteroidal anti-inflammatory drugs
OS	overall survival
p16/CDKN2A	protein16/ cyclin dependent kinase inhibitor 2A
p38 kinases	p38 mitogen-activated protein kinases

PanIN	pancreatic intraepithelial neoplasm
PD	pancreatoduodenectomy or Whipple procedure
PDAC	pancreatic ductal adenocarcinoma
pER-β	phosphorylated estrogen receptor beta
pER-β^{Ser105}	phosphorylated at serine 105 estrogen receptor beta
pJAK	phosphorylated Janus kinase
PPAR	peroxisome proliferator-activated receptor
PPPD	pylorus-preserving pancreaticoduodenectomy or pp-Whipple
pSTAT3	phosphorylated signal transducer and activator of transcription-3
$pSTAT3^{Ser727}$	phosphorylated at serine 727 signal transducer and activator of transcription-3
$pSTAT3^{Tyr705}$	phosphorylated at tyrosine 705 signal transducer and activator of transcription-3
pTNM	pathologic Tumor-Node-Metastasis
SERMs	selective estrogen receptor modulators
SMAD4	small mothers against decapentaplegic homolog-4 transcription factor
STAT3	signal transducer and activator of transcription-3
TMA	tissue micro-arrays
TNM	Tumor-Node-Metastasis
TP	total pancreatectomy
TP53	tumor protein P53
UICC	International Union Against Cancer

Eidesstattliche Versicherung

„Ich, Ioannis Pozios, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Expression of phosphorylated estrogen receptor beta is an independent negative prognostic factor in pancreatic ductal adenocarcinoma“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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15.05.2017

Ioannis Pozios

Anteilserklärung an etwaigen erfolgten Publikationen

Keine.

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publication list

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