Zane Simtniece

PROGNOSTIC FACTORS OF SURGICALLY TREATED PANCREATIC CANCER

Doctoral Thesis
for obtaining the degree of a Doctor of Medicine
Speciality – Pathology

Rīga, 2015

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Supervisors:

Dr. habil. med., Professor **Jānis Gardovskis**

Dr. med., Associate Professor **Ilze Štrumfa**

IEGULDĪJUMS TAVĀ NĀKOTNĒ

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ANNOTATION

According to data provided by the Centre of Health Economics of Latvia, the incidence of pancreatic tumours is increasing: there were 360 diagnosed cases in 2007 but 405 cases in 2010. Similar growth of incidence has been observed worldwide. The 5-year survival of pancreatic cancer is 15% for patients receiving the best possible treatment, including radical surgical treatment, but in the total group of pancreatic cancer, the 5-year survival rate is only 5%. In addition, the treatment results have not improved significantly over last 30 years. Therefore, it is essential to study the biological potential and its underlying mechanisms of pancreatic cancer in order to define possible treatment target molecules and to specify individual prognosis of each patient.

The **aim** of the research work is to detect the morphological and immunohistochemical profile of potentially radically operated pancreatic ductal adenocarcinoma (PDAC) and pancreatic endocrine tumours (PETs) and the clinical and prognostic importance of it in local patients.

Materials and methods. This study was performed as a retrospective, evidence-based morphological and immunohistochemical investigation of potentially radically operated PDACs and PETs, including 14 morphological and 21 immunohistochemical parameters and survival analysis by applying a wide scope of descriptive and analytic statistic methods. The research was carried out in accordance with the Declaration of Helsinki and received approval from the Committee of Ethics of Riga Stradins University.

Results. In the study, 78 cases of potentially radically operated PDAC and 16 cases of PET were selected. The mean age was 63.4 years for PDAC and 59.4 years for PET patients. PDAC had the following characteristics: large tumour size (mean size 3.6 cm), frequent pT3 – 98.7%, pN1 – 67.5%, moderate or poor differentiation in 46.2% and 35.9% of cases, perineural invasion – 87.2% and R1 – 57.5% of cases. PET were characterised by less aggressive characteristics: pT3 – 37.5%, pN1 – 20.0%, R1 – 27.3% of cases; well differentiated endocrine tumours with unclear behaviour predominated (56.3%). Comparing neoplastic cells with the non-neoplastic counterparts, CK 20, CK 5/6, COX-2, Ki-67, p53, p21, cyclin D1, chromogranin A and vimentin were up-regulated in PDAC, but CK 7, CK 19, p63, vimentin, COX-2, Ki-67

and CD44 in PET. In contrast, expression of p27, CK 7, CK 19 and CD56 was lost in PDAC cases.

The median overall survival after potentially radical operation was 11.0 months for PDAC patients, but among PET patients 2/ 14 patients died after 1 and 15 months. Tumour invasion in large blood vessels ($p = 0.013$), necrosis ($p = 0.001$), increased expression of vimentin ($p = 0.002$) and CD44 ($p = 0.018$), decreased expression of p27 ($p = 0.003$) showed prognostic significance in PDAC cases.

Conclusion. The overall survival after potentially radically treated PDAC is poor. At the time of diagnostics, the tumours are already large. PDAC is characterised by high invasiveness and thus able to spread. By immunohistochemical evaluation, the epithelial-mesenchymal transition, increased CD44 expression and loss of p27 had prognostic significance. PET are less aggressive, but decreased E-cadherin expression, up-regulation of CK 19 and vimentin expression can indicate tumour progression and spreading.

ANOTĀCIJA

Pēc Veselības Ekonomikas centra datiem, saslimstība ar aizkuņģa dziedzera vēzi pieaug: 2007. gadā atklāti 360, bet 2010. gadā – 405 gadījumi. Analogu incidences pieaugumu novēro arī citviet pasaulē. Aizkuņģa dziedzera vēža 5 gadu izdzīvotība, pacientam saņemot labāko iespējamo ārstēšanu, t.sk. radikālu ķirurģisku terapiju, ir 15%, bet kopējā aizkuņģa dziedzera vēža grupā 5 gadu izdzīvotība ir tikai 5%, pie tam rezultāti nav būtiski uzlabojušies 30 gadu laikā. Līdz ar to ir būtiski pētīt aizkuņģa dziedzera audzēju bioloģisko potenciālu un to noteicošos mehānismus, lai varētu rast mērķa molekulas iespējamai terapijai un precizēt katra pacienta individuālo prognozi.

Pētījuma mērķis ir noteikt potenciāli radikāli operētu aizkuņģa dziedzera duktālu adenokarcinomu (ADDA) un aizkuņģa dziedzera endokrīnu audzēju (AEA) morfoloģisko un imūnhistoķīmisko profilu un šo pacientu prognozi.

Materiāli un metodes. Pētījums ir retrospektīvs, uz pierādījumiem balstīts potenciāli radikāli operētu ADDA un AEA morfoloģisks un imūnhistoķīmisks izvērtējums, nosakot 14 morfoloģiskus un 21 imūnhistoķīmiskus parametrus un iekļaujot izdzīvotības analīzi. Datu statistiskai apstrādei tika izmantota aprakstošā un analītiskā metode. Pētījums ir veikts saskaņā ar Helsinku deklarāciju un Rīgas Stradiņa universitātes ētikas komitejas atļauju.

Rezultāti. Pētījumā iekļauti 78 potenciāli radikāli operētu ADDA pacientu un 16 AEA pacientu gadījumi. ADDA pacientu vidējais vecums bija 63,4 gadi, AEA pacientiem – 59,4 gadi. ADDA raksturīgas šādas pazīmes: liels audzēja izmērs (vidējais izmērs 3,6 cm), biežs pT3 – 98,7%, pN1 – 67,5%, vidēja vai zema diferenciacijas pakāpe – 46,2% un 35,9% gadījumos attiecīgi, perineirāla invāzija – 87,2% un R1 – 57,5% gadījumos. AEA raksturīgas mazāk agresīvas pazīmes: pT3 – 37,5%, pN1 – 20,0%, R1 – 27,3% gadījumu; visbiežāk tie bija labi diferencēti endokrīni audzēji ar neskaidru potenciālu (56,3%). Salīdzinot neoplastiskās šūnas ar atbilstošiem labdabīgiem audiem, konstatēta CK 20, CK 5/6 un COX-2 jauna ekspresija ADDA gadījumos, bet AEA gadījumos – CK 7, CK 19, p63, vimentīna un COX-2 ekspresija. ADDA audos pastiprinās Ki-67, p53, p21, ciklīna D1, hromogranīna A un vimentīna ekspresija (salīdzinot vidējo pozitīvo šūnu relatīvos daudzumus), bet AEA – Ki-67 un CD44. Savukārt ADDA šūnās samazinās p27, CK 7, CK 19 un CD56 ekspresija.

Mediānais izdzīvotības laiks pēc potenciāli radikālas operācijas bija 11,0 mēneši ADDA pacientiem, bet starp AEA pacientiem 2/ 14 pacienti nomira 1 un 15 mēnešus pēc operācijas. Prognostiska nozīme bija audzēja invāzijai lielajos asinsvados ($p = 0,013$), nekrozei ($p = 0,001$), paaugstinātai vimentīna ($p = 0,002$) un CD44 ($p = 0,018$) ekspresijai, kā arī p27 izzudumam ($p = 0,003$) ADDA gadījumos.

Secinājumi. Potenciāli radikāli operētu aizkuņģa dziedzera duktālas adeno-carcinomas pacientu mediānais izdzīvotības laiks ir īss. Diagnostikas brīdī audzējam bieži ir liels izmērs. Šiem audzējiem raksturīga izteikta invazivitāte un izplatība ārpus aizkuņģa dziedzera audiem. Pēc imūnhistoķīmiskā izvērtējuma, prognostiska nozīme ir epiteliāli-mezenhimālai transformācijai, paaugstinātai CD44 ekspresijai un p27 zudumam. Aizkuņģa dziedzera endokrīni audzēji ir mazāk agresīvi, bet uz agresīvu audzēja progresiju un izplatību norāda samazināta E-kadherīna ekspresija, kā arī iegūta CK 19 un vimentīna ekspresija.

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LIST OF ABBREVIATIONS

AJCC	– American Joint Committee on Cancer
<i>APC</i>	– <i>adenomatous polyposis coli</i> gene
AUC	– area under curve
<i>BRCA1</i>	– breast cancer 1 gene
BRCA1	– breast cancer 1 protein
<i>BRCA2</i>	– breast cancer 2 gene
BRCA2	– breast cancer 2 protein
CD	– cluster of differentiation
CDK	– cyclin dependent kinase
ChrA	– chromogranin A
CI	– confidence interval
CIA	– Confidence Interval Analysis (software)
CK	– cytokeratin
COX	– cyclooxygenase
CRM1	– chromosome region maintenance 1 protein
DNA	– deoxyribonucleic acid
EMT	– epithelial-mesenchymal transition
ERK	– extracellular-regulated kinase
ESMO	– European Society for Medical Oncology
G ₀ ; G ₁ ; G ₂	– Gap 0; Gap 1; Gap 2 (phases of the cell cycle)
G1; G2; G3; G4	– grades (of tumour differentiation)
HE	– haematoxylin and eosin
HPF	– high power field (of the microscope)
IFN- γ	– interferon- γ
IGF	– insulin-like growth factor
IHC	– immunohistochemistry
IQR	– interquartile range
kDa	– kilodalton
LN	– lymph node
M	– Mitosis (a phase of the cell cycle)
MAPK	– mitogen-activated protein kinase

MDM2	– mouse double minute 2 homolog protein
MDMx	– mouse double minute 4 homolog protein
MEN1	– multiple endocrine neoplasia, type 1
MIM	– Mendelian Inheritance in Man: a database of human genes and genetic disorders and traits
MVD	– microvascular density
NA	– not applicable
NCAM	– neural cell adhesion molecule
<i>NF1</i>	– neurofibromin gene
NK	– natural killer (lymphocyte)
NnPD	– non-neoplastic pancreatic ducts
NnPI	– non-neoplastic pancreatic islets
PanIN	– pancreatic intraepithelial neoplasia
PC	– pancreatic cancer
PCNA	– proliferating cell nuclear antigen
PDAC	– pancreatic ductal adenocarcinoma
PDEC	– poorly differentiated endocrine carcinoma
PET	– pancreatic endocrine tumour s. pancreatic neuroendocrine tumour
pM0-1	– designation of the presence or absence of distant metastases in a patient affected by a malignant tumour by pathology examination
pN0-1	– characteristics of regional lymph node status regarding metastases of malignant tumour by pathology examination
pR0-2	– resection margin status regarding presence or absence of malignant tumours; by pathology examination
pRB	– retinoblastoma gene protein
pT1-4	– characteristics of the local tumour spread by pathology examination
RM	– resection margin
ROC	– receiver-operating characteristic
OS	– overall survival
S	– Synthesis (a phase of the cell cycle)
SD	– standard deviation
SE	– shown elsewhere
TGF- β	– transforming growth factor- β
TNF- α	– tumour necrosis factor- α

TNM	– tumour, node, metastasis: classification of malignant tumour by the anatomic disease extent reflecting the local spread of the tumour (T), status or regional lymph nodes regarding tumour metastases (N) and presence of distant metastases (M)
TSC	– tuberous sclerosis complex
TSC1	– tuberous sclerosis, type 1
<i>TSC1</i>	– hamartin gene
TSC2	– tuberous sclerosis, type 2
<i>TSC2</i>	– tuberin gene
UICC	– Union for International Cancer Control
USA	– United States of America
VHL	– von Hippel-Lindau disease
<i>VHL</i>	– von Hippel-Lindau disease gene
VIP	– vasoactive intestinal polypeptide
WDEC	– well differentiated endocrine carcinoma
WDET	– well differentiated endocrine tumour
WHO	– World Health Organisation

INTRODUCTION

Pancreatic cancer (PC) is a major challenge currently for medical science and practical treatment. While it does not rank among the tumours with the highest incidence, PC is nevertheless the fourth leading cause of cancer mortality (Siegel et al., 2012; Ferlay et al., 2013). Among the malignant epithelial pancreatic tumours, pancreatic ductal adenocarcinoma and pancreatic endocrine tumours represent the two most frequent types. Pancreatic ductal adenocarcinoma is well-known for its dismal prognosis. Pancreatic endocrine tumours must be promptly recognised as significantly better survival can be expected (Reid et al., 2014). Endocrine tumours have also attracted marked attention due to the growing incidence (Öberg et al., 2012).

Surgical treatment is the mainstay of PC care. However, the survival of patients affected by pancreatic ductal adenocarcinoma remains low. By exploring the main prognostic factors, there is a possibility of identifying a target mechanism for new treatment, thus transforming the prognostic parameters into predictive factors. Such individualised treatment by assessment of the general molecular pathways is a desired goal (Öberg, 2013). Some prognostic factors such as patient's age or tumour localisation cannot be affected, whereas processes like mitotic activity, epithelial-mesenchymal cell transition or cancer stem cell nature can be influenced and altered. The impact of conventional chemotherapy (gemcitabine, 5-fluorouracil, cisplatin) is low because the tumour cells are resistant to this treatment (Arumugam et al., 2009; Hong et al., 2009; Maginn et al., 2014; Wilson et al., 2014). Fortunately, promising results have appeared regarding the efficiency of the treatment that is directed to a blockage or activation of specific cell mechanisms or to the certain cell-produced compounds. However, the published reports are contradictory (Westphal and Kalthoff, 2003; Jimeno et al., 2009; Chen et al., 2014; Hashim et al., 2014; Maginn et al., 2014; Wilson et al., 2014; Zhang et al., 2014). Therefore, to enhance and improve PC treatment options it is necessary to explore cancer-specific biological mechanisms (Maginn et al., 2014). Various methods are used in tumour research, including immunoblots, *in situ* hybridization or polymerase chain reaction. Immunohistochemistry has also been widely used for tumour studies (Rosai, 2004) to detect the presence of a specific protein in a certain cell.

The proteins that hypothetically can be involved in the carcinogenesis of pancreatic ductal adenocarcinoma and pancreatic endocrine tumours include proliferation markers, notably Ki-67 (McCall et al., 2013), tumour suppressor p53 (Lozano-Leon et al., 2011), DNA repair protein – breast cancer 1 protein (BRCA1) (Beger et al., 2004; Al-Sukhni et al., 2008), cell cycle regulators p21 (Lozano-Leon et al., 2011), p27 (Kim et al., 2014) and cyclin D1 (Guo et al., 2003), anti-apoptotic protein Bcl-2 (Yachida et al., 2012), cell adhesion protein E-cadherin (Hong et al., 2011) and cancer stem cell marker CD44 (Mohammed et al., 2013; Hou et al., 2014; Zhu et al., 2014). The cytokeratin (CK) spectrum including CK 7, CK 20, CK 5/6, CK 19 along with markers of intestinal and squamous differentiation can disclose histogenesis. However, there is a lack of in-depth studies regarding the prognostic role of most markers; controversial findings have been reported in relation to CK 19 (Deshpande et al., 2004; Jonkers et al., 2006; Matros et al., 2006; Schmitt et al., 2007; Zhang et al., 2009; Han et al., 2013; Reid et al., 2014). Neuroendocrine differentiation deserves detailed practical evaluation as well as in-depth studies of the relations with other morphological and immunohistochemical features (Takikita et al., 2009; Öberg et al., 2012). Epithelial-mesenchymal transition by mesenchymal marker vimentin expression (Handra-Luca et al., 2011), cyclooxygenase 2 (COX-2) activation (Juuti et al., 2006; Lozano-Leon et al., 2011), as well as angiogenesis by CD34 visualisation and microvascular density (MVD) measurements (Yamahatsu et al., 2012) also represent reasonable research targets. However, to date significant controversies exist. In addition, most of the studies have been devoted to the detection of a few isolated proteins, thus hindering integrated morphological and immunohistochemical evaluation.

The **aim** of this research work was to detect the morphological and immunohistochemical profile of pancreatic ductal adenocarcinoma and pancreatic endocrine tumours and the clinical and prognostic importance in local patients.

In order to reach this aim, the following **tasks** were conducted:

1. Characterising the morphological structure of pancreatic ductal adenocarcinoma (PDAC) and pancreatic endocrine tumours (PETs), analysing pTNM, grade, manifestations of invasive growth, mitotic activity and necrosis;
2. Assessing the molecular profile of PDAC and PETs by immunohistochemical evaluation, analysing the expression frequency and extent of cell cycle

regulators, markers of endocrine, mesenchymal and stem cell differentiation as well as proteins involved in DNA repair and angiogenesis;

3. Studying intestinal and squamous differentiation along with CK spectrum in PDAC and PETs;
4. Evaluating angiogenesis by MVD in PDAC and PETs;
5. Detecting the survival of surgically treated local patients affected by PDAC and PETs, and the factors influencing it;
6. Analysing the interrelations of the clinical, morphological and molecular parameters.

Scientific assumptions or working hypotheses:

1. The morphological characteristics of PDAC and PETs as a whole group reflect the general health risk degree of potentially radically surgically treated patients. Certain targets for intervention can be identified;
2. Immunohistochemical assessment disclose the changes in protein expression levels in non-neoplastic and corresponding malignant tissues as well as relations between protein level, morphological and clinical findings, and survival. The insights into main molecular pathways can be reached.
3. The survival of surgically treated patients affected by PDAC and PETs in Latvia corresponds with the worldwide experience.

Scientific and practical diagnostic novelty:

1. Within the framework of the present scientific work, molecular markers with equivocal published diagnostic and prognostic value were evaluated in a well-characterised group of primary PCs. The findings will add evidence-based knowledge to the published research data and are outstanding by the wide spectrum of the immunohistochemical profile including 21 target proteins in the same tumour group and by simultaneous comparison of two most frequent pancreatic epithelial malignant tumours and the non-neoplastic counterparts. Regionally, the study represents the first, large and wide morphological study of PC. Regarding the recognised geographic differences in the cancer incidence and morphology, the data present novel findings.
2. The present work has facilitated the practical implementation of the morphological evaluation protocol for examination of the surgical material

containing PC into regular diagnostic practice. Practical recommendations have been reached for routine immunohistochemical evaluation of pancreatic tumours, previously limited to a few episodic examinations.

Personal contribution

The author has performed all stages of the study, including the study design and selection of the immunohistochemical markers, the scientific measurements and statistical analysis. The author performed the immunohistochemical visualisation and is the author of the included gross and microscopic photographs.

Ethical concerns

This research was carried out in accordance with the Declaration of Helsinki and received approval from the Committee of Ethics of Riga Stradins University.

1. LITERATURE REVIEW

1.1. Definition, epidemiology, risk factors and clinical description of the most frequent pancreatic tumours

1.1.1. Characteristics of PDAC

PDAC is a primary malignant pancreatic tumour of epithelial origin, which is defined by the World Health Organisation (WHO) classification as follows: ‘A carcinoma occurring almost exclusively in adults that probably arises from and is phenotypically similar to, pancreatic duct epithelia, with mucin production and expression of characteristic cytokeratin pattern’ (Klöppel et al., 2000). Although PDAC has also been delineated as ‘a malignant tumour derived from ductal epithelia’, it should be emphasised that pancreatic tumours are defined based on the line of differentiation (ductal, endocrine or acinar), not the cell of origin (Jain, 2012)².

PDAC is the most common malignant neoplasm of the pancreas comprising roughly 85–90% of pancreatic tumours (Klöppel et al., 2000; Smyrk, 2009). Thus, epidemiological data on PC provide a quite accurate characterisation of PDAC. There were 378 recorded cases of pancreatic neoplasms in Latvia in 2012 corresponding to 18.6 patients per 100,000 population. Thus, PC ranks in 15th place among malignant tumours. In 2009, 18.0 patients per 100,000 population were diagnosed with PC (SPKC, 2014). In 2012, there were 103,773 estimated new cases of PC in Europe and 43,920 in the United States of America (USA). During the same time period, 104,463 PC-related death cases were expected to occur in Europe and 37,390 in the USA. Among all malignant tumours, except non-melanoma skin cancer, PC ranks 8th in Europe and 10th in the USA by the number of new recorded cases *versus* 4th place for both Europe and the USA regarding death cases (Siegel et al., 2012; Ferlay et al., 2013).

At the time of diagnosis, about 80% of PDAC patients are between 60 and 80 years of age (Klöppel et al., 2000). Different research studies have estimated the median age of PDAC diagnosis as 66.7–72.0 years, the mean age as 63.1 and the age range as 32–90 years (Lim et al., 2003; Cleary et al., 2004; Handra-Luca et al., 2011). No significant gender-related differences in PDAC incidence have been observed. For example, in the study by Lim *et al.*, PDAC was diagnosed in 196 males and 200 females, corresponding to 49.5% and 51.5%, respectively (Lim et al., 2003). In another study, 196 males and 160 females were diagnosed with PDAC, revealing a relative

frequency of 55.1% and 44.9% (Handra-Luca et al., 2011). The gender composition comprised 59.8% males and 40.2% females in the study by Yoon *et al.* (Yoon et al., 2011). However, some studies have specified that among younger patients, PDAC affected men significantly more frequently than women while among older patients the age-adjusted rates were equal for men and women (Andrén-Sandberg et al., 2000).

Epidemiological studies have highlighted the most significant risk factors of PC include family history, age, chronic pancreatitis, smoking, obesity and diabetes mellitus (Kaur et al., 2012). The high risk group of hereditary and familial PC comprises 5–10% of PC patients. Hereditary PC develops within the following syndromes (Klöppel et al., 2000; Lynch et al., 2001; Kaur et al., 2012):

- 1) Lynch syndrome s. hereditary non-polyposis colorectal cancer (phenotype MIM numbers #609310, #120435, #614350 and #614337, inherited mutation in DNA mismatch repair genes *MLH1* [gene MIM number *120436, cytogenetic location 3p22.2], *MSH2* [gene MIM number *609309, cytogenetic location 2p21], *MSH6* [gene MIM number *600678, cytogenetic location 2p16.3], or *PMS2* [gene MIM number *600259, cytogenetic location 7p22.1], increased risk of PC proven by Kastrinos *et al.* (Kastrinos et al., 2009). In addition, Lynch syndrome can be caused by epigenetic silencing of *MSH2* due to partial deletion of *EPCAM* also involving deletion of DNA between *EPCAM* and *MSH2*. *EPCAM* is characterised by the following details: gene MIM number *185535, cytogenetic location 2p21, phenotype MIM number #613244, described in detail by Kuiper *et al.*, 2011);
- 2) Familial atypical multiple mole melanoma-pancreatic cancer syndrome (phenotype MIM number #606719, inactivation of cyclin-dependent kinase inhibitor 2A s. p16 (INK4A) gene *CDKN2A*, cytogenetic location 9p21.3, gene MIM number *600160, reviewed by Harinck *et al.*, 2012);
- 3) Peutz-Jeghers syndrome (phenotype MIM number #175200, mutation in the serine threonine kinase gene *STK11*, cytogenetic location 19p13.3, gene MIM *602216; association with PC described as early as among the first reports by Jeghers *et al.*, 1949 and reconfirmed by Giardiello *et al.*, 1987, among others);
- 4) Cystic fibrosis (phenotype MIM number #219700, mutation of cystic fibrosis transmembrane conductance regulator gene *CFTR*, cytogenetic location 7q31.2, gene MIM number *602421, association with PC reported by Neglia *et al.*, 1995);

- 5) Hereditary breast and ovarian cancer (mutation in the *BRCA2* or *BRCA1* genes: regarding *BRCA1*, gene MIM *113705, cytogenetic location 17q21.31, phenotype MIM number #614320, association between germline *BRCA1* mutation and predisposition to pancreatic adenocarcinoma reported by Al-Sukhni *et al.*, 2008; regarding *BRCA2*, gene MIM *600185, cytogenetic location 13q13.1, phenotype MIM number #613347, the relevant mutations were reported as the most frequent hereditary cause of PC by Murphy *et al.*, 2002);
- 6) The risk of PC is also increased in first-degree blood relatives of patients diagnosed with PC (Klöppel *et al.*, 2000; Lynch *et al.*, 2001; Kaur *et al.*, 2012). Part of the familial PC burden can be attributed to mutations in protooncogene *PALLD*, cytogenetic location 4q32.3, gene MIM number *608092, phenotype MIM number #606856 (Pogue-Geile *et al.*, 2006);
- 7) Patients suffering from hereditary pancreatitis also have significantly increased risk of PC. The PC risk in these persons is up to 57 times higher than in the population, and the cumulative incidence up to the age of 70 years can reach 40% (Lowenfels *et al.*, 1997). Hereditary pancreatitis (phenotype MIM number #167800) is caused by gain-of-function mutations in the serine protease 1 s. cationic trypsinogen gene *PRSS1* (gene MIM number *276000, cytogenetic location 7q34).

Regarding age as a risk factor of PC, most PC cases are diagnosed between the age of 55 and 84 years: 19.1% of cases are observed in the age group between 55 and 64 years, 25.6% between 65 and 74 years, but 30.0% between 75 and 84 years (Kaur *et al.*, 2012).

Chronic pancreatitis is frequently studied as a risk factor of PC. Several studies indicated that patients with chronic pancreatitis have a 14-fold or even 16-fold increased risk of PC (Andrén-Sandberg *et al.*, 2000). Risk factors such as alcohol consumption mostly trigger acute pancreatitis, but repeated bouts of recurrent acute pancreatitis lead to chronic inflammation and fibrosis (Momi *et al.*, 2012).

Other risk factors of chronic pancreatitis include obstruction of the pancreatic ducts by tumours, calculi, preampullary cysts of duodenal wall, posttraumatic scars of pancreatic ducts and *pancreas divisum*. Autoimmune pancreatitis (Figure 1.1.), hereditary pancreatitis and idiopathic pancreatitis also follow a chronic course (Diaconu, 2009).

Figure 1.1. Autoimmune pancreatitis with typical characteristics. A, star-like duct with periductal fibrosis and inflammation; B, infiltration of eosinophils; C, lymphoid follicles in atrophic pancreatic parenchyma; D, storiform fibrosis. Haematoxylin and eosin (HE), original magnification 100x (A, C, D) and 400x (B).

Smokers have about a 2-fold increased risk of PC (Klöppel et al., 2000; Yadav and Lowenfels, 2013). The risk increases with the number of cigarettes smoked and years of exposure; for example, various studies indicated that 10 or more cigarettes per day increased the risk or that 25 or more cigarettes per day resulted in increased PC risk (Andrén-Sandberg et al., 2000).

For alcohol consumers it is difficult to specify the risk of PC development, as alcohol use often overlaps with smoking (Yadav and Lowenfels, 2013). Many research groups have not identified an increased risk of PC due to alcohol usage (Andrén-Sandberg et al., 2000). In contrast, some studies in a non-smoking population indicated that the risk of developing PC increased with 3 or 9 drinks per day (Yadav and Lowenfels, 2013).

Central and overall obesity increased PC risk from 1.4 to 2-fold. In this situation PC development was associated with perturbed energy balance, insulin resistance, hyperinsulinemia, glucose intolerance, development of diabetes, altered adipokine

secretion, chronic inflammation and oxidative stress (Momi et al., 2012). PC can develop in 0.5% of recently diagnosed diabetes patients during 6 years of follow-up. Diabetes is thought to more frequently develop due to the PC. Thus, recently diagnosed diabetes mellitus can be an important symptom of PC, especially in middle-aged, low weight or elderly patients without a family history of diabetes (Andrén-Sandberg et al., 2000; Yadav and Lowenfels, 2013). Certain studies have shown that 45–65% of all PC patients develop diabetes in the early stage (Pannala et al., 2009).

The important role of the above mentioned risk factors in the development of PC is explained by a permanent over-production of a number of inflammatory mediators, which are capable of causing genome damage, altered gene expression, induction of oncogenic signalling pathways to ultimately develop pancreatic intraepithelial neoplasia (PanIN) in which the first stage (PanIN-1) can be observed in 65.5% of chronic pancreatitis patients (Rosty et al., 2003; Momi et al., 2012). Finally, the underlying pathological process progresses in PDAC (Momi et al., 2012).

The unfavourable prognosis of PDAC is promoted by the retroperitoneal location of pancreas and asymptomatic clinical course of tumour in the early stages (Krantz et al., 2012). The most frequently observed clinical symptoms include jaundice, abdominal pain and pain irradiation to back, weight loss, lack of appetite, nausea, vomiting and pruritus. As the symptoms are not specific, there is a broad differential diagnosis with abdominal pathologies, for example, bile duct diseases, abdominal aortic aneurysm and other diseases of the gastrointestinal tract (Allema et al., 1995; McLatchie et al., 2007; Kaur et al., 2012).

The anatomical location of the pancreas also hinders the diagnostic possibilities of PC. Numerous diagnostic visualisation methods could be used in the evaluation of the pancreas such as ultrasonography, endoscopic ultrasonography, magnetic resonance imaging, computed tomography and endoscopic retrograde cholangiopancreatography. However, small tumours can still be missed and diagnosis of early stage PC is difficult using all the listed technologies (Kaur et al., 2012). Endoscopic ultrasonography in combination with computed tomography or magnetic resonance imaging are considered effective. Endoscopic ultrasonography-guided fine-needle aspiration can be superior (Tummala et al., 2011; Kaur et al., 2012).

Serological markers have been explored both for early detection of tumour and assessment of therapy effectiveness. Still, more specific markers are a desirable goal. CA19.9 is one of the most frequently described and applied markers. It is mostly used in

the assessment of PC treatment outcomes. Due to the lack of specificity, CA19.9 is not applicable for diagnostic purposes (Kaur et al., 2012). The plasma level of CA19.9 is elevated in 65% of PC cases, but its expression is also observed in other malignant tumours, such as colorectal, gastric, lung and ovarian carcinoma and uterine cervical squamous cell carcinoma, as well as in benign conditions (hepatitis, liver cirrhosis, cholecystitis, acute cholangitis, pancreatitis, pneumonia, pleural effusion and renal failure). In general, the sensitivity of CA19.9 is 69–98% and specificity is 46–98% (Pavai and Yap, 2003; Kaur et al., 2012). Pancreatic-associated antigen SPan-1 is positive in 81.3% of PC cases and characterised by high sensitivity (81.3%) and specificity parameters (75.6%). Another plasma marker CEACAM1 has a sensitivity of 85.0% and specificity of 98.0% but TSGF reaches sensitivity of 91.6% with specificity of 93.0%. However, these markers also showed positivity in chronic pancreatitis and other non-pancreatic benign conditions (Kaur et al., 2012).

Radical tumour resection represents the most effective treatment of the PDAC. However, at the time of diagnosis it can be applied in only 14.2–15.0% of patients (Niederhuber et al., 1995; Cleary et al., 2004; McLatchie et al., 2007). Higher operability was reported by teams skilled in multivisceral resection. The surgical approach depended on the tumour localisation and spread. The most commonly applied method of curative resection is pancreatoduodenectomy including Whipple operation and pylorus-preserving procedure. Pancreatoduodenectomy has been used in 64.6–91.1% of the previously published cases. Distal pancreatectomy was performed less frequently: 5.7–18.3%. Total pancreatectomy was applied in 3.2–15.9% of cases (Cleary et al., 2004; Yoon et al., 2011).

1.1.2. Characteristics of PETs

PETs are defined as epithelial neoplasms showing predominantly neuroendocrine differentiation (Klimstra et al., 2010). PETs are also known as pancreatic neuroendocrine tumours or islet cell tumours. However, hypothetically, PETs develop from pluripotential stem cell in ductal epithelia or ductal / acinar system (Vortmeyer et al., 2004; Jain, 2012). Occasionally, coexistence of PET and intraductal papillary mucinous pancreatic neoplasm have been reported, also suggesting possible common genesis (Kadota et al., 2013; Tewari et al., 2013). Considering this hypothesis, the designation of islet cell tumour can be inappropriate in at least some cases but the

molecular similarities and differences between PDAC and PETs deserve careful exploration. PETs can secrete some of the hormones or neurotransmitters including insulin, glucagon, gastrin, somatostatin, vasoactive intestinal polypeptide (VIP), pancreatic polypeptide, adrenocorticotrophic hormone, serotonin and calcitonin (Heitz et al., 2004). PETs lacking the clinical evidence of any hormone secretion are designated as non-functional tumours (Halfdanarson et al., 2008).

PETs are rare tumours. However, among all pancreatic neoplasms, PETs comprise 1–2% of cases (Chen, Van Ness et al., 2012). Occasionally, the incidence of PETs has been estimated as high as 3–5% of pancreatic epithelial tumours (Jain, 2012). Thus, PETs represent the second most common group of pancreatic tumours after PDAC (Heitz et al., 2004; Hruban et al., 2009; Chen, Van Ness et al., 2012; Jain, 2012; Morin et al., 2013). The epidemiological data on PET are limited. Few publications have been devoted to the population incidence of PETs. Studies from referral centres may not reflect the true occurrence in the population. In addition, the trend to analyse the whole group of gastroenteropancreatic neuroendocrine tumours has also led to more generalised data. There have been marked differences in diagnostics and reporting over the years due to changing classifications and definitions as well as occasionally controversial histological findings and lack of definite reference for a long time (Nicholls, 1902) before widespread availability of the different possibilities of immunohistochemistry (Asa, 2011; Morin et al., 2013; Miller et al., 2014). It is generally accepted that the incidence of PETs does not exceed 1/ 100,000 (Asa, 2011). In carefully performed population studies, the incidence of PET was considered to be between 0.23/ 100,000 and 0.4/ 100,000 in the population (Eriksson et al., 1989; Watson et al., 1989). Occasionally, an incidence as low as 0.12/ 100,000 has been reported, e.g., in French males (Lepage et al., 2004). Recently, the specialists from the European Society for Medical Oncology (ESMO) have estimated the incidence of PETs as 0.32/ 100,000, a value that slightly exceeds the incidence of gastric endocrine tumours (0.30/ 100,000) and reaches the incidence of small intestinal endocrine tumours ranging from 0.32–1.12/ 100,000 (Öberg et al., 2012). In different autopsy series, PETs have been found in 0.11–2.5% of cases (Halfdanarson et al., 2008). Particularly, in Tokyo Metropolitan Geriatric Hospital, performing autopsy examination of the whole pancreas in 5 mm thick sections, PETs were found in 10% of 60 randomly selected autopsies (Kimura et al., 1991). Thus, autopsy data suggest more frequent occurrence of PETs. However, the clinical relevance of these findings remains unclear as the tumours

have been small, symptomless and mostly not related to the death cause (Kimura et al., 1991).

PETs can develop at any age but more frequently develop between 30 and 60 years of age. The mean age is 51.6–53.0 years, median age is 53–58 years and age range is 17–86 years (Heitz et al., 2004; Bettini et al., 2008; Paik et al., 2008; Scarpa et al., 2010; Ekeblad et al., 2012; Rindi et al., 2012). The narrowest age range is seen in the cases of non-functioning (clinically silent) PETs, respectively, 50–60 years, but the following subtypes of functional PETs are characterised with the widest age range: VIPomas, 20–80 years; and gastrinomas, 20–70 years (Halfdanarson et al., 2008).

PET is characterised with equal incidence between genders (Heitz et al., 2004; Rindi et al., 2012). The proportion of females varies between 45.0–53.1% and of males between 46.9–55.0% of PET cases (Bettini et al., 2008; Ekeblad et al., 2012; Rindi et al., 2012).

There is a lack of detailed information on important risk factors which can be attributed to PET development. Sporadic tumours comprise 80.0–94.6% of PET cases, but the rest develop within hereditary cancer syndromes (Ekeblad et al., 2012; Rindi et al., 2012). Most commonly, PET is associated with multiple endocrine neoplasia type 1 (MEN1, phenotype MIM number #131100, mutation in menin gene *MEN1*, cytogenetic location 11q13.1, gene MIM number *613733). The development of PETs within MEN1 is confirmed by many authors, including Hao *et al.* (Hao et al., 2004). Less commonly, PETs are observed in patients affected by von Hippel-Lindau disease (phenotype MIM number #193300, mutation in *VHL* gene, cytogenetic location 3p25.3, gene MIM number *608537). The association between PETs and von Hippel-Lindau disease is reported by Griffiths *et al.*, 1987 and Gläsker *et al.*, 2006, among others. Very few PETs are described in association with tuberous sclerosis complex (Halfdanarson et al., 2008; Capurso et al., 2012). Tuberous sclerosis complex (TSC) comprises 2 distinct syndromes: TSC1 (phenotype MIM number #191100, mutation in hamartin gene *TSC1*, cytogenetic location 9q34.13, gene MIM number *605284) and TSC2 (phenotype MIM number #613254, mutation in tuberin gene *TSC2*, cytogenetic location 16p13.3, gene MIM number *191092). Regarding these syndromes, several PET patients have been affected by clinically diagnosed TSC (Dworakowska and Grossman, 2009), but *TSC2* mutation has been identified in a few of them (Merritt et al., 2006). In addition, occasional PETs have been reported (Halfdanarson et al., 2008; Capurso et al., 2012) in

association with type 1 neurofibromatosis (phenotype MIM number #162200, mutation in neurofibromin gene *NF1*, cytogenetic location 17q11.2, gene MIM number *61311).

In the case of MEN1, gastrinoma is the most common PET subtype. Among all gastrinomas, 22% of cases are MEN1-attributable (Roy et al., 2000; Brandi et al., 2001). Non-functioning PETs and insulinomas ranked next by frequency in the case of MEN1 syndrome (Brandi et al., 2001; Gibril and Jensen, 2004; Triponez et al., 2006). Characteristically, PET patients with MEN1 are younger than patients with sporadic PET, p value < 0.001 (Rindi et al., 2012). Among all of the patients with von Hippel-Lindau disease, PETs are diagnosed in 17% of patients, and mostly these are non-functioning tumours (Binkovitz et al., 1990; Blansfield et al., 2007). TSC is characterised by pancreatic insulinomas (Dworakowska and Grossman, 2009). When assessing the risk of insulinoma development, higher risk has been found in families with a history of insulinoma and also PETs of different subtypes (Zhan et al., 2013).

Factors such as increased body mass index, smoking and heavy drinking are shown to have a higher predominance in patients with insulinomas compared to control group but multivariate analysis does not show a statistically significant elevated risk for these factors (Zhan et al., 2013).

Similar to the PDAC, the PETs do not always present overt clinical signs and symptoms, thus PETs are divided into functioning and non-functioning tumours, depending on clinical manifestations of hormone production. In addition, the clinical picture can be influenced by the presence of mass lesion or metastatic spread.

Functioning tumours represent up to 30.9% of all PETs (Rindi et al., 2012). Functionally active tumours secrete hormones and create the clinical syndrome. These tumours usually are well-differentiated neoplasms which have not lost the ability to synthesise active substances. Hormonal activity is a great benefit for the differential diagnostic of PET subtypes because occasionally even by combination of gross, microscopic and immunohistochemical evaluation it is hard to specify PET subtypes (Heitz et al., 2004; Hruban et al., 2009). The most common types of functional tumours are insulinoma (27.2–45.0% of all PETs cases, including non- and functioning PETs; 67.1% of functioning tumours), gastrinomas (12.5–30.0% of all PETs), glucagonoma (8% of all PETs), VIPomas (6.4% of all PETs) and somatostatinomas (3.8% of all PETs) (Heitz et al., 2004; Halfdanarson et al., 2008; Rindi et al., 2012). Functioning PETs are more common in females than in males and in younger patients than in elderly patients (Rindi et al., 2012).

Non-functional tumours are clinically silent regarding the characteristic syndrome of hormones, although laboratory investigations can show increased hormone levels in blood. The most common tumours of this type develop from cells which normally produce pancreatic polypeptide and somatostatin. D-cell tumours can develop as well (Heitz et al., 2004). Overall, the proportion of non-functioning tumours varies between 39.7–69.9% (Heitz et al., 2004; Ekeblad et al., 2012; Rindi et al., 2012) of PET cases. Clinical signs and symptoms such as abdominal pain, jaundice, nausea, vomiting or weight loss develop if the tumour has reached a large size and/ or invaded the adjacent organs, and if tumour metastases are already present (Heitz et al., 2004; Bettini et al., 2008; Paik et al., 2008).

Thus, from a diagnostic perspective, the early detection of functioning PETs is based on the symptoms caused by the corresponding hormone. In contrast, diagnosis of non-functional PET follows the same basic protocol as PDAC. The smallest tumour size commonly diagnosed is about 0.5 cm. These tumours are considered to be microadenomas (Heitz et al., 2004).

When choosing treatment options for PETs, radical tumour resection despite the stage of tumour is the first-line therapy (Burns and Edil, 2012). Most typical surgical treatment methods that are applied in PET cases are distal pancreatectomy (more than one third of cases), pancreatoduodenectomy/ Whipple resection (slightly less than one third of cases) or tumour enucleation (about 15% of cases). The middle pancreatectomy is applied less frequently (Bettini et al., 2008; Rindi et al., 2012). Surgical treatment is advocated even in advanced cases. Thus, patients with unresectable liver metastases benefit from longer survival if the primary tumour is removed ($p = 0.010$). The surgical intervention in this situation ensures the 5-year disease specific survival of 82% contrasting with 50% in non-operated patients (Bertani et al., 2014). Procedures such as cytoreductive surgery, radiofrequency ablation and/ or transarterial chemoembolisation have higher significance in the case of locoregional recurrences or hepatic metastases, or in order to decrease symptoms and to prolong patient's survival (Burns and Edil, 2012).

1.2. Pathological characteristics and WHO classification of selected pancreatic tumours

1.2.1. Characteristics of PDAC

PDAC is mostly (60–70% of cases) localised in the head of the pancreas (Klöppel et al., 2000; Hruban et al., 2009; Masugi et al., 2010). Pancreatic body and tail are affected in 15 to 31% of cases (Hruban et al., 2009; Masugi et al., 2010). According to Yoon *et al.*, 79.2% of tumours are localised in the pancreatic head and the neck, 14.0% in the body and 6.7% in the tail (Yoon et al., 2011). Multiple tumours are found in 20% of cases (Rosai, 2004).

Grossly, PDAC is a dense, poorly defined, solid mass, which often obstructs the distal common bile duct and pancreatic duct resulting in more proximal dilatation. The cut surface of PDAC is white or yellowish. Microcystic areas can be observed, but necrosis and haemorrhage are rarely found. Most frequently, at the time of diagnosis the tumour size varies from 1.5 to 5 cm with an average size of 2.5–3.5 cm. Among potentially radically operated PDAC cases, the median size of tumour is 3 cm (Lim et al., 2003; Handra-Luca et al., 2011). The tumours located in the pancreatic body and tail frequently attain a larger size (Klöppel et al., 2000; Hruban et al., 2009).

Microscopically, PDAC is a gland-forming tumour. The glandular shape is usually round or oval and slightly angulated. The glands are lined by a single layer of cubic or cylindrical epithelium. Epithelial layers also can be multiple. Malignant epithelium may grow in papillary and cribriform structures. Presence of mucin is typical (Hruban et al., 2009; Smyrk, 2009). The nuclei of malignant cells show pleomorphism, hyperchromasia, loss of polarity and prominent nucleoli (Hruban et al., 2009). Typically, the ‘inalienable’ component of PDAC is dense stromal fibrosis (desmoplastic stroma), formed by myofibroblasts and over-production of collagen (Smyrk, 2009). Comparison of normal pancreatic parenchyma and PDAC is presented in Figure 1.2.

Figure 1.2. Comparison of normal pancreatic parenchyma and pancreatic ductal adenocarcinoma. A, normal pancreatic parenchyma and ducts (arrows); B, pancreatic ductal adenocarcinoma displaying wide desmoplastic stroma. Haematoxylin and eosin (HE), original magnification 100x.

The properties of malignant glands and neoplastic cells disclose the histological tumour differentiation grades. Regarding PDAC, four histological grades are distinguished: well (G1), moderately (G2) and poorly (G3) differentiated as well as undifferentiated (G4) carcinomas (Klöppel et al., 2000; Guzman and Chejfec, 2007; Edge et al., 2010). The different grades of PDAC and a contrasting normal duct are shown in Figure 1.3.

Well differentiated tumours are characterised by large malignant glands. Between these, there are smaller, mid-size glandular structures (Klöppel et al., 2000), which constitute more than 95% of the neoplastic epithelial component as presented in Figure 1.4. (Guzman and Chejfec, 2007). Typically the glands are tubular or cribriform, occasionally containing small pseudopapillary groups of neoplastic cells without fibrovascular stalk. Mitotic figures are rarely found. Normal pancreatic ducts and areas of parenchyma are seen between the invading malignant glands (Klöppel et al., 2000). Sometimes it is difficult to distinguish well differentiated malignant glands from non-neoplastic ducts. In the following situations, it is necessary to carefully examine the structure of the cells and nuclei. Malignant cells frequently produce mucus and have eosinophilic, light or even clear cytoplasm. The malignant cells can also have a prominent cylindrical shape and larger size than non-malignant epithelium. The nuclei of malignant cells are slightly larger, elongated and have prominent nucleoli (Klöppel et al., 2000). Among all PDACs, G1 adenocarcinoma constitutes 55/ 347 cases of PDAC, corresponding to (recalculated) a proportion of 15.9% (Lim et al., 2003). In another

study, G1 PDAC comprised 19/ 59 cases corresponding to 32.2% of all PDAC cases involved in the study (Dong et al., 2005).

Moderately differentiated PDACs consist of medium-sized tubular glands with variable shape. Incompletely developed glandular structures can be found (Klöppel et al., 2000), but lumen-containing malignant glands represent between 50% and 95% of the neoplastic epithelial component as depicted in Figure 1.5. (Guzman and Chejfec, 2007). G2 carcinomas display greater variations in the nuclear shape and chromatin structure. Mitoses are evident but not ubiquitous. The amount of markedly desmoplastic stroma increases in comparison with G1 PDAC. Mucus production is decreased (Klöppel et al., 2000). Moderately differentiated PDAC has been identified in 21/ 59 cases, corresponding to 35.6% (Dong et al., 2005) or 178/ 347 cases, after recalculation 51.3% of all PDAC cases (Lim et al., 2003).

Poorly differentiated PDACs are characterised by small, irregularly shaped glandular structures that frequently lack lumen and form solid cell groups (Klöppel et al., 2000). In poorly differentiated tumours, glandular complexes constitute only 5–50% of the cancer (Guzman and Chejfec, 2007). Although individual cells can be squamoid (Figure 1.6.), spindled, or anaplastic, these components should not exceed 20% of the tumour (Klöppel et al., 2000). In several studies, the frequency of G3 PDAC varied from 25% to even 44.1% (originally, 157/ 356) of cases (Lim et al., 2003; Dong et al., 2005; Masugi et al., 2010; Handra-Luca et al., 2011).

Undifferentiated PDAC consists mainly of unstructured groups of malignant cells. Fewer than 5% of all tumour cells are arranged into ducts or glands (Guzman and Chejfec, 2007).

Figure 1.3. Normal pancreatic duct with cuboidal epithelium and round nuclei. Haematoxylin and eosin (HE), original magnification 400x.

Figure 1.4. Well differentiated pancreatic ductal adenocarcinoma. Haematoxylin and eosin (HE), original magnification 50x.

Figure 1.5. Moderately differentiated ductal adenocarcinoma. Haematoxylin and eosin (HE), original magnification 100x.

Figure 1.6. Poorly differentiated ductal adenocarcinoma with some squamoid cells (inset). Haematoxylin and eosin (HE), original magnification 100x.

In the grading system of PDAC according to WHO classification, it is recommended to assess tumour differentiation considering several criteria simultaneously: glandular differentiation, mucus production, mitotic count and pleomorphic nuclei. G1, G2 and G3 criteria are shown in Table 1.1. (Klöppel et al., 2000).

Table 1.1.

Histopathological grading of pancreatic ductal adenocarcinoma¹

Tumour grade	Glandular differentiation	Mucin production	Mitoses per 10 HPF	Nuclear features
Grade 1	Well differentiated	Abundant	≤ 5	Little polymorphism, polar arrangement
Grade 2	Moderately differentiated	Irregular	6–10	Moderate polymorphism
Grade 3	Poorly differentiated	Abortive	> 10	Marked polymorphism and increased cell size

¹By Klöppel et al., 2000

Abbreviation in the table: HPF, high power field

Some less common histological types of PDAC include adenosquamous carcinoma, undifferentiated (anaplastic) carcinoma, undifferentiated carcinoma with osteoclast-like giant cells, mucinous non-cystic carcinoma and signet-ring cell carcinoma.

Adenosquamous carcinoma is diagnosed in 3–4% of cases. The main feature is the presence of malignant squamous component accounting for at least 30% of the tumour (Klöppel et al., 2000).

The frequency of undifferentiated (anaplastic) carcinoma (synonyms: sarcomatoid carcinoma, giant cell carcinoma, pleomorphic large cell carcinoma) is 2–7%. The carcinoma is characterised by large, pleomorphic eosinophilic cells and/ or spindled cell component. The neoplastic cells are non-cohesive, and they are supported by scanty fibrous stroma. As a whole, the tumour contains few atypical glands and is characterised by other aggressive features such as high mitotic activity and frequent perineural, intraneural, vascular and lymphovascular invasion (Klöppel et al., 2000).

Undifferentiated carcinoma with osteoclast-like giant cells is a rare tumour type composed of pleomorphic to spindle-shaped cells and scattered non-neoplastic osteoclast-like giant cells. These giant cells usually contain more than 20 uniform small nuclei. Both *in situ* component and invasive carcinoma are frequently observed. Osteoid or unmineralised bone can also develop (Klöppel et al., 2000; Hruban et al., 2009).

Mucinous non-cystic carcinoma (synonyms: colloid or gelatinous carcinoma) is observed in 1–3% of cases. The characteristic feature of this tumour is mucinous component that comprises at least 50% of tumour. The cells are usually well differentiated, cubic and form clumps or strands (Klöppel et al., 2000). This PDAC type is characterised by intestinal immunophenotype (Smyrk, 2009).

Signet-ring cell carcinoma is a very rare type of PDAC which features intracellular mucus production. The prognosis is very poor. Metastatic gastric, colorectal or breast cancer must be excluded (Klöppel et al., 2000; Hruban et al., 2009; Smyrk, 2009).

In PDAC-affected pancreas, neoplastic changes in the ducts are occasionally observed. This lesion, called PanIN, represents the precursor to PDAC. PanIN lesion affects the small terminal (<5 mm) pancreatic ducts. PanIN development is classified into 3 levels depending on the degree of anaplasia (Figure 1.7.). PanIN-1 lesion is characterised by the change of normal ductal epithelium to high columnar epithelium with intracellular mucin. There are two grades of PanIN-1. In PanIN-1A, the epithelial layer is flat, but in PanIN-1B the epithelium is papillary. The epithelial changes in PanIN-2 feature notable cytological atypia and nuclear density. PanIN-3 lesion displays high nuclear density and atypia, pseudopapillary growth, mitotic figures and intraluminal necrosis (Takaori et al., 2006; Macgregor-Das and Iacobuzio-Donahue,

2013). Due to the diverse cellular changes, PanIN-3 is considered to be a carcinoma *in situ* that progresses into invasive adenocarcinoma (Takaori et al., 2006).

Figure 1.7. Pancreatic intraepithelial neoplasia (PanIN). A, PanIN-1A; B, PanIN-2; C, PanIN-3. Haematoxylin and eosin (HE), original magnification 400x.

1.2.2. Characteristics of PETs

Localisation of PET within the pancreas mostly depends on its subtype. In general, about 39.9–48.9% of PETs develop in the head of the pancreas, 23.1% in the body, 35.8% affect pancreatic body and tail and 1.8% invade the whole organ involving the pancreatic head, body and tail (Bettini et al., 2008; Rindi et al., 2012). Multiple PETs occur in 5.1% of cases (Rindi et al., 2012).

Grossly, PET usually is a solitary, sharply delimited mass with pushing borders and a fibrous capsule (Heitz et al., 2004; Hruban et al., 2009). The colour is yellowish-white or brownish-pink. Cystic changes can rarely be found (Heitz et al., 2004). The density of tumour is variable (Hruban et al., 2009). The median size varies in studies from 3.0 to 4.6 cm with size ranging from 0.6–13.5 cm (Bettini et al., 2008; Ekeblad et al., 2012; Rindi et al., 2012). Functional PETs are usually smaller than non-functional tumours which mostly are larger than 2 cm and frequently reach 5 cm (Heitz et al., 2004). PETs exceeding the size of 3 cm are mostly malignant (Heitz et al., 2004). The signs of malignant behaviour also include grossly infiltrative growth as well as widespread haemorrhages and necrosis (Hruban et al., 2009). Interestingly, the tumours in males are statistically significantly larger than in females (Rindi et al., 2012).

Microscopically PETs are quite well recognisable because mostly these are well differentiated tumours with characteristic albeit variable morphological growth patterns. The architecture can display solid, trabecular, gyriform, glandular, tubuloacinar or perivascular pseudorosette patterns (Heitz et al., 2004; Hruban et al., 2009). A normal pancreatic islet and PETs showing different morphological patterns are presented in

Figures 1.8.–1.11. The PET cells are small or of middle size, relatively uniform with finely granular eosinophilic cytoplasm. The nucleus is round or oval and centrally placed with potentially recognisable nucleolus (Heitz et al., 2004; Hruban et al., 2009). Occasionally, less typical morphological traits are found in PETs, for example, mucus secretion, black pigmentation, vacuolated (lipid-filled) cells, clear cells, oncocytes or rhabdoid features (Heitz et al., 2004; Stricker and Kumar, 2010). Poorly differentiated tumours are characterised by pleomorphic cells, hyperchromatic nuclei and invisible nucleoli, nuclear wrapping around one another, distinctly increased nuclear-to-cytoplasmic ratio and high mitotic activity. In addition, a solid growth pattern is characteristic (Heitz et al., 2004; Hruban et al., 2009). The amount of tumour stroma and fibrosis varies between various PETs and also within a single tumour. Assessment of the specific morphological pattern and the amount of fibrosis occasionally allow predicting PET subtype but these morphological features definitely cannot be used as the basis of final diagnosis. Only the presence of amyloid deposits may be accepted as a reliable feature of insulinoma, and psammoma bodies as a sign of somatostatinoma (Heitz et al., 2004). Currently, the morphological diagnostics of PETs can be facilitated via immunohistochemistry with the following neuroendocrine markers applications: synaptophysin, CD57, CD56, neuron specific enolase and chromogranins (Heitz et al., 2004; Hruban et al., 2009). The neuroendocrine differentiation must exceed 50% of the tumour to distinguish it from mixed ductal-endocrine carcinoma showing neuroendocrine differentiation in a third to half of tumour cells (Klöppel et al., 2000) and is usually diffuse (Bellizzi and Stelow, 2009).

Figure 1.8. Normal pancreatic parenchyma with an islet of Langerhans (arrows). Haematoxylin and eosin (HE), original magnification 100x.

Figure 1.9. Pancreatic endocrine tumour displaying trabecular growth pattern. Haematoxylin and eosin (HE), original magnification 100x.

Figure 1.10. Pancreatic endocrine tumour showing solid pattern. Haematoxylin and eosin (HE), original magnification 50x.

Figure 1.11. Pancreatic endocrine tumour with pronounced hyalinosis. Haematoxylin and eosin (HE), original magnification 50x.

As previously stated, well differentiated PETs are characterised by a specific morphological pattern, but poorly differentiated PETs can be difficult to recognise initially as endocrine tumours (Heitz et al., 2004). Males more often have poorly differentiated endocrine carcinomas than females (Hruban et al., 2009).

The exact evaluation of PETs differentiation and behaviour is described in the criteria of the clinico-pathological classification of PET.

1. Well differentiated endocrine tumours (WDET)

1.1. Benign behaviour: confined to the pancreas, non-angioinvasive, no perineural invasion, < 2 cm in diameter, < 2 mitosis/ 10 high power field (HPF) and < 2% Ki-67 positive cells.

1.2. Uncertain behaviour: confined to the pancreas and one or more of the following features: ≥ 2 cm in diameter, 2–10 mitosis/ 10 HPF, > 2% Ki-67 positive cells, angioinvasion, perineural invasion.

2. Well differentiated endocrine carcinoma (WDEC): low grade malignant tumour showing gross local invasion and/ or metastases.

3. Poorly differentiated endocrine carcinoma (PDEC): high grade malignant tumour, > 10 mitosis/ 10 HPF (Heitz et al., 2004).

Among all PETs, frequency of WDET varies between 26–46% of cases. Tumours with uncertain behaviour are more common than PETs with benign behaviour. WDEC represents 47.2–66% of PETs and PDEC comprises about 6.8–10% of PET cases (Bettini et al., 2008; Scarpa et al., 2010; Ekeblad et al., 2012; Rindi et al., 2012).

The most common functional PET subtype is insulinoma. Malignant change is observed in 5–10% of insulinoma cases. Among the remaining less common subtypes,

malignant tumours represent more than 50% of cases. Among non-functioning tumours, more than 50% are malignant (Halfdanarson et al., 2008).

1.3. Tumour growth and spread

1.3.1. pTNM classification and staging

pTNM classification data have the most potent prognostic value, regarding almost all malignant tumours. pTNM classification is based on the tumour anatomical spread, taking into account the following parameters: local spread of the primary tumour, characterised by the size and specified local invasion (pT parameter), regional lymph node status regarding metastases (pN parameter) and presence or absence of distant metastasis which represents pM parameter (Edge et al., 2010). In the 7th edition of TNM classification (2010), the same pTNM system is used for both exocrine and endocrine pancreatic tumours as described below.

pT parameter:

- pTX – Primary tumour cannot be assessed
- pT0 – No evidence of primary tumour
- pTis – Carcinoma *in situ*, including PanIN-3
- pT1 – Tumour limited to the pancreas, 2 cm or less in largest dimension
- pT2 – Tumour limited to the pancreas, more than 2 cm in largest dimension
- pT3 – Tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery
- pT4 – Tumour involves the celiac axis or the superior mesenteric artery (unresectable primary tumour)

pN parameter:

- pNX – Regional lymph nodes cannot be assessed
- pN0 – No regional lymph node metastasis
- pN1 – Regional lymph node metastasis is present

pM parameter:

- pM0 – No distant metastasis
- pM1 – Distant metastasis is present (Edge et al., 2010)

The pTNM characteristics determine the stage of tumour as shown in Table 1.2.

Table 1.2.

Anatomic staging of pancreatic tumours¹

Tumour stage	pT parameter	pN parameter	pM parameter
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage III	T4	any N	M0
Stage IV	any T	any N	M1

¹ By Edge et al., 2010

The previously reported distribution of PDAC as well as PET cases by tumour stage is described in Table 1.3.

Table 1.3.

The distribution of PDAC and PET by tumour stage

Tumour stage	Frequency, %	
	PDAC	PET
IA	8.8	29.5
IB	11.0	15.7
IIA	17.9	7.9
IIB	36.4	13.8
III	13.2	5.3
IV	12.7	27.8
Reference	(Bilimoria et al., 2007)	(Rindi et al., 2012)

Abbreviations in the Table: PDAC, pancreatic ductal adenocarcinoma; PET, pancreatic endocrine tumour.

Along with the pTNM classification, two additional parameters can be specified by morphologic examination, namely G (pG only) and pR characteristics. pG reflects the tumour grade as explained previously. It is defined differently for PDAC and for PET. The pR represents resection margin (RM) status assessed by the following: pR0, negative RM – complete tumour resection with grossly and microscopically negative RMs; pR1, RM grossly negative but microscopically positive for tumour presence; pR2, grossly and microscopically positive RM; pRx, RM cannot be assessed (Edge et al., 2010). R1 status after potentially radical surgical treatment of PDAC is observed in 27–35% (Masugi et al., 2010; Handra-Luca et al., 2011; Sugiura et al., 2013). In some

studies, considering patients who underwent pancreatoduodenectomy, R1 status is observed even in 65.9% cases (Frampton et al., 2013). Regarding PETs, the RM are negative in 68% of cases after potentially radical operation (Pomianowska et al., 2010).

The specific features of tumour growth and spread regarding each tumour type (PDAC and PET) are described in the following sections.

1.3.2. Growth and spread of PDAC

Rarely, PDAC at the time of diagnosis is limited within the pancreas with no invasion into other organs and tissues. Handra-Luca *et al.* has reported 11 pT1 and pT2 *versus* 336 pT3 and 9 pT4 tumours resulting in the following frequency distribution: pT1 and pT2 comprised 3.1% *versus* pT3 with 94.4% and pT4 with 2.5% of cases (Handra-Luca et al., 2011). Retroperitoneal adipose tissues represent the most commonly invaded target including both direct and the characteristic perineural and intraneural spread (Klöppel et al., 2000; Rosai, 2004). The rate of perineural invasion ranges from 90% up to 95% of cases (Rosai, 2004; Masugi et al., 2010). The tumours located in the pancreatic head most frequently invade the duodenum (25% of all PDAC in the head of pancreas), distal common bile duct and ampulla of *Vater* (Rosai, 2004; Hruban et al., 2009). Tumours localised in the body and the tail often have invaded the spleen, stomach, left suprarenal gland, colon and peritoneum; such spread is facilitated by frequently delayed diagnosis (Klöppel et al., 2000).

Invasion in lymphatic ducts is a very common feature in PDAC (Figure 1.12.), observed in up to 96% of resected PDACs. The lymphatic spread results in the following metastases in lymph nodes (Klöppel et al., 2000; Masugi et al., 2010). Microscopic metastases (Figure 1.13.) are found in 75% of pT1 and pT2 cases (Rosai, 2004).

Figure 1.12. Multiple cancer cell groups of pancreatic ductal adenocarcinoma in a lymphatic duct. Haematoxylin and eosin (HE), original magnification 100x.

Figure 1.13. Micrometastasis (arrow) of pancreatic ductal adenocarcinoma in a lymph node. Haematoxylin and eosin (HE), original magnification 100x.

Among the studies analysing potentially curative PDAC resection, frequency of pN1 status varies from 48.7% (recalculated from the original data: pN1 – 193 cases and pN0 – 203 cases) to 80.0% or even up to 85.7% of the cases with the number of metastatic lymph nodes ranging 1–25 and the median number of metastatic lymph nodes reaching 3 (Lim et al., 2003; Masugi et al., 2010; Handra-Luca et al., 2011). In order to assess lymph node status accurately, it is recommended to retrieve at least 12 lymph nodes (Edge et al., 2010). The metastases from pancreatic head cancer most frequently affect the posterior pancreaticoduodenal and the superior pancreatic head lymph nodes, followed by the inferior head and the superior body lymph nodes, as well as the anterior pancreaticoduodenal and the inferior body lymph nodes. Distant lymph node metastases are found in the lymph nodes located in the *ligamentum hepatoduodenale*, at the coeliac trunk and at the root of the superior mesenteric artery as well as in the paraaortic nodes at the level of the renal arteries. If PDAC is situated in the pancreatic body or tail, the superior and inferior body lymph nodes are affected most frequently followed by the tail lymph nodes and the lymph nodes located in the splenic hilus. Less commonly, the PDAC has spread to pleura and lung via lymphatic channels (Klöppel et al., 2000).

Tumour invasion in the blood vessels as well as haematogenous metastases are not a rare finding at the time of PDAC diagnosis (Klöppel et al., 2000). For example, PDAC invasion in the blood vessels has been observed in 176 cases *versus* 170 cases without invasion corresponding to 50.9% *versus* 49.1% (Handra-Luca et al., 2011). In contrast, invasion only in the large blood vessels has been observed in 35% of cases

(Masugi et al., 2010). Haematogenous metastases develop in following frequency sequence: liver $\leq 80\%$ (Figure 1.14.), peritoneum 60%, lungs and pleura 50–70% and suprarenal glands 25%. Less frequently, kidneys, bones, brain and skin are affected (Klöppel et al., 2000; Hruban et al., 2009).

Figure 1.14. Metastasis of pancreatic ductal adenocarcinoma in the liver. The hepatic parenchyma has been highlighted by a star. Haematoxylin and eosin (HE), original magnification 100x.

1.3.3. Growth and spread of PETs

As mentioned, well differentiated PETs are usually a circumscribed mass enclosed by a capsule. Malignant PETs are characterised by invasion into adjacent tissue, blood and lymph vessels as well as by perineural growth (Hruban et al., 2009).

PET mostly invades peripancreatic adipose tissues followed (according to the frequency sequence) by duodenum (Figure 1.15.) and spleen (DeLellis et al., 2004). If the tumour size is $\leq 2\text{cm}$, tumour invasion in adjacent pancreatic tissues is observed in 8% of cases. Analogous invasion is present in 32% of tumours between $> 2\text{cm}$ and $\leq 4\text{cm}$. Tumours larger than 4 cm possess this invasion in 85% of cases (Scarpa et al., 2010). Perineural invasion (Figure 1.16.) can be found in 4/ 33 (12.1%) cases (Chang et al., 2007).

Figure 1.15. Pancreatic endocrine tumour invasion (arrows) in the duodenal mucosa. Haematoxylin and eosin (HE), original magnification 50x.

Figure 1.16. Perineural invasion (arrows) of pancreatic endocrine tumour. Haematoxylin and eosin (HE), original magnification 100x.

The first metastases of PET are usually found in regional lymph nodes, respectively in the peripancreatic, celiac and periaortic nodes (Klöppel et al., 2004; DeLellis et al., 2004). Positive lymph nodes at the time of diagnosis are present in 18.8–47.5% patients (Bettini et al., 2008; Rindi et al., 2012). Among non-functioning tumours, pN1 is found in 30% of cases (Partelli et al., 2013).

Tumour angioinvasion can be observed in 9/ 33 (27%) cases of PETs (Chang et al., 2007). Most frequently, distant metastases develop in the liver (Klöppel et al., 2004; DeLellis et al., 2004). Peritoneal dissemination is less frequent followed by metastases in lungs, adrenal glands and bones (Rosa et al., 2010). In 32.5% (13/ 40) of all PET cases, distant metastases have already developed at the time of diagnosis (Chang et al., 2007). Regarding non-functioning tumours, extranodal metastases are observed in 32.7% of patients at the time of diagnosis (Bettini et al., 2008).

1.4. Tumour profiling by immunohistochemistry

1.4.1. The general characteristics of selected immunohistochemical markers

Immunohistochemistry (IHC) is a tissue evaluation method for diagnostic or research purposes. The technology is based on the application of specific antibodies to search for the target antigen in the tissue sections that are subsequently analysed microscopically to detect deposits of coloured substance identifying the presence and location of the researched antigen.

The following description of IHC markers will include proteins that are involved in the cardinal features of carcinogenesis – cell proliferation, evasion of apoptosis, differentiation along several lines, loss of adhesion and epithelial-mesenchymal transition. Thus, the analysis of cell cycle markers and cell cycle regulatory proteins as well as proteins involved in apoptosis, cell adhesion and epithelial-mesenchymal transition will be provided. Proteins characterising differentiation of epithelial tumours will be described. Immunohistochemical evaluation of angiogenesis will be analysed as well.

Ki-67 is a non-histone nucleoprotein. It ranks among the most important nuclear proteins that indicate active cellular proliferation. Estimates of Ki-67 expressing cells (as percentage of the total cell population) allow for determining growth fraction *sive* proliferation fraction of the particular cell type. Expression of Ki-67 is high in the Gap 2 (G₂) phase of cell cycle as well as in mitosis (M), especially at metaphase (Scholzen and Gerdes, 2000; Hu et al., 2012). Ki-67 expression is also noted in Gap 1 (G₁), synthesis (S) and the other phases of M not limited to the metaphase. It is absent in resting cells that have entered Gap 0 (G₀) or the resting phase (Hu et al., 2012). Although it is considered that cells expressing Ki-67 will divide or are dividing already, some cells can stop the active cell cycle and enter the resting phase. Ki-67 expression is observed in both normal and neoplastic cells regardless of cell type (Scholzen and Gerdes, 2000). In malignant tumours, the proliferation fraction by Ki-67 characterises the rate of tumour growth, progression and spread, the expected efficacy of chemotherapy in a specific setting regarding particular tumour (Luporsi et al., 2012), and the outcome in general (Luporsi et al., 2012), as well as after treatment, e.g. regarding postradiation recurrence of adenocarcinomas (Scholzen and Gerdes, 2000). Thus, it represents both a significant prognostic and predictive factor.

p53 is a nuclear protein coded by tumour suppressor gene *TP53*. Protein p53 accumulates in cells as a response to DNA damage, activation of oncogenes, or hypoxia. Ultimately, apoptosis, cell cycle arrest, modulation of autophagy or senescence occurs (Rosai, 2004; Zilfou and Lowe, 2009; Stricker and Kumar, 2010). Maintaining the genome integrity could be considered the biological function of p53: thus, p53 can arrest cell cycle until complete DNA repair before the replication can be resumed, or induce senescence or cell apoptosis if the DNA damage cannot be fixed (Lenos and Jochemsen, 2011). P53 can also directly affect the DNA repair process by modulating the expression of certain DNA repair genes (Lenos and Jochemsen, 2011). In tumours,

p53 suppresses angiogenesis interfering with hypoxia-induced angiogenesis, inhibiting production of proangiogenic factors and stimulating the synthesis of endogenous angiogenesis inhibitors (Teodoro et al., 2007). ‘The genome guardian’ p53 also counteracts the metabolic switch towards aerobic glycolysis, a process during which tumour cells start to obtain the energy from glycolysis instead of oxidative phosphorylation (Gottlieb and Vousden, 2010).

After p53 has been activated, the further mechanism depends on the cell type, the microenvironment as well as on the particular pathogenetic details of the oncogenesis of each tumour (Zilfou and Lowe, 2009). p53 pathway activation can develop from any of the three following mechanisms. One of them is stress-induced stabilisation of p53. It evolves by inhibiting the ability of mouse double minute 2 homolog protein MDM2 to ubiquitinate. This, in turn, results in decreased degradation of p53. The second mechanism is release of p53 from the MDM2 and MDMx-mediated inhibition. The third mechanism includes activation of specific gene promoters. It is facilitated by the recruitment of molecular cofactors interacting with p53 (Zilfou and Lowe, 2009; Brooks and Gu, 2010).

Generally, the outcomes of p53 activation are directed to cell cycle inhibition, as mentioned above. Cascades of downstream messengers are involved. For example, cell cycle arrest in the checkpoint G₁/ S is mainly achieved by activation of p21, but in the checkpoint G₂/ M is due to activation of 14-3-3 σ and involves cyclin B1/ CDK2 complex. Apoptosis is promoted by induction of pro-apoptotic gene transcription, for example, *BAX*, *FAS*, *p53AIP1*, *Killer/ DR5*, *PIG3* and others. Apoptosis is also activated by transcription-independent mechanisms, as p53 can facilitate release of pro-apoptotic factors by inducing mitochondrial outer membrane permeabilisation. Cell autophagy is induced by p53 transcriptionally activated damage-regulated autophagy modulator gene, which results in producing lysosomal proteins (Zilfou and Lowe, 2009; Stricker and Kumar, 2010). Activated p53 also induces the senescence. However, senescence in turn participates in the p53 inactivation with possible subsequent tumour progression (Zilfou and Lowe, 2009).

In tumours, the tumour suppression function of p53 can be lost by inactivation in protein level as well as by mutation in gene *TP53*. Mutant p53 proteins acquire various oncogenic characteristics that result in cellular growth as well as survival advantages (Rivlin et al., 2011). Accumulation of mutated p53 protein has been observed in 76% of 212 human malignant tumours (Bártek et al., 1991).

Breast cancer susceptibility gene 1 **BRCA1** is a tumour suppressor (Stricker and Kumar, 2010). BRCA1 is a nucleo-cytoplasmic protein that is involved in several nuclear processes, including DNA repair as well as chromatin remodelling, transcription and silencing. In the cytoplasm, BRCA1 protein participates in the activation of cellular apoptotic mechanisms (Starita and Parvin, 2003; Stricker and Kumar, 2010; Wang et al., 2013). Over-expression of BRCA1 is a co-activator for p53, and stabilises p53 (Starita and Parvin, 2003).

p21 and **p27** are cell cycle inhibitors which arrest the cell cycle by binding to cyclin/ cyclin dependent kinases (CDK) complexes (Stricker and Kumar, 2010). Cyclin/ CDK complexes promote cell cycle progress from G₁ to S phase via several different steps (Gartel and Tyner, 2002).

p21 belongs to the Cip/ Kip family of CDK inhibitors (Gartel and Tyner, 2002). As p21^{WAF1/ Cip1} is able to regulate the cell cycle by interaction with cyclin/ CDK complexes, increased expression can induce cell cycle arrest in G₁/ S, as well as G₂/ M checkpoints. However in low concentrations, p21 facilitates the assembly of cyclin D1 and CDKs. Activation of p21 can occur as the response to DNA damage as well as in physiological conditions, for example, during cell differentiation. p21 is expressed in senescent cells (Cazzalini et al., 2010). Induction of p21 can occur by p53 influence or independently of p53 (Dotto, 2000; Gartel and Tyner, 2002; Cazzalini et al., 2010; Stricker and Kumar, 2010). p21 also functions as the inhibitor of p53-dependent apoptosis. DNA damage or stabilisation of p53 can cause over-expression of p53 both with possible p21 activation and activation of pro-apoptotic mechanisms. However, the successive reactions can lead to over-production of p21 and cell cycle arrest, and at the same time the apoptotic processes can be omitted. Apoptosis induced by p53 usually proceeds successfully if there is a defect in p53-dependent activation of p21 or if p53-dependent induction of p21 transcription is suppressed (Gartel and Tyner, 2002). p53-independent activation of p21 also inhibits p53-dependent apoptosis. In these cases various signals act as the activators, for example, transforming growth factor- β (TGF- β), tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ) and histone deacetylase inhibitors. In tumours, some of these listed signals induce p53-independent apoptosis, but as the p21 is activated also, the final reaction is anti-apoptotic, which can be characterised as an unfavourable predictive factor, because the cancer cells are protected from anticancer drug induced apoptosis (Gartel and Tyner, 2002). Thus, inhibiting p21 expression in order to improve the effectiveness of chemotherapy or

tumour irradiation as previously suggested (Tian et al., 2000). However, complexity is added by the challenging observations that in some instances p21 actually promotes apoptosis. This can be due to enforced over-expression of p21 or the nature of apoptotic stimulus, e.g., cisplatin versus methotrexate (Kraljevic Pavelic et al., 2008). The subcellular localisation of p21 (cytoplasmic *versus* nuclear), and the degree of DNA damage can also influence the pro- or antiapoptotic action (Cazzalini et al., 2010).

The functions of p21 are not limited to inactivation of cell cycle in G₁ phase. p21 directly prevents DNA replication by binding to the proliferating cell nuclear antigen (PCNA). Interaction between p21 and p50 may contribute to the inhibition of DNA synthesis; hypothetically p50 and other proteins can facilitate the interaction between p21 and PCNA (Dotto, 2000; Gartel and Tyner, 2002; Cazzalini et al., 2010). p21 takes part in the autophagy and transcription regulation. Regarding participation of p21 in DNA repair, the necessity for further studies has been acknowledged (Cazzalini et al., 2010).

p27 is a cell cycle inhibitor and growth suppressor (Lee and Kim, 2009). The level of p27 is controlled by transcriptional, translational and post-translational mechanisms as well as by postphosphorylation and polyubiquitination of proteins (Lee and Kim, 2009). TGF- β and other environmental factors increase the level of p27 resulting in cell cycle arrest in G₁ phase. As CDK together with cyclin are a positive regulator of cell cycle progression, inhibitory function occurs via p27 binding to the cyclin E/ CDK2 complex or cyclin D/ CDK 4 or CDK6 complexes. Some scientists have proved dual results of p27 binding to CDK4: p27 can be both a CDK4 bound inhibitor, and a CDK4 bound non-inhibitor through p27 conversion by tyrosine phosphorylation under various growth conditions. Regarding tumours, p27 can be considered a tumour suppressor because of its growth inhibitory function. Mutations of the respective gene are rare but p27 protein can undergo inactivation due to impaired synthesis, phosphorylation or accelerated proteolytic degradation due to deranged post-translational modification (Chu et al., 2008). Using IHC, p27 protein is found in the nucleus. Oncogene activation and tumour progression is characterised by decreased p27 expression (Sherr and Roberts, 1999; Sgambato et al., 2000; Lee and Kim, 2009; Stricker and Kumar, 2010; Wander et al., 2011). Cytoplasmic p27 trans-localisation is characterised by cell cycle-independent oncogenic function, which contributes to cancer cell invasion (Wander et al., 2011). p27 trans-localisation develops through p27 interaction with the nuclear export protein chromosome region maintenance 1 protein

CRM1. This interaction also leads to displacement of cyclin D1 from CRM1, resulting in increased nuclear level of cyclin D1 and progression through the cell cycle (Lee and Kim, 2009).

Cyclin D1 is a cell cycle regulatory protein. It can also perform a proto-oncogene function (Tashiro et al., 2007; Shan et al., 2009; Stamatakis et al., 2010). In normal cells, production of cyclin D1 can be induced by cell type-dependent growth factors, such as epithelial growth factor, insulin-like growth factor (IGF) 1 (IGF-1), IGF-2, amino acids and lysophosphatidic acid, androgens, retinoic, gastrin and others (Fu et al., 2004). Cyclin D1 along with its cyclin dependent kinases CDK4 and CDK6 induce phosphorylation of tumour suppressor: retinoblastoma gene protein (pRB). This, in turn, results in pRB inactivation and separating from complex of pRB and E2F transcription factors. The process continues by initiation of DNA synthesis, i.e., G₁ transforms in S phase (Tashiro et al., 2007; Stamatakis et al., 2010). Cyclin D1 has a short half-life, approximately 20 min (Shan et al., 2009). Using IHC, nuclear cyclin D1 expression is observed when cells are in G₁ cell cycle phase, but in DNA-replicating cells cyclin D1 is undetectable (Lukas et al., 1994). The degradation of cyclin D1 is regulated by ubiquitin proteasome mechanism (Stamatakis et al., 2010).

Cyclin D1 acquires the proto-oncogene functions through several mechanisms, such as chromosomal translocation, gene amplification, disruption of normal intercellular trafficking and proteolysis. The result is characterised by increased stability of cyclin D1 (the half-life is longer than normal) and accumulation of cyclin D1 in cells (Shan et al., 2009; Stamatakis et al., 2010). Additionally, in malignant cells cyclin D1 relates to invasive and metastatic potential and resistance to apoptosis. It also has an impact on tumour angiogenesis through various activating signals (Tashiro et al., 2007; Stamatakis et al., 2010).

Bcl-2 is one of the anti-apoptotic proteins from BCL-2 family (Hamacher et al., 2008; Chipuk et al., 2010). BCL-2 family of pro- and anti-apoptotic proteins regulates mitochondrial membrane integrity, endoplasmic reticulum calcium stores, as well as the process of mitochondrial dynamics, apoptosis and autophagy (Chipuk et al., 2010). Bcl-2 is located on the cytoplasmic face of the mitochondrial outer membrane, at the endoplasmic reticulum membrane and the nuclear envelope and may reveal membrane damage (Westphal and Kalthoff, 2003). Bcl-2 regulates apoptosis by mediating cytosolic release of cytochrome C from mitochondria in response to cellular stress

(Hamacher et al., 2008). Over-expression of the Bcl-2 protein inhibits apoptosis having no impact on cell proliferation (Dong et al., 2005).

E-cadherin is a transmembrane glycoprotein, acting as a cell-cell adhesion molecule. It is located on basolateral membranes in the desmosomes of epithelial cells ensuring the apico-basal polarity (Rosai, 2004; Schmalhofer et al., 2009; Stricker and Kumar, 2010). The extracellular part of E-cadherin provides adhesion with other cells, but the intracellular part collaborates with catenins and various regulatory proteins (Schmalhofer et al., 2009; Tian et al., 2011). In carcinogenesis, broken E-cadherin/ β -catenin complex results in decreased cell-cell adhesion and increased ability for cell migration and invasion (Cai et al., 2014; Li, Song et al., 2014; Techasen et al., 2014). Targeting E-cadherin by chemotherapy, e.g., combination of cisplatin and metformin can inhibit migration and invasion of cancer cells (Sun et al., 2014). The loss of E-cadherin and cell-cell adhesion also induce the epithelial-mesenchymal transition (EMT) through β -catenin interaction with TGF- β 1 (Schmalhofer et al., 2009; Tian et al., 2011) and various additional transcription factors, resulting in tumour progression independently of β -catenin (Canel et al., 2013). The known causes of deranged E-cadherin/ β -catenin pathway dysfunction include *CDH1* gene (coding E-cadherin) mutation and promoter hypermethylation, activating mutations of *CTNNB1* gene (coding β -catenin) or inactivating mutations of *adenomatous polyposis coli* gene *APC* or *AXIN1* (Tian et al., 2011).

E-cadherin expression in carcinomas not only correlates inversely with tumour progression but also with the development of metastases (Heimann et al., 2000; Tian et al., 2011; Cai et al., 2014; Techasen et al., 2014; Zhai et al., 2014). However, experiments with cell fusion in cultures yields controversial data (Li, Feng et al., 2014).

CD44 is an integral cell surface glycoprotein, type I transmembrane adhesion molecule (Ringel et al., 2001; Jaggupilli and Elkord, 2012), which controls the interaction between cells and between cell and extracellular molecules, including collagen, fibronectin, laminin, sulphate, heparin and proteoglycans. The CD44-mediated adhesion is relatively weak compared with other mechanisms, such as cadherin-determined adhesion (Sneath and Mangham, 1998). The other functions of CD44, regarding both normal and malignant cells, are related to cell survival, growth control, differentiation, hyaluronate degradation and motility. CD44 is related also to angiogenesis and release of cytokines and chemokines (Sneath and Mangham, 1998; Ringel et al., 2001; Jaggupilli and Elkord, 2012; Sante et al., 2013). CD44 has standard

isoform (CD44s) and 9 variant isoforms (CD44_{v2} – CD44_{v10}). The isoforms develop during the transcription resulting from alternative splicing – the splicing together of variant exons to form mRNA of an isoform (Sneath and Mangham, 1998; Jaggupilli and Elkord, 2012; Sante et al., 2013). The expression of isoform is strongly associated with tissue type, the period of development and growth condition (Sante et al., 2013). Among normal tissue, CD44 is expressed in hematopoietic cells, central nervous system, epithelial cells (epidermis, lung, pancreas, liver), fibroblasts and skeletal muscle (Flanagan et al., 1989; Sneath and Mangham, 1998; Stamenkovic et al., 1989; Rodig et al., 2008). In epithelial tissue, the CD44 expression can vary between cells and layers (Sneath and Mangham, 1998).

The significance of CD44 expression within tumours is variable. Depending on histogenesis, CD44 expression can be maintained from normal cells, or obtained during neoplastic processes, or associated with increased ability to metastasise, or with cancer stem cell status (Sneath and Mangham, 1998; Jaggupilli and Elkord, 2012). Along with CD24, CD133, CD166 and EpCAM, CD44 is one of the cancer stem cell markers in different tumours, including lung, breast, ovarian, prostate, renal, colorectal and pancreatic carcinomas. The cancer stem cells are characterised by enhanced metastatic ability and resistance to therapy. The prognostic value of stem cell markers is still being investigated (Jaggupilli and Elkord, 2012). Interactions between CD44 variants and their ligands can induce production of autocrine growth factors, thus enhancing tumour growth (Sneath and Mangham, 1998). Some studies showed that inactivation of CD44 correlates with metastatic behaviour, whereas other tumours are characterised by the opposite (Sante et al., 2013).

Cytokeratins are water-insoluble intermediate filaments which are found in epithelial cells. By the molecular weight (kilodalton, kDa) and isoelectric pH value, cytokeratins have been distributed into at least 20 well-defined subclasses (Rosai, 2004; Dabbs, 2014), expressed in various types of epithelium (Rosai, 2004). Since cytokeratins usually maintain their expression in malignant tumours and in metastases, these markers are valuable for detection of cell type (Moll et al., 2008). Within the cells, cytokeratins connect the nucleus with the cytoplasmic plaques of the desmosomes creeping through the cytoplasm. Thus, keratins are key factors in the structural integrity and mechanical stability of the epithelial tissues, both by single epithelial cells and by cell-to-cell contact (Moll et al., 2008). The functions of cytokeratins include

maintaining intracellular signalling pathways, epithelial polarity and membrane traffic, regulation of protein synthesis and cell size during wound healing (Moll et al., 2008).

Cytokeratin (CK) 7 is a 54 kDa protein, which belongs to the neutral-basic type B subfamily. CK 7 expression is seen mainly in simple, glandular and transitional epithelium and mesothelia (Moll et al., 1982; Dabbs, 2014). CK 7 is negative in most squamous epithelia, large intestinal epithelium, hepatocytes and prostatic acinar tissue (Dabbs, 2014). Usually CK 7 is observed in lung, breast, ovary, salivary glands, stomach, oesophagus, pancreatobiliary adenocarcinoma, the fibrolamellar variant of hepatocellular carcinoma, uterine endocervical adenocarcinomas, urothelial carcinoma and mesothelioma as well as in neuroendocrine tumors (Lin and Prichard, 2011; Dabbs, 2014).

CK 19 is a 40 kDa protein. It belongs to the acidic type of CKs. CK 19 expression has been observed in almost all epithelium types, including simple epithelia, squamous epithelium, intermediate and basal cells of transitional epithelium as well as luminal, basal and myoepithelial cells in a few complex epithelia. Thus CK 19 is a useful screening marker for epithelial neoplasias (Moll et al., 2008; Dabbs, 2014). Among carcinomas, strong and diffuse expression of CK 19 is observed in colorectal, gastric, pulmonary, ovarian and endometrial adenocarcinoma, PDAC, invasive ductal adenocarcinoma of breast, papillary renal cell carcinoma, malignant mesothelioma and Merkel cell carcinoma (Moll et al., 2008).

CK 20 is a 46 kDa protein, which belongs to the acidic subfamily. However, it is less acidic compared with other CKs of this group. CK 20 is expressed in gastric foveolar epithelium, small and large intestinal epithelium, therefore it is frequently interpreted as a marker of gastrointestinal origin. The expression of CK 20 is also observed in normal urothelium and some neuroendocrine cells, in particular Merkel cells of the skin (Moll et al., 2008). CK 20 expression is found in several adenocarcinomas, including colorectal and small bowel carcinoma, transitional cell and Merkel cell carcinomas and mucinous ovarian tumours. CK 20 can also be present in adenocarcinomas of the biliary system, oesophagus, stomach and pancreas, but is rare in pulmonary or breast adenocarcinomas and squamous cell carcinomas (Moll et al., 1992; Moll et al., 2008; Lin and Prichard, 2011; Dabbs, 2014).

CDX2 is a homeobox transcription factor. Although CDX2 does not belong to CKs, it is frequently considered along with CK 20 due to the parallels in diagnostic applications. CDX2 is an important factor of intestinal epithelium differentiation and

proliferation. Since it is expressed only in the intestinal epithelium (from the duodenum to the rectum), it is useful in differential diagnosis between colorectal cancer and other tumours possessing similar morphological structures (Rosai, 2004; Lin and Prichard, 2011; Dabbs, 2014). Strong and diffuse CDX2 expression is observed in colorectal and duodenal carcinomas. Other tumours, which show more limited or weaker CDX2 positivity, comprise oesophageal carcinoma, gastrointestinal neuroendocrine neoplasms, urinary bladder cancer, ovarian mucinous adenocarcinoma and pancreatic and biliary cancers (Lin and Prichard, 2011; Dabbs, 2014).

CK 5/6 is a combined CK. CK 5 is a high molecular weight protein with a molecular mass of 58 kDa. CK 5 is expressed in the basal, intermediate and superficial cell layers of stratified epithelium, in transitional epithelium as well as in complex epithelium and in mesothelial cells. CK 5 is constantly negative in simple epithelia and in non-epithelial cells. CK 6 is also one of the high molecular weight (56 kDa) proteins. It is a basic type of CK which is expressed in proliferating squamous epithelium (Moll et al., 1982; Moll et al., 1992). In particular, simultaneous expression of both markers CK 5/6 and p63 shows high sensitivity and specificity for squamous differentiation (Dabbs, 2014). Regarding tumours, CK 5/6 is typically expressed in squamous cell carcinomas, transitional cell carcinomas of urinary bladder, epithelial mesotheliomas and undifferentiated large cell carcinomas (Lin and Prichard, 2011; Dabbs, 2014). Less frequently CK 5/6 can be present in endometrial adenocarcinoma, pancreatic, breast and ovarian carcinoma, or cholangiocarcinoma (Dabbs, 2014).

CK 34βE12 comprises a group of high molecular weight (48 – 67 kDa) CKs 1, 5, 10 and 14. The association with CK types 2, 11 and 15 has been noted. Thus, the keratins of basal cells, myoepithelial cells and squamous epithelium can be selectively recognised (Miettinen, 1993; Dabbs, 2014). CK 34βE12 is also observed in duct-derived epithelium (breast, lung, biliary tract and pancreas) and in transitional, ovarian and mesothelial tissues (Dabbs, 2014).

TP63 is a member of the *TP53* gene family. **p63** protein plays an important role in epithelial proliferation and regulation of differentiation (Di Como et al., 2002). Unlike p53, p63 is normally expressed in basal and stem cells of stratified epithelium and myoepithelial cells. Therefore, p63 is also called basal epithelial cell proliferation regulator protein (Rosai, 2004; Lin and Prichard, 2011). Among the malignant tumours, p63 expression is observed in basal cell carcinomas, squamous cell carcinomas and transitional cell carcinomas, including urinary bladder carcinoma, but not in

adenocarcinomas (Di Como et al., 2002; Lin and Prichard, 2011). Interestingly, one of the p53 isoforms (TP53) can induce apoptosis and cell cycle arrest by transactivating p53 target genes, such as *Bax* and *p21^{WAF/CEP1}* (Moll and Slade, 2004; Melino, 2011).

Chromogranin A (ChrA) is an acidic glycoprotein from granin family, found in cytoplasm as the soluble fraction of neurosecretory granules in neural, neuroendocrine and endocrine cells (Helle et al., 2007; Montero-Hadjadje et al., 2008). ChrA contains 439 amino acids. Ten pairs of amino acids represent possible cleavage sites targeted by proteases, for instance, prohormone convertases. Such cleavage results in smaller peptides: pancreastatin, chromostatin, vasostatin and parastatin. These smaller molecules possess intracellular hormone-binding activity, can inhibit the secretion of some hormones, and also have antifungal and antibacterial effects (Dabbs, 2014). Since ChrA is expressed in almost all neuroendocrine tumours, it is considered a 'pan-endocrine' marker (Rosai, 2004; Lin and Prichard, 2011).

CD56, also called neural cell adhesion molecule (NCAM), belongs to glycoprotein family, and is responsible for cell adhesion, differentiation and migration (Dabbs, 2014). CD56 is constitutionally expressed on neurons, astrocytes, Schwann cells, myoblasts and NK lymphocytes, adrenal medulla and *zona glomerulosa*, follicular epithelium of thyroid, proximal renal tubular epithelium, cardiac muscle, hepatocytes, gastric parietal cells and islets of Langerhans (Rosai, 2004; Dabbs, 2014). Regarding tumours, CD56 expression is found in neuroendocrine carcinomas, NK lymphomas, mesotheliomas, follicular thyroid carcinoma, papillary thyroid carcinomas, hepatocellular carcinomas, Wilms tumours and renal cell carcinomas (Rosai, 2004; Dabbs, 2014). As a target in immunohistochemical tumour diagnostics, CD56 is characterised by high sensitivity but low sensitivity (Williams, 2007).

Vimentin is one of the intermediate filaments (57 kDa). It is not cell-type specific, but is normally confined to cells with mesenchymal differentiation, for example, fibroblasts, vascular smooth muscle cells and endothelium (Rosai, 2004; Dabbs, 2014). Among malignant tumours, vimentin expression is observed in renal cell carcinoma, müllerian endometrioid adenocarcinoma, serous ovarian carcinomas, and thyroid follicular carcinomas among others. It is rarely found in endocervical adenocarcinoma, colorectal, breast and prostate adenocarcinoma. Vimentin expression is observed in almost all spindle cell neoplasms, including mesenchymal spindle cell neoplasms as well as sarcomatoid carcinomas (Dabbs, 2014). Vimentin expression in epithelial cells is associated with epithelial-mesenchymal transformation (Masugi et al.,

2010; Krantz et al., 2012). The functions, which depend on vimentin, include cell signalling, cell adhesion and migration (Ivaska et al., 2007). Down-regulation of vimentin inhibits the migration of malignant epithelial cells (McInroy and Määttä, 2007).

CD34 is a single-chain transmembrane protein expressed in immature haematopoietic stem/ progenitor cells, as well as in normal and neoplastic endothelium and in some soft tissue tumours (Ramani et al., 1990; Rosai, 2004). Although CD34 is considered a relatively specific marker of endothelium and its tumours (angiosarcoma and Kaposi sarcoma), it is expressed in other tumours, including leiomyosarcoma, peripheral nerve sheath tumours, epitheloid sarcomas, dermatofibrosarcoma protuberans, spindle cell lipoma, gastrointestinal stromal tumour and solitary fibrous tumour (Dabbs, 2014). The visualisation of endothelium can be helpful in studies of tumour angiogenesis.

COX-2, an inducible subtype of cyclooxygenase (COX), is a prostaglandin endoperoxide synthase-2. It synthesises prostaglandin H that catalyses the synthesis of inflammatory prostaglandins D2, E2, F2 α and I2, as well as thromboxane A2. Cytokines, growth factors, and tumour promoters are known to induce COX activity (Hla and Neilson, 1992; Grösch et al., 2006; Sahin et al., 2009; Rizzo, 2011). Smoking is also one of the COX-2 releasing factors and thus could be associated with tumour pathogenesis and progression (Huang and Chen, 2011). COX-2 stimulates the stromal cells, fibroblasts and vascular cells to release growth factors, angiogenic proteins and chemokines (Rizzo, 2011), thus facilitating oncogenesis. COX-2 promotes cell growth, neovascularisation, inhibits apoptosis, decreases cell adhesion and increases metastatic spread. The role of COX-2 is also supported by the successful application of COX-2 inhibitors in prophylaxis and by possible application as a treatment supplement regarding certain cancers (Masferrer et al., 2000; Stratton and Alberts, 2002; Grösch et al., 2006; Harris, 2009; Huang and Chen, 2011; Rizzo, 2011).

1.4.2. Immunohistochemical profile of PDAC

The immunohistochemical profile of the tumour could reveal the most characteristic features of carcinogenesis as well as possible targets for treatment. Regarding the serious prognosis of PDAC, such studies are of utmost importance. The most important findings are summarised here. Thus far, controversies still exist. The

total tumour profile also necessitates that the analysis of the association between different markers would optimally be performed simultaneously in the same cohort thus minimising bias due to technological diversity, different genetic background or risk factors.

The data that have been reported on the expression of cell cycle markers in PDAC and in non-neoplastic pancreatic ducts (NnPD) are shown in Tables 1.4. and 1.5.

Table 1.4.

Immunohistochemical expression of cell cycle markers in PDAC and NnPD

Marker	Positive cases in PDAC (%)	Positive cases in NnPD (%)	Cut-off value (%)	References
Ki-67	94.3	16.7	5	(Hu et al., 2012)
p53	41–60	0–10	5	(Kawesha et al., 2000; Xu et al., 2006; Lozano-Leon et al., 2011)
	32.7–54	NI	10	(Sarela et al., 2002; Lee et al., 2005; Chen, Zheng et al., 2012)
p21	20.7–25	0	5	(Hu et al., 1998; Kawesha et al., 2000; Lozano-Leon et al., 2011)
	79	9	10	(Biankin et al., 2001; Biankin et al., 2002)
p27	30–49	100	5	(Juuti et al., 2003; Rahman et al., 2003; Fukumoto et al., 2004; Mielko et al., 2006)
Cyclin D1	50–72	0	5	(Kawesha et al., 2000; Biankin et al., 2002; Maitra et al., 2003)
	77.8	0	10	(Culhaci et al., 2005; Li et al., 2005)
Bcl-2	35.6–55	100	5	(Sinicrope et al., 1996; Campani et al., 2001; Dong et al., 2005)

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; NnPD, non-neoplastic pancreatic ducts; NI, no information found

In PDAC, the proliferation activity by **Ki-67** expression ranged from 0.5–97% with a mean value of 41.5% (Linder et al., 1997; Culhaci et al., 2005). The median expression of Ki-67 in PDAC cells was 29.7% (Linder et al., 1997). Ki-67 expression was considerably up-regulated in PDAC in comparison with NnPD, $p < 0.001$ (Karamitopoulou et al., 2010). Increased Ki-67 expression correlated with the development of metastasis in the lymph nodes ($p = 0.002$), tumour grade ($p = 0.008$) and clinical stage ($p = 0.003$) (Hu et al., 2012). However, Ki-67 expression does not distinguish between the pT stages (Karamitopoulou et al., 2010). There was also no

correlation between the expression of Ki-67 and positive/ negative p53 expression (Linder et al., 1997).

Regarding **p53**, the immunohistochemical expression of p53 protein showed a statistically significant higher mean value in PDAC than in NnPD, $p < 0.001$. The mean number of p53-positive cells was 29.4% in PDAC, but 4.8% among normal cells (Karamitopoulou et al., 2010). With a 1% cut-off, p53 was observed over a wide range in 46–81.1% of PDAC cases (Linder et al., 1997; Oshima et al., 2013). p53 expression showed a significant association with tumour size and local spread. However, the data are controversial. Among PDAC exceeding 5 cm, 90% of cases showed negative p53 expression, $p < 0.01$ (Kawesha et al., 2000). In contrast, considerably higher p53 expression has been reported in late pT tumours (pT3, pT4) than in early pT carcinoma (pT1, pT2), $p = 0.015$ (Karamitopoulou et al., 2010). A statistically significant association ($p = 0.022$) also existed between p53 expression and tumour differentiation (Oshima et al., 2013). A significant correlation has been found between the p53 expression and Bcl-2 up-regulation, $p = 0.026$ (Chen, Zheng et al., 2012).

p21 was upregulated in PDAC as the carcinoma possessed a higher mean expression than NnPD, $p < 0.001$ (Karamitopoulou et al., 2010). The expression of p21 did not correlate with the patient's age, tumour size with 5 cm cut-off, pT parameter, lymph node status, tumour stage and grade or resection line status (Hu et al., 1998; Kawesha et al., 2000; Karamitopoulou et al., 2010).

Regarding **p27**, the mean expression in PDAC was lower than in NnPD; the difference was statistically significant, $p < 0.001$ (Karamitopoulou et al., 2010). With cut-off values of 5% or 1%, similar frequency of p27 expression was observed: 43.8% according to the original reported data, 14/ 32 PDAC cases (Lu et al., 1999; Fukumoto et al., 2004; Mielko et al., 2006). In NnPD, p27 invariably showed positive expression in 10% or more of epithelial cells (Lu et al., 1999; Rahman et al., 2003). There was a statistically significant association between positive/ negative p27 expression and tumour size ($p = 0.013$): among the tumours not exceeding a diameter of 2 cm, p27 expression was more frequent than in tumours larger than 2 cm (Lu et al., 1999). p27 expression was considerably lower in high pT (T3, T4) and poorly differentiated tumours (G3, G4) than in early pT (T1, T2) and well differentiated tumours (G1, G2). The respective p values were $p = 0.029$ and $p = 0.02$ (Culhaci et al., 2005; Karamitopoulou et al., 2010). There are no known correlations between p27 expression and other clinical or morphological parameters, including gender, status of lymph

nodes, distant metastases, tumour stage, tumour invasion in blood vessels and/ or perineural invasion (Culhaci et al., 2005).

Cyclin D1 expression correlated with the size of the tumour ($p = 0.03$) and perineural invasion (Lebe et al., 2004; Culhaci et al., 2005). No associations with clinical and morphological parameters are reported (Kawesha et al., 2000; Culhaci et al., 2005).

Bcl-2 positive expression was found in 12–24% of PDAC cases applying a 10% cut-off value (Nio et al., 2001²; Sarela et al., 2002). In meta-analysis of trials in which the total number of patients reached 314, the mean frequency of positive Bcl-2 expression was 33% with a range of 12–67% of PDAC cases (Smith et al., 2011).

A significant loss of **E-cadherin**, leading to E-cadherin expression in less than 5% of cells was seen in 2% of PDAC cases (Hong et al., 2011). E-cadherin expression in less than 10% of cells was observed in 66% of PC cases (Nakajima et al., 2004), but less than 90% in 46.4% (original data, 58/ 125) of PDAC cases (Shimamura et al., 2003). Compared with well differentiated tumours, E-cadherin expression was significantly decreased in poorly differentiated tumours (p value was variable, ranging from 0.007–0.02). The loss of E-cadherin was particularly evident in the tumour areas rich in single invasive neoplastic cells; the difference was statistically significant, $p = 0.006$ (Shimamura et al., 2003; Masugi et al., 2010; Hong et al., 2011). Altered expression of E-cadherin correlated with an increased incidence of metastases in lymph nodes ($p < 0.05$) and liver, $p < 0.01$ (Li et al., 2003). There was no association between E-cadherin expression and other clinical and pathologic characteristics of the patient and tumour (Pryczynicz et al., 2010; Hong et al., 2011). E-cadherin presented a convincing negative correlation with vimentin expression, $p = 0.0024$ (Javle et al., 2007).

Regarding **CD44**, positive expression was found in 53.8–85% of PDAC cases (Gansauge et al., 1995; Immervoll et al., 2011; Tajima et al., 2012). A statistically significant correlation was found between CD44 expression and lymph node status ($p = 0.036$). Among the cases lacking CD44, 41% of patients were free of lymph node metastasis (N0). In contrast, only 15% of patients with positive expression of CD44 lacked lymph node metastases (Immervoll et al., 2011).

Table 1.5.

Immunohistochemical expression of CKs and CDX2 in PDAC and NnPD

Marker	Positive cases in PDAC (%)	Positive cases in NnPD (%)	Cut-off value (%)	References
CK 7	85.2–100	100	5	(Schüssler et al., 1992; Chu et al., 2000; Goldstein and Bassi, 2001; Hamidov et al., 2011; Bayrak et al., 2012; Liu et al., 2012)
CK 19	75–100	95–100	5	(Schüssler et al., 1992; Hamidov et al., 2011; Liu et al., 2012)
CK 20	11.5–62	0	5	(Chu et al., 2000; Goldstein and Bassi, 2001; Hamidov et al., 2011; Bayrak et al., 2012; Liu et al., 2012)
CDX2	0–22	10	5	(Chu et al., 2005; Park et al., 2007; Bayrak et al., 2012; Liu et al., 2012)

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; NnPD, non-neoplastic pancreatic ducts; CK, cytokeratin.

In several studies, **CK 7** expression in more than 75% or 90% of the cells was observed in 97% or 72.2% (13/ 18) PC cases, respectively (Schüssler et al., 1992; Goldstein and Bassi, 2001). CK 7 expression decreased in the areas of squamous differentiation within PDAC (Schüssler et al., 1992). There was no association between CK 7 expression and cancer localisation, tumour stage and grade (Bayrak et al., 2012).

The amount of **CK 19** positive cells exceeded 90% in all PDAC cases. CK 19 expression decreased in the areas with squamous differentiation (Schüssler et al., 1992).

Controversial estimates are published regarding **CK 20**. In the study by Bayrak *et al.*, CK 20 expression in PDAC was invariably below 50% of cells (Bayrak et al., 2012), while Goldstein and Bassi found 9% of cases showed positive expression of CK20 in more than 75% of PDAC cells (Goldstein and Bassi, 2001).

Among **CDX2** stained cases, 36.1% of PDAC and 100% of NnPD cases were positive if the cut-off value is 1% and weakly reactive cases are included (Xiao et al., 2014).

Few articles are devoted to the expression of **CK 5/6** in PDAC and in NnPD. Surprisingly, CK 5/6 is found in 38% of PDAC cases (Chu and Weiss, 2002).

The expression of high-molecular weight **CK 34 β E12** has not been studied in PDAC or NnPD. In other adenocarcinomas, positive expression has been observed. Tubular breast cancer shows positive CK 34 β E12 expression in 50% of cases and

tubulo-lobular carcinoma in 59% of cases with a 1% cut-off value (Esposito et al., 2007).

p63 expression in PDACs and in NnPD is still only occasionally investigated. p63 expression was observed in 68.2% of PDAC cases (Ito et al., 2001). Studying p63 deltaNp63 isoform (DNp63), a negative reaction was observed in the 'classical' (ductal) PDAC structures, but positive expression was observed in the areas with squamous differentiation (Basturk et al., 2005). Similarly, both p63 and DNp63 were absent from NnPD, but DNp63 showed 100% expression in the ducts with squamous metaplasia (Basturk et al., 2005; Brody et al., 2009). p63 and DNp63 were more frequently studied in squamous cell carcinoma of the pancreas characterised by positive expression of both markers in 100% of cases (Basturk et al., 2005; Brody et al., 2009).

ChrA expression has been reported in 11% of PDAC cases (Linder et al., 2006). However, ChrA positive cells in PDAC were scattered in small amounts usually not exceeding < 1%. Occasionally, up to 10% of neoplastic cells expressed ChrA (Kamisawa et al., 1996; Sakaki et al., 2002). In NnPDs, ChrA positive expression has been reported in 87.5% (original data, 21/ 24) of cases. Positive cells were scattered evenly with the density of 1/ 10 cells. The amount of ChrA positive cells increased in hyperplastic areas (Sakaki et al., 2002).

Controversial data are reported regarding the amount of endocrine cells in the invasive areas of tumour and its metastasis. Sakaki *et al.* proved that in the areas of tumour invasion, where pancreatic islets were absent, ChrA expression did not appear (Sakaki et al., 2002). In comparison, in another study the presence of endocrine cells was identified in the areas of invasion, also including the perineural space (Pour et al., 1993). Wider endocrine cell population has been observed in well differentiated carcinomas, but significantly less ChrA expression was seen in poorly differentiated tumours, $p < 0.05$ (Kamisawa et al., 1996). ChrA positive expression in well, moderate and poorly differentiated PDAC was as follows: 93.3%, 88.9% and 40% of all PDAC cases (Sakaki et al., 2002). The high frequency of neuroendocrine differentiation in that study was contradictory to the reported expression of 11% of PDAC cases reported by Linder *et al.*, 2006.

Similarly to ChrA expression, the positive expression of **CD56** was seen in scattered cells or their groups in NnPD. In chronic pancreatitis, expression of CD56 in the luminal cells increased. In invasive PDAC, positive expression of CD56 was not displayed (Fujisawa et al., 2003; Naito et al., 2006).

Without specifying the marker, negative/ positive neuroendocrine expression did not correlate with age, tumour differentiation, size, enhanced tumour invasion or lymph node lesions (Tezel et al., 2000).

Vimentin expression was observed in 45% of PDAC cases with a range of positive cells from 1–95% and a median of 1% of PDAC cells. In 27.5% of cases vimentin expression was seen in more than 10% of cells. Vimentin expression depends on the tumour differentiation degree. In poorly differentiated tumours, especially in the areas of single invasive malignant cell infiltration ($p = 0.001$), vimentin expression was higher than in well differentiated tumours, $p = 0.01$ (Masugi et al., 2010; Handra-Luca et al., 2011). At the same time, there was no statistically significant association between increased vimentin expression and local tumour spread, as revealed by comparative analysis of early pT1 and pT2 tumours or advanced tumours pT3 and pT4 (Handra-Luca et al., 2011). Vimentin expression had a trend toward cells with reduced E-cadherin expression (Masugi et al., 2010).

MVD, detected by **CD34**, most often is described as the mean count of vessels per microscope field of vision $\pm$ standard deviation (SD) and range of the count of vessels. The following results have been reported: 1) mean MVD 120.8 ± 2.6 , range 29.0–351.0 vessels (microscopic parameters: magnification 200x, area $0.64 \text{ mm}^2/\text{field}$), 2) mean MVD 58.6 ± 17.4 , range 33.0–117.0 vessels (200x, $0.785 \text{ mm}^2/\text{field}$), 3) mean MVD 50.8 ± 21.7 (200x, $0.128 \text{ mm}^2/\text{field}$) and 4) mean MVD 101.06 ± 70.2 ; range, 19–391 (200x, $0.949 \text{ mm}^2/\text{field}$) (Ikeda et al., 1999; Esposito et al., 2004; Giannopoulos et al., 2008; Yamahatsu et al., 2012). Thus, the measurements are highly controversial.

There was no correlation between the number of CD34-positive vessels (by cut-off $< 99/ \geq 99$ vessels) and tumour stage or invasion in blood and lymph vessels (Yamahatsu et al., 2012). Another research group, using another endothelial marker CD31, reported a correlation that the MVD was greater in poorly and moderate differentiated tumours than in well differentiated tumours (van der Zee et al., 2011).

Regarding **COX-2**, controversial findings have been published. Positive expression of COX-2 in PDAC has been described as highly variable: 21.5–36% of PDAC cases with 5% cut-off value, 56% cases with 10% cut-off value and even 80.5% cases with 20% cut-off value (Juuti et al., 2006; Matsubayashi et al., 2007; Hermanova et al., 2010; Lozano-Leon et al., 2011). In contrast, expression of COX-2 has not been observed in NnPD (Lozano-Leon et al., 2011).

COX-2 expression was found significantly ($p = 0.03$) more frequently in tumours measuring at least ($\geq$) 3 cm in diameter. In contrast, other researchers have reported no association between the frequency of COX-2 expression and the tumour size categories ≤ 2 / $2-4$ / > 4 cm (Juuti et al., 2006; Matsubayashi et al., 2007). COX-2 expression inversely correlated with distant metastasis ($p = 0.026$), as positive expression of COX-2 was found in 39% of M0 patients, but no positive results were present in M1 patients (Juuti et al., 2006).

There were limited amounts of published data regarding **BRCA1** expression. In the PubMed database selection rounds (by searching combinations: “BRCA1” OR “BRCA1 gene” OR “BRCA1 protein” AND “pancreatic ductal adenocarcinoma” OR “pancreatic cancer” OR “pancreatic carcinoma” OR “pancreas” OR “pancreatic” AND “immunohistochemi*”, “BRCA1” AND “pancreatic cancer”), four articles were identified. Only one article had the target information that BRCA1 nuclear expression in more than $> 50\%$ of cells was observed in 50% of PC cases (Beger et al., 2004). *BRCA1* gene methylation has been reported in 60.3% of PDAC cases (Peng et al., 2006). In contrast, *BRCA1* gene methylation was detected in only 10.8% of PDAC cases among Ashkenazi Jewish patients (Lucas et al., 2013). By tissue microarray analysis, cytosolic *BRCA1* distribution was associated with a higher pathologic stage, $p = 0.006$ (Wang et al., 2013).

1.4.3. Immunohistochemical profile of PET

Despite the invariable use of IHC in the diagnostics of PETs, only limited research studies have been devoted to simultaneous complex evaluation of multiple markers in PETs and non-neoplastic pancreatic islets (NnPI).

Ki-67 is an essential immunohistochemical marker of PET evaluation. Positive Ki-67 was seen in all types of PETs (Imam et al., 2000) and can be expected in any neoplastic process. The expression range was very variable: 1–40% of neoplastic cells with a median count of positive cell reaching mostly 2% (Beghelli et al., 1998; Chetty et al., 2008; Piani et al., 2008; Rindi et al., 2012; Scarpa et al., 2010). Ki-67 expression below 5% was observed in 87.5% of PET cases (Yamamoto et al., 2004²). The mean number of Ki-67 positive cells in benign PET can reach 5.55% with a range of 0.3–4.2% of cells. In contrast, malignant PET was characterised by the mean number of Ki-67 positive cells reaching 17.4% but the range as 1.5–40.0% of tumour cells

(Beghelli et al., 1998; Imam et al., 2000). Increased Ki-67 expression had a strong statistically significant association with more frequent development of tumour metastases (Ohike and Morohoshi, 2005). Different Ki-67 positive expression thresholds (5%, 10% and 25%) have been described for the evaluation of the risk of metastases (Clarke et al., 1997; Jorda et al., 2003; Yamamoto et al., 2004²).

In the pancreatic islets, **p53** expression was negative (Liu et al., 2012). In contrast, the amount of positive PETs varied between 0–41.7% of cases (recalculated from original data, 5/ 12 cases) (Lee, 1996; Tomita, 1997; Beghelli et al., 1998; Lam and Lo, 1998; Sato et al., 2000; Proca and Frankel, 2008). Evaluating the different subtypes of PDEC, p53 expression was invariably (100%) observed in small cell neuroendocrine carcinoma and frequently (90%) in large cell neuroendocrine carcinoma (Yachida et al., 2012).

Considering **p21** and **p27**, the expression of these proteins have not been evaluated directly in PETs. In gastro-entero-pancreatic neuroendocrine tumours, positive expression of p21 was seen in 60.6% of cases and p27 expression was maintained in 49.7% of cases (Lee et al., 2014). p21 expression did not differ between benign and malignant, differentiated and non-differentiated endocrine tumours (Canavese et al., 2001). In NnPI, all cells expressed p27 (Lu et al., 1999).

Nuclear expression of **cyclin D1** statistically significantly differed between benign PET (including benign and uncertain behaviour) and malignant PET ($p = 0.003$), i.e. 17% *versus* 67% (Chang et al., 2007). In malignant tumours, 71% of WDEC and 57% of PDEC cases presented cyclin D1 reactivity (Chang et al., 2007). Cyclin D1 expression showed statistically significant associations with both mitotic rate ($p = 0.047$) and proliferative activity ($p = 0.01$). Tumours with proliferative fraction of 2% or more showed cyclin D1 expression more frequently (Chang et al., 2007).

Anti-apoptotic protein **Bcl-2** was expressed in 53% of all PET cases (originally, 16/ 30 and 15/ 28 cases) (Proca and Frankel, 2008; Yachida et al., 2012). Bcl-2 was positive in 18.2% of benign PETs (originally, 2/ 11 cases). There were no positive cases among G1 (< 2 mitosis/ 10 HPF and/ or Ki-67 index $\leq 2\%$) PETs, but in G2 PETs (2–20 mitosis/ 10 HPF and/ or Ki-67 labelling index 3–20%), 40% of cases were positive (Yachida et al., 2012). Among malignant PETs, Bcl-2 showed expression in 45% of cases (originally, 9/ 20 cases) (Proca and Frankel, 2008). Bcl-2 positive expression had a significant association with higher mitotic activity ($p = 0.0002$) and proliferation activity ($p = 0.0001$) (Yamahatsu et al., 2012).

Regarding **E-cadherin**, loss of cell adhesion by E-cadherin expression was observed in 50–59.6% of PETs cases (Chetty et al., 2008; Fendrich et al., 2012).

CD44 was not expressed in NnPI, but was invariably positive in PETs (Chaudhry et al., 1994; Imam et al., 2000). A trend towards a correlation existed between CD44-positive tumours and development of metastases in lymph nodes (Chaudhry et al., 1994).

Regarding the CK spectrum, **CK 7** was expressed in 50% of PET cases, while not observed in NnPI (Schüssler et al., 1992; Cai et al., 2001; Liu et al., 2012).

Considering **CK 19**, the findings were similar with the distribution of CK 7. CK 19 usually was negative in NnPI. However, in Schüssler *et al.*, 2/ 21 PET cases showed positive expression (Schüssler et al., 1992; Zhang et al., 2009; Liu et al., 2012; Han et al., 2013). In PETs, CK 19 expression was observed in 60–70% of cases (Zhang et al., 2009; Han et al., 2013), and has been associated with non-functioning tumours and metastases in regional lymph nodes (Han et al., 2013).

The expression of **CK 20** and **CDX2** in NnPI and PET showed a close correlation between the markers. Both markers were negative in NnPI. Regarding PETs, 33% of cases expressed CK 20 and 30–37.5% of cases expressed CDX2 (Cai et al., 2001; Saqi et al., 2005; Denby et al., 2012; Liu et al., 2012). CDX2 nuclear staining was seen in 5–40% of PET cells (Denby et al., 2012).

Neither **CK 34βE12** nor **CK 5/6** have been investigated in the normal endocrine pancreatic tissues or benign or malignant PETs. Regarding **p63**, information is lacking about p63 expression in both pancreatic neuroendocrine cells and PETs.

Due to the diagnostic requirements, **ChrA** and **CD56** were detected in many studies. The strongest positivity was presented by ChrA which was invariably positive in PETs without differences by grade and biological behaviour (Burford et al., 2009; Yachida et al., 2012). In contrast, CD56 has been found in 79–100% of PETs, also lacking a statistically significant expression difference between benign and malignant PETs, 70% cases *versus* 84% cases, respectively (original data, 7/10 *versus* 16/19) (Burford et al., 2009; Yachida et al., 2012).

Vimentin expression was detected in 25% of PET cases (Kimura et al., 1990).

There were no extensive immunohistochemical examinations of **BRCA1** expression in PET. By polymerase chain reaction, allelic loss of *BRCA1* was found in 2/ 22 insulinoma cases (Hrasćan et al., 2008).

MVD in PETs (determined by IHC visualisation of endothelium) varied between studies. In different studies, MVD has been described by the following parameters: the mean number of vessels, the median number of vessels and range of vessel number: 1) 311 vessels, 276 vessels, range 26–792 vessels/ mm² (microscopic parameters: magnification x25 objective, area 0.442 mm²/field) (Couvelard et al., 2005); 2) 288.3 vessels, 268.0 vessels, 132.3–625.0 vessels/ field (200x, 0.95 mm²/ field) (Takahashi et al., 2007).

The average vascularity in WDET was higher compared to WDEC and PDEC (Couvelard et al., 2005). Higher MVD has also been detected in large tumours, necrotic tumours, in fibrotic focus, in tumours exhibiting high mitotic activity, high proliferative fraction and/ or liver metastases (Couvelard et al., 2005).

The data concerning **COX-2** expression in PETs were highly controversial. COX-2 expression was invariably observed in NnPI by Jutti *et al.*, 2006 and Okami *et al.*, 2002. In contrast, Ohike and Morohoshi, 2001 and Koliopanos *et al.*, 2001 described COX2 expression in NnPI as negative or slightly positive (Koliopanos et al., 2001; Ohike and Morohoshi, 2001). Regarding PETs, COX-2 expression was positive in 65–100% of all PETs cases, including all functioning and 42.9% (in original article, 6/ 14 cases) of non-functioning PETs (Ohike and Morohoshi, 2001; Okami et al., 2002). In one of these studies, the expression difference between functioning and non-functioning PETs was statistically significant, $p < 0.05$ (Okami et al., 2002). Another research group reported a statistically insignificant trend for different expression rates in benign *versus* malignant PETs, namely 73.3% *versus* 50% of cases, respectively (Okami et al., 2002; Bergmann et al., 2009).

1.5. Survival and prognostic factors

1.5.1. Characteristics of PDAC

The prognosis of PDAC patients is dismal. The median overall survival (OS) after surgical treatment of PDAC was between 12.2–21 months (Kawesha et al., 2000; Cleary et al., 2004; Hamidov et al., 2011; An et al., 2012). Five-year survival rate after surgical treatment of PDAC patients varied between 8.0–16% (Niederhuber et al., 1995; Cleary et al., 2004; Hamidov et al., 2011; Handra-Luca et al., 2011). Three-year survival rate was 10–34.3% (Niederhuber et al., 1995; Lim et al., 2003; An et al., 2012). Two-year survival rate was 15–24%, but one-year survival rate varied between 38–48%

(Niederhuber et al., 1995; An et al., 2012). The first-month mortality was 7.6% (Lim et al., 2003).

In the cases of unresectable primary tumour, five-year survival rate was about 4%, but considering the stage of the tumour it can vary between 0.6–3.8 %. Three-year OS rate was 6% (Niederhuber et al., 1995; Bilimoria et al., 2007).

Variables or characteristics of a patient which predict distant relapse and survival are prognostic factors. Since PDAC is characterised by a serious prognosis, there has been extensive search for clinical, morphological and genetic factors that can affect patients' survival. Evaluation of morphological factors and biomarker expression can be informative for diagnostic purposes, estimation of the progression of illness and evaluation of the prognosis. The factors that have frequently been associated with patients' prognosis are described below.

Some studies have shown a difference between the gender incidence of PDAC. However, there was no OS difference between males and females (Andrén-Sandberg et al., 2000; Kawesha et al., 2000; Lim et al., 2003; Masugi et al., 2010; Handra-Luca et al., 2011; An et al., 2012).

There were seldom studies which proved the impact of age on OS. For example, patients' survival with age ≤ 62 years exceeded those with age > 62 years ($p = 0.042$), respectively, one-, two- and five-year survival were 51%, 25% and 8% *versus* 39%, 19% and 6% (Juuti et al., 2006). The more often targeted age range spanned between 60 and 65 years (Lu et al., 1999; Lim et al., 2003; Masugi et al., 2010; An et al., 2012).

A better prognosis has been detected in patients with pancreatic head tumour in comparison to other localisations ($p = 0.045$), for example, one-, two- and five-year survival was 47%, 24% and 8% *versus* 31%, 8% and 0% (Juuti et al., 2006). Median OS parameters of PDAC which were localised in the body and tail of pancreas were 14.2 months with 3-year and 5-year survival as 25.8% and 6.5% respectively (Rohan et al., 2013).

One of the most described while at the same time also most controversial prognostic factor is tumour size. Some studies have shown the following rule: the larger the tumour, the worse prognosis. Median OS for the patients with tumour size ≤ 2 cm was 37.8 months and 3-year survival was 51.3%, whereas for patients with tumour size > 2 cm median OS was 14.8 months and 3-year survival was 28.8%, $p = 0.002$ (Lim et al., 2003). A similar difference in results was observed between the groups of tumour size ≤ 2 / $2-4$ / > 4 cm ($p = 0.049$), respectively; one-, two- and five-year survival was

67%, 23% and 0% *versus* 53%, 27% and 11% *versus* 17%, 10%, 3% (Juuti et al., 2006). There was also a difference between OS when the borderline size 3 cm was used. For example, in patients with a tumour size less than 3 cm the median OS varied between different publications from 19.6–23.9 months, but, for tumours exceeding the size of 3 cm the median OS varied from 13–14.8 months. The corresponding p values ranged 0.0002–0.03 (Lim et al., 2003; Mielko et al., 2006; Masugi et al., 2010; Handra-Luca et al., 2011).

The tumour size was one of the determining criteria of pT parameter. The tumour invasion in the surrounding tissues was another defining criterion. Accordingly, pT was described as a prognostic indicator. A statistically significant difference in survival rates was observed between patients with pT3 tumour versus early pT parameters (pT1, pT2), $p = 0.004$ (Oshima et al., 2013). Detailed expansion of prognostic indicators by pT values (pT1, pT2, pT3; pT4) were the following: one-, two- and five year survival corresponded to 71%, 29% and 0% (pT1) *versus* 46%, 32%, 11% (pT2) *versus* 47%, 26%, 8% (pT3) *versus* 31%, 3%, 3% (pT4), $p = 0.045$ (Juuti et al., 2006).

The prognosis was undoubtedly poor for the patients with a wider spread of the tumour mostly described by pN and pM parameters. In patients with N0 status of lymph nodes, the median OS varied from 17.9–26.4 months, but in N1 patients OS varied from 8.9–16.63 months with a three-year survival of 37.9% *versus* 29.1% respectively (p value varied from 0.0002–0.05) (Kawesha et al., 2000; Lim et al., 2003; Masugi et al., 2010; Handra-Luca et al., 2011; An et al., 2012; Oshima et al., 2013). OS difference regarding one-, two- and five-year survival in M0 and M1 patients was 48%, 24% and 7% *versus* 11%, 0% and 0%, $p < 0.001$ (Juuti et al., 2006).

The status of RM is a potentially adjustable and significant prognostic marker. In the cases of pR0, the median patients' OS was 20.3–25 months contrasting with 10–14.0 months in the cases of pR1; in the studies p value varied from < 0.001 –0.039 (Mielko et al., 2006; Handra-Luca et al., 2011; An et al., 2012; Konstantinidis et al., 2013). Additionally, if the tumour was located 0–1 mm from the resection line, patients survival was similar to the direct cases of R1 (OS was 16 and 14 months) when compared to patients with a tumour localisation more 1 mm from RM (OS was 35 months), $p < 0.001$ (Konstantinidis et al., 2013).

The average results of OS between tumour differentiation grades both confirm and deny pG parameter prognostic value. The median survival in G3 *versus* G1 and G2 tumours was 5–13.01 *versus* 19–21.89 months; the corresponding p value ranged from

< 0.01–0.006 (Mielko et al., 2006; Masugi et al., 2010; Handra-Luca et al., 2011). Extended OS analysis between the patients with G1 and G2 *versus* G3 tumours was the following: the one-, two- and five-year survival was 79%, 43% and 29% *versus* 48%, 25% and 7% *versus* 27%, 8% and 0%, $p < 0.001$ (Juuti et al., 2006).

A few more statistically significant morphological prognostic factors included perineural invasion ($p = 0.029$) and tumour invasion in lymph nodes ($p = 0.008$) (Masugi et al., 2010; Oshima et al., 2013).

In an effort to understand the factors of PDAC development, growth and spread, not only have morphological features been evaluated but many studies have explored the molecular changes of PDAC cells.

Among IHC markers, **Ki-67** prevails. Comparing patients with negative and positive Ki-67 expression by selected cut-off level of 10%, the one-year survival results were 65.8% (95% CI = 50–77) *versus* 28% (95% CI = 12–46) as reported by Karamitopoulou *et al.*, 2010 (Karamitopoulou et al., 2010). Median OS also statistically significantly differed if Ki-67 expression cut-off values were 20% and 45%, respectively, 66.3 months *versus* 18.8 months ($p < 0.05$) and 16.0 months *versus* 8.0 months with a corresponding $p = 0.021$ (Linder et al., 1997; Yamamoto et al., 2004).

Controversial findings were reported regarding the impact of **p53** on survival. Most studies have not proven the prognostic value of aberrant p53 protein expression at a 5% or 10% cut-off, but Linder *et al.* showed that patients with negative p53 expression survived longer (median OS 16.0 months) than patients with positive p53 expression (8.7 months), $p < 0.001$ (Linder et al., 1997; Kawesha et al., 2000; Nio et al., 2001; Karamitopoulou et al., 2010; Jamieson et al., 2011).

The reverse role of **p27** protein expression has been identified. Patients with positive p27 expression in PDAC tended to survive longer than patients with a loss of p27 expression (p value varied between 0.008 – 0.04) (Lu et al., 1999; Fukumoto et al., 2004). The five-year survival in p27-positive and negative cases was 20% *versus* 3.6%, $p = 0.03$ (Juuti et al., 2003). However, Jamieson *et al.* using meta-analysis showed that p27 expression had no statistically significant prognostic value ($p = 0.47$) (Jamieson et al., 2011).

According to the same meta-analytic study, a statistically significant association was found between OS and **Bcl-2** expression ($p < 0.0001$) (Jamieson et al., 2011). Median post-surgical survival in patients with Bcl-2 positive *versus* Bcl-2 negative tumours was 14.3 (95% CI = 8.1–26.4) *versus* 7.3 (95% CI = 2.4–18.5) months, at a 5%

cut-off (Dong et al., 2005). Surprisingly, contradictory results have been reported as well. Thus patients with Bcl-2 expression in $\leq 25\%$ in tumour cells had longer survival than patients with Bcl-2 positive expression $> 25\%$ in tumour cells, $p = 0.02$ (ShengMian et al., 2009).

A statistically convincing prognostic value has been found in a loss of **E-cadherin** expression and appearance of **vimentin** expression; the survival of these patients was shorter with p values between 0.001–0.0021 (Shimamura et al., 2003; Javle et al., 2007; Handra-Luca et al., 2011; Jamieson et al., 2011). More accurately characterised prognostic features showed that patients with intact expression (positive E-cadherin expression in 100% of cells) had longer survival than patients with partially lost expression (6–99% of cells expressing E-cadherin) and lost E-cadherin expression ($<5\%$ of cells expressing E-cadherin), $p = 0.002$ with a median survival of 18.5 months *versus* 12.7 months *versus* 5.5 months (Hong et al., 2011). Median OS in cases showing positive expression of vimentin (at a 10% cut-off) was 12.49 months, but in negative cases median OS reached 19.72 months, $p < 0.01$ (Handra-Luca et al., 2011).

From the CK spectrum (CK 7, CK 19, CK 20, CK 34 β E12, CK 5/6) and CDX2, **CK 20** was the only marker which has been prognostically evaluated and has demonstrated association with OS; the association was statistically significant with $p = 0.02$ (Jamieson et al., 2011).

Without specifying an **endocrine marker**, in general, neuroendocrine cells have been identified more often in PDAC patients with longer survival ($p < 0.05$), but isolated ChrA expression did not show a statistically significant association with better prognosis (Pour et al., 1993; Tezel et al., 2000; Linder et al., 2006).

In a number of studies **MVD** has shown insignificant prognostic value. In contrast, Ikeda *et al.* has found that median OS was shorter in groups with vascular density over 58.6 vessels per field in comparison with patients with ≤ 58.6 vessels per field (200x, 0.785 mm²/field); the median OS was 6.3 *versus* 18.8 months, $p < 0.001$ (Ikeda et al., 1999; Esposito et al., 2004; Yamahatsu et al., 2012; Zorretto et al., 2013).

Patients with better prognosis were characterised by negative **COX-2** expression ($p = 0.002$ –0.019), with a median survival of 13.2–20 (95% CI = 18–25) *versus* 8.1–15 (95% CI = 12–17) months, but one-, two- and five-year survival was 51%, 32% and 8% *versus* 34%, 5% and 5% respectively (Juuti et al., 2006; Matsubayashi et al., 2007; Hermanova et al., 2010; Jamieson et al., 2011).

1.5.2. Characteristics of PET

In PET cases, survival is better in comparison with PDAC. Five- and ten-years survival rate after operations showed an average of 78–80.6% and 54.5–65% (Takahashi et al., 2007; Zhang et al., 2009). In malignant PETs, the OS was 21 months (Roland et al., 2012).

Statistically significant risk factors affecting the survival were infiltrating tumour borders ($p < 0.001$), tumour necrosis ($p < 0.001$) and perineural invasion ($p = 0.042$) (Zhang et al., 2009). Survival rate was significantly shorter for the patients with surgical resection lines addressed as R2 rather than R0 or R1. At the same time, there was no statistically significant difference in survival rates among patients with status R0 or R1 (Pomianowska et al., 2010).

Between IHC markers, strong prognostic significance was attributed to elevated Ki-67 expression, loss of p27 expression, positive CK 19 and lower MVD as detected by CD34 IHC (Chang et al., 2000; Deshpande et al., 2004; Couvelard et al., 2005; Schmitt et al., 2007; Takahashi et al., 2007; Pomianowska et al., 2010; Han et al., 2013; Lee et al., 2014). The prognostic threshold of **Ki-67** is 2%. Patients with positive Ki-67 expression below 2% survived longer compared to patients who had Ki-67 expression $\geq 2\%$ ($p = 0.003$) (Pomianowska et al., 2010). An analogous role has been noted by Boyar Cetinkaya *et al.*, 2014 (Boyar Cetinkaya et al., 2014). Ki-67 expression retains its prognostic value in complex clinical situations when radical tumour removal is not possible, e.g., in patients undergoing multimodal treatment for unresectable liver metastases with/ without primary tumour resection (Bertani et al., 2014). In the case of **CK 19** negative expression, patient 5-year survival rate was 100%, but CK 19 positive expression was 68.4%, p value < 0.001 (Zhang et al., 2009). **COX-2** expression might possibly be utilised as useful additional chemotherapeutic targets in PETs (Bergmann et al., 2009).

2. MATERIALS AND METHODS

2.1. The study quintessence and ethical principles

This study was performed as a retrospective, evidence-based morphological and immunohistochemical investigation of PDAC and PETs including survival analysis. It also comprised a prospectively recruited group in order to create and validate the protocol for the morphologic investigation of surgically resected pancreatic tumours. The study design was appropriate for profound evaluation of morphological factors and expression of immunohistochemical markers by analyses of retrospective data and it enabled determining the impact of a diagnostic protocol in an investigation of surgical material in the prospective part of the study. The research was carried out in accordance with the Declaration of Helsinki and received approval from the Committee of Ethics of Riga Stradins University.

2.2. Patient identification

The study comprised 94 pancreatic tumours. Seventy-eight consecutive cases of potentially radically operated PDAC and sixteen consecutive cases of PETs were identified by archive search in a single university hospital from January 2004 to January 2014. The inclusion criteria comprised verified unequivocal morphological diagnosis of either PDAC or PET, respectively, in the tissue material submitted after a potentially curative operation. Patients, who had a pancreatic tumour with a different histogenesis or cancer of equivocal origin, as well as those who only underwent biopsy or received preoperative chemotherapy or radiotherapy were excluded from the study.

Patient's demographic information (age at the time of tumour diagnostics; gender) as well as the treatment data (preoperative treatment, type and date of surgery) were acquired from the medical histories and surgical reports, submitted by the Department of Surgery.

2.3. The study design

The location of the tumour within the pancreatic gland and the type of pancreatic surgery were detected during gross pathological examination and verified by the surgical reports, obtained from the Department of Surgery. The gross morphological data for the study were obtained from pathological reports via a standardised approach as described below. The microscopic assessment was performed using the diagnostic pathology slides. These slides were entirely re-assessed for the present research in order to verify the diagnosis and to perform scientific measurements. The complete algorithm of the study is depicted in the Figure 2.1.

2.4. Surgical pathology evaluation

The pathological data were obtained via uniform gross and microscopic examination of pancreatic surgery materials.

2.4.1. Gross examination

The gross examination included the evaluation of submitted organs, the tumour and the lymph nodes. At first, the surgical material was characterised by the description and three-dimensional measurements of each submitted organ, section or soft tissues (stomach, adipose tissue along the lesser and greater gastric curvature, duodenum, omentum, common bile duct and distal part of hepatic bile duct, gallbladder and cystic duct, pancreas, spleen, peripancreatic soft tissue, other).

Further, the tumour was evaluated and described in detail, including the clarification of tumour localisation within the pancreas (pancreatic head *versus* corpus *versus* tail *versus* specified extensive spread within several of the listed compartments), measurement of the distance between the invasive front of tumour and the closest RM, tumour size by the largest diameter, assessment of the tumour colour, tumour consistency by density description and tumour margins (rounded, pushing *versus* infiltrative).

Figure 2.1. The algorithm of data processing

The localisation and number of lymph nodes (LN) were detected within the following groups: peripancreatic LN, pancreatoduodenal LN as well as LN of the lesser and greater gastric curvature and other LN.

The gross data constituted the basis for further evaluation of the tumour including location, origin and assessment of pTNM as well as for the identification of the type of surgery and status of resection lines.

2.4.2. Tissue processing and microscopic examination

During grossing, the following tissue samples were submitted for processing and subsequent microscopic analysis. In order to evaluate the tumour and to perform more accurate tumour size measurements in microscopic specimens, consecutive sections of whole pancreatic tissue were obtained with a distance of 5 mm. The RM were completely submitted for microscopic investigation, including proximal and distal RM of stomach and duodenum, respectively, as well as RM of blood vessels, RM of common hepatic bile duct, RM of pancreas, peripancreatic RM (marked with Alcian-blue) and other, if evident. All identified LN were entirely submitted for the microscopic investigation. In addition, tissue samples were obtained from all the surgically removed organs as well as from the ampulla, the confluence of common and hepatic bile ducts and the *papilla duodeni major*. The obtained tissue slices were treated with the technological cascade as described further and shown in Figure 2.2.

The tissue samples that were dissected during grossing were fixed in neutral buffered 10% formalin (Sigma-Aldrich, Saint Louis, United States of America) and processed by increasing grades of 2-propanol (Sigma-Aldrich), followed by incubation in Histograde xylene (J.T.Baker, Deventer, the Netherlands) and paraplast (Diapath S.r.l., Belgamo, Italy) in the vacuum infiltration processor Tissue-Tek® VIP™ 5 (Sakura Seiki Co., Ltd., Nagano, Japan). The processed tissue was then embedded in paraplast (Diapath S.r.l.) using tissue embedding system TES 99 (Medite GmbH, Burgdorf, Germany). After embedding, tissue samples from the obtained paraffin blocks were cut in four-micron-thick sections by microtome Microm HM 360 (Thermo Fisher Scientific, Inc., Waltham, USA) on glass slides (Menzel-Glaser, Braunschweig, Germany). The slides were stained with haematoxylin and eosin by automated tissue stainer TST 44 (Medite Medizintechnik, Burgdorf, Germany) and covered by cover

glass (Biosigma, Cona, Italy) using automated cover slipper (Dako, Glostrup, Denmark) and Pertex (Histolab, Gothenburg, Sweden) covering medium.

Figure 2.2. The technological cascade. A, Vacuum infiltration processor; B, Paraffin embedding system; C, Electronic rotary microtome; D, Automated tissue stainer; E, Equipment for microscopy and computed morphometry.

Standard slides, stained by haematoxylin and eosin, were examined under a light microscope (Leica DM500, Wetzlar, Germany) to obtain the following four categories of data:

1. Characteristics of the primary pancreatic tumour;

2. Presence of cancer metastases, other malignant tumour or reactive changes in the LN;
3. Status of all the RM;
4. Characteristics of the tissue samples of the submitted organs or parts of them.

Regarding pancreatic tumours, the following parameters were evaluated by the obtained data: histological type of tumour, tumour characteristics by pTNM classification (pT for local tumour invasion: Tis–T4 or Tx; pN for LN metastasis: N0, N1, Nx; pM for distant metastasis: M0, M1), tumour biological potential by differentiation grade for PDAC or by clinico-pathological classification of differentiation and behaviour for PETs, status of RM (R0, R1, R2 or Rx), mitotic activity, intravascular invasion, peri- and intra-neural invasion, lymphatic vessel invasion and tumour necrosis.

The tumour histological type was estimated and classified according to the World Health Organization (WHO) classification of tumours of the exocrine pancreas and tumours of the endocrine pancreas (Klöppel et al., 2000; Heitz et al., 2004). The pTNM staging was performed in accordance with the Seventh Edition of the AJCC Cancer Staging Handbook. The pTNM staging is described in the literature review section devoted to tumour growth and spread: pTNM classification and staging (Edge et al., 2010).

Regarding the biological potential, PDAC were classified into G1 – well differentiated; G2 – moderately differentiated and G3 – poorly differentiated tumours as described in the Cancer Grading Manual and shown in table 2.1. (Guzman and Chejfec, 2007).

Table 2.1.

The grade of differentiation of PDAC

Histologic grade	The grade of differentiation	The amount of ductal or glandular structures in cancer	Comments
G1	Well-differentiated adenocarcinoma	> 95%	Tumour cells are mostly arranged into ducts or glands
G2	Moderately differentiated adenocarcinoma	50–95%	Ducts and glands are more numerous than solid cords and nests
G3	Poorly differentiated adenocarcinoma	5–50%	Solid nests and cords are more numerous than the well-formed ducts and glands

(Guzman and Chejfec, 2007)

The biological potential of PETs was estimated by the differentiation and behaviour according to clinicopathological classification (further in text, tumour grade). Differentiation grade of PET was based on WHO classification of tumours of the endocrine pancreas, as described in literature review section devoted to pathological characteristics and WHO classification of selected pancreatic tumours: Characteristics of PET (Heitz et al., 2004). The following categories were distinguished: well differentiated endocrine tumours with benign behaviour, well differentiated endocrine tumours with uncertain behaviour, well differentiated endocrine carcinoma and poorly differentiated endocrine carcinoma.

The following RM have been identified grossly and were examined grossly and microscopically for tumour invasion: proximal and distal RM of stomach and duodenum, RM of blood vessels, RM of common hepatic bile duct, RM of pancreas, peripancreatic RM (marked with Alcian-blue as shown in Figure 2.3.), other.

The status of RM was classified as R0, R1, R2 or Rx by the following criteria: R0, negative RM – by gross and microscopic evidence, no cancer cells are found in RM; R1, microscopic residual tumour in the RM – any resection line is microscopically positive for cancer presence despite negative gross appearance; R2, grossly positive resection line – any of the resection lines contain grossly evident tumour mass and the cancer presence in resection line is confirmed by microscopic assessment; Rx, RM cannot be assessed.

Figure 2.3. Operation material of pancreatoduodenectomy with stained peripancreatic resection margin (Alcian-blue)

1 – gall bladder, 2 – duodenum, 3 – head of the pancreas stained by Alcian-blue

To assess the mitotic activity, mitoses were counted in 10 consecutive high-power fields with magnification 400x (i.e., 40x objective and 10x ocular lenses; 0.65 mm² per field) within the mitotically most active areas. The activity was expressed as mitotic count in 10 high-power fields.

The peri- and intra-neural invasion as well as intravascular invasion were assessed as a categorical variable: present or absent. The invasion into large or small blood vessels was assessed separately. Superior mesenteric vein and portal vein were included among the large vessels. The small blood vessels comprised all other intrapancreatic and peripancreatic blood vessels. Lymphatic vessel invasion was classified in mutually exclusive categories as present or absent. The spread of tumour necrosis was measured as the relative area of tumour occupied by necrosis. The measurement was performed in all microscopic slides and the average value was used to characterise the tumour.

The gross photographs were obtained by Sony Cyber-shot digital camera (Sony Electronics Inc., San Diego, USA). The morphological and immunohistochemical images were taken by the Kappa image base program (KAPPA opto-electronics Inc., USA) using Axiolab (Zeiss, Oberkochen, Germany) microscope as the optical system and Kappa CF 11 DSP camera (KAPPA opto-electronics Inc., USA).

2.5. Elaboration of the diagnostic protocol

The diagnostic protocol for the examination of surgical specimens of pancreatic tumours was created for the present study based on the generally accepted principles (guidelines) of gross description and surgical pathology dissection and the necessary information for full diagnostic evaluation according to WHO classification and AJCC Cancer Staging system (Klöppel et al., 2000; Westra et al., 2003; Heitz et al., 2004; Edge et al., 2010).

2.6. Immunohistochemical visualisation and assessment

Immunohistochemical visualisation of the researched antigens was performed on formalin-fixed paraffin-embedded pancreatic tumours and non-neoplastic control tissues. From each case, a representative block of formalin-fixed paraffin-embedded tumour tissue was selected for immunohistochemical visualisation, based on the

presence of a viable tumour with tissue architecture and atypia corresponding to the most widespread grade in the analysable case. Pancreatic tissues from tumour-free resection lines were used to detect the immunophenotype of non-neoplastic control tissues. Complete necrosis or disappearance of tumour tissue in the deeper sections prompted exclusion from immunohistochemical evaluation. Thus, the number of assessed cases was recorded for each antigen.

For IHC, three-micrometre-thick sections were cut by electronic rotary microtome Microm HM 360 on electrostatically charged glass slides (Histobond, Marienfeld, Germany) and subjected to deparaffinisation in graded alcohols (Sigma-Aldrich) and Histograde xylene (J.T.Baker). Heat-induced antigen retrieval was performed in a microwave oven (3x5 min.) using basic TEG (pH 9.0) buffer (DAKO, Glostrup, Denmark). After blocking endogenous peroxidase (Sigma-Aldrich), the sections were incubated with primary antibodies at room temperature in the magnetic incubation tray.

The clonality, species origin, species specificity, working dilution and incubation time of the applied primary antibodies are listed in Table 2.2. The bound primary antibodies were detected by enzyme-conjugated polymeric visualisation system EnVision linked with horseradish peroxidase. 3,3'-diaminobenzidine was used as chromogen, followed by counterstaining implying Meyer's haematoxylin. The stained slides were covered by cover glass (Biosigma). All IHC reagents were produced by DAKO, Glostrup, Denmark. Positive and negative quality controls were performed and reacted appropriately.

Table 2.2.

The applied panel of primary antibodies: characteristics of antibody and dilution

Antigen	Antibody characteristics	Clone	Dilution	Incubation, min.
Ki-67 protein	Monoclonal mouse Ab against human Ag	MIB-1	1:100	60
p53 protein	Monoclonal mouse Ab against human Ag	DO-7	1:400	60
p21 ^{WAF1/Cip1} protein	Monoclonal mouse Ab against human p21	SX118	1:25	60
p27 ^{Kip1} protein	Monoclonal mouse Ab against human p27	SX53G8	1:50	60
Cyclin D1	Monoclonal rabbit Ab against human Ag	EP12	1:500	60

Table 2.2. (continued)				
Antigen	Antibody characteristics	Clone	Dilution	Incubation, min.
Bcl-2 oncoprotein	Monoclonal mouse Ab against human Ag	124	1:800	60
E-cadherin	Monoclonal mouse Ab against human Ag	NCH-38	1:50	60
Phagocytic glycoprotein1 CD44	Monoclonal mouse Ab against human Ag	DF1485	1:50	60
Cytokeratin 7	Monoclonal mouse Ab against human Ag	OV-TL 12/30	1:800	60
Cytokeratin 19	Monoclonal mouse Ab against human Ag	RCK108	1:200	60
Cytokeratin 20	Monoclonal mouse Ab against human Ag	Ks 20.8	1:200	60
CDX2	Monoclonal mouse Ab against human Ag	DAK-CDX2	1:50	60
Cytokeratin 5/6	Monoclonal mouse Ab against human Ag	D5/16 B4	1:100	60
Cytokeratin, high molecular weight	Monoclonal mouse Ab against human Ag	34 β E12	1:400	60
p63 protein	Monoclonal mouse Ab against human Ag	DAK-p63	1:200	60
Chromogranin A	Monoclonal mouse Ab against human Ag	DAK-A3	1:1000	60
CD56	Monoclonal mouse Ab against human Ag	123C3	1:100	60
Vimentin	Monoclonal mouse Ab	V9	1:200	60
CD34 class II	Monoclonal mouse Ab against human Ag	QBEnd10	1:1	30
COX-2	Monoclonal mouse Ab against human Ag	CX-294	1:200	60
BRCA1	Monoclonal mouse Ab	GLK-2	1:50	60

Abbreviations in the table: Ab, antibody; Ag, antigen; CD, cluster of differentiation; BRCA1, breast cancer 1.

Expression of immunohistochemical markers was evaluated by light microscopy using high-power magnification 400x (i.e., 40x objective and 10x ocular lenses; 0.65 mm² per field). The expression of the IHC markers was evaluated according to three levels regarding intensity: low, moderate and high intensity. The marker expression was considered positive if the expression intensity was moderate or high. The intensity levels are shown in Figure 2.4. using the example of CD44 expression.

Figure 2.4. The levels of intensity of CD44 expression in pancreatic ductal adenocarcinoma and non-neoplastic pancreatic duct. A, no expression; B, low intensity; C, moderate intensity; D, high intensity. Immunoperoxidase, anti-CD44, original magnification 400x.

Further evaluating the immunohistochemical reactivity, four hundred sequential cells were estimated in each case. The relative amount of positive cells was expressed as a percentage. For a case to be considered positive, the relative amount of positive cells had to reach the cut-off value. The cut-off value was different for each marker and was based on other studies published in international cited journals. The target structure of marker labelling and cut-off value are shown in Table 2.3.

Table 2.3.

Antigen characteristics by target structures and cut-off value for positive case

Antigen	Target structure of cell	Cut-off value	References
Ki-67 protein*	nucleus	1% and 10%	(Linder et al., 1997; Imam et al., 2000; Culhaci et al., 2005; Chang et al., 2007; Karamitopoulou et al., 2010; Sanaat et al., 2013)

Table 2.3. (continued)			
Antigen	Target structure of cell	Cut-off value	References
p53 protein	nucleus	5%	(Sinicrope et al., 1996; Kawesha et al., 2000; Lozano-Leon et al., 2011; Smith et al., 2011; Liu et al., 2012)
p21 ^{WAF1/Cip1} protein	nucleus	5%	(Hu et al., 1998; Kawesha et al., 2000; Kourea et al., 2003; Tiezzi et al., 2007; Lozano-Leon et al., 2011)
p27 ^{Kip1} protein	nucleus	5%	(Saitou et al., 2005; Mielko et al., 2006; Wiksten et al., 2008)
Cyclin D1	nucleus	10%	(Culhaci et al., 2005; Kanthan et al., 2010)
Bcl-2 oncoprotein	cytoplasm	5%	(Sinicrope et al., 1996; Dong et al., 2005; Jamieson et al., 2011; Smith et al., 2011)
E-cadherin	cell surface membrane and/or cytoplasm	10%	(Arumugam et al., 2009; Brody et al., 2009; Burford et al., 2009; Sethi et al., 2011; Markiewicz et al., 2012; Toru and Bilezikçi, 2012; Saito et al., 2013)
CD44	cell surface membrane	30%	(Tajima et al., 2012)
CK 7	cytoplasm	5%	(Schüssler et al., 1992; Chu et al., 2000; Goldstein and Bassi, 2001; Bayrak et al., 2012; Liu et al., 2012)
CK 19	cytoplasm	5%	(Schüssler et al., 1992; Liu et al., 2012)
CK 20	cytoplasm	5%	(Chu et al., 2000; Goldstein and Bassi, 2001; Bayrak et al., 2012; Liu et al., 2012)
CDX2	nucleus	5%	(Bayrak et al., 2012; Liu et al., 2012)
CK 5/6	cytoplasm	5%	(Chu and Weiss, 2002)
CK 34βE12	cytoplasm	5%	**
p63 protein	nucleus	10%	(Brody et al., 2009; Oncel et al., 2011; Koyuncuer, 2013)
Chromogranin A	cytoplasm, granular staining pattern	1%	(Kimura et al., 2000; Takikita et al., 2009; Canzonieri et al., 2012)
CD56	cytoplasm and/or membrane	1%	**
Vimentin	cytoplasm	10%	(Burford et al., 2009; Sethi et al., 2011; Markiewicz et al., 2012)
COX-2	cytoplasm	5%	(Juuti et al., 2006; Lozano-Leon et al., 2011)
BRCA1	cytoplasm and/or nucleus	10%	(Gachechiladze et al., 2013; Markowska et al., 2013)

Abbreviations in the table: CD, cluster of differentiation; CK, cytokeratin; BRCA1, breast cancer 1

*Ki-67: The number of Ki-67 positive cells was determined as a fraction (%) of the total number of target cells, also called Ki-67 index or proliferation fraction.

**due to the lack of accurate information about evaluation of CK 34βE12 and CD56 expression in tissue and also due to the lack of articles about the evaluation of marker expression precisely in the pancreatic tissue, the cut-off value was accepted as 5% for CK 34βE12, analogous to other cytokeratins, and 1% for CD56, analogous to chromogranin A.

To detect the MVD, endothelial differentiation was highlighted by CD34 expression (Figure 2.5.). Cells labelled by CD34 displayed staining confined to the cell

surface membrane. MVD was evaluated in 4 hot spots of the target tissue of each case. The hot spots were identified by scanning the whole slide with low-power magnification 100x (i.e., 10x objective lens and 10x ocular lens). Individual microvessels were counted on a 400x field (i.e. 40x objective lens and 10x ocular lens; 0.65 mm² per field). Vessels with a clearly defined lumen or well-defined linear vessel shape were taken into account. The MVD was calculated as mean amount of microvessels per one high-power field. The number of microvessels per mm² was further calculated.

Figure 2.5. Immunohistochemical visualisation of microvessels (arrows) in a pancreatic endocrine tumour. Immunoperoxidase, anti-CD34, original magnification 400x.

2.7. Survival and prognostic factors

The last update about the survival of each patient was performed in February 2014. The follow-up was the time interval between the date of surgery and the last date of update about the survival, or date of death due to pancreatic tumour, or the date when the patient was lost from cohort for another reason. Censoring occurred if patients were alive at last follow-up or died due to other reasons. The median follow-up was 10.5 months.

The hypothesised clinical, morphological and immunohistochemical prognostic factors were selected for further evaluation according to literature studies. The following parameters were studied in relation to the survival: demographic characteristics (gender, age), surgery (type of operation), the whole spectrum of morphological characteristics (pTNMGR, grade, peri- and intra-neural invasion,

intravascular and lymphatic invasion, mitotic count, necrosis) and the whole immunophenotype including all the researched tumour antigens as well as MVD. The survival analysis was performed separately for PDAC and PET.

2.8. Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics Version 20.0 statistical software package (International Business Machines Corp., Armonk, New York, USA). The confidence interval (CI) was invariably calculated by Confidence Interval Analysis (CIA) software (Altman et al., 2000).

Before statistical analysis the assumption check of normality using Shapiro-Wilk test was performed. The descriptive data were expressed as mean $\pm$ standard deviation (SD), median with interquartile range (IQR) or relative frequency with 95% CI.

Descriptive statistical methods including descriptive and cross tabulation with Pearson's Chi-square, bivariate correlation including Spearman's rank correlation coefficient, non-parametric method including Mann-Whitney U-test and Kruskal-Wallis one way analysis of variance by ranks were used. For data with statistically significant differences, receiver-operating characteristic (ROC) line analysis was used to determine the impact of the independent variable. The cut-off value of MVD for survival analysis was determined by ROC line analysis as well. The post-hoc analysis with Bonferroni correction was used to determine differences between 3 or more groups. For survival analysis Kaplan-Meier method was used with the log-rank test and Cox proportional hazards model. P-values of ≤ 0.05 were considered statistically significant for all analyses.

3. RESULTS

3.1. Patients and surgical approach

3.1.1. Characteristics of PDAC cases

During the evaluated time period (2004–2014), potentially curative resection of PDAC was performed in 78 patients, including 41/ 78 (52.6%; 95% CI = 41.6–63.3) women and 37/ 78 (47.4%; 95% CI = 36.7–58.4) men. The age of the patients ranged from 36 to 81 years. The mean age $\pm$ standard deviation (SD) was 63.4 ± 11.0 (95% CI = 60.9–65.9) years, the median value was 67.0 (IQR = 18.3) years. Women's mean age $\pm$ SD was 66.0 ± 10.0 (95% CI = 62.8–69.2) years; median age was 68.0 (IQR = 13.5) years. Men's mean age $\pm$ SD was 60.5 ± 11.6 (95% CI = 56.6–64.4) years; median age was 59.0 (IQR = 17.5) years. The patients were older than 65 years in 41/ 78 cases (52.6%; 95% CI = 41.6–63.3), including 26/ 41 women (63.4%; 95% CI = 48.1–76.4) and 15/ 41 men (36.6%; 95% CI = 23.6–51.9). The age distribution of PDAC patients is presented in the Figure 3.1.

Figure 3.1. The age distribution of PDAC patients by five-year intervals

Pancreatoduodenectomy was performed in 62/ 78 (79.5%; 95% CI = 69.3–87.0) cases, total pancreatectomy in 6/ 78 (7.7%; 95% CI = 3.6–15.8) cases, distal

pancreatectomy in 9/ 78 (11.5%; 95% CI = 6.2–20.5) cases and Beger procedure in 1/ 78 (1.3%; 95% CI = 0.2–6.9) case. In 8/ 78 (10.3%; 95% CI = 5.3–19.0) cases splenectomy also was performed and in 2/ 78 (2.6%; 95% CI = 0.7–8.9) cases this included pancreatoduodenectomy, in 2/ 78 (2.6%; 95% CI = 0.7–8.9) cases with total pancreatectomy, and 4/ 78 (5.1%; 95% CI = 2.0–12.5) patients underwent splenectomy along with distal pancreatectomy. The operation material of pancreatoduodenectomy and distal pancreatectomy with splenectomy are shown in Figure 3.2.

Figure 3.2. The operation material for pancreatic ductal adenocarcinoma. A, after pancreatoduodenectomy; B, after pancreatoduodenectomy and distal pancreatectomy with splenectomy.

1 – stomach, 2 – gall bladder, 3 – duodenum, 4 – head of the pancreas,
5 – tail of the pancreas, 6 – spleen

Liver metastases of PDAC were simultaneously removed in 3/ 78 (3.9%; 95% CI = 1.3–10.7) cases.

The surgical activity for PDAC treatment by the number of operations per year is shown in the Figure 3.3.

Figure 3.3. Surgical activity for the treatment of PDAC, 2004–2013, by number of operations per year

PDAC was localised mostly in the head of the pancreas, respectively in 65/ 78 (83.3%; 95% CI = 73.5–90.0) cases. The tumour had spread throughout the head and the body of pancreas in 3/ 78 (3.9%; 95% CI = 1.3–10.7) cases. Carcinoma was limited to the body of the pancreas in 4/ 78 (5.1%; 95% CI = 2.0–12.5) cases and showed wide spread throughout the body and the tail in 5/ 78 (6.4%; 95% CI = 2.8–14.1) cases. The tumour was situated just in the tail of the pancreas in 1/ 78 (1.3%; 95% CI = 0.2–6.9) case.

3.1.2. Characteristics of PET cases

During the study period, 16 patients underwent potentially curative surgical treatment for PETs. In this group, women were significantly more frequently affected than the men: 12/ 16 (75.0%; 95% CI = 50.5–89.8) *versus* 4/ 16 (25.0%;

95% CI = 10.2–49.5). The age of all patients ranged from 47 to 78 years. The mean $\pm$ SD age was 59.4 ± 9.2 (95% CI = 54.5–64.3) years with a median of 56.5 (IQR = 16) years. Women's mean age $\pm$ SD was 59.7 ± 10.2 (95% CI = 53.2–66.2), median was 56.5 (IQR = 18) years. Men's mean age $\pm$ SD was 58.5 ± 6.0 (95% CI = 49.0–68.0), median was 57.0 (IQR = 11) years. The patients were older than 65 years in 5/ 16 cases (31.3%; 95% CI = 14.2–55.6), including 4/ 5 women (80.0%; 95% CI = 37.6–96.4) and 1/ 5 men (20.0%; 95% CI = 3.6–62.5).

The type of operation included pancreatoduodenectomy in 2/ 16 (12.5%; 95% CI = 3.5–36.0) cases, total pancreatectomy in 1/ 16 (6.3%; 95% CI = 1.1–28.3) cases, distal pancreatectomy in 7/ 16 (43.8%; 95% CI = 23.1–66.8) cases and enucleation of tumour in 6/ 16 (37.5%; 95% CI = 18.5–61.4) cases. The splenectomy was carried out in 5/ 16 (31.3%; 95% CI = 14.2–55.6) cases including 4/ 5 (80.0%; 95% CI = 37.6–96.4) cases along with distal pancreatectomy and 1/ 5 (20.0%; 95% CI = 3.6–62.5) case with total pancreatectomy. Distant metastases were found and simultaneously removed in 2/ 16 (12.5%; 95% CI = 3.5–36.0) cases. The gross view of operation material, namely distal pancreatectomy with splenectomy, containing PET, is shown in Figure 3.4.

Figure 3.4. Operation material of distal pancreatectomy with splenectomy of pancreatic endocrine tumour

1 – pancreatic tissue, 2 – pancreatic endocrine tumour,
3 – spleen

Regarding the anatomic localisation of PET, the cases were equally distributed into the following four groups: tumour located in the head of the pancreas, in the body of the pancreas, tumour spreading throughout the body and the tail and tumour located in the tail of the pancreas. There were 4/ 16 (25.0%; 95% CI = 10.2–49.5) cases in each of these groups.

3.2. Morphology

3.2.1. Characteristics of PDAC cases

The data on the largest tumour diameter were available in 61/ 78 (78.2%; 95% CI = 67.8–85.9) cases of all PDACs. The tumour size as characterised by the largest diameter ranged from 1.5 to 9 cm. The mean tumour size $\pm$ SD was 3.6 ± 1.4 (95% CI = 3.2–4.0) cm, while the median value was 3.4 (IQR = 2) cm. The tumour was larger than 2 cm in 56/ 61 (91.8%; 95% CI = 82.2–96.5) cases.

All PDACs were invasive; there were no cases of carcinoma *in situ* (pTis). Regarding the local spread of the tumour, pT3 was the dominant finding (Table 3.1); the extrapancreatic invasion most frequently affected peripancreatic fat tissue and duodenum (Figures 3.5. and 3.6.). Two cases lacked information about the tumour size as well as about tumour invasion in surrounding extrapancreatic tissues, so the pT parameter in these cases was classified as pTx.

Figure 3.5. Invasion of pancreatic ductal adenocarcinoma (arrows) in the peripancreatic fat tissue. Haematoxylin and eosin (HE), original magnification 100x.

Figure 3.6. Invasion of pancreatic ductal adenocarcinoma (arrows) in the submucosa of duodenum. Haematoxylin and eosin (HE), original magnification 50x.

Analysis of LN was performed in 77/ 78 cases (98.7%; 95% CI = 93.1–99.8). In these cases, the number of assessed LN ranged from 1 to 58. The mean number of retrieved LN $\pm$ SD was 19.4 ± 15.1 (95% CI = 16.0–22.8), and the median value was 15.0 (IQR = 24.0) in all 77 cases. The amount of LN affected by cancer metastases (Figure 3.7.) ranged from 1 to 13 and the mean amount $\pm$ SD was 3.6 ± 3.2 (95% CI = 2.7–4.5), while the median value was 3.0 (IQR = 3.8).

If less than 12 LN were evaluated, the rate of pN1 was 14/ 28 (50.0%; 95% CI = 32.6–67.4). In contrast, the rate of pN1 was 38/ 49 (77.6%; 95% CI = 64.1–87.0) in cases where 12 or more LN were examined. Particularly, the rate of pN1 was 17/ 23 (73.9%; 95% CI = 53.5–87.5) in cases where 12–23 LN were examined and 21/ 26 (80.8%; 95% CI = 62.1–91.5) cases if more than 24 LN were detected. The regional LN were not examined by the pathologist (pNx) in 1/ 78 (1.3%; 95% CI = 0.2–6.9) case.

Microscopic tumour presence in the RM (Figure 3.8.) was a frequent finding occurring in 57.5% (95% CI = 46.1–68.2) cases. Peripancreatic RM has been identified as the area of significantly highest positivity risk, followed by pancreatic RM (Table 3.1.). There were no R2 cases in the study group. The status of RM was unspecified (pRx) in 5/ 78 (6.4%; 95% CI = 2.8–14.1) cases.

Figure 3.7. Metastasis of pancreatic ductal adenocarcinoma (arrows) in the lymph node. Haematoxylin and eosin (HE), original magnification 100x.

Figure 3.8. The gland of pancreatic ductal adenocarcinoma exhibiting perineural invasion in stained (Alcian-blue) resection margin (arrows). Haematoxylin and eosin (HE), original magnification 100x.

The full results of pTNM parameters, tumour grade and status of RM are shown in Table 3.1. After complete evaluation of pTNM parameters, the tumour stage was identified.

Table 3.1.

**The characteristics of pTNM parameters, tumour differentiation and status of
RM in PDACs**

Variable	Proportion, %	95% CI for proportion
pT characteristics		
pT1	1.3	0.2–7.1
pT2	0.0	0.0–4.8
pT3	98.7	92.9–99.8
The invaded structure in pT3 cases		
Peripancreatic fat tissue	100	87.5–100
Duodenum	59.3	40.7–75.5
Ampulla of <i>Vater</i> or sphincter of <i>Oddi</i>	55.6	37.3–72.4
Common bile duct	25.9	13.2–44.7
Large blood vessels ¹	14.8	5.9–32.5
Large intestine	3.7	0.7–18.3
pN characteristics		
pN0	32.5	23.1–43.5
pN1	67.5	56.5–76.9
pM characteristics		
pM0	96.2	89.3–98.7
pM1	3.8	1.3–10.7
Tumour differentiation		
G1	17.9	11.0–27.9
G2	46.2	35.5–57.1
G3	35.9	26.2–47.0
RM		
Negative	42.5	31.8–53.9
Positive	57.5	46.1–68.2
Location of invaded RM		
Pancreatic RM	38.1	20.8–59.1
Peripancreatic RM	81.0	60.0–92.3
RM of common or hepatic bile duct	23.8	10.6–45.1
RM of blood vessel	4.8	0.9–22.7

¹ Excluding the celiac axis and the superior mesenteric artery

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; CI, confidence interval; pT1, tumour limited to the pancreas, 2cm or less in greatest dimension; pT2, tumour limited to the pancreas, more than 2 cm in greatest dimension; pT3, tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least 1 node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; G1, well differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma; RM, resection margins (Guzman and Chejfec, 2007; Edge et al., 2010).

After complete evaluation of pTNM parameters, the tumour stages were the following: IA in 1/ 75 (1.3%; 95% CI = 0.3–7.2) cases, IB none, IIA in 21/ 75 (28.0%; 95% CI = 19.1–39.0) cases, IIB in 49/ 75 (65.3%; 95% CI = 54.1–75.1) cases, III in 1/ 75 (1.3%; 95% CI = 0.3–7.2) cases and IV in 3/ 75 (4.0%; 95% CI = 1.4–11.1) cases.

The tumour stage remained unspecified in 3 patients, including a case lacking morphological data about LN status and 2 cases of unspecified tumour size in combination with lacking evidence of tumour invasion in surrounding extrapancreatic tissue.

The other morphological findings included at least one mitosis per 10 HPF in PDAC identified in 77/ 78 (98.7%; 95% CI = 93.1–99.8) cases. In the mitotically active cases, the mitotic count ranged from 1 to 12. The mean mitotic count of all cases $\pm$ SD was 3.7 ± 2.5 (95% CI = 3.1–4.3) mitoses and median value was 3.0 (IQR = 3) mitoses. According to tumour grade, in well differentiated PDAC cases the mean count of mitosis $\pm$ SD was 1.7 ± 1.2 (95% CI = 1.0–2.4) and median was 1.5 (IQR = 1); in moderately differentiated PDAC, it was 3.1 ± 1.9 (95% CI = 2.5–3.7) with a median of 3.0 (IQR = 2); in poorly differentiated PDAC, it was 5.5 ± 2.6 (95% CI = 4.5–6.5) with a median of 5.0 (IQR = 2) as shown in Figure 3.9.

Figure 3.9. Poorly differentiated pancreatic ductal adenocarcinoma with mitotic figures (arrows). Haematoxylin and eosin (HE), original magnification 400x.

Intravascular invasion (Figure 3.10.) was found in 30/ 78 (38.5%; 95% CI = 28.5–49.6) cases, including tumour invasion in large blood vessels in 6/ 78 (7.7%; 95% CI = 3.6–15.8) cases and invasion in small vessels in 25/ 78 (32.1%; 95% CI = 22.8–43.0). In addition, perineural invasion was found in 68/ 78 (87.2%; 95% CI = 78.0–92.9) cases and intraneural invasion in 28/ 78 (35.9%; 95% CI = 26.2–47.0) cases (Figure 3.11.). The lymph vessels were invaded (Figure 3.12.) in 29/ 78 (37.2%; 95% CI = 27.3–48.3) cases. Tumour necrosis (Figure 3.13.) was detected in

21/ 78 (26.9%; 95% CI = 18.3–37.7) cases, ranging from 0.4% to 37% of the neoplastic tissues. The mean relative spread of necrosis $\pm$ SD was 10.4 ± 9.2 (95% CD = 6.2–14.6) and the median value was 10.0 (IQR = 15.3).

Figure 3.10. Invasion of pancreatic ductal adenocarcinoma (arrows) in the blood vessel. Haematoxylin and eosin (HE), original magnification 50x.

Figure 3.11. Perineural and intraneural invasion of pancreatic ductal adenocarcinoma. Green arrows, pancreatic ductal adenocarcinoma; yellow arrow, nerve. Haematoxylin and eosin (HE), original magnification 50x.

Figure 3.12. Invasion of pancreatic ductal adenocarcinoma in the lymph vessel (arrow). Haematoxylin and eosin (HE), original magnification 100x.

Figure 3.13. Necrosis (star) of pancreatic ductal adenocarcinoma. Haematoxylin and eosin (HE), original magnification 100x.

3.2.2. Characteristics of PET cases

The PET size ranged between 1.2 and 12.5 cm. The mean tumour size $\pm$ SD was 3.9 ± 3.2 (95% CI = 2.2–5.6) cm and the median value was 2.9 (IQR = 4.3) cm. The tumour diameter exceeded 2 cm in 9/ 16 (56.3%; 95% CI = 33.2–76.9) cases. Since the size was stated in all PET cases, the pT parameter was also certain in all cases

(Table 3.2). Tumour invasion in peripancreatic fat tissue and in duodenum was frequently present (Figure 3.14. and 3.15.).

Figure 3.14. Invasion of pancreatic endocrine tumour (arrow) in the peripancreatic fat tissue. Haematoxylin and eosin (HE), original magnification 50x.

Figure 3.15. Invasion of pancreatic endocrine tumour (arrow) in the submucosa of duodenum. Haematoxylin and eosin (HE), original magnification 50x.

The number of assessed LN ranged from 1 to 33. The mean count of LN $\pm$ SD was 6.2 ± 10.0 (95% CI = 0.0–13.6) with a median of 1.5 (IQR = 6). The number of LN affected by metastases (Figure 3.16.) ranged from 1 to 2. The pNx status was issued in 6/ 16 (37.5%; 95% CI = 18.5–61.4) cases.

Distant metastases were identified by morphologic investigation in 2/ 16 (12.5%; 95% CI = 3.5–36.0) cases, and both were located in the liver. Regarding resection lines, there was a trend towards less frequent occurrence of R1 (Figure 3.17.). In 5/ 16 (31.3%; 95% CI = 14.2–55.6) cases the pRx status was issued.

Figure 3.16. Metastasis of pancreatic endocrine tumour (arrows) in the lymph node. Haematoxylin and eosin (HE), original magnification 50x.

Figure 3.17. Pancreatic endocrine tumour in stained (Alcian-blue) resection margin (arrows). Haematoxylin and eosin (HE), original magnification 100x.

The summary of pTNM parameters, tumour grade and status of RM in PETs is represented in Table 3.2.

Table 3.2.

The characteristics of pTNM parameters, tumour grade and status of RM in PETs

Variable	Proportion, %	95% CI for proportion
pT characteristics		
pT1	25.0	10.2–49.5
pT2	37.5	18.5–61.4
pT3	37.5	18.5–61.4
The invaded structure in pT3 cases		
Peripancreatic fat tissue	83.3	43.7–97.0
Duodenum	33.3	9.7–70.0
Ampulla of <i>Vater</i> or sphincter of <i>Oddi</i>	16.7	3.0–56.4
Stomach and spleen	16.7	3.0–56.4
pN characteristics		
pN0	80.0	49.0–94.3
pN1	20.0	5.7–51.0
pM characteristics		
pM0	87.5	64.0–96.5
pM1	12.5	3.5–36.0
Tumour grade		
Well differentiated endocrine tumour, benign behaviour	6.2	1.1–28.3
Well differentiated endocrine tumour, unclear behaviour	56.3	33.2–76.9
Well differentiated endocrine carcinoma	31.3	14.2–55.6
Poorly differentiated endocrine carcinoma	6.2	1.1–28.3
RM		
Negative	72.7	43.4–90.3
Positive	27.3	9.8–56.6
Location of the invaded RM		
Pancreatic RM	66.7	20.8–93.9
Peripancreatic RM	33.3	6.2–79.2
RM of stomach	33.3	6.2–79.2

Abbreviations in the table: PET, pancreatic endocrine tumour; CI, confidence interval; pT1, tumour limited to the pancreas, 2cm or less in greatest dimension; pT2, tumour limited to the pancreas, more than 2 cm in greatest dimension; pT3, tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least 1 node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; RM, resection margins (Heitz et al., 2004; Edge et al., 2010).

The tumour stages were as follows: IA in 2/ 10 (20.0%; 95% CI = 5.7–51.0) cases, IB in 3/ 10 (30.0%; 95% = 10.8–60.3), IIA in 2/ 10 (20.0%; 95% CI = 5.7–51.0)

cases, IIB in 1/ 10 (10.0%; 95% CI = 1.8–40.4) cases, III none and IV in 2/ 10 (20.0%; 95% CI = 5.7–51.0) cases. The tumour stage was unspecified in 6 cases because the LN had not been submitted for pathological examination in these cases.

For other morphological characteristics, mitotic activity (Figure 3.18.) was found in 10/ 16 (62.5%; 95% CI = 38.6–81.5) cases. The mitotic count in these cases ranged from 1 to 13. The mean number $\pm$ SD reached 4.0 ± 3.8 (95% CI = 1.3–6.7) mitoses, and the median was 3.0 (IQR = 4.0) mitoses per 10 HPF.

Figure 3.18. The mitotic figures (arrows) in the pancreatic endocrine tumour. Haematoxylin and eosin (HE), original magnification 400x.

The number of mitoses $\pm$ SD (including mitotically inactive cases) by tumour grade was the following: in well differentiated endocrine tumours, benign behaviour (single case) had 1.0 mitosis; in well differentiated endocrine tumours, unclear behaviour had a mean of 1.1 ± 1.5 (95% CI = 0.0–2.3) and median of 0.0 (IQR = 3); in well differentiated endocrine carcinomas, the mean was 3.2 ± 3.1 (95% CI = 0.0–7.0), with a median of 3.0 (IQR = 6) and in poorly differentiated endocrine carcinomas, 13.0 mitoses in the single case for the study group.

Intravascular invasion was found in 8/ 16 (50.0%; 95% CI = 28.0–72.0) cases and was limited to small blood vessels. Perineural invasion was present in 2/ 16 (12.5%; 95% CI = 3.5–36.0) cases. Intraneural invasion was not detected for any case. The lymphatic vessels contained tumour emboli in 2/ 16 (12.5%; 95% CI = 3.5–36.0) cases. Necrosis was invariably absent from PETs.

3.3. Immunohistochemical profile of PDAC, PETs, NnPD and NnPI

The expression of immunohistochemical markers including Ki-67, p53, p21, p27, cyclin D1, Bcl-2, CK 7, CK 19, CK 20, CDX2, CK 5/6, CK 34 β E12, p63, E-cadherin, CD44, ChrA, CD56, vimentin, COX-2 and BRCA1, were assessed in PDACs, in NnPD as well as in PETs and non-neoplastic islets of the pancreas.

The evaluation of cell cycle markers was started with the analysis of proliferation activity as the comprehensive result and also one of the cardinal tumour features. The proliferation activity was significantly more marked in PDAC than in NnPD or normal and neoplastic endocrine cells. In addition, PETs showed higher proliferative activity than NnPI as evident in Table 3.3.

Similarly to proliferation activity, the expression of aberrant p53 protein was significantly more frequent in PDAC than in NnPD or non-neoplastic or neoplastic endocrine cells. The difference was observed in both analysing cases with any level of p53 expression or using the selected cut-off as described in the materials and methods section. Any level of positive p53 expression was found in 73.0% (95% CI = 61.9–81.8) of PDAC cases, but only in 12.3% (95% CI = 6.6–21.8) of NnPD. In PETs, p53 positive expression was found in 26.7% (95% = 10.9–52.0) of cases, lower than in PDAC. By the selected cut-off level, 67.6% (95% CI = 56.3–77.1) PDAC were positive in contrast to 4.1% (95% CI = 1.4–11.4) of NnPD. This difference remained significant by analysis of the mean amount of positive cells.

Expression of p21 was significantly more frequent in PDAC than in NnPD or PETs, i.e., 95.9% (95% CI = 88.6–98.6) *versus* 30.4% (95% CI = 20.9–42.1) *versus* 71.4% (95% CI = 45.4–88.3). The mean number of p21 expressing cells confirmed these differences (Table 3.3.).

The expression of p27 was common in all types of analysed tissues (Table 3.3.). However, the mean number of p27 positive cells was significantly lower in PDAC (28.2%; 95% CI = 24.2–32.2) than in PET (62.6%; 95% CI = 46.6–78.6) or NnPD (42.3%; 95% CI = 39.0–45.6), or in pancreatic islets (75.3%; 95% CI = 64.5–86.1).

Significantly increased cyclin D1 expression was observed in PDAC compared with NnPD. The mean relative number of cyclin D1 positive cells was 19.3% (95% CI = 16.4–22.2) in PDAC but in NnPD was 2.6% (95% CI = 1.3–3.9). High levels were also found in normal and neoplastic endocrine tissues (Table 3.3.)

Positive expression of Bcl-2 was not found in non-neoplastic and neoplastic endocrine cells. It was rare in PDAC (1.3%, 95% CI = 0.2–7.2). In NnPD, Bcl-2 expression was observed in 8/ 73 (11.0%; 95% CI = 5.7–20.2) cases.

The full results of cell cycle marker evaluation are represented in Table 3.3. and in Figure 3.19.

Table 3.3.

Expression of cell cycle marker proteins in non-neoplastic and neoplastic pancreatic tissues

Variable	PDAC	NnPD	PET	NnPI
Ki-67				
Evaluated cases	75	73	15	15
No. of cases with any level of Ki-67 expression; %; 95% CI	75 100 95.1–100	13 17.8 10.7–28.1	15 100 79.6–100	0 0.0 0.0–20.4
Range of Ki-67 expressing cells (%)	1–65	1–4	1–12	0
Mean of Ki-67 expressing cells $\pm$ SD (%); 95% CI	23.2 $\pm$ 15.0 19.7–26.7	1.8 $\pm$ 0.9 1.3–2.3	3.4 $\pm$ 3.4 1.5–5.3	0 $\pm$ 0 0.0–0.0
Median of Ki-67 expressing cells (%); IQR	18.0; 21.0	2.0; 1.0	2.0; 3.0	0.0
No. of positive Ki-67 status; %; 95% CI (cut-off 1%)	75 100 95.1–100	13 17.8 10.7–28.1	15 100 79.6–100	0 100 0.0–20.4
No. of negative Ki-67 status; %; 95% CI (cut-off 1%)	0 0.0 0.0–4.9	60 82.2 71.9–89.3	0 0.0 0.0–20.4	15 100 79.6–100
No. of positive Ki-67 status; %; 95% CI (cut-off 10%)	63 84.0 74.1–90.6	0 0.0 0.0–5.0	2 13.3 3.7–37.9	0 100 0.0–20.4
No. of negative Ki-67 status; %; 95% CI (cut-off 10%)	12 16.0 9.4–25.9	73 100 95.0–100	13 86.7 62.1–96.3	15 100 79.6–100
p53				
Evaluated cases	74	73	15	15
No. of cases with any level of p53 expression; %; 95% CI	54 73.0 61.9–81.8	9 12.3 6.6–21.8	4 26.7 10.9–52.0	2 13.3 3.7–37.9
Range of p53 expressing cells (%)	2–97	1–10	2–58	1–8
Mean of p53 expressing cells $\pm$ SD (%); 95% CI	46.8 $\pm$ 28.6 39.0–54.6	4.4 $\pm$ 3.5 1.7–7.1	34.5 $\pm$ 26.1 7.0–76.0	4.5 $\pm$ 4.9 0.0–48.5
Median of p53 expressing cells (%); IQR	45.5; 51	3.0; 7.0	39.0; 49.0	4.5; 3.5
No. of positive p53 status; %; 95% CI	50 67.6 56.3–77.1	3 4.1 1.4–11.4	3 20.0 7.1–45.2	1 6.7 1.2–29.8
No. of negative p53 status; %; 95% CI	24 32.4 22.9–43.7	70 95.9 88.6–98.6	12 80.0 54.8–93.0	14 93.3 70.2–98.8

Table 3.3. (continued)				
Variable	PDAC	NnPD	PET	NnPI
p21				
Evaluated cases	73	69	14	13
No. of cases with any level of p21 expression; %; 95% CI	70 95.9 88.6–98.6	21 30.4 20.9–42.1	10 71.4 45.4–88.3	5 38.5 17.7–64.5
Range of p21 expressing cells (%)	1–65	1–13	4–30	1–23
Mean of p21 expressing cells $\pm$ SD (%); 95% CI	26.2 $\pm$ 17.1 22.1–30.3	4.3 $\pm$ 3.7 2.6–6.0	8.1 $\pm$ 9.5 1.3–14.9	11.2 $\pm$ 9.0 0.0–22.4
Median of p21 expressing cells (%); IQR	25.5; 29.0	3.0; 4.5	3.5; 12.5	8.0; 17.0
No. of positive p21 status; %; 95% CI	62 84.9 75.0–91.4	5 7.2 3.1–15.9	4 28.6 11.7–54.7	4 30.8 12.7–57.6
No. of negative p21 status; %; 95% CI	11 15.1 8.6–25.0	64 92.8 84.1–96.9	10 71.4 45.4–88.3	9 69.2 42.4–87.3
p27				
Evaluated cases	74	68	14	13
No. of cases with any level of p27 expression; %; 95% CI	74 100 95.1–100	68 100 94.7–100	14 100 78.5–100	13 100 77.2–100
Range of p27 expressing cells (%)	3–82	12–67	16–98	38–99
Mean of p27 expressing cells $\pm$ SD (%); 95% CI	28.2 $\pm$ 17.4 24.2–32.2	42.3 $\pm$ 13.5 39.0–45.6	62.6 $\pm$ 27.7 46.6–78.6	75.3 $\pm$ 17.9 64.5–86.1
Median of p27 expressing cells (%); IQR	23.0; 25.0	42.0; 18.0	66.5; 49.0	79.0; 23.0
No. of positive p27 status; %; 95% CI	68 91.9 83.4–96.2	68 100 94.7–100	14 100 78.5–100	13 100 77.2–100
No. of negative p27 status; %; 95% CI	6 8.1 3.8–16.6	0 0.0 0.0–5.4	0 0.0 0.0–21.5	0 0.0 0.0–22.8
Cyclin D1				
Evaluated cases	74	71	12	13
No. of cases with any level of cyclin D1 expression; %; 95% CI	67 90.5 81.7–95.3	7 9.9 4.9–19.0	9 75.0 46.8–91.1	11 84.6 57.8–95.7
Range of cyclin D1 expressing cells (%)	1–45	1–5	2–90	1–39
Mean of cyclin D1 expressing cells $\pm$ SD (%); 95% CI	19.3 $\pm$ 11.7 16.4–22.2	2.6 $\pm$ 1.4 1.3–3.9	30.9 $\pm$ 32.3 6.1–55.7	20.4 $\pm$ 13.9 11.1–29.7
Median of cyclin D1 expressing cells (%); IQR	21.0; 20.0	2.0; 2.0	25.0; 57.0	23.0; 31.0
No. of positive cyclin D1 status; %; 95% CI	48 64.9 53.5–74.8	0 0.0 0.0–5.1	5 41.7 19.3–68.1	8 61.5 35.5–82.3
No. of negative cyclin D1 status; %; 95% CI	26 35.1 25.2–46.5	71 100 94.9–100	7 58.3 32.0–80.7	5 38.5 17.7–64.5

Table 3.3. (continued)				
Variable	PDAC	NnPD	PET	NnPI
Bcl-2				
Evaluated cases	75	73	15	15
No. of cases with any level of Bcl-2 expression; %; 95% CI	1 1.3 0.2–7.2	8 11.0 5.7–20.2	0 0.0 0.0–20.4	0 0.0 0.0–20.4
Range of Bcl-2 expressing cells (%)	13	6–45	0	0
Mean of Bcl-2 expressing cells $\pm$ SD (%); 95% CI	NA	17.5 $\pm$ 13.6 6.1–28.9	0 $\pm$ 0 0.0–0.0	0 $\pm$ 0 0.0–0.0
Median of Bcl-2 expressing cells (%); IQR	NA	13.5; 20.0	0	0
No. of positive Bcl-2 status; %; 95% CI	1 1.3 0.2–7.2	8 11.0 5.7–20.2	0 0.0 0.0–20.4	0 0.0 0.0–20.4
No. of negative Bcl-2 status; %; 95% CI	74 98.7 92.8–99.8	65 89.0 79.8–94.3	15 100 79.6–100	15 100 79.6–100

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; NnPD, non-neoplastic pancreatic ducts; PET, pancreatic endocrine tumour; NnPI, non-neoplastic pancreatic islets; No., number; CI, confidence interval; SD, standard deviation; IQR, interquartile range; NA, not applicable.

Figure 3.19. Immunohistochemical visualisation of cell cycle markers in pancreatic ductal adenocarcinoma (PDAC), non-neoplastic pancreatic ducts (NnPD), pancreatic endocrine tumour (PET) and non-neoplastic pancreatic islets (NnPI). A–C, nuclear expression of Ki-67: A, in PDAC; B, in NnPD; C, in PET. D and E, nuclear expression of p53: D, in PDAC; E, in PET. F–I, nuclear expression of p21: F, in PDAC; G, in NnPD; H, in PET; I, in NnPI. J–M, nuclear expression of p27: J, in PDAC; K, in NnPD; L, in PET; M, in PET. N–R, nuclear expression of cyclin D1: N, in PDAC; O, in NnPD; P, in PET; R, in NnPI. S, cytoplasmic expression of Bcl-2. Immunoperoxidase, A–C, anti-Ki-67; D and E, anti-p53; F–I, anti-p21; J–M, anti-p27; N–R, anti-cyclin D1; S, anti-Bcl-2. Original magnification 100x (B, C, L, R) and 400x (A, D–K, M–P, S).

Regarding both cell adhesion markers, E-cadherin and CD44 showed significant differences between non-neoplastic investigated structures, i.e., E-cadherin expression in NnPD was more frequent than in islets: 97.0% (95% CI = 89.8–99.2) *versus* 64.3% (95% CI = 38.8–83.7) of cases. Significantly more frequent expression was also found in PDAC than in PETs by the applied cut-off. CD44 positivity was also more marked in NnPD than in NnPI with a mean of 39.9% (95% CI = 34.5–45.3) *versus* 13.8% (95% CI = 7.0–20.6) of CD44 expressing cells. It was also more frequent, 59.4% (95% CI = 47.6–70.2) *versus* 7.7% (95% CI = 1.4–33.3), in NnPD and NnPI cases, respectively. The results of cell adhesion marker expression are summarised in Table 3.4., and findings of the immunohistochemical visualisation are shown in Figure 3.20.

Table 3.4.

Expression of cell adhesion markers in non-neoplastic and neoplastic pancreatic tissues

Variable	PDAC	NnPD	PET	NnPI
E-cadherin				
Evaluated cases	75	67	14	14
No. of cases with any level of E-cadherin expression; %; 95% CI	74 98.7 92.8–99.8	65 97.0 89.8–99.2	13 92.9 68.5–98.7	9 64.3 38.8–83.7
Range of E-cadherin expressing cells (%)	5–99	3–100	5–95	8–96
Mean of E-cadherin expressing cells $\pm$ SD (%); 95% CI	62.9 $\pm$ 30.3 55.9–69.9	69.7 $\pm$ 31.7 61.8–77.6	49.6 $\pm$ 34.7 28.6–70.6	54.9 $\pm$ 27.2 34.0–75.8
Median of E-cadherin expressing cells (%); IQR	69.0; 57.0	85.0; 53.0	54.0; 76.0	56.0; 42.0
No. of positive E-cadherin status; %; 95% CI	72 96.0 88.9–98.6	61 91.0 81.8–95.8	10 71.4 45.4–88.3	8 57.1 32.6–78.6
No. of negative E-cadherin status; %; 95% CI	3 4.0 1.4–11.1	6 9.0 4.2–18.2	4 28.6 11.7–54.7	6 42.9 21.4–67.4
CD44				
Evaluated cases	73	69	14	13
No. of cases with any level of CD44 expression; %; 95% CI	63 86.3 76.6–92.4	67 97.1 90.0–99.2	8 57.1 32.6–78.6	12 92.3 66.7–98.6
Range of CD44 expressing cells (%)	2–98	2–91	14–98	2–35
Mean of CD44 expressing cells $\pm$ SD (%); 95% CI	37.2 $\pm$ 23.6 31.3–43.1	39.9 $\pm$ 22.2 34.5–45.3	48.8 $\pm$ 33.6 20.7–76.9	13.8 $\pm$ 10.7 7.0–20.6
Median of CD44 expressing cells (%); IQR	34.0; 37.0	36.0; 30	43.0; 67.0	10.50; 19.0
No. of positive CD44 status; %; 95% CI	33 45.2 34.3–56.6	41 59.4 47.6–70.2	5 35.7 16.3–61.2	1 7.7 1.4–33.3
No. of negative CD44 status; %; 95% CI	40 54.8 43.4–65.7	28 40.6 29.8–52.4	9 64.3 38.8–83.7	12 92.3 66.7–98.6

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; NnPD, non-neoplastic pancreatic ducts; PET, pancreatic endocrine tumour; NnPI, non-neoplastic pancreatic islets; No., number; CI, confidence interval; SD, standard deviation; IQR, interquartile range; NA, not applicable.

Figure 3.20. Immunohistochemical visualisation of cell adhesion markers in pancreatic ductal adenocarcinoma (PDAC), non-neoplastic pancreatic ducts (NnPD), pancreatic endocrine tumour (PET) and non-neoplastic pancreatic islets (NnPI). A–E, membranous expression of E-cadherin: A, in PDAC; B, in NnPD (green arrow) and in NnPI (yellow arrows); C, in NnPD; D, in PET; E, in NnPI (arrows). F–I, membranous expression of CD44: F, in PDAC; G, in NnPD; H, in PET; I, in NnPI. Immunoperoxidase, A–E, anti-E-cadherin; F–I, anti-CD44. Original magnification 100x (B, H) and 400x (A, C–G and I).

CK spectrum as a characteristic of tumour histogenesis and differentiation was examined along with CDX2 and marker of squamous differentiation p63 (Table 3.5.). PDAC showed an identical spectrum with NnPD regarding CK 7 and CK 19 and high molecular weight CK both by any level of expression and frequency of positive expression by the defined cut-off level. However, the CK 7 as well as CK 19 showed significantly decreased expression in PDAC compared with NnPD by the mean number of positive cells: regarding CK 7, expression was found in 91.5% (95% CI = 88.1–94.9) of cells in PDAC, but 98.0% (95% CI = 96.8–99.2) of NnPD cells. The mean number of CK 19 positive cells was 86.8% (95% CI = 83.7–89.9) of PDAC cells but 95.7 (95% CI = 94.0–97.4) of cells in NnPD.

NnPD were completely negative regarding CK 20, CK 5/6 and p63. In comparison with NnPD, PDAC had elevated frequency of all three markers. The

difference at cut-off level was significant for CK 20 and p63 but not for CK 5/6. Despite a difference in CK 20, the expression of CDX2 was as frequent in PDAC as in NnPD. PET occasionally expressed CK 7, CK 19 and p63, but NnPI did not show any positivity in immunohistochemical reactions with markers of CK spectrum, CDX2 and p63. However, only CK 19 in PETs was characterised by statistically significant increased frequency of positive expression compared with NnPI, 42.9% (95% CI = 21.4–67.4) *versus* 0% (95% CI = 0.0–20.4). Statistically significant lower expression by mean number of positive cells was also observed in PETs compared with PDAC cases in immunohistochemical reactions regarding CK 7, CK 19 and p63. The full results of evaluation of CK spectrum, CDX2 and p63 are represented in Table 3.5. and in Figure 3.21.

Table 3.5.

Characteristics of CK spectrum, intestinal and squamous differentiation in non-neoplastic and neoplastic pancreatic tissues

Variable	PDAC	NnPD	PET	NnPI
CK 7				
Evaluated cases	74	71	14	13
No. of cases with any level of CK 7 expression; %; 95% CI	74 100 95.1–100	71 100 94.9–100	2 14.3 4.0–39.9	0 0.0 0.0–22.8
Range of CK 7 expressing cells (%)	5–100	65–100	13–18	0
Mean of CK 7 expressing cells $\pm$ SD (%); 95% CI	91.5 $\pm$ 14.7 88.1–94.9	98.0 $\pm$ 5.0 96.8–99.2	15.5 $\pm$ 3.5 0.0–47.0	0 $\pm$ 0 0.0–0.0
Median of CK 7 expressing cells (%); IQR	97.0; 12.0	100; 2.0	15.5; 2.5	0
No. of positive CK 7 status; %; 95% CI	73 98.6 92.7–99.8	71 100 94.9–100	2 14.3 4.0–39.9	0 0.0 0.0–22.8
No. of negative CK 7 status; %; 95% CI	1 1.4 0.2–7.3	0 0.0 0.0–5.1	12 85.7 60.1–96.0	13 100 77.2–100
CK 19				
Evaluated cases	75	71	14	15
No. of cases with any level of CK 19 expression; %; 95% CI	75 100 95.1–100	71 100 94.9–100	6 42.9 21.4–67.4	0 0.0 0.0–20.4
Range of CK 19 expressing cells (%)	41–100	66–100	1–92	0
Mean of CK 19 expressing cells $\pm$ SD (%); 95% CI	86.8 $\pm$ 13.5 83.7–89.9	95.7 $\pm$ 7.2 94.0–97.4	22.8 $\pm$ 34.5 0.0–59.0	0 $\pm$ 0 0.0–0.0
Median of CK 19 expressing cells (%); IQR	91.0; 16.0	98.0; 5.0	10.0; 33.0	0

Table 3.5. (continued)				
Variable	PDAC	NnPD	PET	NnPI
No. of positive CK 19 status; %; 95% CI	75 100 95.1–100	71 100 94.9–100	4 28.6 11.7–54.7	0 0.0 0.0–20.4
No. of negative CK 19 status; %; 95% CI	0 0.0 0.0–4.9	0 0.0 0.0–5.1	10 71.4 45.4–88.3	15 100 79.6–100
CK 20				
Evaluated cases	75	72	14	15
No. of cases with any level of CK 20 expression; %; 95% CI	27 36.0 26.1–47.3	0 0.0 0.0–5.1	0 0.0 0.0–21.5	0 0.0 0.0–20.4
Range of CK 20 expressing cells (%)	2–80	0	0	0
Mean of CK 20 expressing cells $\pm$ SD (%); 95% CI	13.7 $\pm$ 15.5 7.6–19.8	0 $\pm$ 0 0.0–0.0	0 $\pm$ 0 0.0–0.0	0 $\pm$ 0 0.0–0.0
Median of CK 20 expressing cells (%); IQR	8.0; 15.0	0	0	0
No. of positive CK 20 status; %; 95% CI	17 22.7 14.7–33.3	0 0.0 0.0–5.1	0 0.0 0.0–21.5	0 0.0 0.0–20.4
No. of negative CK 20 status; %; 95% CI	58 77.3 66.7–85.3	72 100 94.9–100	14 100 78.5–100	15 100 79.6–100
CDX2				
Evaluated cases	75	72	14	14
No. of cases with any level of CDX2 expression; %; 95% CI	9 12.0 6.4–21.3	15 20.8 13.1–31.6	0 0.0 0.0–21.5	0 0.0 0.0–21.5
Range of CDX2 expressing cells (%)	8–31	2–37	0	0
Mean of CDX2 expressing cells $\pm$ SD (%); 95% CI	16.1 $\pm$ 7.5 10.3–21.9	18.1 $\pm$ 9.6 12.8–23.4	0 $\pm$ 0 0.0–0.0	0 $\pm$ 0 0.0–0.0
Median of CDX2 expressing cells (%); IQR	15.0; 12.0	17.0; 14.0	0	0
No. of positive CDX2 status; %; 95% CI	9 12.0 6.4–21.3	14 19.4 12.0–30.0	0 0.0 0.0–21.5	0 0.0 0.0–21.5
No. of negative CDX2 status; %; 95% CI	66 88.0 78.7–93.6	58 80.6 70.0–88.1	14 100 78.5–100	14 100 78.5–100
CK 5/6				
Evaluated cases	75	67	15	14
No. of cases with any level of CK 5/6 expression; %; 95% CI	10 13.3 7.4–22.8	0 0.0 0.0–5.4	0 0.0 0.0–20.4	0 0.0 0.0–21.5
Range of CK 5/6 expressing cells (%)	2–17	0	0	0
Mean of CK 5/6 expressing cells $\pm$ SD (%); 95% CI	8.7 $\pm$ 5.8 4.6–12.8	0 $\pm$ 0 0.0–0.0	0 $\pm$ 0 0.0–0.0	0 $\pm$ 0 0.0–0.0
Median of CK 5/6 expressing cells (%); IQR	7.0; 11.0	0	0	0

Table 3.5. (continued)				
Variable	PDAC	NnPD	PET	NnPI
No. of positive CK 5/6 status; %; 95% CI	5 6.7 2.9–14.7	0 0.0 0.0–5.4	0 0.0 0.0–20.4	0 0.0 0.0–21.5
No. of negative CK 5/6 status; %; 95% CI	70 93.3 85.3–97.1	67 100 94.6–100	15 100 79.6–100	14 100 78.5–100
CK 34βE12				
Evaluated cases	75	67	14	14
No. of cases with any level of CK 34βE12 expression; %; 95% CI	73 97.3 90.8–99.3	66 98.5 92.0–99.7	0 0.0 0.0–21.5	0 0.0 0.0–21.5
Range of CK 34βE12 expressing cells (%)	5–99	5–98	0	0
Mean of CK 34βE12 expressing cells ± SD (%); 95% CI	54.9 ± 28.8 48.2–61.6	46.4 ± 21.0 41.2–51.6	0 ± 0 0.0–0.0	0 ± 0 0.0–0.0
Median amount of CK 34βE12 expressing cells (%); IQR	54.0; 52	47.0; 29.0	0	0
No. of positive CK 34βE12 status; %; 95% CI	72 96.0 88.9–98.6	65 97.0 89.8–99.2	0 0.0 0.0–21.5	0 0.0 0.0–21.5
No. of negative CK 34βE12 status; %; 95% CI	3 4.0 1.4–11.1	2 3.0 0.8–10.3	14 100 78.5–100	14 100 78.5–100
p63				
Evaluated cases	75	72	14	14
No. of cases with any level of p63 expression; %; 95% CI	28 37.3 27.3–48.7	0 0.0 0.0–5.1	4 28.6 11.7–54.7	0 0.0 0.0–21.5
Range of p63 expressing cells (%)	1–75	0	2–7	0
Mean of p63 expressing cells ± SD (%); 95% CI	22.4 ± 19.0 15.0–29.8	0 ± 0 0.0–0.0	3.3 ± 2.5 0.0–7.3	0 ± 0 0.0–0.0
Median of p63 expressing cells (%); IQR	21.5; 34	0	2.0; 4.0	0
No. of positive p63 status; %; 95% CI	18 24.0 15.8–34.8	0 0.0 0.0–5.1	0 0.0 0.0–21.5	0 0.0 0.0–21.5
No. of negative p63 status; %; 95% CI	57 76.0 65.2–84.3	72 100 94.9–100	14 100 78.5–100	14 100 78.5–100

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; NnPD, non-neoplastic pancreatic ducts; PET, pancreatic endocrine tumour; NnPI, non-neoplastic pancreatic islets; CK, cytokeratin; No., number; CI, confidence interval; SD, standard deviation; IQR, interquartile range; NA, not applicable.

Figure 3.21. Immunohistochemical visualisation of cytokeratin spectrum, CDX2 and squamous differentiation markers in pancreatic ductal adenocarcinoma (PDAC), non-neoplastic pancreatic ducts (NnPD), pancreatic endocrine tumour (PET) and non-neoplastic pancreatic islets (NnPI). A–C, cytoplasmic expression of CK 7: A, in PDAC; B, in NnPD. Note the lack of expression in NnPI; C, in PET. D–G, cytoplasmic expression of CK 19: D, in PDAC; E, in NnPD; F, in PET; G, in NnPD. Note the lack of expression in NnPI. H, cytoplasmic expression of CK 20 in PDAC. I and J, nuclear expression of CDX2: I, in PDAC; J, in NnPD. K, cytoplasmic expression of CK 5/6 in PDAC. L and M, cytoplasmic expression of CK 34βE12: L, in PDAC; M, in NnPD. N and O, nuclear expression of p63: N, in PDAC; O, in PET. Immunoperoxidase, A–C, anti-CK 7; D–G, anti-CK 19; H, anti-CK 20; I and J, anti-CDX2; K, anti-CK 5/6; L and M, anti-CK 34βE12; N and O, anti-p63. Original magnification 100x (A, D–F) and 400x (B, C, G–O).

ChrA was studied as an endocrine marker. There was no difference between PDAC and NnPD and also between PET and NnPI regarding proportion of cases with any level of ChrA expression, but a convincing difference was identified between exocrine (PDAC, NnPD) and endocrine tissues (PET, NnPI): 40.0% (95% CI = 28.6–52.6), 30.0% (95% CI = 19.9–42.5) and 100% (95% CI = 79.6–100), 100% (95% CI = 79.6–100) of cases, respectively. The mean number of ChrA expressing cells also showed a significant difference between exocrine and endocrine structures (Table 3.6.). PDAC possessed a significantly higher mean number of endocrine cell than NnPD: 7.6% (95% CI = 4.1–11.1) and 1.2% (95% CI = 1.0–1.4) of cells, respectively. Regarding the diagnostic value of ChrA in the differential diagnosis of PDAC and PET by WHO criteria, the sensitivity was 100.0% (95% CI = 79.6–100.0) and specificity also 100.0% (95% CI = 94.0–100.0). The positive and negative predictive values were 100% (95% CI = 79.6–100.0) and 100.0% (95% CI = 94.0–100.0), respectively.

In contrast to ChrA, significantly more CD56 positive cells were found in NnPD than in PDAC. Mean number of CD56 expressing cells was 26.6% (95% CI = 21.9–31.3) cells in NnPD but 4.3% (95% CI = 2.1–6.5) in PDAC. In addition, a significantly higher number of NnPD cells expressed CD56 than ChrA.

The full results characterising neuroendocrine differentiation in pancreatic tissues and neoplasms are represented in Table 3.6. and Figure 3.22.

Table 3.6.

Expression of neuroendocrine markers in non-neoplastic and neoplastic pancreatic tissues

Variable	PDAC	NnPD	PET	NnPI
Chromogranin A				
Evaluated cases	60	60	15	15
No. of cases with any level of chromogranin A expression; %; 95% CI	24 40.0 28.6–52.6	18 30.0 19.9–42.5	15 100 79.6–100	15 100 79.6–100
Range of chromogranin A expressing cells (%)	1–28	1–2	93.0–100	98–100
Mean of chromogranin A expressing cells $\pm$ SD (%); 95% CI	7.6 $\pm$ 8.4 4.1–11.1	1.2 $\pm$ 0.4 1.0–1.4	97.9 $\pm$ 2.2 96.7–99.1	99.4 $\pm$ 0.7 99.0–99.8
Median of chromogranin A expressing cells (%); IQR	5.0; 8.5	1.0; 0.0	98.0; 4.0	100; 1.0

Table 3.6. (continued)				
Variable	PDAC	NnPD	PET	NnPI
No. of positive chromogranin A status; %; 95% CI	24 40.0 28.6–52.6	18 30.0 19.9–42.5	15 100 79.6–100	15 100 79.6–100
No. of negative chromogranin A status; %; 95% CI	36 60.0 47.4–71.4	42 70.0 57.5–80.1	0 0.0 0.0–20.4	0 0.0 0.0–20.4
CD56				
Evaluated cases	60	60	14	14
No. of cases with any level of CD56 expression; %; 95% CI	24 40.0 28.6–52.6	54 90.0 79.9–95.3	14 100 78.5–100	14 100 78.5–100
Range of CD56 expressing cells (%)	1–17	1–55	89–100	98.0–100
Mean of CD56 expressing cells $\pm$ SD (%); 95% CI	4.3 $\pm$ 5.2 2.1–6.5	26.6 $\pm$ 17.3 21.9–31.3	97.7 $\pm$ 3.2 95.9–99.5	99.1 $\pm$ 0.8 98.6–99.6
Median of CD56 expressing cells (%); IQR	2; 4.5	22.5; 33.0	98.5; 3.3	99.0; 2.0
No. of positive CD56 status; %; 95% CI	24 40.0 28.6–52.6	54 90.0 79.9–95.3	14 100 78.5–100	14 100 78.5–100
No. of negative CD56 status; %; 95% CI	36 60.0 47.4–71.4	6 10.0 4.7–20.2	0 0.0 0.0–21.5	0 0.0 0.0–21.5

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; NnPD, non-neoplastic pancreatic ducts; PET, pancreatic endocrine tumour; NnPI, non-neoplastic pancreatic islets; CD, cluster of differentiation; No., number; CI, confidence interval; SD, standard deviation; IQR, interquartile range; NA, not applicable.

Positive expression of mesenchymal intermediate filament vimentin in the epithelial component was observed in PDACs, NnPDs as well as in PETs. In NnPDs it was significantly lower than in both tumours, with the mean number of vimentin expressing cells reaching 5.2% (95% CI = 2.7–7.7) in NnPD, 33.1% (95% CI = 17.6–48.6) in PDAC and 37.7% (95% CI = 8.1–67.3) in PET.

COX-2 positive cells were found only in tumour tissue. PDAC and PET showed a similar number of cases with any level of COX-2 expression, but PDAC were characterised by significantly higher COX-2 expression than PET, as the mean number of COX-2 expressing cells was 23.8% (95% CI = 17.7–29.9) in PDAC *versus* 6.2% (95% CI = 0.0–13.0) in PET.

Cytoplasmic expression of BRCA1 in all investigated structures was a rare finding. The most frequent and extensive expression was found in NnPI, showing positive expression in 26.7% (95% CI = 10.9–52.0) of cases and a high mean number of positive cells in 51.5% (95% CI = 30.3–72.7).

The full results characterising the expression of vimentin, COX-2 as well as BRCA1 protein are represented in Table 3.7. and shown in Figure 3.22.

Table 3.7.

Characteristics of mesenchymal markers, COX-2 and BRCA1 in non-neoplastic and neoplastic pancreatic tissues

Variable	PDAC	NnPD	PET	NnPI
Vimentin				
Evaluated cases	74	72	14	15
No. of cases with any level of vimentin expression; %; 95% CI	20 27.0 18.2–38.1	10 13.9 7.7–23.7	6 42.9 21.4–67.4	0 0.0 0.0–20.4
Range of vimentin expressing cells (%)	3–98	2–12	10–82	0
Mean of vimentin expressing cells $\pm$ SD (%); 95% CI	33.1 $\pm$ 33.1 17.6–48.6	5.2 $\pm$ 3.5 2.7–7.7	37.7 $\pm$ 28.2 8.1–67.3	0 $\pm$ 0 0.0–0.0
Median of vimentin expressing cells (%); IQR	19.0; 48.0	3.5; 6.0	28.0; 53.0	0
No. of positive vimentin status; %; 95% CI	12 16.2 9.5–26.2	1 1.4 0.3–7.5	5 35.7 16.3–61.2	0 0.0 0.0–20.4
No. of negative vimentin status; %; 95% CI	62 83.8 73.8–90.5	71 98.6 92.5–99.8	9 64.3 38.8–83.7	15 100 79.6–100
COX-2				
Evaluated cases	74	69	14	13
No. of cases with any level of COX-2 expression; %; 95% CI	39 52.7 41.5–63.7	0 0.0 0.0–5.3	5 35.7 16.3–61.2	0 0.0 0.0–22.8
Range of COX-2 expressing cells (%)	2–83	0	3–16	0
Mean of COX-2 expressing cells $\pm$ SD (%); 95% CI	23.8 $\pm$ 18.8 17.7–9.9	0 $\pm$ 0 0.0–0.0	6.2 $\pm$ 5.5 0.0–13.0	0 $\pm$ 0 0.0–0.0
Median of COX-2 expressing cells (%); IQR	21.0; 22.0	0	4.0; 8.0	0
No. of positive COX-2 status; %; 95% CI	33 44.6 33.8–55.9	0 0.0 0.0–5.3	1 7.1 1.3–31.5	0 0.0 0.0–22.8
No. of negative COX2 status; %; 95% CI	41 55.4 44.1–66.2	69 100 94.7–100	13 92.9 68.5–98.7	13 100 77.2–100
BRCA1 (nuclear expression)				
Evaluated cases	76	67	15	15
No. of cases with any level of BRCA1 expression; %; 95% CI	0 0.0 0.0–4.8	1 1.5 0.3–8.0	0 0.0 0.0–20.4	0 0.0 0.0–20.4
Range of BRCA1 expressing cells (%)	0	12	0	0
Mean of BRCA1 expressing cells $\pm$ SD (%); 95% CI	0 $\pm$ 0 0.0–0.0	NA	0 $\pm$ 0 0.0–0.0	0 $\pm$ 0 0.0–0.0

Table 3.7. (continued)				
Variable	PDAC	NnPD	PET	NnPI
Median of BRCA1 expressing cells (%); IQR	0	NA	0	0
No. of positive BRCA1 status; %; 95% CI	0 0.0 0.0–4.8	1 1.5 0.3–8.0	0 0.0 0.0–20.4	0 0.0 0.0–20.4
No. of negative BRCA1 status; %; 95% CI	76 100 95.2–100	66 98.5 92.0–99.7	15 100 79.6–100	15 100 79.6–100
BRCA1 (cytoplasmic expression)				
Evaluated cases	76	67	15	15
No. of cases with any level of BRCA1 expression; %; 95% CI	4 5.3 2.1–12.8	1 1.5 0.3–8.0	2 13.3 3.7–37.9	4 26.7 10.9–52.0
Range of BRCA1 expressing cells (%)	2–17	20	4–31	32–62
Mean of BRCA1 expressing cells $\pm$ SD (%); 95% CI	10.8 $\pm$ 7.5 0.0–22.7	NA	17.5 $\pm$ 19.1 0.0–100.0	51.5 $\pm$ 13.3 30.3–72.7
Median of BRCA1 expressing cells (%); IQR	12.0; 13.8	NA	17.5; 13.5	56.0; 23.0
No. of positive BRCA1 status; %; 95% CI	2 2.6 0.7–9.1	1 1.5 0.3–8.0	1 6.7 1.2–29.8	4 26.7 10.9–52.0
No. of negative BRCA1 status; %; 95% CI	74 97.4 90.9–99.3	66 98.5 92.0–99.7	14 93.3 70.2–98.8	11 73.3 48.0–89.1

Abbreviations in the table: COX-2, cyclooxygenase 2; BRCA1, breast cancer 1 protein; PDAC, pancreatic ductal adenocarcinoma; NnPD, non-neoplastic pancreatic ducts; PET, pancreatic endocrine tumour; NnPI, non-neoplastic pancreatic islets; No., number; CI, confidence interval; SD, standard deviation; IQR, interquartile range; NA, not applicable.

Figure 3.22. Immunohistochemical visualisation of neuroendocrine, mesenchymal, angiogenesis-related markers and BRCA1 in pancreatic ductal adenocarcinoma (PDAC), non-neoplastic pancreatic ducts (NnPD), pancreatic endocrine tumour (PET) and non-neoplastic pancreatic islets (NnPI). A–D, cytoplasmic expression (granular staining pattern) of chromogranin A: A, in PDAC; B, in NnPD; C, in PET; D, in NnPI. E–H, membranous and cytoplasmic CD56 expression: E, in PDAC; F, in NnPD; G, in PET; H, in NnPI. I–K, cytoplasmic expression of vimentin: I, in PDAC; J, lack of expression in NnPD; K, in PET. L and M, cytoplasmic expression of cyclooxygenase 2 (COX-2): L, in PDAC; M, in PET. N, nuclear expression of BRCA1 in NnPD; O, cytoplasmic expression of BRCA1 in NnPI. Immunoperoxidase, A–D, anti-chromogranin A; E–H, anti-CD56; I–K, anti-vimentin; L and M, anti-COX-2; N and O, anti-BRCA1. Original magnification 100x (D, G, H, K) and 400x (A–C, E, F, I, J, L – O).

In addition, PETs were characterised by marked heterogeneity of immunohistochemical markers in certain cases: heterogeneity of p27 in 2/ 14 (14.3%; 95% CI = 4.0–39.9) cases with p27 expression from none to 77% of cells per high power field, heterogeneity of COX-2 in 1/ 14 (7.1%; 95% CI = 1.3–31.5) case with expression from none to 37% of cells per high power field, heterogeneity of CK 7 in 2/ 14 (14.3%; 95% CI = 4.0–39.9) cases with CK 7 expression from none to 34% of cells per high power field, heterogeneity of CK19 in 4/ 14 (28.6%; 95% CI = 11.7–54.7) cases with CK 19 expression from none to 63% of cells per high power field, heterogeneity of E-cadherin in 7/ 14 (50.0%; 95% CI = 26.8–73.2) cases with

expression from none to 100% of cells per high power field. The heterogeneity of CD44 as well as of vimentin appeared in 4/ 14 (28.6%; 95% CI = 11.7–54.7) cases. The relative amount of positive cells per high power field ranged 0–76% in case of vimentin and 0 – 61% regarding CD44 expression.

The MVD was determined following immunohistochemical visualisation of CD34 to highlight the endothelial cells. In PDACs the MVD (Figure 3.23. and 3.24.) ranged from 11 to 144 vessels per one high power field. The mean MVD $\pm$ SD was 48.7 ± 22.1 (95% CI = 43.4–53.9) and the median value was 44.5 (IQR = 30) vessels per one high power field within PDAC.

The normal pancreatic parenchyma was characterised by the following MVD (Figures 3.25. and 3.26.): the range was 14–163 vessels, close to the characteristics of PDAC. However, the mean MVD $\pm$ SD was 82.3 ± 20.9 (95% CI = 77.2–87.4) and the median value was 81.0 (IQR = 29) vessels per one high power field, reaching significantly higher values than in the stroma of PDAC.

Figure 3.23. The blood vessels (arrows) in pancreatic ductal adenocarcinoma. Haematoxylin and eosin (HE), original magnification 400x.

Figure 3.24. Immunohistochemical visualisation of vessels (arrows) in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CD34, original magnification 100x.

Figure 3.25. The blood vessels (arrows) in normal pancreatic parenchyma. Haematoxylin and eosin (HE), original magnification 400x.

Figure 3.26. Immunohistochemical visualisation of vessels (arrows) in normal pancreatic parenchyma. Immunoperoxidase, anti-CD34, original magnification 100x.

PETs were characterised by 21–128 vessels per one high power field (Figures 3.27. and 3.28.). The mean MVD $\pm$ SD was 70.4 ± 24.2 (95% CI = 56.4–84.4), while the median value was 75.5 (IQR = 43) vessels. In non-neoplastic islets of pancreas, the MVD ranged from 28 to 168 vessels per one high power field. The mean MVD $\pm$ SD in NnPI (Figure 3.29. and 3.30.) was 79.5 ± 31.1 (95% CI = 60.7–98.3) and median value was 81.0 (IQR = 45) vessels per one high power field.

Figure 3.27. The blood vessels (arrows) in pancreatic endocrine tumour. Haematoxylin and eosin (HE), original magnification 400x.

Figure 3.28. Immunohistochemical visualisation of vessels (arrows) in pancreatic endocrine tumour. Immunoperoxidase, anti-CD34, original magnification 100x.

Figure 3.29. The blood vessels (arrows) in non-neoplastic pancreatic islet. Haematoxylin and eosin (HE), original magnification 400x.

Figure 3.30. Immunohistochemical visualisation of vessels (yellow arrows) in non-neoplastic pancreatic islet (green arrows). Immunoperoxidase, anti-CD34, original magnification 100x.

3.4. Association between clinical and morphological findings and immunohistochemical variables

3.4.1. Characteristics of PDAC cases

Patients' age showed a trend towards a difference between women and men according to Mann-Whitney U test ($z = -1.913$; $p = 0.056$). A significant association was found between gender and age ≤ 65 or > 65 years according to Pearson's chi-squared test ($\chi^2 = 4.081$; $p = 0.043$) as depicted in Figure 3.31. Thus, the development of tumours occurred at an older age for females in the studied group; there was no significant association between gender and tumour size ($z = -0.435$; $p = 0.663$) or with stage ($\chi^2 = 4.373$; $p = 0.358$) suggesting that the age difference was not associated with delayed diagnostics in women.

Figure 3.31. The distribution of women and men in age groups ≤65 and >65 years

Tumour size smaller than or exceeding 2 cm showed a trend towards association with pN0 and pN1 status ($\chi^2 = 3.564$; $p = 0.059$), respectively. A convincing association was shown with the status of resection lines pR0 and pR1 ($\chi^2 = 9.421$; $p = 0.002$). Thus, tumours greater than 2 cm were characterised by more frequent pN1 and R1 status.

A statistically significant difference of examined LN was found between pN0 and pN1 using Mann-Whitney U test, indicating that more pN1 cases were found if a greater number of LN was evaluated ($z = -2.460$; $p = 0.014$), with a weak impact according to ROC curve analysis (area under curve (AUC) = 0.674; 95% CI = 0.542–0.805; $p = 0.014$). Statistically significant associations were found between pN1 characteristics and positive status of RM ($\chi^2 = 4.296$; $p = 0.038$), lower differentiation degree ($\chi^2 = 7.090$; $p = 0.029$), tumour invasion in small blood vessels ($\chi^2 = 12.737$; $p < 0.001$), intravascular tumour invasion ($\chi^2 = 7.399$; $p = 0.007$), perineural invasion ($\chi^2 = 3.973$; $p = 0.046$) and tumour invasion in lymphatic vessels ($\chi^2 = 4.919$; $p = 0.027$).

In patients with LN metastasis, the tumour was characterised by statistically significantly higher mitotic count than in pN0 group using Mann-Whitney U test ($z = -2.397$; $p = 0.017$) with a weak impact using ROC curve analysis (AUC = 0.667; 95% CI = 0.545–0.789; $p = 0.018$).

Presence of distant metastases showed associations with tumour invasion in large blood vessels ($\chi^2 = 15.282$; $p < 0.001$), intravascular invasion ($\chi^2 = 4.992$; $p = 0.025$) and intraneural invasion ($\chi^2 = 5.571$; $p = 0.018$).

The tumour stage showed an association with tumour invasion in large blood vessels ($\chi^2 = 28.046$; $p < 0.001$) as well as in small blood vessels ($\chi^2 = 11.850$; $p = 0.019$), intravascular invasion ($\chi^2 = 13.041$; $p = 0.011$) and perineural invasion ($\chi^2 = 9.550$; $p = 0.049$). Analysing stage IIA and IIB cancers, additional results showed that tumour differentiation grade was different between both stages ($\chi^2 = 6.022$; $p = 0.049$), with a lower differentiation grade in IIB cancers.

The status of RM (pR1) showed a convincing association with perineural invasion ($\chi^2 = 13.908$; $p < 0.001$) and trend towards an association with intraneural invasion ($\chi^2 = 3.589$; $p = 0.058$). The difference in the frequency of perineural invasion (absent/ present) between pR0 and pR1 groups is shown in Figure 3.32.

Figure 3.32. The frequency of perineural invasion in the pR0 and pR1 pancreatic ductal adenocarcinoma

Abbreviations in the figure: pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins.

Lower degree of tumour differentiation showed associations with invasion in small type blood vessels ($\chi^2 = 8.432$; $p = 0.015$), intravascular invasion ($\chi^2 = 10.266$; $p = 0.006$) and perineural invasion ($\chi^2 = 8.021$; $p = 0.018$). Interestingly, there was no correlation between tumour grade and invasion in large blood vessels ($\chi^2 = 2.945$; $p = 0.229$).

The mean ranks of mitotic count showed a different distribution between tumour differentiation grades ($\chi^2 = 30.866$; $p < 0.001$). The post-hoc analysis with Bonferroni correction was used to determine differences between groups, presented in Figure 3.33.

Figure 3.33. The difference of mitotic count (by mean ranks) between tumour differentiation grades in pancreatic ductal adenocarcinoma

Abbreviations in the figure: G1, well differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma

No significant correlations were found between tumour size and mitotic count, tumour necrosis as well as number of LN with metastasis ($p > 0.050$). The correlation coefficients and p values are shown in Table 3.8. The full results of associations between clinical and morphological findings are summarised in Table 3.8.

Table 3.8.

The associations between clinical and morphological findings in PDAC by z/χ^2 and p values

Variables	Female/ male	Patients' age (years)	Patients' age $\leq 65/ > 65$ years	Tumour localisa- tion	Tumour size (cm)	Tumour size $\leq 2/ > 2$ cm	pN0/ pN1
Patients' age (years)	-1.913 0.056	NA	NA	SE	NA	SE	SE
Patients' age $\leq 65/ > 65$ years	4.081 0.043	NA	NA	SE	SE	SE	SE
Tumour localisation	4.972 0.262	4.451 0.348	8.479 0.076	NA	SE	SE	SE
Tumour size (cm)	-0.435 0.663	0.046 0.728 ¹	-0.749 0.454	0.123 7.254	NA	NA	SE
Tumour size $\leq 2\text{cm}/ > 2\text{cm}$	0.124 0.725	-0.118 0.906	0.076 0.782	1.334 0.856	NA	NA	SE
pN0/ pN1	0.961 0.327	-0.811 0.417	1.271 0.259	1.215 0.876	-0.858 0.391	3.564 0.059	NA
No. of lymph nodes with metastases	-0.355 0.723	-0.205 0.145 ¹	-1.379 0.168	2.543 0.637	-0.028 0.854 ¹	-0.867 0.386	NA
pM0/ pM1	0.463 0.496	-1.430 0.153	0.463 0.496	0.624 0.960	-0.610 0.542	0.185 0.667	1.501 0.221
Tumour stage	4.373 0.358	0.282 0.052	5.065 0.281	2.770 1.000	3.718 0.445	12.882 0.012	75.000 0.000
pR0/ pR1	0.043 0.835	-1.089 0.276	0.114 0.736	7.530 0.110	-1.703 0.089	9.421 0.002	4.296 0.038
Tumour grade	3.152 0.207	1.142 0.565	2.484 0.289	12.994 0.112	1.286 0.526	1.590 0.452	7.090 0.029
Mitotic count	-0.318 0.751	0.034 0.767 ¹	-1.358 0.174	3.319 0.506	-0.103 0.429 ¹	-0.626 0.531	-2.397 0.017
Invasion in large blood vessels (P/ A)	2.468 0.116	-0.075 0.940	2.468 0.116	1.300 0.861	-0.819 0.413	0.486 0.486	0.912 0.340
Invasion in small blood vessels (P/ A)	1.082 0.298	-0.123 0.902	0.174 0.676	3.773 0.438	-1.509 0.131	1.353 0.245	12.737 0.000
Intravascular invasion (P/ A)	0.129 0.720	-0.149 0.881	1.081 0.298	3.851 0.427	-1.809 0.070	0.672 0.412	7.399 0.007
Perineural invasion (P/ A)	3.463 0.063	-1.212 0.226	2.342 0.126	2.294 0.682	-0.119 0.905	1.008 0.315	3.973 0.046
Intraneural invasion (P/ A)	1.651 0.199	-0.297 0.766	0.367 0.544	4.853 0.303	-0.808 0.419	0.001 0.975	0.305 0.581
Invasion in lymph vessels (P/ A)	1.108 0.292	-1.263 0.207	0.340 0.560	5.143 0.273	-0.732 0.464	0.610 0.435	4.919 0.027
Area of necrosis (%)	-1.250 0.211	0.013 0.954 ¹	-0.857 0.391	4.133 0.247	-0.071 0.785 ¹	NA ²	-1.036 0.300
Tumour necrosis (P/ A)	1.086 0.297	-1.066 0.287	1.086 0.297	3.395 0.494	-1.600 0.110	2,104 0.147	1.422 0.233

Table 3.8. (continued)

Variables	pM0/ pM1	Tumour stage	Tumour stage IIA/ IIB	pR0/ pR1	Tumour grade	Number of mitosis
Tumour stage	75.000 0.000	NA	NA	SE	SE	SE
pR0/ pR1	2.309 0.129	8.083 0.089	3.624 0.057	NA	SE	SE
Tumour grade	1.494 0.474	13.333 0.101	6.022 0.049	2.340 0.310	NA	SE
Mitotic count	-0.447 0.655	5.925 0.205	-1.887 0.059	-0.169 0.865	30.866 0.000	NA
Invasion in large blood vessels (P/ A)	15.282 0.000	28.046 0.000	2.007 0.157	1.781 0.182	2.945 0.229	-1.365 0.172
Invasion in small blood vessels (P/ A)	0.002 0.961	11.850 0.019	10.734 0.001	0.361 0.548	8.432 0.015	-1.409 0.159
Intravascular invasion (P/ A)	4.992 0.025	13.041 0.011	6.000 0.014	1.255 0.263	10.266 0.006	-1.746 0.081
Perineural invasion (P/ A)	0.459 0.498	9.550 0.049	1.720 0.190	13.908 0.000	8.021 0.018	-1.819 0.069
Intraneural invasion (P/ A)	5.571 0.018	6.378 0.173	0.074 0.785	3.589 0.058	2.726 0.256	-0.216 0.829
Invasion in lymph vessels (P/ A)	1.161 0.281	5.009 0.286	2.759 0.097	0.405 0.525	0.542 0.762	-0.196 0.844
Area of necrosis (%)	-1.245 0.213	3.06 0.382	-0.580 0.562	-1.036 0.300	4.694 0.096	0.318 0.160 ¹
Tumour necrosis (P/ A)	0.065 0.799	4.813 0.307	1.820 0.177	0.628 0.428	0.670 0.715	-0.370 0.711

¹ Statistical analysis by Spearman's correlation (r_s ; p).

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; NA, not applicable; NA², not applicable because tumour necrosis was detected in all tumours with size greater than 2 cm; SE, shown elsewhere; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins - no cancer cells are found in resection margins; pR1, positive resection margins - cancer cells are found in resection margins; stage IIA, pT3/N0/M0; stage IIB, any pT/ N1/M0; P, present; A, absent.

The immunohistochemically assessed proliferation fraction by Ki-67, p53 protein, cell cycle regulatory markers p21, p27, cyclin D1, cell adhesion proteins E-cadherin and CD44 as well as CK spectrum, markers of squamous, intestinal, mesenchymal neuroendocrine differentiation and miscellaneous markers showed different correlations with clinical and morphological findings. Mutual associations between immunohistochemical markers were disclosed as well.

Regarding cell cycle markers, higher proliferation fraction by Ki-67 expression and by Mann-Whitney U test (Table 3.9., Figure 3.35.) was found in PDAC exhibiting perineural invasion ($z = -1.974$; $p = 0.004$) with a moderate effect ($AUC = 0.704$; 95% CI = 0.550–0.857; $p = 0.049$). Ki-67 had weak, positive correlations (Table 3.10.)

with amount of LN with metastases ($r_s = 0.322$; $p = 0.023$) and mitotic count ($r_s = 0.236$; $p = 0.047$). Regarding all other IHC markers, Ki-67 showed weak correlations (Table 3.11.) with p21 ($r_s = 0.280$; $p = 0.016$), p27 ($r_s = 0.314$; $p = 0.006$), CD44 ($r_s = 0.281$; $p = 0.016$) and CK 34 β E12 ($r_s = 0.305$; $p = 0.008$).

Expression of p53 correlated with patients' age ($r_s = 0.258$; $p = 0.026$) as shown in Table 3.10. Higher p53 expression using Mann-Whitney U test (Table 3.9.) was identified in patients older than 65 years ($z = -2.624$; $p = 0.009$) with a weak difference (AUC = 0.676; 95% CI = 0.554–0.798; $p = 0.009$) as well as in lower differentiated tumours ($\chi^2 = 8.673$; $p = 0.013$). The post-hoc analysis with Bonferroni correction was used to determine differences between grades as presented in Figure 3.34.

Figure 3.34. The difference of p53 expression (%) by mean ranks between various tumour differentiation grades in pancreatic ductal adenocarcinoma

Abbreviations in the figure: G1, well differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma

The difference in mean ranks of p53 expression (Figure 3.36.) was also found between groups with and without tumour invasion in large blood vessels ($z = -2.020$; $p = 0.043$) with a moderate impact (AUC = 0.748; 95% CI = 0.558–0.937; $p = 0.045$). Notably, patients with lower p53 expression had more frequent tumour invasion in large blood vessels. The opposite situation was observed regarding mean ranks of p53 expression between groups with and without tumour invasion in small blood vessels

($z = -1.982$; $p = 0.047$). Higher p53 expression was observed in PDAC invading small blood vessels. However, there was no impact according to ROC analysis ($p = 0.050$).

There was also significantly higher p53 expression according to Mann-Whitney U test in tumours with perineural invasion ($z = -2.021$; $p = 0.043$) with a moderate effect (AUC = 0.707; 95% CI = 0.583–0.830; $p = 0.045$).

Expression of p53 showed correlations (Table 3.11.) with p21 ($r_s = 0.337$; $p = 0.004$), p27 ($r_s = 0.276$; $p = 0.018$) and CDX2 ($r_s = 0.292$; $p = 0.012$).

Figure 3.35. High nuclear expression of Ki-67 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-Ki-67, original magnification 400x.

Figure 3.36. High nuclear expression of p53 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-p53, original magnification 400x.

The p21 expression using Mann-Whitney U test (Table 3.9.) was higher in patients older than 65 years ($z = -1.981$; $p = 0.048$) with a weak impact (AUC = 0.635; 95% CI = 0.508–0.762; $p = 0.048$) as shown in Figure 3.37. The patients with lower p21 expression more frequently had tumours exceeding a size of 2 cm ($z = -1.982$; $p = 0.047$) with a moderate impact (AUC = 0.770; 95% CI = 0.437–1.000; $p = 0.048$) as presented in Figure 3.38.

Figure 3.37. The difference of p21 expression (%) by mean rank analysis between patients affected by pancreatic ductal adenocarcinoma aged ≤ 65 versus > 65 years

Figure 3.38. The difference of p21 expression (%) by mean rank analysis between patients affected by pancreatic ductal adenocarcinoma ≤ 2 cm versus > 2 cm

Among IHC markers (Table 3.11.), p21 showed weak associations with cyclin D1 ($r_s = 0.318$; $p = 0.006$), CD44 ($r_s = 0.265$; $p = 0.024$), CK 19 ($r_s = 0.316$; $p = 0.007$), CDX2 ($r_s = 0.233$; $p = 0.047$), p63 ($r_s = 0.242$; $p = 0.039$), vimentin ($r_s = 0.303$; $p = 0.010$) and trend towards an association with CK 34 β E12 ($r_s = 0.225$; $p = 0.056$). As described previously, presence of p21 was also associated with Ki-67 ($r_s = 0.280$; $p = 0.016$) and p53 ($r_s = 0.337$; $p = 0.004$). Expression of p21 is shown in Figure 3.39.

Expression of p27 (Figure 3.40.) had no correlations with certain clinical and morphological findings. However, p27 had a weak correlation with CK 19 ($r_s = 0.271$; $p = 0.020$) and moderate with CK 34 β E12 ($r_s = 0.406$; $p < 0.001$). In addition, the correlations with Ki-67 ($r_s = 0.314$; $p = 0.006$) and p53 ($r_s = 0.276$; $p = 0.018$) were observed, as described previously.

The mean ranks of cyclin D1 were different between groups with and without tumour invasion in large blood vessels ($z = -1.982$; $p = 0.047$) with a moderate effect (AUC = 0.745; 95% CI = 0.617–0.873; $p = 0.048$). Consequently, patients with lower cyclin D1 had more frequent tumour invasion in large blood vessels. The mean ranks of cyclin D1 differed in tumours with absent/ present necrosis ($z = -2.071$; $p = 0.038$) with a weak impact (AUC = 0.657; 95% CI = 0.503–0.811; $p = 0.039$). Thus, patients had more frequent tumour necrosis if the cyclin D1 expression was lower. Correlation analysis showed a moderate association of cyclin D1 expression with the area of necrosis ($r_s = 0.594$; $p = 0.006$). The expression of cyclin D1 is shown in Figure 3.41.

The mean ranks of CD44 expression were higher in pN1 patients than in pN0 group ($z = -2.577$; $p = 0.010$) with a weak impact (AUC = 0.689; 95% CI = 0.557–

0.821; $p = 0.010$). Mean ranks of CD44 expression had higher expression in tumours with perineural invasion compared with those lacking perineural invasion ($z = -2.781$; $p = 0.005$). Weak, positive correlations of CD44 were found with tumour grade ($r_s = 0.266$; $p = 0.023$) and mitotic count ($r_s = 0.256$; $p = 0.034$). Regarding mutual immunophenotype analysis, CD44 showed a trend towards a negative correlation with MVD ($r_s = -0.234$; $p = 0.054$) as well as correlations with Ki-67 ($r_s = 0.281$; $p = 0.016$), p21 ($r_s = 0.265$; $p = 0.024$), CK 34 β E12 ($r_s = 0.507$; $p < 0.001$), p63 ($r_s = 0.334$; $p = 0.004$) and COX-2 ($r_s = 0.249$; $p = 0.035$). However, there was no statistically significant correlation with p53 ($r_s = 0.186$; $p = 0.117$) and CK 5/6 ($r_s = 0.161$; $p = 0.174$). The expression of CD44 is shown in Figure 3.42.

The full results of differences regarding the clinical and morphological findings by mean ranks of percentage of expressed cell cycle and adhesion markers in PDAC and their values are summarised in Table 3.9.

Table 3.9.

The values of differences (z/χ^2 ; p) in clinical and morphological findings by mean ranks of cell cycle and adhesion markers in PDAC

Independent variable	Dependent variable						
	Ki-67	p53	p21	p27	Cyclin D1	E-cadherin	CD44
Female/ male	-1.089 0.276	-0.180 0.837	-1.746 0.081	-0.255 0.799	-0.146 0.884	-0.409 0.682	-0.575 0.556
Patients' age $\leq 65/ > 65$ years	-0.425 0.671	-2.624 0.009	-1.981 0.048	-0.661 0.509	-0.374 0.708	-1.531 0.126	-0.990 0.322
Tumour localisation	3.685 0.450	5.099 0.277	1.120 0.891	1.461 0.833	7.379 0.117	4.938 0.294	6.111 0.192
Tumour size ≤ 2 cm/ > 2 cm	-0.735 0.462	-1.291 0.197	-1.982 0.047	-0.414 0.679	-0.444 0.657	-1.022 0.307	-0.046 0.963
pN0/ pN1	-1.658 0.097	-0.904 0.366	-0.981 0.327	-0.190 0.849	-0.172 0.863	-0.092 0.926	-2.577 0.010
pM0/ pM1	-0.622 0.534	-0.208 0.836	-0.403 0.687	-0.329 0.742	-1.221 0.222	-0.920 0.358	-0.710 0.478
Tumour stage	5.388 0.250	2.137 0.711	5.570 0.234	3.569 0.467	4.157 0.385	1.584 0.812	6.867 0.143
pR0/ pR1	-1.613 0.107	-0.078 0.938	-0.651 0.515	-0.686 0.493	-0.992 0.321	-0.041 0.967	-0.116 0.907
Tumour grade	0.034 0.983	8.673 0.013	0.297 0.862	0.628 0.730	0.270 0.874	1.108 0.575	5.101 0.078
Invasion in large blood vessels (P/ A)	-0.713 0.476	-2.020 0.043	-0.593 0.553	-0.327 0.744	-1.982 0.047	-0.860 0.390	-1.197 0.231
Invasion in small blood vessels (P/ A)	-1.813 0.070	-1.982 0.047	-1.456 0.146	-0.300 0.764	-0.532 0.595	-0.244 0.807	-1.267 0.205

Table 3.9. (continued)							
Independent variable	Dependent variable						
	Ki-67	p53	p21	p27	Cyclin D1	E-cadherin	CD44
Intravascular invasion (P/ A)	-1.344 0.179	-0.850 0.395	-1.240 0.215	-0.127 0.899	-0.504 0.614	-0.376 0.707	-0.722 0.471
Perineural invasion (P/ A)	-1.974 0.004	-2.021 0.043	-0.630 0.529	-0.223 0.823	-1.159 0.246	-0.416 0.677	-2.781 0.005
Intraneural invasion (P/ A)	-0.309 0.757	-0.284 0.777	-0.334 0.738	-1.597 0.110	-1.529 0.126	-0.088 0.930	-1.246 0.213
Invasion in lymph vessels (P/ A)	-0.991 0.322	-0.391 0.695	-0.289 0.772	-0.654 0.513	-0.643 0.520	-0.681 0.496	-0.670 0.503
Tumour necrosis (P/ A)	-0.174 0.862	-0.535 0.593	-1.003 0.301	-0.554 0.580	-2.071 0.038	-1.499 0.134	-1.164 0.244

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; CD, cluster of differentiation; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins; P, present; A, absent.

The full results of correlations between cell cycle and adhesion markers and clinical and morphological findings are shown in Table 3.10.

Table 3.10.

Correlations between cell cycle/ adhesion markers and clinical and morphological findings in PDAC by r/r_s and p values

Variable	Patients' age	Tumour size	LN MTS	Tumour stage	Tumour grade	Mitotic count	Necrosis ¹ (%)
Ki-67	0.075 0.525	0.054 0.683	0.322 0.023	0.072 0.545	-0.006 0.958	0.236 0.047	-0.119 0.618
p53	0.258 0.026	-0.190 0.152	-0.180 0.210	0.039 0.744	0.199 0.089	0.051 0.674	0.391 0.088
p21	0.160 0.175	-0.213 0.109	-0.060 0.680	0.028 0.816	-0.053 0.655	0.076 0.530	-0.168 0.478
p27	0.163 0.164	-0.024 0.856	0.141 0.330	-0.100 0.405	-0.078 0.506	-0.042 0.728	0.227 0.336
Cyclin D1	0.131 0.266	0.126 0.345	-0.041 0.776	-0.083 0.487	-0.027 0.822	0.011 0.931	0.594 0.006
E-cadherin	0.208 0.073	0.002 0.990	0.061 0.674	0.039 0.741	0.097 0.410	-0.037 0.761	-0.172 0.469
CD44	0.152 0.198	0.134 0.317	0.026 0.859	0.213 0.074	0.266 0.023	0.256 0.034	0.142 0.551

¹ By the area of necrosis within the tumour.

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; LN MTS, number of lymph nodes with metastases; CD, cluster of differentiation.

Figure 3.39. Nuclear expression of p21 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-p21, original magnification 400x.

Figure 3.40. Nuclear expression of p27 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-p27, original magnification 400x.

The full results of correlations between cell cycle and adhesion markers and all other IHC markers in PDAC are summarised in Table 3.11.

Table 3.11.

Correlations between cell cycle and adhesion markers and the entire immunohistochemical profile in PDAC by r/r_s and p values

Variable	Ki-67	p53	p21	p27	Cyclin D1	E-cadherin	CD44
Ki-67	NA	0.002 0.988	0.280 0.016	0.314 0.006	-0.043 0.718	-0.092 0.433	0.281 0.016
p53	0.002 0.988	NA	0.337 0.004	0.276 0.018	0.118 0.322	0.042 0.725	0.186 0.117
p21	0.280 0.016	0.337 0.004	NA	0.189 0.113	0.318 0.006	-0.006 0.959	0.265 0.024
p27	0.314 0.006	0.276 0.018	0.189 0.113	NA	0.116 0.327	0.061 0.608	0.217 0.065
Cyclin D1	-0.043 0.718	0.118 0.322	0.318 0.006	0.116 0.327	NA	0.004 0.976	0.047 0.695
E-cadherin	-0.092 0.433	0.042 0.725	-0.006 0.959	0.061 0.608	0.004 0.976	NA	0.104 0.383
CD44	0.281 0.016	0.186 0.117	0.265 0.024	0.217 0.065	0.047 0.695	0.104 0.383	NA
CK 7	0.097 0.413	0.020 0.869	0.091 0.448	0.073 0.538	0.091 0.442	0.141 0.232	0.046 0.700
CK 19	0.197 0.090	0.137 0.244	0.316 0.007	0.271 0.020	-0.055 0.641	0.142 0.225	0.053 0.656
CK 20	-0.040 0.730	0.086 0.464	0.090 0.446	0.082 0.488	-0.068 0.566	0.040 0.733	0.056 0.636
CDX2	0.113 0.333	0.292 0.012	0.233 0.047	0.209 0.074	0.213 0.068	0.124 0.290	0.055 0.647

Table 3.11. (continued)							
Variable	Ki-67	p53	p21	p27	Cyclin D1	E-cadherin	CD44
CK 5/6	0.126 0.280	0.012 0.920	-0.041 0.733	0.145 0.217	-0.056 0.633	0.002 0.988	0.161 0.174
CK 34 β E12	0.305 0.008	0.130 0.270	0.225 0.056	0.406 0.000	-0.013 0.915	0.109 0.351	0.507 0.000
p63	0.110 0.349	0.089 0.448	0.242 0.039	0.027 0.820	0.174 0.138	0.062 0.597	0.334 0.004
ChrA	-0.036 0.881	-0.097 0.685	0.052 0.827	-0.014 0.955	-0.222 0.347	-0.114 0.633	0.157 0.510
CD56	-0.095 0.689	-0.051 0.830	0.068 0.777	-0.006 0.980	-0.207 0.381	-0.072 0.763	0.120 0.614
Vimentin	0.085 0.472	-0.091 0.442	0.303 0.010	-0.049 0.684	0.132 0.268	-0.184 0.120	0.217 0.069
MVD (CD34)	-0.134 0.269	-0.044 0.717	-0.170 0.167	-0.032 0.793	-0.051 0.678	-0.054 0.664	-0.234 0.054
COX-2	0.086 0.469	0.142 0.232	-0.065 0.590	-0.052 0.662	-0.079 0.508	-0.099 0.400	0.249 0.035
BRCA1 ¹	0.011 0.927	-0.203 0.082	-0.044 0.709	-0.109 0.353	0.008 0.946	-0.220 0.058	0.038 0.747

¹ Cytoplasmic expression.

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; CD, cluster of differentiation; NA, not applicable; CK, cytokeratin; ChrA, chromogranin A; MVD, microvascular density; COX-2, cyclooxygenase 2; BRCA1, breast cancer 1 protein.

Figure 3.41. Nuclear expression of cyclin D1 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-cyclin D1, original magnification 400x.

Figure 3.42. Membranous expression of CD44 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CD44, original magnification 400x.

The analysis of CK spectrum comprised evaluation of CK 7, CK 19, CK 20, CK 5/6 and CK 34 β E12. Along with the CK spectrum, squamous differentiation was assessed by p63 expression and intestinal by CDX2 expression. The following associations with clinical and morphological parameters as well as with other IHC markers were found.

The expression of CK 7 by mean rank analysis showed a difference regarding tumour localisation ($\chi^2 = 9.677$; $p = 0.046$). CK 7 had a moderate, positive association with expression of CK 19 ($r_s = 0.413$; $p < 0.001$) and weak correlation with CK 34 β E12 ($r_s = 0.381$; $p = 0.001$) as well as moderate, negative correlations with ChrA ($r_s = -0.507$; $p = 0.022$) and CD56 ($r_s = -0.483$; $p = 0.031$). The expression of CK 7 is shown in Figure 3.43.

Expression of CK 19 (Figure 3.44.) did not correlate with any clinical or morphological findings, but CK 19 had a moderate association with CK 34 β E12 expression ($r_s = 0.429$; $p < 0.001$). There were also correlations with the expression of p21 ($r_s = 0.316$; $p = 0.007$), p27 ($r_s = 0.271$; $p = 0.020$) and CK 7 ($r_s = 0.413$; $p < 0.001$), as noted previously.

Figure 3.43. Cytoplasmic expression of cytokeratin 7 (CK 7) in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CK 7, original magnification 400x.

Figure 3.44. Cytoplasmic expression of cytokeratin 19 (CK 19) in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CK 19, original magnification 400x.

Expression of CK 20 (Figure 3.46.) showed a weak, negative association with number of LN affected by metastases ($r_s = -0.290$; $p = 0.041$). Among the immunohistochemical findings, expression of CK 20 correlated with CDX2 ($r_s = 0.343$; $p = 0.003$) and CK 5/6 ($r_s = 0.306$; $p = 0.008$).

The patient groups ≤ 65 and > 65 years old had a difference in mean ranks of CDX2 expression ($z = -2.296$; $p = 0.022$), but there was no impact by ROC analysis ($p = 0.195$). The mean ranks of CDX2 expression were also different between groups with and without tumour invasion in lymph vessels ($z = -2.477$; $p = 0.013$), but also without a statistically significant impact using ROC analysis ($p = 0.162$). CDX2 correlated with the expression of cell cycle regulator proteins p53 ($r_s = 0.292$; $p = 0.012$) and p21 ($r_s = 0.233$; $p = 0.047$). Nuclear CDX2 expression is shown in Figure 3.47.

The CK 34 β E12 expression by mean ranks showed a difference between the following tumours: with/ without intravascular invasion ($z = -2.062$; $p = 0.039$) with a weak impact ($AUC = 0.642$; 95% CI = 0.515–0.769; $p = 0.039$), with/ without perineural invasion ($z = -2.234$; $p = 0.025$) with a moderate effect ($AUC = 0.731$; 95% CI = 0.566–0.895; $p = 0.026$), with/ without intraneural invasion ($z = -2.269$; $p = 0.023$) with a weak effect ($AUC = 0.659$; 95% CI = 0.525–0.792; $p = 0.023$) and with/ without tumour necrosis ($z = -2.642$; $p = 0.008$) with a moderate impact ($AUC = 0.700$; 95% CI = 0.562–0.839; $p = 0.008$). The differences in mean ranks of CK 34 β E12 expression between the above mentioned groups are shown in Figure 3.45. Within the spectrum of immunohistochemical markers, CK 34 β E12 showed correlations with Ki-67 ($r_s = 0.305$; $p = 0.008$), p27 ($r_s = 0.406$; $p < 0.001$), CK 7 ($r_s = 0.381$; $p = 0.001$), CK 19 ($r_s = 0.429$; $p < 0.001$) and CD44 expression ($r_s = 0.507$; $p < 0.001$). The expression of CK 34 β E12 is shown in Figure 3.49.

Figure 3.45. The differences of CK 34 β E12 expression (%) by mean ranks between the following morphological groups of PDAC: 3.45.A, with absent/ present intravascular invasion; 3.45.B, with absent/ present perineural invasion; 3.45.C, with absent/ present intraneural invasion; 3.45.D, with absent/ present tumour necrosis.

Abbreviations in the figure: PDAC, pancreatic ductal denocarcinoma; CK, cytokeratin.

The difference of CK 5/6 expression by mean ranks was found in groups of variable tumour localisation ($\chi^2 = 10.183$; $p = 0.037$). In 7/ 10 (70.0%; 95% CI = 39.7–89.2) of CK 5/6 positive cases, the tumour was localised in the head of pancreas, in 2/ 10 (20%; 95% CI = 5.7–51.0) cases PDAC spread through corpus and tail of pancreas and in 1/ 10 (10%; 95% CI = 1.8–40.4) case it was localised in the pancreatic corpus. The difference in mean ranks of CK 5/6 expression was also found in tumours with/ without intraneural invasion ($z = -1.975$; $p = 0.048$). However, there was no impact according to ROC curve analysis ($p = 0.265$). There was also a trend towards a difference between tumours with and without necrosis ($z = -1.964$; $p = 0.050$).

CK 5/6 correlated with CK 20 ($r_s = 0.306$; $p = 0.008$) and COX-2 ($r_s = 0.238$; $p = 0.041$) but lacked a correlation with p63 protein ($r_s = 0.074$; $p = 0.530$) or high molecular weight CK 34 β E12 ($r_s = 0.171$; $p = 0.142$). Expression of CK 5/6 is shown in Figure 3.48.

Figure 3.46. Diffuse cytoplasmic expression of CK 20 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CK 20, original magnification 400x.

Figure 3.47. Nuclear expression of CDX2 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CDX2, original magnification 400x.

Figure 3.48. Cytoplasmic expression of CK 5/6 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CK 5/6, original magnification 400x.

Figure 3.49. Cytoplasmic expression of CK 34βE12 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CK 34βE12, original magnification 400x.

Expression of p63 (Figure 3.50.) showed weak, positive correlations with p21 ($r_s = 0.242$; $p = 0.039$) and CD44 ($r_s = 0.334$; $p = 0.004$).

Figure 3.50. Nuclear expression of p63 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-p63, original magnification 400x.

The full results of differences among clinical and morphological findings by mean ranks of percentage of cells of expressed CK and squamous markers in PDAC and their values are summarised in Table 3.12.

Table 3.12.

The values of differences (z/χ^2 ; p) in clinical and morphological findings by mean ranks of CK, intestinal and squamous differentiation markers in PDA

Independent variable	Dependent variable						
	CK 7	CK 19	CK 20	CDX2	CK 5/6	CK 34 β E12	p63
Female/ male	-0.867 0.386	-1.005 0.315	-1.385 0.166	-0.621 0.535	-1.364 0.172	-0.919 0.358	-0.324 0.746
Patients' age $\leq 65/ > 65$ years	-1.138 0.255	-1.925 0.054	-0.816 0.414	-2.296 0.022	-1.214 0.225	-0.526 0.599	-0.232 0.816
Tumour localisation	9.677 0.046	6.295 0.178	3.072 0.546	1.697 0.791	10.183 0.037	6.940 0.139	4.089 0.394
Tumour size ≤ 2 cm/ > 2 cm	-0.300 0.764	-0.423 0.673	-0.461 0.645	-0.727 0.467	-0.778 0.436	-1.742 0.081	-0.078 0.938
pN0/ pN1	-1.377 0.169	-0.463 0.644	-0.569 0.569	-0.651 0.515	-0.641 0.522	-0.346 0.729	-0.152 0.879
pM0/ pM1	-1.609 0.108	-1.137 0.256	-1.275 0.202	-0.647 0.518	-0.647 0.518	-0.473 0.636	-0.218 0.827
Tumour stage	3.544 0.471	2.790 0.593	4.065 0.397	2.233 0.693	1.350 0.853	1.389 0.846	1.194 0.879
pR0/ pR1	-0.079 0.937	-0.804 0.421	-0.391 0.696	-0.224 0.807	-0.665 0.506	-1.502 0.133	-0.121 0.903
Tumour grade	4.161 0.125	1.690 0.430	0.550 0.760	0.637 0.727	0.297 0.862	3.741 0.154	0.966 0.617
Invasion in large blood vessels (P/ A)	-0.141 0.887	-1.203 0.229	-0.318 0.750	-0.934 0.350	-0.934 0.350	-1.377 0.168	-0.495 0.621
Invasion in small blood vessels (P/ A)	-0.931 0.352	-0.364 0.716	-0.205 0.838	-1.700 0.089	-0.252 0.801	-1.772 0.076	-0.641 0.522
Intravascular invasion (P/ A)	-1.113 0.266	-0.773 0.439	-0.190 0.849	-1.195 0.232	-0.193 0.847	-2.062 0.039	-0.514 0.607
Perineural invasion (P/ A)	-0.743 0.458	-0.800 0.424	-0.047 0.962	-1.054 0.292	-0.173 0.862	-2.234 0.025	-1.127 0.260
Intraneural invasion (P/ A)	-0.191 0.849	-1.829 0.067	-1.382 0.167	-0.430 0.667	-1.975 0.048	-2.269 0.023	-0.044 0.965
Invasion in lymph vessels (P/ A)	-0.774 0.439	-1.226 0.220	-1.159 0.246	-2.477 0.013	-0.376 0.707	-0.408 0.683	-0.921 0.357
Tumour necrosis (P/ A)	-0.634 0.526	-0.048 0.962	-1.060 0.289	-1.189 0.235	-1.964 0.050	-2.642 0.008	-0.110 0.912

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; CK, cytokeratin; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins; P, present; A, absent.

The full results of correlations between IHC markers and clinical and morphological findings are shown in Table 3.13.

Table 3.13.

Correlation between CK, intestinal and squamous differentiation markers and clinical and morphological findings in PDAC by r/r_s and p value

Variable	Patients' age	Tumour size	LN MTS	Tumour stage	Tumour grade	Mitotic count	Necrosis ¹ (%)
CK 7	-0.126 0.283	0.186 0.163	-0.037 0.801	0.146 0.220	0.003 0.983	-0.017 0.887	0.057 0.810
CK 19	0.148 0.205	0.019 0.883	0.058 0.691	0.005 0.965	0.092 0.434	0.091 0.453	-0.213 0.368
CK 20	0.089 0.447	0.106 0.424	-0.290 0.041	-0.016 0.892	-0.026 0.825	-0.034 0.776	-0.320 0.169
CDX2	0.221 0.057	0.009 0.944	0.191 0.183	0.097 0.414	0.092 0.432	-0.150 0.212	-0.120 0.614
CK 5/6	-0.149 0.201	0.098 0.458	-0.002 0.987	-0.110 0.353	0.035 0.765	-0.008 0.944	-0.138 0.563
CK 34βE12	0.073 0.534	0.031 0.816	0.170 0.238	-0.085 0.474	0.187 0.108	0.130 0.279	-0.058 0.807
p63	0.068 0.564	-0.015 0.909	0.155 0.284	-0.027 0.821	0.113 0.333	-0.206 0.085	0.074 0.756

¹ By the area of necrosis within the tumour.

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; LN MTS, number of lymph nodes with metastases; CK, cytokeratin.

The complete set of correlations between the CK spectrum, intestinal and squamous differentiation and the entire immunophenotype in PDAC is summarised in Table 3.14.

Table 3.14.

Correlations between CK, intestinal and squamous differentiation markers and the entire immunohistochemical profile in PDAC by r/r_s and p values

Variable	CK 7	CK 19	CK 20	CDX2	CK 5/6	CK 34βE12	p63
Ki-67	0.097 0.413	0.197 0.090	-0.040 0.730	0.113 0.333	0.126 0.280	0.305 0.008	0.110 0.349
p53	0.020 0.869	0.137 0.244	0.086 0.464	0.292 0.012	0.012 0.920	0.130 0.270	0.089 0.448
p21	0.091 0.448	0.316 0.007	0.090 0.446	0.233 0.047	-0.041 0.733	0.225 0.056	0.242 0.039
p27	0.073 0.538	0.271 0.020	0.082 0.488	0.209 0.074	0.145 0.217	0.406 0.000	0.027 0.820
Cyclin D1	0.091 0.442	-0.055 0.641	-0.068 0.566	0.213 0.068	-0.056 0.633	-0.013 0.915	0.174 0.138
E-cadherin	0.141 0.232	0.142 0.225	0.040 0.733	0.124 0.290	0.002 0.988	0.109 0.351	0.062 0.597
CD44	0.046 0.700	0.053 0.656	0.056 0.636	0.055 0.647	0.161 0.174	0.507 0.000	0.334 0.004

Table 3.14. (continued)							
Variable	CK 7	CK 19	CK 20	CDX2	CK 5/6	CK 34βE12	p63
CK 7	NA	0.413 0.000	−0.001 0.997	−0.035 0.768	0.165 0.160	0.381 0.001	0.055 0.641
CK 19	0.413 0.000	NA	0.047 0.687	0.029 0.802	0.030 0.800	0.429 0.000	0.068 0.560
CK 20	−0.001 0.997	0.047 0.687	NA	0.343 0.003	0.306 0.008	0.157 0.180	−0.025 0.832
CDX2	−0.035 0.768	0.029 0.802	0.343 0.003	NA	0.215 0.063	0.032 0.788	0.149 0.203
CK 5/6	0.165 0.160	0.030 0.800	0.306 0.008	0.215 0.063	NA	0.171 0.142	0.074 0.530
CK 34βE12	0.381 0.001	0.429 0.000	0.157 0.180	0.032 0.788	0.171 0.142	NA	0.203 0.081
p63	0.055 0.641	0.068 0.560	−0.025 0.832	0.149 0.203	0.074 0.530	0.203 0.081	NA
ChrA	− 0.507 0.022	−0.085 0.720	0.390 0.089	−0.193 0.415	0.002 0.992	−0.201 0.397	0.024 0.920
CD56	− 0.483 0.031	−0.003 0.989	0.408 0.074	−0.207 0.380	0.002 0.992	−0.231 0.326	−0.002 0.993
Vimentin	0.017 0.884	0.039 0.740	0.001 0.994	−0.019 0.870	0.215 0.068	0.161 0.173	0.103 0.385
MVD (CD34)	−0.050 0.685	−0.093 0.442	0.116 0.337	0.120 0.324	−0.181 0.133	−0.221 0.065	0.001 0.995
COX-2	−0.049 0.679	−0.142 0.227	0.050 0.674	0.153 0.194	0.238 0.041	0.000 0.999	0.007 0.950
BRCA1 ¹	−0.074 0.532	−0.019 0.873	−0.172 0.139	−0.087 0.456	−0.087 0.456	−0.137 0.242	−0.043 0.715

¹ Cytoplasmic expression.

Abbreviations in the table: PDAC, pancreatic ductal adenocarcinoma; CK, cytokeratin; NA, not applicable; CD, cluster of differentiation; ChrA, chromogranin A; MVD, microvascular density; COX-2, cyclooxygenase 2; BRCA1, breast cancer 1 protein.

Expression of ChrA as well as expression of CD56 (Figure 3.51.) showed moderate correlations with vascular density: $r_s = 0.490$; $p = 0.033$ and $r_s = 0.522$; $p = 0.022$. There was also a strong correlation between both endocrine markers ($r_s = 0.985$; $p < 0.001$). ChrA negatively correlated with tumour stage ($r_s = -0.445$; $p = 0.049$). Both ChrA and CD56 had negative correlations with CK 7 ($r_s = -0.507$; $p = 0.022$ and $r_s = -0.483$; $p = 0.031$).

Patients with distant metastasis had convincing higher mean ranks of vimentin expression (Figure 3.52.) than M0 patients ($z = -2.538$; $p = 0.011$) with a very strong impact (AUC = 0.915; 95% CI = 0.832–0.999; $p = 0.046$). Mean ranks of vimentin expression were higher for patients with later tumour stages ($\chi^2 = 10.857$; $p = 0.028$). Regarding immunophenotype, vimentin expression correlated with p21 ($r_s = 0.303$; $p = 0.010$).

A difference was found in mean ranks of COX-2 expression between LN status pN0 and pN1 ($z = -2.073$; $p = 0.038$) with a weak impact ($AUC = 0.644$; $95\% \text{ CI} = 0.512\text{--}0.776$; $p = 0.049$). COX-2 expression was higher in pN1 patients. All three PDAC which possessed distant metastases also showed COX-2 positive expression with the percentage of positive cells ranging from 11.0–25.0%. The trend towards a difference in mean ranks of COX-2 expression was found for tumour grade ($\chi^2 = 5.805$; $p = 0.055$) and between groups with absent/ present necrosis ($z = -1.886$; $p = 0.059$). Weak correlations were found between COX-2 and CK 5/6 ($r_s = 0.238$; $p = 0.041$) expression and between COX-2 and CD44 ($r_s = 0.249$; $p = 0.035$). Expression of COX-2 is shown in Figure 3.53.

Mean ranks of cytoplasmic BRCA1 expression showed a difference between patients with and without distant metastasis ($z = -2.275$; $p = 0.023$) without a statistically significant impact using ROC analysis ($p = 0.379$). BRCA1 expression (Figure 3.54.) showed a trend towards a correlation with E-cadherin expression ($r_s = -0.220$; $p = 0.058$).

The full results of differences in clinical morphological findings by mean ranks of neuroendocrine and mesenchymal differentiation and angiogenesis in PDAC are summarised in Table 3.15.

Table 3.15.

The values of differences (z/χ^2 ; p) in clinical and morphological findings by mean ranks of neuroendocrine and mesenchymal differentiation, angiogenesis and BRCA1 in PDAC

Independent variable	Dependent variable					
	ChrA	CD56	Vimentin	MVD	COX-2	BRCA1
Female/ male	-1.119 0.263	-1.523 0.128	-1.182 0.237	-0.736 0.462	-1.059 0.289	-1.102 0.270
Patients' age ≤ 65/ > 65 years	-0.000 1.000	-0.258 0.797	-1.157 0.247	-0.696 0.487	-0.429 0.668	-1.169 0.242
Tumour localisation	2.402 0.493	2.154 0.541	5.263 0.261	4.478 0.345	2.089 0.719	3.501 0.478
Tumour size ≤ 2 cm/ > 2 cm	-0.538 0.591	-0.691 0.490	-0.115 0.908	-0.908 0.364	-0.403 0.687	-0.531 0.595
pN0/ pN1	-1.867 0.062	-1.763 0.078	-0.695 0.487	-0.535 0.593	-2.073 0.038	-0.321 0.748
pM0/ pM1	-0.783 0.433	-0.784 0.433	-2.538 0.011	-0.943 0.346	-1.507 0.132	-2.275 0.023
Tumour stage	3.815 0.148	3.452 0.178	10.857 0.028	4.341 0.362	6.070 0.194	5.088 0.278
pR0/ pR1	-0.885 0.376	-0.793 0.428	-0.117 0.860	-1.285 0.199	-0.166 0.868	-1.352 0.176
Tumour grade	4.381 0.112	4.107 0.128	0.425 0.809	0.272 0.873	5.805 0.055	1.495 0.473

Table 3.15. (continued)						
Independent variable	Dependent variable					
	ChrA	CD56	Vimentin	MVD	COX-2	BRCA1
Invasion in large blood vessels (P/ A)	NA	NA	-1.214 0.225	-1.626 0.104	-1.026 0.305	-1.344 0.179
Invasion in small blood vessels (P/ A)	-0.850 0.395	-0.807 0.420	-0.138 0.890	-0.444 0.657	-0.678 0.498	-1.429 0.153
Intravascular invasion (P/ A)	-0.850 0.395	-0.807 0.420	-0.967 0.333	-0.480 0.631	-0.304 0.761	-0.577 0.564
Perineural invasion (P/ A)	-0.783 0.433	-0.784 0.433	-0.278 0.781	-0.860 0.390	-1.531 0.126	-0.748 0.455
Intraneural invasion (P/ A)	-0.172 0.864	-0.172 0.864	-1.657 0.098	-0.887 0.375	-1.253 0.210	-0.570 0.568
Invasion in lymph vessels (P/ A)	-1.263 0.206	-1.483 0.138	-0.643 0.521	-0.468 0.640	-0.345 0.730	-0.525 0.600
Tumour necrosis (A/ P)	-0.071 0.943	-0.071 0.943	-1.503 0.133	-0.722 0.470	-1.886 0.059	-0.030 0.976

Abbreviations in the table: BRCA1, breast cancer 1 protein; PDAC, pancreatic ductal adenocarcinoma; ChrA, chromogranin A; CD, cluster of differentiation; MVD, microvascular density; COX-2, cyclooxygenase 2; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins - no cancer cells are found in resection margins; pR1, positive resection margins - cancer cells are found in resection margins; NA, not applicable; P, present; A, absent.

The full results of correlations between percentages of expressed IHC markers and clinical and morphological findings are shown in Table 3.16.

Table 3.16.

Correlations between neuroendocrine and mesenchymal differentiation, angiogenesis and BRCA1 and clinical and morphological findings in PDAC by r/r_s; and p value

Variable	Patients' age	Tumour size	LN MTS	Tumour stage	Tumour grade	Mitotic count	Necrosis ¹ (%)
ChrA	-0.023 0.925	-0.104 0.671	-0.108 0.690	-0.445 0.049	-0.026 0.913	-0.033 0.889	NA
CD56	0.002 0.994	-0.121 0.623	-0.165 0.542	-0.425 0.062	-0.010 0.967	-0.035 0.884	NA
Vimentin	-0.181 0.125	0.020 0.884	-0.113 0.440	-0.059 0.622	0.070 0.556	0.111 0.360	0.066 0.783
MVD	-0.038 0.758	-0.126 0.359	0.041 0.783	0.062 0.614	0.011 0.925	-0.232 0.060	0.112 0.639
COX-2	-0.058 0.624	0.024 0.858	0.033 0.822	0.218 0.065	0.222 0.057	0.135 0.266	0.399 0.081
BRCA1²	-0.183 0.114	0.104 0.430	0.005 0.970	0.097 0.410	0.023 0.845	0.042 0.727	0.140 0.556

¹ By the area of necrosis within the tumour.

² Cytoplasmic expression.

Abbreviations in the table: BRCA1, breast cancer 1 protein; PDAC, pancreatic ductal adenocarcinoma; LN MTS, number of lymph nodes with metastases; ChrA, chromogranin A; NA, not applicable; MVD, microvascular density; COX-2, cyclooxygenase 2.

Figure 3.51. Membranous and cytoplasmic expression of CD56 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CD56, original magnification 400x.

Figure 3.52. Cytoplasmic expression of vimentin in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-vimentin, original magnification 400x.

The full results of correlations between IHC markers in PDAC are summarised in Table 3.17.

Table 3.17.

Correlations between neuroendocrine and mesenchymal differentiation, angiogenesis and BRCA1 and the entire immunohistochemical profile in PDAC by r/r_s and p values

Variable	ChrA	CD56	Vimentin	MVD	COX-2	BRCA1 ¹
Ki-67	-0.036 0.881	-0.095 0.689	0.085 0.472	-0.134 0.269	0.086 0.469	0.011 0.927
p53	-0.097 0.685	-0.051 0.830	-0.091 0.442	-0.044 0.717	0.142 0.232	-0.203 0.082
p21	0.052 0.827	0.068 0.777	0.303 0.010	-0.170 0.167	-0.065 0.590	-0.044 0.709
p27	-0.014 0.955	-0.006 0.980	-0.049 0.684	-0.032 0.793	-0.052 0.662	-0.109 0.353
Cyclin D1	-0.222 0.347	-0.207 0.381	0.132 0.268	-0.051 0.678	-0.079 0.508	0.008 0.946
E-cadherin	-0.114 0.633	-0.072 0.763	-0.184 0.120	-0.054 0.664	-0.099 0.400	-0.220 0.058
CD44	0.157 0.510	0.120 0.614	0.217 0.069	-0.234 0.054	0.249 0.035	0.038 0.747
CK 7	-0.507 0.022	-0.483 0.031	0.017 0.884	-0.050 0.685	-0.049 0.679	-0.074 0.532
CK 19	-0.085 0.720	-0.003 0.989	0.039 0.740	-0.093 0.442	-0.142 0.227	-0.019 0.873
CK 20	0.390 0.089	0.408 0.074	0.001 0.994	0.116 0.337	0.050 0.674	-0.172 0.139
CDX2	-0.193 0.415	-0.207 0.380	-0.019 0.870	0.120 0.324	0.153 0.194	-0.087 0.456
CK 5/6	0.002 0.992	0.002 0.992	0.215 0.068	-0.181 0.133	0.238 0.041	-0.087 0.456
CK 34βE12	-0.201 0.397	-0.231 0.326	0.161 0.173	-0.221 0.065	0.000 0.999	-0.137 0.242

Table 3.17. (continued)						
Variable	ChrA	CD56	Vimentin	MVD	COX-2	BRCA1 ¹
p63	0.024 0.920	−0.002 0.993	0.103 0.385	0.001 0.995	0.007 0.950	−0.043 0.715
ChrA	NA	0.985 0.000	0.038 0.878	0.490 0.033	−0.217 0.358	0.427 0.061
CD56	0.985 0.000	NA	0.012 0.962	0.522 0.022	−0.198 0.403	0.337 0.146
Vimentin	0.038 0.878	0.012 0.962	NA	0.129 0.277	−0.020 0.865	0.129 0.227
MVD (CD34)	0.490 0.033	0.522 0.022	0.129 0.277	NA	0.018 0.883	−0.054 0.655
COX-2	−0.217 0.358	−0.198 0.403	−0.020 0.865	0.018 0.883	NA	0.056 0.638
BRCA1¹	0.427 0.061	0.337 0.146	0.129 0.227	−0.054 0.655	0.056 0.638	NA

¹ Cytoplasmic expression.

Abbreviations in the table: BRCA1, breast cancer 1 protein; PDAC, pancreatic ductal adenocarcinoma; ChrA, chromogranin A; CD, cluster of differentiation; MVD, microvascular density; COX-2, cyclooxygenase 2; CK, cytokeratin; NA, not applicable.

Figure 3.53. Cytoplasmic expression of cyclooxygenase 2 (COX-2) in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-COX-2, original magnification 400x.

Figure 3.54. Cytoplasmic expression of BRCA1 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-BRCA1, original magnification 400x.

3.4.2. Characteristics of PET cases

The analysis of patient's age showed a statistically significant difference in mean ranks by age in pN0 and pN1 groups ($z = -2.089$; $p = 0.037$) with a very strong impact of age for LN status (AUC = 1.000; 95% CI = 1.000–1.000; $p = 0.037$).

The tumour size by mean ranks was higher in patients with distant metastases than in the group without metastasis ($z = -2.234$; $p = 0.025$) with a very pronounced effect (AUC = 1.00; 95% CI = 1.00–1.00; $p = 0.026$) as presented in Figure 3.55.

Figure 3.55. The pancreatic endocrine tumour size difference (by mean rank analysis) in accordance with the presence of distant metastasis. 3.55.A, boxplot with p value; 3.55.B, ROC curve analysis

Abbreviations in the figure: ROC, receiver-operating characteristic; AUC, area under curve; CI, confidence interval

The mean ranks of tumour size were also higher in patients' affected by tumour invasion in blood vessels ($z = -2.006$; $p = 0.045$) with a moderate impact ($AUC = 0.79$; $95\% \text{ CI} = 0.55\text{--}1.00$; $p = 0.046$) as represented in Figure 3.56.

Figure 3.56. The pancreatic endocrine tumour size difference (by mean ranks) by tumour intravascular invasion 3.56.A, boxplot with p value; 3.56.B, ROC curve analysis

Abbreviations in the figure: ROC, receiver-operating characteristic; AUC, area under curve; CI, confidence interval.

There was an association between tumour size with a cut-off of 2 cm (i.e., ≤ 2 / > 2 cm) and presence or absence of tumour invasion in blood vessels ($\chi^2 = 6.349$; $p = 0.012$). The pT parameter showed an association with tumour grade ($\chi^2 = 18.667$; $p = 0.005$) and invasion in blood vessels ($\chi^2 = 10.667$; $p = 0.005$).

The presence of distant metastases showed associations with the following variables: status of RM pR0/ pR1 ($\chi^2 = 6.519$; $p = 0.011$), tumour grade ($\chi^2 = 8.686$; $p = 0.034$) and mitotic count ($z = -2.293$; $p = 0.022$).

A statistically significant difference between groups was found for mean ranks of mitotic count between status pR0 and pR1 ($z = -2.380$; $p = 0.017$) with a very strong impact (AUC = 0.979; 95% CI = 0.901–1.000; $p = 0.019$). There was also a trend towards an association between the resection line status and tumour grade ($\chi^2 = 5.958$; $p = 0.051$). Tumour invasion in blood vessels (absent/ present) showed an association with tumour grade ($\chi^2 = 9.778$; $p = 0.021$) as well as with the mitotic count ($z = -2.275$; $p = 0.023$) with a strong effect (AUC = 0.828; 95% CI = 0.612–1.000; $p = 0.027$). The associations between clinical and morphological findings are summarised in Table 3.18.

Table 3.18.

The values of differences (z/χ^2 ; p) in PET by mean ranks of clinical and morphological findings

Independent variable	Dependent variable					
	Female/ male	Patients' age (years)	Patients' age $\leq 65/ > 65$ years	Tumour localisation	Tumour size (cm)	Tumour size $\leq 2/ > 2$ cm
Patients' age (years)	-0.182 0.855	NA	NA	SE	SE	SE
Patients' age $\leq 65/ > 65$ years	0.097 0.755	NA	NA	SE	SE	SE
Tumour localisation	2.667 0.446	1.703 0.636	0.873 0.832	NA	SE	SE
Tumour size (cm)	-0.427 0.670	-0.136 0.614 ¹	-0.626 0.531	3.315 0.346	NA	NA
Tumour size $\leq 2\text{cm}/ > 2\text{cm}$	0.085 0.771	-0.849 0.396	0.780 0.377	2.794 0.425	NA	NA
pT	1.778 0.411	0.116 0.944	0.097 0.953	6.000 0.423	4.886 0.087	7.196 0.027
pN0/ pN1	1.071 0.301	-2.089 0.037	3.750 0.053	1.667 0.644	-0.524 0.600	1.071 0.301
pM0/ pM1	0.762 0.383	0.000 1.000	0.374 0.541	2.286 0.515	-2.234 0.025	1.778 0.182
Tumour stage	2.063 0.724	3.436 0.488	3.056 0.549	9.722 0.640	6.608 0.158	6.825 0.145
pR0/ pR1	0.076 0.782	-0.205 0.838	0.076 0.782	1.757 0.624	-1.841 0.066	1.547 0.214
Tumour grade	3.437 0.329	3.489 0.322	2.967 0.397	9.422 0.399	3.624 0.305	3.719 0.293
Mitotic count	-0.688 0.491	-0.316 0.232 ¹	-0.409 0.683	1.508 0.680	0.468 0.067 ¹	-1.747 0.081

Table 3.18. (continued)						
Independent variable	Dependent variable					
	Female/ male	Patients' age (years)	Patients' age ≤65/ >65 years	Tumour localis- ation	Tumour size (cm)	Tumour size ≤2/ >2 cm
Invasion in small blood vessels (P/A)	0.000 1.000	-0.685 0.494	0.291 0.590	2.000 0.572	-2.006 0.045	6.349 0.012
Perineural invasion (P/A)	0.762 0.383	-0.557 0.577	0.374 0.541	6.857 0.077	-0.239 0.811	1.778 0.182
Invasion in lymph vessels (P/A)	0.762 0.383	-0.159 0.873	0.374 0.541	2.286 0.515	-1.117 0.264	1.778 0.182

Table 3.18. (continued)

Independent variable	Dependent variable						
	pT	pN0/ pN1	pM0/ pM1	Tumour stage	pR0/ pR1	Tumour grade	Mitotic count
pT	NA	SE	SE	SE	SE	SE	SE
pN0/ pN1	2.500 0.287	NA	SE	SE	SE	SE	SE
pM0/ pM1	3.810 0.149	1.406 0.236	NA	SE	SE	SE	SE
Tumour stage	20.000 0.010	6.875 0.143	10.000 0.040	NA	SE	SE	SE
pR0/ pR1	4.950 0.084	0.321 0.571	6.519 0.011	6.750 0.150	NA	SE	SE
Tumour grade	18.667 0.005	5.313 0.070	8.686 0.034	13.750 0.089	5.958 0.051	NA	SE
Mitotic count	4.022 0.134	-0.928 0.353	-2.293 0.022	7.336 0.119	-2.380 0.017	4.749 0.191	NA
Invasion in small blood vessels (P/A)	10.667 0.005	1.667 0.197	2.286 0.131	7.222 0.125	3.438 0.064	9.778 0.021	-2.275 0.023
Perineural invasion (P/A)	3.810 0.149	1.406 0.236	0.327 0.568	6.875 0.143	0.637 0.425	5.029 0.170	-0.737 0.461
Invasion in lymph vessels (P/A)	3.810 0.149	1.406 0.236	2.939 0.086	6.875 0.143	0.637 0.425	5.029 0.170	-0.983 0.326

¹ Statistical analysis by Spearman's correlation (r_s ; p).

Abbreviations in the table: PET, pancreatic endocrine tumour; NA, not applicable; SE, shown elsewhere; pT, T parameter of pTNM classification; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one lymph node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins - no cancer cells are found in resection margins; pR1, positive resection margins - cancer cells are found in resection margins; P, present; A, absent.

The evaluation of the immunohistochemical profile in the clinical and morphological context was performed, analysing the difference in mean ranks of IHC markers between clinical and morphological findings as well as correlations between clinical and morphological variables and percentage of cells of expressed IHC markers.

The mean ranks of Ki-67 expression had just a trend towards a difference between tumours with/ without distant metastases ($z = -1.922$; $p = 0.055$) and between groups with RM status pR0/ pR1 ($z = -1.885$; $p = 0.059$). Ki-67 expression (Figure 3.57.) showed moderate correlations with tumour size ($r_s = 0.584$; $p = 0.022$) and grade ($r_s = 0.588$; $p = 0.021$), but strong associations with tumour stage ($r_s = 0.688$; $p = 0.028$) and mitotic count ($r_s = 0.660$; $p = 0.007$). Among IHC markers, Ki-67 correlated with CK 19 ($r_s = 0.572$; $p = 0.033$) and vimentin ($r_s = 0.543$; $p = 0.045$). There was also a trend towards a negative correlation with CD44 ($r_s = -0.531$; $p = 0.051$).

Figure 3.57. Nuclear expression of Ki-67 in pancreatic endocrine tumour. Immunoperoxidase, anti-Ki-67, original magnification 400x.

All p53 positive cases were well differentiated endocrine tumours with unclear behaviour, pT1 and pT2 with size ranging from 1.2 to 3.0 cm. Mean ranks of p53 were higher in PETs without invasion in blood vessels compared with tumours invading blood vessels ($z = -2.079$; $p = 0.038$). However, there was no significant impact by ROC analysis ($p = 0.105$). Similarly, mean ranks of p53 were higher in tumours lacking perineural invasion compared with PETs showing perineural invasion ($z = -2.079$; $p = 0.038$), also without a statistically significant effect by ROC analysis ($p = 0.497$).

Strong, positive correlations were found between p53 expression and E-cadherin ($r_s = 0.655$; $p = 0.011$) as well as with p63 ($r_s = 0.993$; $p < 0.001$) and MVD ($r_s = 0.705$; $p = 0.005$).

Expression of p21 did not show correlations with morphological findings, but p21 expression correlated moderately with E-cadherin ($r_s = 0.541$; $p = 0.046$). No associations with clinical and morphological findings or expression of IHC markers

were found regarding p27 expression. Cyclin D1 showed a moderate, positive correlation with vascular density ($r_s = 0.578$; $p = 0.049$).

A difference in E-cadherin expression by mean ranks was observed between tumour pT characteristics ($\chi^2 = 6.128$; $p = 0.047$). The significance of the difference is shown in Figure 3.58. The difference was found in mean ranks of E-cadherin expression between groups with/ without tumour invasion in blood vessels ($z = -3.016$; $p = 0.003$) with a very strong impact (AUC = 0.980; 95% CI = 0.917–1.000; $p = 0.003$). Thus, patients with lower expression of E-cadherin had more frequent tumour intravascular invasion (Figure 3.59.).

Figure 3.58. The difference of E-cadherin expression (%) by mean ranks between variable pT characteristics in pancreatic endocrine tumour.

Abbreviations in the figure: PET, pancreatic endocrine tumour; pT1, tumour limited to the pancreas, 2cm or less in greatest dimension; pT2, tumour limited to the pancreas, more than 2 cm in greatest dimension; pT3, tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery.

Figure 3.59. The difference of E-cadherin expression (%) by mean ranks between PETs with absent and present tumour invasion in small blood vessels.

A difference regarding E-cadherin expression was also found between groups with/ without tumour perineural invasion ($z = -3.016$; $p = 0.003$), but there was no significant impact using ROC analysis ($p = 0.715$). A trend towards a difference was found for tumour size ≤ 2 / > 2 cm ($z = -1.945$; $p = 0.052$). E-cadherin expression also correlated with tumour pT characteristics ($r_s = -0.655$; $p = 0.011$) and grade ($r_s = -0.680$; $p = 0.007$). Among IHC markers, E-cadherin showed a strong, positive association with MVD ($r_s = 0.701$; $p = 0.005$). Correlations with p53 ($r_s = 0.655$; $p = 0.011$), p21 ($r_s = 0.541$; $p = 0.046$) and p63 expression ($r_s = 0.665$; $p = 0.009$) were also detected. Regarding CD44 expression, there was a trend towards a negative correlation with proliferation activity ($r_s = -0.531$; $p = 0.051$), as described above.

The full results of differences in mean ranks of expressed cell cycle and adhesion markers among groups of clinical and morphological findings are summarised in Table 3.19.

Table 3.19.

The value of difference (z/χ^2 ; p) in mean ranks of cell cycle and adhesion markers among clinical and morphological findings in PET

Independent variable	Dependent variable						
	Ki-67	p53	p21	p27	Cyclin D1	E-cadherin	CD44
Female/ male	-0.940 0.347	-1.340 0.180	-0.645 0.519	-1.136 0.256	-0.514 0.607	-0.142 0.887	-0.589 0.556
Patients' age ≤65/ >65 years	-0.126 0.900	-0.943 0.346	-1.013 0.311	-0.335 0.738	-0.771 0.441	-0.871 0.384	-1.320 0.187
Tumour localisation	1.222 0.748	7.659 0.054	2.065 0.559	2.738 0.434	4.916 0.178	3.488 0.322	1.559 0.669
Tumour size ≤2cm/ >2cm	-1.786 0.074	-1.188 0.235	-0.720 0.472	-0.778 0.437	-0.600 0.549	-1.945 0.052	-0.067 0.946
pT	3.810 0.149	5.287 0.071	1.017 0.601	0.540 0.763	4.322 0.115	6.128 0.047	0.939 0.625
pN0/ pN1	-0.532 0.595	-0.500 0.617	-0.298 0.766	-0.293 0.770	-0.298 0.766	-0.294 0.769	-1.223 0.221
pM0/ pM1	-1.922 0.055	-0.872 0.383	-0.093 0.926	-0.917 0.359	-1.409 0.159	-1.100 0.271	-0.761 0.447
Tumour stage	6.481 0.166	2.333 0.675	1.770 0.778	2.044 0.728	4.069 0.397	1.866 0.760	3.721 0.445
pR0/ pR1	-1.885 0.059	-0.908 0.364	-1.042 0.298	-0.342 0.732	0.000 1.000	-0.457 0.648	-0.354 0.723
Tumour grade	5.088 0.165	4.324 0.229	3.543 0.315	1.618 0.655	1.041 0.791	6.906 0.075	1.388 0.708
Invasion in small blood vessels (P/ A)	-1.846 0.065	-2.079 0.038	-0.842 0.400	-0.064 0.949	-0.727 0.467	-3.016 0.003	-0.200 0.842
Perineural invasion (P/ A)	-1.846 0.065	-2.079 0.038	-0.093 0.926	-0.734 0.463	-1.084 0.279	-3.016 0.003	-0.200 0.842
Invasion in lymph vessels (P/ A)	-0.786 0.432	-0.872 0.383	-1.850 0.064	-1.650 0.099	-1.084 0.279	-1.284 0.199	-0.476 0.634

Abbreviations in the table: PET, pancreatic endocrine tumour; CD, cluster of differentiation; pT, T parameter of pTNM classification; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins; P, present; A, absent.

The full results for correlations between cell cycle and adhesion markers and clinical and morphological findings in PET are shown in Table 3.20.

Table 3.20.

Correlations between cell cycle and adhesion markers and clinical and morphological findings in PET (r/r_s and p values)

Variable	Patients' age	Tumour size	pT	Tumour stage	Tumour grade	Mitotic count
Ki-67	-0.265 0.339	0.584 0.022	0.493 0.062	0.688 0.028	0.588 0.021	0.660 0.007
p53	-0.025 0.929	-0.373 0.171	-0.278 0.316	-0.178 0.623	-0.381 0.161	-0.385 0.156
p21	0.165 0.572	-0.119 0.686	-0.226 0.438	-0.217 0.574	-0.134 0.647	0.167 0.568
p27	0.080 0.786	-0.391 0.167	-0.111 0.705	-0.274 0.476	0.080 0.786	0.041 0.890
Cyclin D1	0.102 0.751	-0.088 0.784	-0.045 0.889	-0.235 0.543	-0.282 0.374	-0.482 0.112
E-cadherin	0.250 0.388	-0.456 0.102	-0.655 0.011	-0.425 0.254	-0.680 0.007	-0.409 0.147
CD44	0.463 0.095	-0.242 0.404	0.257 0.376	-0.098 0.801	0.246 0.397	-0.439 0.116

Abbreviations in the table: PET, pancreatic endocrine tumour; pT, T parameter of pTNM classification; CD, cluster of differentiation.

The IHC visualisation of expression of aberrant p53 oncoprotein and cell cycle and regulator protein p21 in PET is presented in Figures 3.60. and 3.61.

Figure 3.60. Nuclear expression of p53 in pancreatic endocrine tumour.
Immunoperoxidase, anti-p53, original magnification 400x.

Figure 3.61. Nuclear expression of p21 in pancreatic endocrine tumour.
Immunoperoxidase, anti-p21, original magnification 400x.

Full results of correlations between cell cycle and adhesion markers and the entire immunophenotype in PET are summarised in Table 3.21.

Table 3.21.

**Correlations between cell cycle and adhesion markers and the entire
immunohistochemical profile in PET by r/r_s and p values**

Variable	Ki-67	p53	p21	p27	Cyclin D1	E-cadherin	CD44
Ki-67	NA	-0.059 0.835	0.328 0.253	-0.307 0.286	-0.290 0.360	-0.325 0.257	-0.531 0.051
p53	-0.059 0.835	NA	0.504 0.066	0.011 0.970	0.338 0.283	0.655 0.011	-0.056 0.849
p21	0.328 0.253	0.504 0.066	NA	0.289 0.317	0.389 0.212	0.541 0.046	-0.327 0.253
p27	-0.307 0.286	0.011 0.970	0.289 0.317	NA	0.166 0.607	0.007 0.982	0.481 0.082
Cyclin D1	-0.290 0.360	0.338 0.283	0.389 0.212	0.166 0.607	NA	0.505 0.094	0.249 0.435
E-cadherin	-0.325 0.257	0.655 0.011	0.541 0.046	0.007 0.982	0.505 0.094	NA	-0.074 0.803
CD44	-0.531 0.051	-0.056 0.849	-0.327 0.253	0.481 0.082	0.249 0.435	-0.074 0.803	NA
CK 7	0.117 0.691	-0.253 0.383	-0.013 0.965	0.170 0.561	0.293 0.356	-0.112 0.702	0.440 0.116
CK 19	0.572 0.033	-0.512 0.061	-0.083 0.779	0.015 0.960	-0.281 0.377	-0.489 0.076	0.093 0.753
p63	-0.070 0.812	0.993 0.000	0.521 0.056	0.039 0.895	0.281 0.376	0.665 0.009	-0.079 0.787
ChrA	-0.002 0.995	-0.028 0.921	0.145 0.621	-0.224 0.441	0.408 0.189	0.258 0.373	-0.320 0.265
CD56	0.098 0.739	-0.017 0.953	0.062 0.841	0.377 0.204	0.206 0.544	-0.054 0.861	0.021 0.946
Vimentin	0.543 0.045	-1.47 0.617	-0.063 0.831	-0.118 0.689	-0.398 0.200	-0.426 0.129	-0.239 0.411
MVD (CD34)	-0.295 0.307	0.705 0.005	0.344 0.229	0.000 0.100	0.578 0.049	0.701 0.005	0.112 0.702
COX-2	0.111 0.706	0.127 0.665	0.043 0.884	0.193 0.508	-0.004 0.990	0.039 0.896	0.310 0.281
BRCA1¹	-0.161 0.565	0.093 0.742	-0.18 0.714	0.355 0.213	-0.176 0.584	-0.080 0.787	0.115 0.696

¹ Cytoplasmic expression.

Abbreviations in the table: PET, pancreatic endocrine tumour; CD, cluster of differentiation; NA, not applicable; CK, cytokeratin; ChrA, chromogranin A; MVD, microvascular density; COX-2, cyclooxygenase 2; BRCA1, breast cancer 1 protein.

The IHC visualisation of expression of cell cycle and adhesion markers in PET is presented in Figures 3.62. – 3.65.

Figure 3.62. Predominantly nuclear expression of p27 in pancreatic endocrine tumour. Immunoperoxidase, anti-p27, original magnification 400x.

Figure 3.63. Nuclear expression of cyclin D1 in pancreatic endocrine tumour. Immunoperoxidase, anti-cyclin D1, original magnification 400x.

Figure 3.64. Membranous expression of E-cadherin in pancreatic endocrine tumour. Immunoperoxidase, anti-E-cadherin, original magnification 400x.

Figure 3.65. Membranous expression of CD44 in pancreatic endocrine tumour. Immunoperoxidase, anti-CD44, original magnification 400x.

Regarding the CK spectrum and markers of squamous and intestinal differentiation, the negative markers were excluded from correlation analysis. Thus, CK 7, CK 19 and p63 were evaluated in relation to clinical and morphological data and the full immunohistochemical profile.

CK 7 expression (Figure 3.68.) showed positive correlations with COX-2 ($r_s = 0.711$; $p = 0.004$) and CK 19 ($r_s = 0.607$; $p = 0.021$). A statistically significantly different distribution of mean ranks of CK 19 was found between the following groups: tumour pT characteristics ($\chi^2 = 8.068$; $p = 0.018$) with a significant difference between pT1 and pT3 ($p = 0.022$) as well as between pT2 and pT3 ($p = 0.004$). Thus, CK 19 expression was higher in pT3 PETs. There was also a significantly different distribution of mean ranks of CK 19 between resection line status pR0 and pR1 ($z = -2.234$;

$p = 0.025$) with a very strong impact ($AUC = 0.952$; 95% CI = 0.817–1.000; $p = 0.030$) and between tumours with/ without invasion in blood vessels ($z = -2.335$; $p = 0.020$) with a strong effect ($AUC = 0.837$; 95% CI = 0.604–1.000; $p = 0.035$). CK 19 expression was higher in pR1 than in pR0 tumours and in tumours invading blood vessels (Figure 3.66.). Similarly, differences were also observed between PETs with perineural invasion compared with the group lacking perineural invasion ($z = -2.224$; $p = 0.026$) as depicted in Figure 3.67., with a very strong impact ($AUC = 0.958$; 95% CI = 0.847–1.000; $p = 0.045$). There was also a difference between PETs with/ without invasion in lymph vessels ($z = -2.022$; $p = 0.043$) but there was no impact according to ROC analysis ($p = 0.068$). The higher mean ranks of CK 19 expression showed a trend towards more frequent tumour size exceeding 2 cm ($z = -1.930$; $p = 0.054$) and lower tumour differentiation by grade assessment ($\chi^2 = 7.803$; $p = 0.050$).

Figure 3.66. The difference of cytokeratin 19 (CK 19) expression (%) by mean ranks between pancreatic endocrine tumours regarding invasion in blood vessels.

Figure 3.67. The difference of cytokeratin 19 (CK 19) expression (%) by mean ranks between pancreatic endocrine tumours regarding perineural invasion.

Using Spearman's correlation analysis, expression of CK 19 (Figure 3.69.) correlated with the following: tumour pT characteristics ($r_s = 0.654$; $p = 0.011$), tumour stage ($r_s = 0.730$; $p = 0.025$) and grade ($r_s = 0.744$; $p = 0.002$) as well as with the mitotic count ($r_s = 0.675$; 0.008). CK 19 expression also showed a trend towards correlation with tumour size ($r_s = 0.528$; $p = 0.052$). Among the IHC markers, CK 19 expression showed correlations with proliferation activity ($r_s = 0.572$; $p = 0.033$), CK 7 ($r_s = 0.607$; $p = 0.021$) and vimentin expression ($r_s = 0.551$; $p = 0.041$). There was also a negative correlation with MVD ($r_s = -0.639$; $p = 0.014$).

Figure 3.68. Cytoplasmic expression of cytokeratin 7 (CK 7) in pancreatic endocrine tumour. Immunoperoxidase, anti-CK 7, original magnification 400x.

Figure 3.69. Cytoplasmic expression of cytokeratin 19 (CK 19) in pancreatic endocrine tumour. Immunoperoxidase, anti-CK 19, original magnification 400x.

A difference was found in p63 expression for mean ranks between tumours with/without invasion in blood vessels ($z = -2.256$; $p = 0.024$). However, there was no significant impact according to ROC analysis ($p = 0.074$). p63 expression (Figure 3.70.) correlated with E-cadherin ($r_s = 0.665$; $p = 0.009$) as well as with MVD ($r_s = 0.688$; $p = 0.007$) and p53 ($r_s = 0.993$; $p < 0.001$), with a trend towards a correlation with p21 ($r_s = 0.521$; $p = 0.056$) and CK 19 ($r_s = -0.516$; $p = 0.059$).

Figure 3.70. Nuclear expression of p63 in pancreatic endocrine tumour. Immunoperoxidase, anti-p63, original magnification 400x.

The data characterising the associations between the CK 7, CK 19 and p63 and clinical and morphological parameters of PETs are shown in Tables 3.22. and 3.23.

Table 3.22.

The value of difference (z/χ^2 ; p) in mean ranks of CK and p63 between clinical and morphological findings in PET

Independent variable	Dependent variable		
	CK 7	CK 19	p63
Female/ male	-0.580 0.580	-0.313 0.754	-1.427 0.154
Patients' age ≤ 65/ > 65 years	-0.547 0.584	-0.517 0.605	-0.841 0.400
Tumour localisation	5.385 0.146	0.731 0.866	6.375 0.095
Tumour size ≤2cm/ >2cm	-1.271 0.204	-1.930 0.054	-1.547 0.122
pT	2.872 0.238	8,068 0.018	4.594 0.101
pN0/ pN1	-1.203 0.229	-0.893 0.372	-0.535 0.593
pM0/ pM1	-0.599 0.549	-1.820 0.069	-0.921 0.357
Tumour stage	6.000 0.199	6.621 0.157	2.000 0.736
pR0/ pR1	-0.488 0.626	-2.234 0.025	-0.982 0.326
Tumour grade	3.877 0.275	7.803 0.050	5.091 0.165
Invasion in small blood vessels (P/ A)	-1.468 0.142	-2.335 0.020	-2.256 0.024
Perineural invasion (P/ A)	-1.468 0.142	-2.224 0.026	-0.921 0.357
Invasion in lymph vessels (P/ A)	-1.648 0.099	-2.022 0.043	-0.921 0.357

Abbreviations in the table: PET, pancreatic endocrine tumour; CK, cytokeratin; pT, T parameter of pTNM classification; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins; P, present; A, absent.

Table 3.23.

Correlations between CK and p63 and clinical and morphological findings in PET by r/r_s and p value

Variable	Patients' age	Tumour size	pT	Tumour stage	Tumour grade	Mitotic count
CK 7	0.181 0.536	0.044 0.882	0.433 0.122	0.281 0.464	0.387 0.171	0.165 0.573
CK 19	-0.020 0.947	0.528 0.052	0.654 0.011	0.730 0.025	0.744 0.002	0.675 0.008
p63	-0.039 0.895	-0.420 0.135	-0.404 0.152	-0.281 0.464	-0.425 0.130	-0.403 0.153

Abbreviations in the table: PET, pancreatic endocrine tumour; pT, T parameter of pTNM classification; CK, cytokeratin.

Full results of correlations between cytokeratins and p63 and all other IHC markers in PET are summarised in Table 3.24.

Table 3.24.

**Correlations between CK and p63 and the entire immunophenotype
in PET by r/r_s and p value**

Variable	CK 7	CK 19	p63
Ki-67	0.117 0.691	0.572 0.033	-0.070 0.812
p53	-0.253 0.383	-0.512 0.061	0.993 0.000
p21	-0.013 0.965	-0.083 0.779	0.521 0.056
p27	0.170 0.561	0.015 0.960	0.039 0.895
Cyclin D1	0.293 0.356	-0.281 0.377	0.281 0.376
E-cadherin	-0.112 0.702	-0.489 0.076	0.665 0.009
CD44	0.440 0.116	0.093 0.753	-0.079 0.787
CK 7	NA	0.607 0.021	-0.255 0.379
CK 19	0.607 0.021	NA	-0.516 0.059
p63	-0.255 0.379	-0.516 0.059	NA
ChrA	-0.144 0.623	0.009 0.976	-0.081 0.783
CD56	-0.065 0.834	0.149 0.628	-0.014 0.963
Vimentin	-0.102 0.729	0.551 0.041	-0.197 0.500
MVD (CD34)	-0.213 0.464	-0.639 0.014	0.688 0.007
COX-2	0.711 0.004	0.484 0.079	0.163 0.577
BRCA1 ¹	-0.166 0.571	-0.335 0.241	0.130 0.659
Variable	CK7	CK19	P63

¹ Cytoplasmic expression.

Abbreviations in the table: PET, pancreatic endocrine tumour; CK, cytokeratin; CD, cluster of differentiation; ChrA, chromogranin A; MVD, microvascular density; COX-2, cyclooxygenase 2; BRCA1, breast cancer 1 protein.

Regarding the neuroendocrine markers ChrA and CD56, no correlations with any clinical or morphological features were found. Vimentin expression showed just a

trend towards correlation with tumour size ($r_s = 0.531$; $p = 0.051$) as well as correlations with the proliferation activity ($r_s = 0.543$; $p = 0.045$) and CK 19 ($r_s = 0.551$; $p = 0.041$).

The mean ranks of MVD were different between tumours with/ without invasion in blood vessels ($z = -2.113$; $p = 0.035$) with a strong impact ($AUC = 0.837$; 95% CI = 0.610–1.000; $p = 0.035$). Cases featuring intravascular invasion had lower mean ranks of MVD (Figure 3.71.).

Figure 3.71. The difference of microvascular density by mean ranks among pancreatic endocrine tumours with respect to the invasion in blood vessels

The vascular density also showed a correlation with mitotic count ($r_s = -0.697$; $p = 0.006$). MVD correlated with expression of p53 ($r_s = 0.705$; $p = 0.005$), cyclin D1 ($r_s = 0.578$; $p = 0.049$), E-cadherin ($r_s = 0.701$; $p = 0.005$), and p63 ($r_s = 0.688$; $p = 0.007$). There was a negative correlation with CK 19 ($r_s = -0.639$; $p = 0.014$).

Mean ranks of COX-2 expression were different between tumours with/ without perineural invasion ($z = -2.557$; $p = 0.011$) with a very strong impact ($AUC = 1.000$; 95% CI = 1.000–1.000; $p = 0.028$) as well as with/ without invasion in lymph vessels ($z = -2.024$; $p = 0.043$) but without a significant impact ($p = 0.083$). PETs showing either perineural invasion or invasion in lymph vessels had higher COX-2 expression than groups without corresponding invasion (Figures 3.72. and 3.73.). A strong, positive correlation was found between COX-2 expression and CK 7 ($r_s = 0.711$; $p = 0.004$).

Figure 3.72. The difference in COX-2 expression (%) by mean ranks between pancreatic endocrine tumours regarding perineural invasion.

Figure 3.73. The difference in COX-2 expression (%) by mean ranks between pancreatic endocrine tumours regarding invasion in lymph vessels.

There was a moderate, negative correlation between BRCA1 (cytoplasmic expression) and tumour size ($r_s = -0.592$; $p = 0.020$). The full results of differences in mean ranks of IHC markers between clinical and morphological findings in PET and their values are summarised in Table 3.25.

Table 3.25.

The value of difference (z/χ^2 ; p) in mean ranks of neuroendocrine and mesenchymal markers, angiogenesis and BRCA1 between clinical and morphological findings in PET

Independent variable	Dependent variable					
	ChrA	CD56	Vimentin	MVD (CD34)	COX-2	BRCA1 ¹
Female/ male	-1.201 0.230	-0.163 0.871	-0.157 0.875	-1.134 0.257	-0.330 0.741	-0.883 0.377
Patients' age ≤65/ >65 years	-1.127 0.260	-1.394 0.163	-0.444 0.657	-0.134 0.894	-0.545 0.586	-1.035 0.301
Tumour localisation	2.136 0.545	0.949 0.814	1.559 0.669	3.307 0.347	2.442 0.486	5.893 0.117
Tumour size ≤2cm/ >2cm	-0.237 0.813	-1.336 0.181	-1.646 0.100	-1.811 0.070	-0.377 0.706	-1.565 0.118
pT	0.094 0.954	0.007 0.997	1.892 0.388	2.830 0.243	1.305 0.521	1.131 0.568
pN0/ pN1	-1.729 0.084	-0.153 0.878	-0.459 0.646	-0.293 0.770	-0.870 0.384	0.000 1.000
pM0/ pM1	-0.434 0.664	-1.528 0.127	-1.518 0.129	-1.647 0.100	-0.107 0.915	-1.090 0.276
Tumour stage	2.632 0.621	2.655 0.617	6.545 0.162	3.044 0.550	4.941 0.293	0.000 1.000
pR0/ pR1	-1.348 0.178	-1.541 0.123	-0.708 0.479	-0.800 0.424	-0.902 0.367	-0.612 0.540

Table 3.25. (continued)						
Independent variable	Dependent variable					
	ChrA	CD56	Vimentin	MVD (CD34)	COX-2	BRCA1 ¹
Tumour grade	1.673 0.643	1.999 0.573	2.184 0.535	2.139 0.544	2.808 0.422	0.561 0.905
Invasion in small blood vessels (P/ A)	-0.591 0.554	-0.068 0.946	-0.921 0.357	-2.113 0.035	-0.746 0.456	-0.196 0.845
Perineural invasion (P/ A)	-0.347 0.728	-0.095 0.924	-0.921 0.357	-0.732 0.464	-2.557 0.011	-0.574 0.566
Invasion in lymph vessels (P/ A)	-0.347 0.728	-0.095 0.924	-0.405 0.686	-1.830 0.067	-2.024 0.043	-0.574 0.566

¹ Cytoplasmic expression.

Abbreviations in the table: BRCA1, breast cancer 1 protein; PET, pancreatic endocrine tumour; ChrA, chromogranin A; CD, cluster of differentiation; MVD, microvascular density; COX-2, cyclooxygenase 2; pT, T parameter of pTNM classification; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins; P, present; A, absent.

Full results of correlation analysis between IHC markers and clinical and morphological findings in PET are represented in Table 3.26.

Table 3.26.

Correlations between neuroendocrine and mesenchymal markers, angiogenesis and BRCA1 and clinical and morphological findings in PET by r/r_s and p value

Variable	Patients' age	Tumour size	pT	Tumour stage	Tumour grade	Mitotic count
ChrA	-0.256 0.357	0.048 0.866	-0.080 0.778	-0.170 0.638	-0.187 0.504	-0.091 0.747
CD56	-0.381 0.179	0.338 0.237	0.022 0.940	0.336 0.376	0.146 0.618	0.315 0.272
Vimentin	-0.381 0.178	0.531 0.051	0.325 0.256	0.321 0.399	0.409 0.147	0.412 0.143
MVD (CD34)	0.106 0.718	-0.512 0.061	-0.267 0.356	-0.248 0.520	-0.403 0.153	-0.697 0.006
COX-2	-0.080 0.786	-0.074 0.803	0.271 0.349	0.518 0.153	0.267 0.356	0.255 0.380
BRCA1¹	-0.327 0.234	-0.592 0.020	-0.016 0.955	NA	0.097 0.731	-0.028 0.921

¹ Cytoplasmic expression.

Abbreviations in the table: BRCA1, breast cancer 1 protein; PET, pancreatic endocrine tumour; pT, T parameter of pTNM classification; ChrA, chromogranin A; CD, cluster of differentiation; MVD, microvascular density; COX-2, cyclooxygenase 2; NA, not applicable.

The IHC visualisation of neuroendocrine and mesenchymal markers is shown in Figures 3.74. – 3.77.

Figure 3.74. Membranous and cytoplasmic expression of CD56 in pancreatic endocrine tumour. Immunoperoxidase, anti-CD56, original magnification 100x.

Figure 3.75. Cytoplasmic expression of chromogranin A in pancreatic endocrine tumour. Immunoperoxidase, anti-chromogranin A, original magnification 100x.

Figure 3.76. Cytoplasmic expression of vimentin in pancreatic endocrine tumour. Immunoperoxidase, anti-vimentin, original magnification 100x.

Figure 3.77. Cytoplasmic expression of vimentin in pancreatic endocrine tumour. Immunoperoxidase, anti-vimentin, original magnification 400x.

The full results of correlations between expressed IHC markers in PET are summarised in Table 3.27.

Table 3.27.

**Correlations between neuroendocrine and mesenchymal markers, angiogenesis and
BRCA1 and the entire immunophenotype in PET by r/r_s and p value**

Variable	ChrA	CD56	Vimentin	MVD (CD34)	COX-2	BRCA ¹
Ki-67	-0.002 0.995	0.098 0.739	0.543 0.045	-0.295 0.307	0.111 0.706	-0.161 0.565
p53	-0.028 0.921	-0.017 0.953	-1.47 0.617	0.705 0.005	0.127 0.665	0.093 0.742
p21	0.145 0.621	0.062 0.841	-0.063 0.831	0.344 0.229	0.043 0.884	-0.18 0.714
p27	-0.224 0.441	0.377 0.204	-0.118 0.689	0.000 0.100	0.193 0.508	0.355 0.213
Cyclin D1	0.408 0.189	0.206 0.544	-0.398 0.200	0.578 0.049	-0.004 0.990	-0.176 0.584
E-cadherin	0.258 0.373	-0.054 0.861	-0.426 0.129	0.701 0.005	0.039 0.896	-0.080 0.787
CD44	-0.320 0.265	0.021 0.946	-0.239 0.411	0.112 0.702	0.310 0.281	0.115 0.696
CK 7	-0.144 0.623	-0.065 0.834	-0.102 0.729	-0.213 0.464	0.711 0.004	-0.166 0.571
CK 19	0.009 0.976	0.149 0.628	0.551 0.041	-0.639 0.014	0.484 0.079	-0.335 0.241
p63	-0.081 0.783	-0.014 0.963	-0.197 0.500	0.688 0.007	0.163 0.577	0.130 0.659
ChrA	NA	0.165 0.573	0.249 0.391	0.249 0.391	-0.307 0.286	0.249 0.391
CD56	0.165 0.573	NA	0.209 0.493	0.209 0.493	0.128 0.653	0.209 0.493
Vimentin	0.249 0.391	0.209 0.493	NA	-0.349 0.221	-0.142 0.628	-0.336 0.240
MVD (CD34)	0.196 0.503	-0.200 0.513	-0.349 0.221	NA	-0.158 0.589	0.237 0.415
COX-2	-0.307 0.286	0.128 0.653	-0.142 0.628	-0.158 0.589	NA	0.006 0.983
BRCA1¹	-0.120 0.669	0.111 0.705	-0.336 0.240	0.237 0.415	0.006 0.983	NA

¹ Cytoplasmic expression.

Abbreviations in the table: BRCA1, breast cancer 1 protein; PET, pancreatic endocrine tumour; ChrA, chromogranin A; CD, cluster of differentiation; MVD, microvascular density; COX-2, cyclooxygenase 2; CK, cytokeratin; NA, not applicable.

The IHC visualisation of angiogenesis-related markers and BRCA1 is shown in Figures 3.78. and 3.79.

Figure 3.78. Cytoplasmic expression of cyclooxygenase 2 (COX-2) in pancreatic endocrine tumour. Immunoperoxidase, anti-COX-2, original magnification 400x.

Figure 3.79. Cytoplasmic expression of BRCA1 in pancreatic endocrine tumour. Immunoperoxidase, anti-BRCA1, original magnification 400x.

3.5. Survival

3.5.1. Characteristics of PDAC cases

The survival data analysis has been performed for 74 patients. Four patients were lost for follow-up. Among the follow-up patients, 20/ 74 (27.0%; 95% CI = 18.2–38.1) patients were alive, but 54/ 74 (73.0%; 95% CI = 61.9–81.8) patients had died during the observation time. The overall median survival time was 11.0 (95% CI = 7.6–14.4) months. The Kaplan-Meier survival line is shown in Figure 3.80.

Within the first month after the operation, 4/ 74 (5.4%; 95% CI = 2.1–13.1) patients died, but 70/ 74 (94.6%; CI = 86.9–97.9) patients remained alive. Six months after the operation, 23/ 67 (34.3%; 95% CI = 24.1–46.3) patients had died and 44/ 67 (65.7%; 95% CI = 53.7–75.9) were alive. Twelve months after operation, 38/ 66 (57.6%; 95% CI = 45.6–68.8) patients had died, but 28/ 66 (42.4%; 95% CI = 31.2–54.4) patients were alive. Eighteen months after operation, 47/ 64 (73.4%; 95% CI = 61.5–82.7) patients had died, but 17/ 64 (26.6%; 95% CI = 17.3–38.5) were alive. Twenty-four months after, 49/ 61 (80.3%; 95% CI = 68.7–88.4) patients had died, but 12/ 61 (19.7%; 95% CI = 11.6–31.3) patients were alive. Within a 30 month period, 51/ 59 (86.4%; 95% CI = 75.5–93.0) patients had died, but 8/ 59 (13.6%; 95% CI = 7.0–24.5) were alive. Thirty-six months after, 52/ 58 (89.7%; 95% CI = 79.2–95.2) patients had died, but 6/ 58 (10.3%; 95% CI = 4.8–20.8) patients were alive.

Figure 3.80. Kaplan-Meier survival line of patients affected by pancreatic ductal adenocarcinoma

3.5.2. Characteristics of PET cases

The survival analysis was performed with 14 patients. During the observation time, 2/ 14 (14.3%; 95% CI = 4.0–39.9) patients had died: full 1 month and 15 months after operation. The overall median survival time was impossible to detect due to the small study group and rarity of undesirable events. The Kaplan-Meier survival line is shown in Figure 3.81.

Figure 3.81. Kaplan-Meier survival line of patients affected by pancreatic endocrine tumour

3.6. Associations between survival and clinical and morphological prognostic findings

3.6.1. Prognostic characteristics of PDAC cases

A trend towards a difference in overall survival (OS) was found by tumour differentiation grade (log-rank, $p = 0.050$). The median OS of patients with G1 tumour was 18.0 (95% CI = 8.1–28.0) months, G2 was 13.0 (95% CI = 9.0–17.0) months, but G3 was only 6.0 (95% CI = 2.2–9.8) months. The corresponding Kaplan-Meier lines are shown in Figure 3.82.

Figure 3.82. Kaplan-Meier survival lines by tumour differentiation grade of pancreatic ductal adenocarcinoma

G1, well differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma;
G3, poorly differentiated adenocarcinoma.

The distribution of OS was statistically significantly different in relation to tumour invasion in large blood vessels (log-rank, $p = 0.013$). Median OS was 12.0 (95% CI = 7.8–16.2) months for patients without such invasion, contrasting with a median OS of 3.0 (95% CI = 0.7–5.3) months in patients affected by tumour invasion in large blood vessels (Figure 3.83.).

Figure 3.83. Kaplan-Meier survival lines by pancreatic ductal adenocarcinoma invasion in arge blood vessels

Although tumour invasion in large blood vessels and development of distant metastasis showed a convincing association ($\chi^2 = 15.282$; $p < 0.001$), the three patients with distant metastasis survived 1, 3 and 22 months.

A statistically significant difference in median OS was also found regarding the presence of tumour necrosis (log-rank, $p = 0.001$) with a median OS of 13.0 (95% CI = 9.3–16.8) months for patients lacking necrosis whereas a median OS of 4.0 (95% CI = 1.4–6.6) months in patients exhibiting tumour necrosis (Figure 3.84.).

Figure 3.84. Kaplan-Meier survival lines by tumour necrosis in pancreatic ductal adenocarcinoma

Several tests between median OS and clinical or morphological findings showed statistically non-significant differences, but after assessing the Kaplan-Meier lines, the visual trend towards a difference between abruptness of the lines was perspicuous. These observations included the following features: LN status pN0 *versus* pN1 (log-rank, $p=0.201$), tumour stage IIA *versus* stage IIB (log-rank, $p=0.184$) and RM status pR0 *versus* pR1 (log-rank, $p=0.081$). Kaplan-Meier lines are shown in Figures 3.85. and 3.86.

Figure 3.85. Kaplan-Meier survival lines by status of lymph nodes in cases of pancreatic ductal adenocarcinoma.

Abbreviations in the figure: pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least 1 node.

Figure 3.86. Kaplan-Meier survival lines by tumour stages IIA and IIB in cases of pancreatic ductal adenocarcinoma.

Abbreviations in the figure: IIA, pT3N0M0; IIB, pT1-3N1M0.

Regarding RM status, median OS was 14.0 (95% CI = 8.9–19.1) months for patients with negative RM and 10.0 (95% CI = 6.8–13.2) months for patients with positive RM. The same trend was observed comparing PDAC with and without perineural invasion (log-rank, $p=0.088$). Kaplan-Meier lines are shown in Figures 3.87. and 3.88.

Figure 3.87. Kaplan-Meier survival lines by status of resection margins in cases of pancreatic ductal adenocarcinoma.

Abbreviations in the figure: pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins.

Figure 3.88. Kaplan-Meier survival lines by tumour perineural invasion in cases of pancreatic ductal adenocarcinoma.

The parameters with a statistically non-significant survival difference are summarised in Table 3.28.

Table 3.28.

Statistically non-significant OS differences between clinical and morphological findings in pancreatic ductal adenocarcinoma

Patients' groups	p value
Female/ male	0.151
Patients' age ≤ 65 / > 65 years	0.424
Type of operation	0.603
Tumour localisation	0.075
Tumour size ≤ 2 / > 2 cm	0.226
Tumour invasion in small blood vessels (P/ A)	0.628
Intravascular invasion (P/ A)	0.127
Intraneural invasion (P/ A)	0.072
Invasion in lymph vessels (P/ A)	0.553

Abbreviations in the table: OS, overall survival; P, present; A, absent.

3.6.2. Prognostic characteristics of PET cases

A difference in OS was not found between clinical and morphological findings. The summarised results are shown in Table 3.29. Follow-up time of patients with distant metastases was 13 and 15 months and both patients were alive at time of renewing survival data.

Table 3.29.

Statistically non-significant OS differences by clinical and morphological findings in pancreatic endocrine tumours

Patients' groups	p value
Female/ male	0.284
Patients' age ≤ 65 / > 65 years	0.431
Tumour localisation	0.640
Tumour size ≤ 2 / > 2 cm	0.208
pT characteristics	0.216
pN0/ pN1	0.486
pM0/ pM1	0.606
Tumour stage	0.080
pR0/ pR1	0.520
Tumour grade	0.314
Invasion in blood vessels (P/ A)	0.138
Perineural invasion (P/ A)	0.195
Invasion in lymph vessels (P/ A)	0.389

Abbreviations in the table: OS, overall survival; pT, T parameter of pTNM classification; pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least one node; pM0, distant metastases are absent; pM1, distant metastases are present as evidenced by pathology examination; pR0, negative resection margins – no cancer cells are found in resection margins; pR1, positive resection margins – cancer cells are found in resection margins; P, present; A, absent.

3.7. Association between survival and immunohistochemical variables

3.7.1. Immunohistochemical prognostic profile of pancreatic ductal adenocarcinoma

In PDAC, a statistically significant median survival difference was found between patients regarding vimentin expression (log-rank, $p = 0.002$) with a median OS of 14.0 (95% CI = 10.7–17.3) months for patients lacking vimentin expression whereas the median survival was 4.0 (95% CI = 1.7–6.3) months for patients with vimentin expression in PDAC.

A statistically significant difference in median survival was found for CD44 expression in PDAC (log-rank, $p = 0.018$). The median OS was 15.0 (95% CI = 9.6–20.4) months in patients lacking CD44 expression whereas the median OS was 8.0 (95% CI = 6.0–10.0) months in patients showing CD44 expression in PDAC.

Concerning cell cycle regulator proteins, a statistically significant difference in median OS was found between patients with negative or positive p27 expression (log-rank, $p = 0.003$). The median OS was 3.0 (95% CI = 1.9–4.1) months in patients with negative p27 expression. In contrast, the median OS was 12.0 (95% CI = 7.9–16.1) months in patients with positive p27 expression. However, a statistically significant median survival difference was not found between groups with negative or positive expression of Ki-67 with a cut-off value of 10% ($p = 0.350$).

According to multivariate analysis using the Cox proportional hazards model to assess the impact of morphological and immunohistochemical factors on survival in univariate analysis, the variable models of tumour invasion in large blood vessels and necrosis as well as positive vimentin and CD44 and negative p27 expression showed an impact for a higher risk for shorter survival (Table 3.30). The impact using Cox proportional hazards was not found ($p > 0.05$) if the following models were used: positive/ negative RM and tumour stages, including isolated analysis between stages IIA and IIB.

Evaluating tumour stage (IIA *versus* IIB) in association with either tumour invasion in blood vessels (absent/ present) or tumour necrosis (absent/ present), or vimentin, CD44 and p27 status (negative/ positive), the Cox proportional hazards model did not disclose a statistically significant risk of shorter survival ($p > 0.050$). In contrast, in the Cox proportional hazards model including all stages, tumour necrosis (absent/ present) or vimentin and p27 or CD44 (negative/ positive), the significance of impact on survival was shown by all parameters (Table 3.30). Such an association was not found if the model comprised tumour stage (including all stages) and tumour invasion in large blood vessels (absent/ present). Including the RM status (negative/ positive) and any of the above described factors in the model, there was no evidence for a higher risk of shorter survival ($p > 0.050$).

Thus, multivariate analysis using Cox proportional hazards model confirmed invasion in large blood vessels and vimentin, CD44 and p27 expression as independent prognostic factors regarding survival.

Table 3.30.

Cox proportional hazards models in the assessment of morphological and immunohistochemical characteristics of pancreatic ductal adenocarcinoma

No.of model	Variable	Hazards ratio	CI of hazards ratio	p value
1.	Invasion in large blood vessels (P/ A)	4.156	1.489–11.600	0.007
	Vimentin (N/ P)	2.204	1.062–4.573	0.034
	CD44 (N/ P)	2.209	1.209–4.035	0.010
	p27 (N/ P)	0.310	0.125–0.771	0.012
2.	Tumour necrosis (P/ A)	3.155	1.628–6.117	0.001
	Vimentin (N/ P)	2.827	1.380–5.789	0.011
	p27 (N/ P)	0.310	0.125–0.765	0.004
3.	Tumour necrosis (P/ A)	2.598	1.362–4.956	0.004
	CD44 (N/ P)	1.790	1.008–3.178	0.047
4.	Vimentin (N/ P)	2.231	1.074–4.634	0.031
	CD44 (N/ P)	1.894	1.059–3.387	0.031
	p27 (N/ P)	0.316	0.127–0.791	0.014
5.	Tumour stage (any)	1.592	1.118–2.268	0.010
	Necrosis (A/ P)	3.048	1.611–5.766	0.001
6.	Tumour stage (any)	1.514	1.092–2.099	0.013
	Vimentin (N/ P)	3.087	1.475–6.460	0.003
	p27 (N/ P)	0.384	0.157–0.940	0.036
7.	Tumour stage (any)	1.468	1.004–2.145	0.047
	CD44 (N/ P)	1.783	1.009–3.152	0.046
8.	Status of resection margin (N/ P)	2.035	1.110–3.730	0.022
	Vimentin (N/ P)	3.085	1.468–6.483	0.003

Abbreviations in the table: No., number; P, present; A, absent; N, negative status of marker expression; P, positive status of marker expression.

Using the ROC curve analysis and subsequent log-rank test, a trend towards a median survival difference was found between groups exhibiting $< 55/ \geq 55$ vessels per high-power field in PDAC. Median survival was 8.0 months (95% CI = 4.4–11.6) for patients with less than 55 vessels per high power field, contrasting with the median survival of 14 months (95% CI = 11.4–16.6) in patients with 55 or more vessels per high power field in PDAC.

Less than 55 vessels per one high power field were present in 43/ 70 (61.4%; 95% CI = 46.9–72.2) of PDAC cases. Fifty-five or more vessels per one high power field of PDAC were identified in 27/ 70 (38.6%; 95% CI = 27.8–53.1) cases.

The IHC visualisation with low and high MVD is shown in Figures 3.89. and 3.90.

Figure 3.89. Immunohistochemical visualisation of vessels in poorly differentiated pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CD34, original magnification 100x.

Figure 3.90. Immunohistochemical visualisation of vessels in well differentiated pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CD34, original magnification 100x.

A statistically insignificant survival difference was found regarding negative/positive expression of other IHC markers. The results are shown in Table 3.31.

Table 3.31.

Statistically non-significant OS difference by IHC marker expression in pancreatic ductal adenocarcinoma

IHC marker	Value, p
p53	0.424
p21	0.784
Cyclin D1	0.081
E-cadherin	0.774
CK 20	0.465
CDX2	0.605
CK 5/6	0.192
p63	0.311
Chromogranin A	0.923
CD56	0.923
COX-2	0.412
BRCA1	0.081

Abbreviations in the table: OS, overall survival; IHC, immunohistochemical; CK, cytokeratin; CD, cluster of differentiation; COX-2, cyclooxygenase 2; BRCA1, breast cancer 1 protein.

3.7.2. Prognostic evaluation of the immunohistochemical profile in PETs

A statistically significant difference in OS was not found between positive/negative groups regarding any IHC marker expression. The data are summarised in Table 3.32.

Table 3.32.

Statistically non-significant OS differences by immunophenotype in pancreatic endocrine tumours

Target protein	p value
p53	0.511
p21	0.617
Cyclin D1	0.844
E-cadherin	0.379
CD44	0.317
COX-2	0.642
BRCA1	0.659

Abbreviations in the table: OS, overall survival; CD, cluster of differentiation; COX-2, cyclooxygenase 2; BRCA1, breast cancer 1 protein.

3.8. Diagnostic protocol for the examination of surgical specimens containing pancreatic carcinoma

The efficiency of gross as well as microscopic examination of pancreatic operation material was analysed in cases of PDAC. Tumour size was detected in 66.7% (95% CI = 53.0–78.0) cases by free approach statistically significantly contrasting with invariable measurements by protocol approach, namely 100% (95% CI = 87.5–100). Tumour pT parameter was not defined in 7.8% (95% CI = 3.1–18.5) by free description. Such inadequacy was completely avoided by protocol use: 0.0% (95% CI = 0.0–12.5) cases when protocol was used. LN were not investigated in one case during the research time due to a non-protocol approach. Less than 12 LN were examined in 52.0% (95% CI = 38.5–65.2) cases with a median of 10 (IQR = 10) examined LN by free approach versus 7.4% (95% CI = 2.1–23.4) cases and a median of 32 (IQR = 17) examined LN when protocol was used. The information about status of RM was not determined in 9.8% (95% CI = 4.3–21.0) cases by free description. No cases of pRx were observed using protocol. In all protocol-assessed cases, the peripancreatic RM was stained with Alcian-blue for more accurate examination (Figures 3.91. and 3.92.).

Figure 3.91. Operation material of pancreatoduodenectomy with stained peripancreatic resection margin

1 – PDAC, 2 – pancreatic tissue, 3 – gall bladder,
4 – duodenum

Figure 3.92. Invasion of pancreatic ductal adenocarcinoma in fat tissue and in stained (Alcian-blue) resection margin (arrows). Haematoxylin and eosin (HE), original magnification 400x.

The elaborated diagnostic protocol contained patient's identification, basic clinical and surgical data, gross investigation of each submitted organ or part of it (total 11), RM (total 6 RM with peripancreatic RM marked with Alcian-blue), pathologic process and assessed LN. Subsequently, data about tumour histologic type, pTNMGR parameters, vascular and neural invasion, necrosis, intraepithelial neoplasia, inflammation, desmoplasia and immunohistochemical visualisation were filled out using the created ready-to-use form containing the necessary options.

4. DISCUSSION

PC does not rank among the tumours with the highest incidence, but, nevertheless, PC is the fourth leading cause of cancer mortality (Siegel et al., 2012; Ferlay et al., 2013). By exploring the main prognostic factors, there was a possibility to identify a target mechanism for a new treatment, thus transforming the prognostic parameters into predictive factors. Some prognostic factors such as patient's age or tumour localisation cannot be affected, whereas processes like mitotic activity, epithelial-mesenchymal cell transition or cancer stem cell nature can be influenced and altered. The impact of conventional chemotherapy (gemcitabine, 5-fluorouracil, cisplatin) is low because the tumour cells are resistant to this treatment (Arumugam et al., 2009; Hong et al., 2009; Maginn et al., 2014; Wilson et al., 2014). Fortunately, positive results have been found in the efficiency of treatment that is directed to a blockage or activation of specific cell mechanisms or to the certain cell produced compounds. However, contradictory results exist (Westphal and Kalthoff, 2003; Jimeno et al., 2009; Chen et al., 2014; Hashim et al., 2014; Maginn et al., 2014; Wilson et al., 2014; Zhang et al., 2014). Therefore, to enhance and improve PC treatment options it is necessary to explore cancer-specific biological mechanisms (Maginn et al., 2014). Several methods are used in these types of studies, including immunoblots, *in situ* hybridization or polymerase chain reaction. IHC also has been widely used for these studies (Rosai, 2004) and was also applied in the present research.

Within this study, the prognostic factors were classified into two large groups: clinical and morphological prognostic factors including analysis of patients and surgical approach as well as morphological findings and biomolecular parameters by IHC markers. In order to assess the prognostic significance of each parameter it is important to be aware of the general patient survival results.

4.1. Overall survival

In this study, the median OS of the evaluated Latvian patients affected by PDAC and treated by a potentially radical operation was 11.0 (95% CI = 7.6–14.4) months which did not differ from other studies that analysed patients from the United Kingdom, Sweden, Canada and China (Kawesha et al., 2000; Cleary et al., 2004; An et al., 2012).

The three-, two- and one-year survival rates in different studies mostly lacked statistically significant differences (Niederhuber et al., 1995; Lim et al., 2003; An et al., 2012). In the present study these survival rates were 10.3% (95% CI = 4.8–20.8), 19.7% (95% CI = 11.6–31.3) and 42.4% (95% CI = 31.2–54.4), respectively. Only Lim *et al.* reported a statistically significantly longer three-year survival rate of 34.3% (Lim et al., 2003).

The 1-month mortality, which is considered to be perioperative mortality, of the target population in this study after a potentially radical surgical treatment of PDAC (5.4%; 95% CI = 2.1–13.1) was statistically analogous to the results obtained by Lim *et al.* in the USA (7.6%). The team of researchers evaluated the role of prognostic parameters using both approaches: including and excluding the perioperative mortality, and reached the agreement that there was no difference between results (Lim et al., 2003). From the published data, the patients with non-operated PDAC experienced very short survival. Taking into account the tumour stage, the five-year survival rate varied between 0.6–3.8% (Bilimoria et al., 2007).

In comparison to PDAC patients, patients diagnosed with PET had statistically significantly more favourable survival rates. In the present study, during the observation time, 73.0% of PDAC patients had died, but 14.3% of PET patients had died. However, the Canadian study reported a mean OS of 225.3 months (Morin et al., 2013). In the case of PET, five-, three-, two- and one-year survival rate varied between 42.1–56.0%, 53.0%, 61.7% and 72.3%, which is two, three or even five times longer than in the studied group of PDAC. Even a ten-year survival rate was presented as 24% (Hurtuk et al., 2011). Takahashi *et al.* observed a five-year survival rate of 78% and ten-year survival rate of even 65% of patients affected by PET, although the inclusion criteria of patients for the study were the same as for the present study (Takahashi et al., 2007).

4.2. Patients and surgical approach

Gender. This study along with other reported data confirmed that PDAC affects males and females in equal proportions. In addition, there were no differences between genders regarding survival, any morphological findings or IHC marker expression (Kawesha et al., 2000; Lim et al., 2003; Masugi et al., 2010; Handra-Luca et al., 2011; Yoon et al., 2011; An et al., 2012). Only Andrén-Sandberg *et al.* reported that within the younger age group PDAC was more commonly observed in males (Andrén-Sandberg

et al., 2000). Such an association was also found in the present study. In the age group ≤ 65 years there was a predominance of males but PDAC in females was more frequent in the age group > 65 years ($p = 0.043$). Of note, the average lifespan in Latvia was 73 years (gender-specifically: women – 78, men – 68 years) in 2012, but 70 years (women – 76, men – 65 years) in 2004, when the data collection of present study started (CSDB, 2012). Within this study, PC was invariably stated as the reason for death, but it would be appropriate to also consider the general processes of aging and other diseases which can affect the patient's overall condition with increasing age. If the length of men's lifetime ranges 65–68 (as mentioned above), then in the age group above 65 years the women's prevalence was higher than men's. In addition, more frequent occurrence of diabetes mellitus in females results from CD44-pathway associated PC over prolonged time course (Mohammed et al., 2013).

Although various articles noted that the PET incidence was also equal in both sexes, in this study women accounted for 75.0% (95% CI = 50.5–89.8) of all surgically treated PET patients (Heitz et al., 2004; Takahashi et al., 2007; Bettini et al., 2008; Haynes et al., 2011; Ekeblad et al., 2012; Rindi et al., 2012; Morin et al., 2013). The difference in survival by gender was not detected either in this study, or in other studies (Paik et al., 2008; Zhang et al., 2009; Scarpa et al., 2010).

Age. The median age of PDAC patients was similar between this and several other studies (Cleary et al., 2004; Handra-Luca et al., 2011), but a difference was found between the median age of patients in Latvia and the patients in USA, 67.0 (IQR = 18.3) years *versus* 72 years (Lim et al., 2003), respectively.

In this study, the mean age was not significantly different between PDAC and PET patients. However, the observed mean age of PET patients differed from other studies. In three other studies, the mean age of PET patients (53, 52, 51.6 and 48 years) was slightly lower than in this study (59.4 ± 9.2 ; 95% CI = 54.5–64.3 years) which could be explained by the fact that the group in the present study consisted only of an adult population (over 18 years) but in the other two studies, age group or range of patients' age was not specified and paediatric patients could have been included, which was displayed in the other two studies; the range of patients' ages were 17–76 years and 14–78 years, respectively (Lam and Lo, 1997; Paik et al., 2008; Rindi et al., 2012; Morin et al., 2013).

The age of patients generally was not included in both PDAC and PET prognostic indicators as substantiated by OS analysis. The survival rates did not differ

between patients age groups using the cut-off of 60, 65, 72 years (Lu et al., 1999; Lim et al., 2003; Masugi et al., 2010; An et al., 2012). The same conclusion was reached in the present study with a cut-off at 65 years.

Surgical treatment. No statistically significant survival difference was found between the groups of PDAC patients depending on the type of surgery performed (Lim et al., 2003). Slight differences were observed in the frequency of selection between operation types. For example, in this study pancreatoduodenectomy was performed in 79.5% (95% CI = 69.3–87.0) of PDAC cases but in other studies it was performed either less frequently (64.6%) or, on the contrary, even more often, i.e., 91.1% of cases (Cleary et al., 2004; Yoon et al., 2011). In some cohorts, e.g., in the study performed by Yoon *et al.*, total pancreatectomy was performed more frequently: 15.9% *versus* 7.7% (95% CI = 3.6–15.8) in the present study, respectively (Yoon et al., 2011).

PET cases are characterised by greater differences in the selection of operation type than PDAC patients. In this study, pancreatoduodenectomy was performed less frequently than in Takahashi *et al.*'s studies, respectively, 12.5% (95% CI = 3.5–36.0) cases *versus* 51.4% after recalculation (original article, 19/ 37) (Takahashi et al., 2007). Bettini *et al.* showed similar results with the present study – 34.8% (original article 39/ 112) cases (Bettini et al., 2008). Tumour enucleation was used considerably more often (37.5%: 95% CI = 18.5–61.4 of PET cases) in Latvian patients compared to the studies in which patients were collected from Japan (10.8% cases by recalculation from original article), Italy (12.5%; original data 14/ 112), 2 USA studies (6 and 6.5%) and in the patients group from Germany, France, Italy and Netherlands; 17.3% cases (Takahashi et al., 2007; Bettini et al., 2008; Haynes et al., 2011; Hurtuk et al., 2011; Rindi et al., 2012). In the present study, central pancreatic resection was not applied, whereas in other studies it was performed in 6.4–12.5% of cases (Bettini et al., 2008; Haynes et al., 2011; Rindi et al., 2012). On the whole, many factors can influence the surgical options of the operation type. These factors include tumour localisation, size, extent of invasion, availability of surgical equipment and surgeons' experience.

4.3.Morphological findings

Tumour localisation. Both this study and other studies showed that the most common localisation of PDAC was the head of pancreas. In the present study it was observed even more frequently: 83.3% (95% CI = 73.5–90.0) compared to 60–70% of

cases in other studies. Consequently, the other localisations of the tumour proportionally were less frequent (Klöppel et al., 2000; Rosai, 2004; Hruban et al., 2009; Masugi et al., 2010; Yoon et al., 2011).

Juuti *et al.* showed that PDAC of the pancreatic head was characterised by better survival than in the cases of other localisations ($p = 0.045$). However, survival rates in this study did not show statistically significant differences between tumour localisations in accordance with the findings by Masugi *et al.* (Juuti et al., 2006; Masugi et al., 2010).

Within this study, PETs were significantly less frequently localised in the pancreatic head (25.0%; 95% CI = 10.2–49.5) in comparison with PDAC. Consequently, pancreatoduodenectomy was performed statistically less frequently in the cases of PET (12.5%; 95% CI = 3.5–36.0) compared to cases of PDAC (79.5%; 95% CI = 69.3–87.0).

Considering that density of the islets of Langerhans is greater in the tail of pancreas, more frequent development of PETs in the tail of pancreas could be expected. However, PET frequency was equally balanced among the different anatomic compartments of the pancreas (Dälmane, 2004; Bettini et al., 2008; Rindi et al., 2012).

Tumour size. It has been reported that tumours located in the pancreatic tail and body were larger than the tumours localised in the head of pancreas (Klöppel et al., 2000). Nevertheless, in the present study this hypothesis was not confirmed either in PDAC or PET cases. The median size of PDAC was similar to the other studies (Lim et al., 2003; Handra-Luca et al., 2011). However, larger tumours were observed more frequently. Thus, tumours exceeding 2 cm in the largest diameter comprised 91.8% (95% CI = 82.2–96.5) of PDAC cases in the present study but only 73% cases in the group analysed by Kawesha *et al.* (Kawesha et al., 2000).

Despite these differences, Kawesha *et al.* and the present study indicated that there was no association between survival and tumour size (Kawesha et al., 2000). However, a large number of studies demonstrated that patients with larger tumours (cut-off values of either 2 cm or 3 cm have been applied) have shorter survival (Lim et al., 2003; Cleary et al., 2004; Juuti et al., 2006; Mielko et al., 2006; Masugi et al., 2010; Handra-Luca et al., 2011). In order to estimate the relation between tumour size and survival several precautions exist. The study group must be sufficiently large and the follow-up time must be reliable. In addition, for precise analysis of tumour size impact on survival it is necessary to detect tumour size very accurately. In the present study, the tumour size was additionally specified microscopically, especially in the cases when

tumour size during the gross examination was around 2 cm which is a critical parameter for pT determination. Thus, the obtained data could have high relevance. However, the analysis was hindered by the small number of tumours measuring 2 cm or less in the largest diameter (only 8.2%).

Tumour size (using the cut-off of largest diameter of 2 cm) proved to also be significant regarding the RM status. In this study, positive surgical resection lines were statistically more frequently observed if the PDAC size was larger than 2 cm, compared to smaller tumour sizes ($p = 0.002$).

Within the study, tumour size range was broader in the PET cases rather than PDAC cases, respectively 1.2–12.5 cm *versus* 1.5–9 cm. In PETs, the median size was slightly smaller than in PDAC cases, 2.9 (IQR = 4.3) cm *versus* 3.4 (IQR = 2) cm respectively, and the tumours exceeding 2 cm in the largest diameter were observed statistically significantly less frequently in PET cases (56.3%; 95% CI = 33.2–76.9) than in PDAC cases (91.8%; 95% CI = 82.2–96.5). The statistically significant difference between the results described above could be explained by hormone secretion in a subset of PETs leading to early symptoms and diagnosis of these cases leading to increased fraction of small tumours, not exceeding the diameter of 2 cm. In addition, PDAC grows faster than PET as indicated by the significantly higher proliferation activity in PDAC than in PET: mean of Ki-67 expressing cells $\pm$ SD was $23.2\% \pm 15.0$ (95% CI = 19.7–26.7) of cells *versus* $3.4\% \pm 3.4$ (95% CI = 1.5–5.3) of cells respectively.

In comparison with other studies, the data on PET size, regarding both median size and range, in this study were very similar to published results (Takahashi et al., 2007; Bettini et al., 2008; Chetty et al., 2008; Haynes et al., 2011; Ekeblad et al., 2012; Kim et al., 2012; Rindi et al., 2012).

Some studies have discovered prognostic significance of PET size. Five-year survival rate of the patients was statistically significantly better (100%) in patients with tumour size ≤ 1.5 cm, but dropped to 86% for tumour size 1.6–2.0 cm, 71% regarding tumour size 2.1–3.0 cm, 83% regarding tumour size 3.1–5.0 cm and 48% regarding tumours larger than 5.0 cm (Kishi et al., 2014). Thus, patients affected by tumours smaller than 1.5 cm had statistically better survival. Similar results were also displayed in Ferrone *et al.*, where five-year survival rate was 100% if the tumour size was < 2 cm and 90% if the tumour size was ≥ 2 cm ($p < 0.001$), excluding poorly differentiated endocrine carcinomas (Ferrone et al., 2007).

Thus, it has been suggested that patients with tumours smaller than 1.5 cm can even undergo observation. However, authors also warned that risk of distant metastases increased sharply with larger PET size (Kishi et al., 2014). This was in accordance with data obtained in the present study that showed an association between tumour size, distant metastases ($p = 0.025$), and invasion in large blood vessels ($p = 0.045$), the precursor lesion of distant metastases. In addition, Kishi *et al.* also found that larger size of PET correlated with the metastases in LN (Kishi et al., 2014). In present study, the trend towards such an association was found in PDAC cases ($p = 0.059$, regarding the cut-off at 2 cm of largest diameter) but not among PETs ($p = 0.600$). According to the literature, PETs reaching a definite cut-off size were characterised by significantly increased risk of lymphogenous metastases. However, the value of cut-off diameter varied between studies from 2 to 2.5 cm (Haynes et al., 2011; Kim et al., 2012). In Kim *et al.*, the smallest tumour giving rise to metastases measured only 1.2 cm (Kim et al., 2012). The correlation between lower tumour differentiation and the trend towards larger tumour size (Kim et al., 2012) or possibility of invasive growth have also been reported (Scarpa et al., 2010).

Considering the molecular basis of PET growth, the tumour size showed an association with Ki-67 and trend towards vimentin expression. PETs larger than 2 cm showed a trend towards associations with E-cadherin and CK 19 expression. Thus, proliferation, epithelial – mesenchymal transformation, loss of cell adhesion enhancing the invasiveness and stem cell immunophenotype are the pathways of increasing tumour mass.

pT parameter. The PDAC cases most often were detected and treated surgically as pT3, which meant that the cancer had invaded extrapancreatic tissues (Edge et al., 2010). pT3 cancers constituted 82% and 94% of cases in Lim *et al.* and Handra-Luca *et al.*, respectively, but in the present study comprised 98.7% (95% CI = 92.9 – 99.8) of cases (Lim et al., 2003; Handra-Luca et al., 2011). The peripancreatic adipose tissues represented the highest target for pT3 PDAC invasion (Klöppel et al., 2000; Rosai, 2004). In this study tumour invasion in adipose tissues were detected in all pT3 cases.

In particular, some research groups have proved that this unfavourable finding reflected on patient survival (Juuti et al., 2006; Oshima et al., 2013). Contrary reports were published as well, demonstrating no survival rate difference between pT parameter even in large PDAC groups comprising 396 and 240 patients (Lim et al., 2003; An et al., 2012). It should be noted that Juuti *et al.* and Lim *et al.* used pT parameter criteria

from the UICC 1997 TNM classification, presented in Table 4.1. (Lim et al., 2003; Juuti et al., 2006). In contrast, An *et al.* applied AJCC 2008 TNM classification that defined pT analogously to the AJCC 2010 TNM classification, used in the present study.

Table 4.1.

UICC TNM classification for pancreatic cancer (1997)¹

T1	Tumour limited to the pancreas
T1a	Tumour 2 cm or less in greatest dimension
T1b	Tumour > 2 cm in greatest dimension
T2	Tumour extends directly to any of the following: duodenum, bile duct, or peripancreatic tissues
T3	Tumour extends directly to any of the following: stomach, spleen, colon, or adjacent large vessels
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M0	No distant metastasis
M1	Distant metastasis
Stage grouping	
Stage I	T1,2 N0 M0
Stage II	T3 N0 M0
Stage III	anyT N1 M0
Stage IV	anyT anyN M1

¹ By Balzano et al., 1997

Abbreviations in the table: UICC, Union for International Cancer Control; TNM – classification of cancer spread by T (tumour), N (nodes) and M (metastasis) parameters

In the target group of Latvian patients, the predominance of pT3 over other T parameters was so high it precluded successful search for correlations with the clinical and morphological findings. Within this study PDAC was characterised by statistically less common pT2 and more frequent pT3 parameter finding than PET cases.

Regarding PETs, pT parameter frequency distribution between pT1 (25.0%; 95% CI = 10.2–49.5), pT2 (37.5%; 95% CI = 18.5–61.4) and pT3 (37.5%; 95% CI = 18.5–61.4) was not statistically significantly different. The occurrence of pT3 tumours was in line with Takahashi *et al.*, i.e., 32.4% of PET cases (Takahashi et al., 2007). The most common reason for pT3 in PETs was analogous to PDAC as represented by invasion in peripancreatic adipose tissue.

Prognostically significant survival difference has been reported between PET groups of T1 and T2 *versus* T3 or T4 as well as between T3 *versus* T4 (Scarpa et al., 2010). It was attributed to the tumour invasion in extrapancreatic tissue and in large arteries in the case of pT3 and pT4, while pT1 and pT2 tumours were situated in the

pancreatic tissue. The present study has provided more pathogenetically based explanations disclosing a significant association between pT and tumour grade ($\chi^2 = 18.667$; $p = 0.005$). Thus, one reason for increasing pT parameter is that cancer cells become more anaplastic, i.e., increasing tumour grade and also developing the ability to invade surrounding tissue and the blood vessels ($\chi^2 = 10.667$; $p = 0.005$).

pN parameter. In the present study and in most other studies, the occurrence of N1 was observed in more than half of the PDAC cases (Masugi et al., 2010; Handra-Luca et al., 2011). In contrast, pN1 finding was statistically significantly less frequent in PET cases, for example, 20.0% (95% CI = 5.7–51.0) PET *versus* 67.5% (95% CI = 56.5–76.9) PDAC cases in the present study in accordance with other observations (Takahashi et al., 2007; Bettini et al., 2008; Chetty et al., 2008; Haynes et al., 2011; Hurtuk et al., 2011; Kim et al., 2012; Rindi et al., 2012; Partelli et al., 2013). In PDAC patients, the median number of positive LN was 3 (IQR = 3.8) but in PET cases positive LN ranged from 1 to 2. The median amount of evaluated LN in PET patients was 1.5 (IQR = 6) which was considerably less than in PDAC cases (15.0; IQR = 24.0 LN). At the same time malignant tumours comprised almost 40% of all PET cases. An association was detected between the amount of evaluated LN and positive LN ($z = -2.460$; $p = 0.014$) which demonstrated the importance of accurate pancreatic tumour examination, because the reported prognostic value of LN status in both tumour cases (PDAC and PET) is high. Many articles have reported the mean OS in N1 cases as approximately twice as short compared to N0 cases, for example, 8.9 *versus* 17.9 months in PDAC. Similarly, in pN1 *versus* pN0 PETs, the five-year survival rate was 55.1% *versus* 91.1% (Kawesha et al., 2000; Masugi et al., 2010; Scarpa et al., 2010; Handra-Luca et al., 2011; Haynes et al., 2011; An et al., 2012; Oshima et al., 2013; Partelli et al., 2013). Thus, the diagnostic protocol was created for these purposes within the framework of the present study. The protocol resulted in statistically significantly more exact data as concluded from CI analysis.

The present study disclosed the possible pathogenetic mechanisms of the association between positive LN status and significant impact on the survival rate by demonstrating that pN1 PDAC also had other aggressive characteristics like poor differentiation ($\chi^2 = 7.090$; $p = 0.029$), increased mitotic activity ($z = -2.397$; $p = 0.017$), tumour invasion in the blood vessels ($\chi^2 = 7.399$; $p = 0.007$) and perineural growth ($\chi^2 = 3.973$; $p = 0.046$).

pM parameter. The tumour invasion in blood vessels and development of distant metastases have been reported as important prognostic factors both in PDAC and PET (Deshpande et al., 2004; Juuti et al., 2006; Bettini et al., 2008; Ekeblad et al., 2008; Zhang et al., 2009; Scarpa et al., 2010). In this study, PDAC patients' median survival was 12.0 (95% CI = 7.8–16.2) months and 3.0 (95% CI = 0.7–5.3) months without and with tumour invasion in large type blood vessels respectively. The difference was statistically significant, $p = 0.013$.

Although tumour invasion in blood vessels was observed in 38.5% (95% CI = 28.5–59.6) of PDAC, surgically removed distant metastases in potentially radically operated PDAC patients at the time of diagnosis was found in 3.8% (95% CI = 1.3–10.7) of cases. The difference could be attributable to the case selection for surgical treatment but also explained by the time course of disease as the invasion in blood vessels should precede the distant spread. Survival rate results indicated that patients' survival decreased dramatically in M1 cases and two-year survival was 0% (Juuti et al., 2006). This conclusion was also confirmed by the present study.

Regarding PETs, the survival of patients with distant metastases was better compared to PDAC cases. Distant metastases in potentially radical operations have been reported in 10.8–27.0% of PET cases (Takahashi et al., 2007; Chetty et al., 2008; Haynes et al., 2011). In the present study, the result was similar – 12.5% (95% CI = 3.5–36.0) of PET cases. Interestingly, Ekeblad *et al.* reported a frequent occurrence of M1 in 60% of PET cases. The respective study was conducted in an intrahospital Endocrine Oncology Unit and, possibly due to the relevant patient referral, the rate of well differentiated neuroendocrine carcinoma was 71% (Ekeblad et al., 2008) exceeding the frequency of 31.3% (95% CI = 14.2–55.6) observed in the present study. The median survival of M1 cases was 39.5 months, contrasting with a better survival of 58.9 months in patients lacking LN or distant metastases (Bettini et al., 2008). In the present study both PET patients with M1 also were alive for more than one year. Vascular invasion, representing the precursor lesion of haematogenous metastases, has been observed in about half of the PET cases both in the literature (Takahashi et al., 2007) and also in the present study (50%; 95% CI = 28.0–72.0). The development of distant metastases was associated with worse PET grade ($\chi^2 = 8.686$; $p = 0.034$) and increased mitotic activity ($z = -2.293$; $p = 0.022$).

Tumour stage. Complex assessment of all three pTNM parameters resulted in the tumour stage. In the published PDAC studies, IIB stage, characterised by presence

of LN metastases was dominate. In the present study group IIB stage was statistically more frequent (65.3%; 95% CI = 54.1–75.1) than in Bilimoria *et al.* (36.4%), which included 18,743 PC patients. Subsequently IIA stage was the second most common estimate in both the Bilimoria *et al.* and present study; these patients had tumour invasion in extrapancreatic tissues but no metastases (Bilimoria *et al.*, 2007). Lim *et al.* presented a trend towards a survival difference between stage II and stage III ($p = 0.05$) by the UICC 1997 TNM classification. However, the characteristics of these stages were quite close to IIA and IIB using AJCC 2010 TNM classification (Lim *et al.*, 2003).

The spread of tumour was the limiting determinant of late tumour stages. PDAC stage demonstrated association with tumour invasion in both large and small blood vessels. Between stage IIA and stage IIB tumours, the differentiation degree differed ($\chi^2 = 6.022$; $p = 0.049$).

Rindi *et al.* and also the present study showed that PETs were characterised by dispersed stages lacking a predominant stage that would be observed more frequently than others (Rindi *et al.*, 2012). Similarly, Morin *et al.* described the following PET stage distribution: I stage – 39% cases; IIA – 22%; IIB – 15%, IIIB – 8%, IV – 16% cases according to the 7th edition of the AJCC/ UICC TNM staging system designated for PNETs (Morin *et al.*, 2013). The analysis of survival rate between groups showed a significant difference ($p = 0.001$) between PET stages: I – 217.5 months, IIA – 113.3 months, IIB – 64.0 months, IIIB 45.0 months and IV – 49.0 months (Morin *et al.*, 2013)

RM. In order to assess the completeness of surgical treatment as well as the risk of tumour recurrence and patient's prognosis, it is very important to examine surgical RM carefully. Acknowledging this, Alcian-blue staining of RM was implemented in the present study, especially to evaluate the peripancreatic RM status (which was also the most frequent positive RM in PDACs) and PET enucleation operation material. In comparison to other PDAC studies, in the present study group R1 was diagnosed statistically more frequently: 27–35% versus 57.5% (95% CI = 46.1–68.2) cases, but it has not been demonstrated as a prognostically important factor (Masugi *et al.*, 2010; Handra-Luca *et al.*, 2011; Sugiura *et al.*, 2013).

Although some studies failed to demonstrate significance of RM condition in survival rate, there were large scale studies which have disclosed such a correlation (Kawesha *et al.*, 2000; Masugi *et al.*, 2010; Sugiura *et al.*, 2013). These studies showed that in the cases of negative RM, median OS was noticeably higher (even up to 35 months) than in the whole group of potentially radically operated PDAC patients in the

present study (11.0 months; 95% CI = 7.6–14.4) and also other studies, and that statistically significantly shorter survival was observed in patients with R1 status, e.g, 10–14 months (Mielko et al., 2006; Hamidov et al., 2011; Handra-Luca et al., 2011; Konstantinidis et al., 2013; Sugiura et al., 2013).

As previously stated, peripancreatic RM was the most commonly affected RM. During the study it was noted that the reason for positive RM was tumour complex location around the nerves or in the nerves which was also strongly confirmed by the correlation between perineural invasion of PDAC and positive RM in this study ($\chi^2 = 13.908$; $p < 0.001$). Overall, perineural invasion was one of the most typical features of PDAC. Several studies demonstrated an association or trend towards an association that patients with perineural invasion have shorter survival but because there was a correlation between R1 and shorter survival, perineural invasion and shorter survival and R1 and perineural invasion, there was also a higher probability that determining factor of survival influence was positive RM and not the perineural invasion directly (Mielko et al., 2006; Masugi et al., 2010; Handra-Luca et al., 2011; An et al., 2012; Konstantinidis et al., 2013; Sugiura et al., 2013). From a practical standpoint, further studies could be suggested to detect such morphological context of perineural invasion that should be qualified as equal to pR1, a finding that could be difficult to detect due to a large resection surface. The pure presence of perineural invasion would be too frequent a feature, being present in 87.2% (95% CI = 78.0–92.9). The evaluation algorithm could employ the other variables that were shown to be associated with R1, namely, tumour size exceeding 2 cm ($\chi^2 = 9.421$; $p < 0.002$) and pN1 ($\chi^2 = 4.296$; $p < 0.038$). Surprisingly, the immunohistochemical profile, including cell adhaesion markers and epithelial-mesenchymal transformation, has not demonstrated such an association.

Regarding PETs, both in the present study and literature, R0 can be reached in 68–87.1% of patients (Pomianowska et al., 2010; Haynes et al., 2011). In the present study, the rate of R0 (72.7%; 95% CI = 43.4–90.3) exceeded the frequency of R1 (27.3; 95% CI = 9.7–56.6) although the difference did not gain statistical significance.

No survival differences between R0/ R1 were shown in PETs by Pomianowska *et al.* (Pomianowska et al., 2010). Kim *et al.* revealed an interesting fact that recurrence of PET in patients with R0 status was observed in 23.1% of cases and in 11.1% of cases patients died because of the tumour (Kim et al., 2012). However, Haynes *et al.* and Chung *et al.* demonstrated that in the R0 cases survival was statistically significantly

longer than in the R1 cases: the five-year survival rate was 91.2% *versus* 55.0%, respectively (Chung et al., 2007; Haynes et al., 2011).

The other PET studies have not found an association between perineural invasion and R1, but shorter survival has been reported in patients with perineural invasion (Deshpande et al., 2004; Zhang et al., 2009). Perineural invasion has been described in 15/ 37 (40.5%) of PET cases (Takahashi et al., 2007), representing more frequent occurrence than in the present study (12.5%; 95% CI = 3.5 – 36.0). In the present study, pR1 status in PETs was not associated with perineural invasion but with mitotic count ($\chi^2 = -2.380$; $p = 0.017$); there was also a trend towards an association with the tumour grade ($\chi^2 = 5.958$; $p = 0.051$).

Grade of tumour differentiation. One of the parameters showing a relatively close correlation with both morphological and IHC parameters was tumour differentiation grade. Unfortunately, several studies did not specify which tumour differentiation grade classification was used thus hindering the interpretation. Nevertheless, the general trends can still be compared.

The G parameter distribution was similar in this and the other published studies. PDAC were mostly moderately differentiated (up to 51.3 % in the literature; 46.2%; 95% CI = 35.5–57.1 in the present study), followed by poorly differentiated (up to 44.1% in the literature; 35.9%; 95% CI = 26.2–47.0 in the present study). Well-differentiated PDAC were less common, comprising up to 32.2% of cases in the literature and 17.9% (95% CI = 11.0–27.9) in present study (Lim et al., 2003; Dong et al., 2005; Handra-Luca et al., 2011).

Poorly differentiated PDAC had significantly shorter survival (the median OS varied between 5–13.0 months) compared to patients with well or moderately differentiated PDAC, experiencing a median OS between 19–21.89 months (Mielko et al., 2006; Masugi et al., 2010; Handra-Luca et al., 2011). In the present study, a trend towards different survival (log-rank; $p = 0.050$) between patients with varying tumour differentiation grades was observed, respectively, 18 (95% CI = 8.1–28.0), 13 (95% CI = 9.0–17.0) and 6 (95% CI = 2.2–9.8) months between G1, G2 and G3 adenocarcinomas. In Lim *et al.*, no survival differences were revealed between G2 and G3, but patients with G1 tumours had significantly longer survival than in the case of G2 ($p = 0.004$) or G3 (0.002) carcinomas (Lim et al., 2003).

Some studies have demonstrated that tumour differentiation grade had no prognostic value (Kawesha et al., 2000; An et al., 2012). Nevertheless, the fact that

poorly differentiated tumours more frequently invaded blood vessels ($\chi^2 = 10.266$; $p = 0.006$) and grew perineurally ($\chi^2 = 8.021$; $p = 0.018$), substantiated the unfavourable nature of this finding. Similar results were revealed in PETs, i.e., the poorer tumour grade, the more frequent was tumour invasion in blood vessels ($\chi^2 = 9.778$; $p = 0.021$).

The grade distribution in PETs was the following. According to several authors, well differentiated endocrine carcinomas predominated and comprised 47.2–66% of all PETs, followed by well differentiated endocrine tumours (Bettini et al., 2008; Scarpa et al., 2010; Ekeblad et al., 2012; Rindi et al., 2012). Ekeblad *et al.* showed that well differentiated endocrine carcinomas accounted for 71% of PET cases, however, as was noted, the study was conducted in the Endocrine Oncology Unit of hospital leading to a specific cohort (Ekeblad et al., 2008). The patient enrolment in other studies (including Bettini et al., 2008; Scarpa et al., 2010; Haynes et al., 2011; Rindi et al., 2012) was analogous to the present study.

In the present study, well differentiated endocrine carcinomas comprised 31.3% (95% CI = 14.2–55.6) of PETs. Although this occurrence was not statistically significantly different from that reported, with the exception of Ekeblad *et al.*, 2008, well differentiated endocrine tumours with uncertain behaviour were detected more frequently by Haynes *et al.* and also the present study, comprising 51.8% (Haynes et al., 2011) and 56.3% (95% CI = 33.2–76.9) of all PET cases, respectively.

The identified grade distribution in local patients affected by PETs predicted long survival because the 5- and 10-year survival in well differentiated endocrine tumours with benign behaviour was 88.8% and 67.7%, respectively, and in well differentiated endocrine tumours with uncertain behaviour was 92.5% and 77.8%, respectively. Both previously described tumour grades accounted for 62.3% of PET cases in the present study. In contrast, both malignant PET groups (well differentiated endocrine carcinoma and poorly differentiated endocrine carcinoma) were characterised by 5- and 10-year survival rates of 49.8% and 16.6%, respectively (Haynes et al., 2011). In the present study, a direct association between the survival and PET grade was not identified. This could be attributable to a low number of the observed undesirable events. However, similar conclusions were reported by other authors. Assessing survival by WHO 2010 gastroenteropancreatic neuroendocrine tumours classification principles, survival showed no differences between G1, G2 and G3 PETs (Morin et al., 2013).

Necrosis. Tumour necrosis was observed in 26.9% (95% CI = 18.3–37.7) of PDAC cases. There was also a significant correlation between presence of necrosis and survival rates (log-rank, $p = 0.001$), i.e., median OS for patients lacking tumour necrosis was 13.0 (95% CI = 9.3–16.8) months, but for patients exhibiting tumour necrosis it was 4.0 (95% CI = 1.4–6.6) months.

Thus, significant prognostic value of necrosis has been identified. However, to provide adequate diagnostic accuracy assessing the extent of necrosis, both gross and microscopic measurements of necrosis would be required. The tumour tissue must also be entirely submitted for microscopic analysis.

Although in the present study PETs lacked necrosis, Deshpande *et al.* and Zhang *et al.* reported the presence of tumour necrosis and survival association with it. Thus, the median survival was 41 months in patients exhibiting PET necrosis, but 173 months in the group lacking tumour necrosis; $p = 0.006$ by Demir *et al.* (Deshpande *et al.*, 2004; Zhang *et al.*, 2009; Demir *et al.*, 2011). The survival differences can be attributed to grade distribution, i.e., poorly differentiated endocrine carcinomas were characterised by extensive areas of necrosis, but well differentiated endocrine tumours did not have necrotic foci (Klimstra *et al.*, 2010; Verbeke, 2010).

4.4. Immunohistochemical profile of PDAC and PETs

In the present study, a wide spectrum of IHC marker expression was analysed in order to ascertain which PDAC and PET molecular mechanisms were affected most frequently in the local target population, as well as to compare the tumour immunoprofile with non-neoplastic pancreatic tissues.

Ki-67. Increase in cell proliferation fraction was a characteristic feature of the tumour. As Ki-67 protein level in proliferating cells is sufficiently high for immunohistochemical detection, it has been analysed in different tumours. Determination of proliferation fraction of the relative amount of Ki-67 positive cells is essential for grading of PET (Dabbs, 2014). In PDAC increased proliferation fraction was described as a negative prognostic factor (Linder *et al.*, 1997; Yamamoto *et al.*, 2004; Karamitopoulou *et al.*, 2010; Jamieson *et al.*, 2011).

In PDAC this feature was reported in almost all cases and a relative amount of proliferative active cells can be as high as 97% (Culhaci *et al.*, 2005; Hu *et al.*, 2012). In this study the highest proliferation fraction among PDAC cases was 65.0%. The median

value of the relative amount of positive cells was comparatively low, i.e., 18.0% (IQR = 21.0). Several other researchers, for example, Linder *et al.* reported the median value of 29.7% (Linder et al., 1997). In the present study, rare Ki-67 positive cells were found also in NnPDs in agreement with Hu *et al.* (Hu et al., 2012).

Several research teams have reported statistically significant differences in PDAC survival rates by Ki-67 expression. However, the employed cut-off values have been as different: 10%, 20% and 45% (Linder et al., 1997; Yamamoto et al., 2004; Karamitopoulou et al., 2010; Jamieson et al., 2011). Stanton *et al.* and ShengMian *et al.*, however, did not show survival differences between Ki-67 expression positive and negative cases (Stanton et al., 2003; ShengMian et al., 2009). In the present study this correlation, using the cut-off value of 10%, also had no prognostic significance.

However, in the present study, an association was found between higher Ki-67 expression and higher number of LN metastases in PDAC ($r_s = 0.322$; $p = 0.023$). Hu *et al.* found higher Ki-67 expression correlated ($p = 0.002$) with increase of N1 cases (Hu et al., 2012). In the present study, there was also a higher mean rank of proliferation fraction by Ki-67 expression in PDAC with perineural invasion ($z = -1.974$; $p = 0.004$) indicating increased aggressiveness of malignant cells. Regarding the correlation between increased Ki-67 expression and tumour grade, contrary results were found by Hu *et al.* and Linder *et al.*: the correlation was confirmed by one team and denied by the other (Linder et al., 1997; Hu et al., 2012). Correlation between Ki-67 and tumour differentiation grade in PDAC was not present in this study. The diversity of results can be affected by different grading systems used to characterise the tumour differentiation. For example, Hu *et al.* used WHO classification including mitotic count (per 10 HPF) as one of the diagnostic criteria. Naturally, a close correlation between the mitotic activity and proliferation fraction can be expected (Hu et al., 2012). The correlation between mitotic activity and Ki-67 positive expression was also observed in this study ($r_s = 0.236$; $p = 0.047$) but in this study tumour differentiation grade was determined by the relative fraction of glandular structures and degree of cellular pleomorphism by Cancer Grading Manual (Guzman and Chejfec, 2007) in order to conform to internationally accepted classification and also to avoid data doubling. Linder *et al.* did not specify their classification system (Linder et al., 1997). The expected correlation (which was also confirmed) was found in the present study that increased cell proliferation in PDAC involved defects in both cell cycle inhibitors p21 and p27 as

disclosed by statistically significant correlations: $r_s = 0.280$; $p = 0.016$ regarding p21 and $r_s = 0.314$; $p = 0.006$, regarding p27.

In PETs, Ki-67 expression was observed in all tumours with a median relative amount of positive cells of 2% (IQR = 3.0), which was in agreement with other authors and was lower than in PDAC (18.0; IQR = 21.0) in the present study (Beghelli et al., 1998; Imam et al., 2000; Chetty et al., 2008; Piani et al., 2008; Scarpa et al., 2010; Rindi et al., 2012). On the other hand, positive expression of Ki-67 was invariably absent from NnPI.

Increased Ki-67 expression, i.e., at least above 2%, was usually judged as an unfavourable prognostic factor of PET. However, this was not confirmed by multivariate analysis performed by Chang *et al.* (Chang et al., 2000; Deshpande et al., 2004; Kishi et al., 2014). It is recommended to assign the PET grade by complex assessment of mitotic rate and Ki-67 issuing the grade on the basis of the highest value. Such approach allows for classifying the frequent discordant cases (McCall et al., 2013) that occur despite the general correlation between mitotic activity and proliferation rate by Ki-67.

Regarding elevated Ki-67 expression in PET, the most convincing correlation was described by more frequent metastatic spread (Yamamoto et al., 2004²; Jorda et al., 2003; Ohike and Morohoshi, 2005). In this study there was only a trend towards an association between increased Ki-67 expression and development of distant metastases ($z = -1.922$; $p = 0.055$) but at the same time there was also a strong correlation with tumour stage ($r_s = 0.688$; $p = 0.028$) and a moderately strong association with tumour size ($r_s = 0.584$; $p = 0.022$). Unfortunately, along with a higher ability for cell division, PET cells were characterised by newly acquired features: mesenchymal differentiation by vimentin expression ($r_s = 0.543$; $p = 0.045$) and CK 19 expression ($r_s = 0.572$; $p = 0.033$). Both markers were not observed in NnPI cells. In addition, both markers were characterised by adverse behaviour, indicated by correlations between increased CK 19 expression and many morphological findings, such as higher pT characteristic ($r_s = 0.654$; $p = 0.011$), tumour stage ($r_s = 0.730$; $p = 0.025$) and grade ($r_s = 0.744$; $p = 0.002$), tumour invasion in blood vessels ($z = -2.335$; $p = 0.020$), lymph vessels ($z = -2.022$; $p = 0.008$), perineural invasion ($z = -2.224$; $p = 0.026$) and increased mitotic count ($r_s = 0.675$; $p = 0.047$). Both CK 19 as well as vimentin showed a trend with larger tumour size.

Interestingly, in PET cases there was a trend towards a negative correlation ($r_s = -0.531$; $p = 0.051$) between Ki-67 and CD44 expression, but in PDAC the correlation was positive ($r_s = 0.281$; $p = 0.016$). Supposedly, in PDAC CD44 could be as a marker for cancer stem cell nature, but this association is controversial in PET cases. For Chaudhry *et al.*, increased CD44 expression correlated with increased rate of LN metastases (Chaudhry *et al.*, 1994).

p53. Normal p53 protein is found within cells in small quantities due to a very short half-life (Dabbs, 2014), so in normal pancreatic ducts p53 expression mostly was negative. Very weak reactivity in some cells has been reported (Lin and Prichard, 2011). Gene *TP53* mutations can result in synthesis of aberrant p53 protein that has a longer half-life than normal p53 protein and accumulates in cells. The presence of aberrant p53 can be proven by anti-p53 antibodies as intensive, diffuse p53 expression. It must be emphasised that there was no direct correlation between p53 protein expression and gene (Dabbs, 2014). Thus, p53 IHC and *TP53* mutation analysis provide two different levels of molecular examination. Among all pancreatic malignant tumours, p53 positive expression mostly but not invariantly was observed in PDAC and in PanIN-3 lesion (Lin and Prichard, 2011; Macgregor-Das and Iacobuzio-Donahue, 2013).

In the evaluated group, p53 protein expression was observed in 67.6% (95% CI = 56.3–77.1) of PDAC cases in compliance with the report of 60% by Liu *et al.* (Liu *et al.*, 2012). Kawesha *et al.* and Lozano-Leon *et al.* showed lower frequency of p53-expressing cases: 41% and 50% (Kawesha *et al.*, 2000; Lozano-Leon *et al.*, 2011). In contrast, an extensive meta-analysis including 952 PDAC patients concluded that p53 expression was observed in all cases (Smith *et al.*, 2011). Although p53 positive expression is considered to be a sign of malignant change and in pancreatic tissues it starts the expression mostly in high-grade PanINs (PanIN-3 lesion), it was occasionally also detected in the normal ducts in both this study in 4.1% (95% CI = 1.4–11.4) of cases and other studies with similar results (Takaori *et al.*, 2006; Xu *et al.*, 2006; Liu *et al.*, 2012). Only Lozano-Leon *et al.* did not find p53 expression in any NnPD (Lozano-Leon *et al.*, 2011). This observation has high practical value as a warning sign evaluating immunocytochemical findings of pancreatic fine needle aspirates in the case of questionable pancreatic malignancy.

Although p53 was observed in many tumours and also in a significant fraction of PDAC cases, however, based on both extensive meta-analysis studies and separated

studies, p53 protein expression cannot be used as a PDAC prognostic factor (Bártek et al., 1991; Kawesha et al., 2000; Nio et al., 2001; Karamitopoulou et al., 2010; Jamieson et al., 2011).

It was observed in the present study that PDAC with decreased p53 expression was a characteristic feature of more frequent invasion in large blood vessels ($z = -2.020$; $p = 0.043$). In contrast, invasion in small blood vessels was significantly associated with positive expression of aberrant p53 protein ($z = -1.982$; $p = 0.047$). Kawesha *et al.* found that 90% of tumours larger than 5 cm lacked p53 expression (Kawesha et al., 2000). Thus, it is possible that aberrant p53 expression appears in early defects of gene *TP53* which correlates with early and invasive tumour stages but after progression of tumour mutagenic processes and becoming more aggressive, p53 expression disappears due to additional silencing mutations. It could also be the reason why there has not been a correlation found between p53 expression and survival.

The p53 protein was statistically less frequent in PET than in PDAC, i.e., 20.0% (95% CI = 7.1–45.2) cases in this study were assessed as positive (Wick and Graeme-Cook, 2001). Contradictory results were observed between the present study and the research work of Yachida *et al.* Yachida *et al.* described p53 expression as present only in malignant PETs but in this study p53 expression was seen in all well differentiated endocrine tumours with unclear behaviour lacking invasion in surrounding tissues (Yachida et al., 2012). It is possible that a well differentiated endocrine tumour with uncertain behaviour has a malignant nature but not yet invasive growth, similarly to PanIN-3 lesion in PDAC. Since p53 expression was observed only in 4 PETs, the correlation analysis would lack meaningful statistical value. Other researchers have also reported rare occurrence of *TP53* mutations (1/ 22 tumours) and immunohistochemical expression of p53 protein in PETs denying the prognostic significance of these changes due to rare occurrence (Jonkers et al., 2006).

At the same time, Liu *et al.* reported that p53 was not observed in any NnPI cases. However, in this study two cases expressed p53 in 1% and 8% of non-neoplastic, possibly reactive cells (Liu et al., 2012).

p21. The p21 protein is a cell cycle inhibitor which is involved in the cell cycle control (cell cycle progression, apoptosis and transcription) of normal as well as of malignant cells. It can be induced by p53-dependent and independent mechanisms (Ciccarelli et al., 2005; Cazzalini et al., 2010). Increased expression of p21 in cancer cells is intriguing because p21 has a dual function: inhibition and positive modulation; it

can play anti- and pro-apoptotic function, depending on the nature of the apoptotic stimulus (Dotto, 2000; Abbas and Dutta, 2009; Cazzalini et al., 2010). Loss of p21 expression may be a reason for increased proliferative activity, and as associated with tumour cells which contained a non-functional, mutated p53 or even did not have p53 (el-Deiry et al., 1993; Cazzalini et al., 2010). By IHC the antibody labels the cells which express p21^{WAF1/Cip} (Noda et al., 2002).

In PDAC, in the present study, positive expression of the p21 was statistically more frequent (84.9% of cases; 95% CI = 75.0–91.4) compared to several other studies which demonstrated the relative frequency of positive cases as 20.7–25%, although the applied cut-off value between all studies was equal, i.e., 5% (Hu et al., 1998; Kawesha et al., 2000; Lozano-Leon et al., 2011). However, the data obtained in the present study were in close correlation with results reported by Biankin *et al.* This team found positive expression of p21 in 79% of cases with a cut-off of 10% (Biankin et al., 2002).

In some studies p21 expression was not detected in any of NnPD cases. In the present study its expression was displayed only in distinct cells in 21/ 69 (30.4%; 95% CI = 20.9–42.1) NnPD cases, of which the threshold of positive expression in at least 5% cells was reached in 7.2%; 95% CI = 3.1–15.9 cases (Hu et al., 1998; Lozano-Leon et al., 2011).

In the present study, an association between decreased expression of p21 and larger PDAC size (exceeding 2 cm) was identified ($z = -1.982$; $p = 0.047$). This could be interpreted as confirmation of the theory advanced by Cazzalini *et al.* and el-Deiry *et al.* and stating that cell proliferation increases if p21 is decreased (el-Deiry et al., 1993; Cazzalini et al., 2010). However, a positive correlation between p21 and Ki-67 was shown in PDAC ($r_s = 0.280$; $p = 0.016$) as well. Thus, p21 positive cases are proliferating more actively, but p21 negative cases are larger, hypothetically suggesting involvement of another p21-dependent function – increased apoptosis. Lack of apoptosis in p21-negative cases could eventually result in larger cell mass despite lower proliferation. Resistance to apoptosis also would protect tumour cells from chemotherapy. Unfortunately, the expression of p21 showed a correlation with stem cell marker CD44 ($r_s = 0.265$; $p = 0.024$), known to be a negative prognostic factor associated with treatment resistance. The p21 protein expression also correlated with epithelial-mesenchymal transition by vimentin ($r_s = 0.303$; $p = 0.010$), identified in the present study as a negative prognostic factor regarding the OS ($p = 0.002$). From the literature, patients with larger tumours size also experienced shorter survival (Lim et al.,

2003; Cleary et al., 2004; Juuti et al., 2006; Mielko et al., 2006; Masugi et al., 2010; Handra-Luca et al., 2011). Thus, p21 is in the centre of a complicated molecular network of undesirable outcomes, possibly balancing them. Not surprisingly, the net prognostic value is controversial. Although meta-analysis showed the prognostic value of p21 expression in PDAC, there were studies that did not confirm a significant association between expression and survival (Kawesha et al., 2000; Karamitopoulou et al., 2010; Jamieson et al., 2011).

Similarly as aberrant p53 expression, p21 expression was observed in a higher fraction of PDAC cells in the patients older than 65 years ($z = -1.981$; $p = 0.048$). Both these markers showed a weak positive correlation with CDX2 expression suggesting an association with intestinal – type differentiation.

In PETs, positive p21 expression was less frequent than in PDAC. In PET and in NnPI, positive p21 expression was observed in 4/ 14 cases (28.6%; 95% CI = 11.7–54.7) and 4/ 13 cases (30.8%; 95% CI = 12.7–57.6), correspondingly.

p27. p27 is a cyclin-cyclin dependent kinase inhibitor and tumour suppressor. It inhibits cell cycle progression due to the ability to mediate G_1 arrest by several pathways (Sherr and Roberts, 1999; Koff, 2006). In cancer cells, immunohistochemical expression of p27 was decreased due to impaired synthesis or accelerated degradation of the relevant protein, functionally leading to p27 inactivation (Koff, 2006; Chu et al., 2008).

In this study, positive cases regarding PDAC and NnPD occurred with similar frequency but the mean relative amount of positive cells was significantly lower in PDAC (28.2% of cells; 95% CI = 24.2–32.2) than in NnPD (42.3% of cells; 95% CI = 39.0–45.6), indicating that p27 inhibition was one of the carcinogenetic pathways in PDAC. In the present study, p27 positive PDAC cases were statistically significantly more frequent (91.9%; 95% CI = 83.4–96.2 of cases) than in other studies (41 – 49% of cases) with an equal cut-off value (Fukumoto et al., 2004; Mielko et al., 2006). Hypothetically, p27 pathway damage accompanied with loss of the relevant protein expression are less frequent in local patients. Nevertheless, in the present study it had prognostic value regarding survival (log-rank, $p = 0.003$).

In PET and in NnPI, p27 expression was observed in all cases without a statistically significant difference in the mean amount of positive cells, i.e., 62.6% (95% CI = 46.6–78.6) and 75.3% (95% CI = 64.5–86.1) respectively. In contrast, Lu *et al.* noted that p27 was observed in all NnPI cells (Lu et al., 1999).

The p27 could be one of the most important prognostic markers, because many PDAC and PET studies including the present study showed that patients with retained high p27 expression have considerably longer survival than patients with decreased p27 expression (Lu et al., 1999; Chang et al. 2000; Juuti et al., 2003; Fukumoto et al., 2004; Kim et al., 2014).

In large PDAC exceeding a diameter of 2 cm, more cases of p27 loss have been identified (Lu et al., 1999). Likewise, Karamitopoulou *et al.* showed that late stage PDAC having high pT also present with significantly lower p27 expression (Karamitopoulou et al., 2010). In this study association with clinical and morphological parameters was not found but it was observed that p27, in the same way as p21, positively correlated with CK19 positive expression in PDAC ($r_s = 0.271$; $p = 0.020$ and $r_s = 0.316$; $p = 0.007$).

Cyclin D1. As mentioned previously, the transcriptional regulator protein cyclin D1 forms a complex with CDK. T complex sustains the regulation of G₁ phase to the S phase of the cell cycle (Dabbs, 2014). In malignant cells, cyclin D1 stability is changed. Thus, the level of cyclin D1 can grow due to an affected degradation mechanism, resulting in cell cycle progression (Alao, 2007).

In general, the fraction of cyclin D1 positive PDAC cases was similar in this (64.9%; 95% CI = 53.5–74.8) and other studies (Kawesha et al., 2000; Biankin et al., 2002). In NnPD, distinct cells showed cyclin D1 reactivity; but none of the 7 (9.9%; 95% CI = 4.9–19.0) cyclin D1 reactive cases reached the set 5% cut-off value. Hypothetically, the up-regulation of cyclin D1 could also be related to the association with new-onset diabetes and PC. It is known that SOX6, a down-regulated transcription factor in pancreatic islets of hyperinsulinemic obese mice, suppresses cyclin D1 levels due to interaction with promoter activity. Thus, hyperinsulinemic diabetes can involve SOX6 pathway-associated up-regulation of cyclin D1 (Iguchi et al., 2007).

From the obtained data, no direct prognostic value of cyclin D1 was found regarding survival in accordance with other researchers (Kawesha et al., 2000). However, Culhaci *et al.* and Lebe *et al.* found that cyclin D1 in PDAC correlated with both tumour size and perineural invasion, but lacked correlation with gender, tumour stage, status of LN, tumour differentiation, distant metastases and invasion in blood vessels (Kawesha et al., 2000; Lebe et al., 2004; Culhaci et al., 2005). In the present study, results were contrary to expectations, i.e., that decreased cyclin D1 expression is characteristic in tumours with invasion in large blood vessels ($z = -1.982$; $p = 0.047$). It

could be explained by the fact that tumours characterised by increased invasiveness and cell migration more frequently undergo EMT. At the same time cytoskeleton changes by EMT and cell division are incompatible. It is proven that these cells are characterised by activated p21, decreased level of cyclin D1 and induced resistance to apoptosis (Karamitopoulou et al., 2010). Thus, decreased expression of cyclin D1 cannot be routinely considered as a favourable sign in tumours.

Decreased cyclin D1 expression was found also in PDAC exhibiting tumour necrosis ($z = -2.071$; $p = 0.038$). The presence of necrosis, in turn, was demonstrated to be a prognostic factor regarding the survival (log-rank, $p = 0.001$).

The endocrine pancreas was different from the exocrine pancreatic tissues regarding cyclin D1, respectively, the frequency of cyclin D1 positive cases was without a statistically significant difference between NnPI (61.5% of cases; 95% CI = 35.5–82.3) and PET (41.7% of cases; 95% CI = 19.3–68.1) cases, and the median relative amount of positive cells was also similar: 23.0% (IQR = 31.0) and 25.0% (IQR = 57.0), respectively. In contrast, Chang *et al.* convincingly presented that proportion of cyclin D1 positive cases was significantly higher in malignant PETs, paralleling increased mitotic activity and proliferation fraction (Chang et al., 2007). Interestingly, in an animal study using a mouse model, scientists induced cyclin D1 expression in the pancreatic β -cells, resulting in pancreatic islet hyperplasia, but this hyperplasia did not change blood glucose levels and glucose tolerance tests while no diabetes or endocrine cell tumour developed during 2 years. It showed that over-expression of cyclin D1 could be also non-tumorigenic (Zhang et al., 2005). Involvement of cyclin D1 pathways in the regulation of pancreatic endocrine cell physiology was also shown by other scientists (Iguchi et al., 2007).

Although cyclin D1 did not show prognostic value, in several studies it was regarded an important predictive factor, because the investigation of treatment was based on the possibility of cyclin D1 degradation (Alao, 2007). The relevance of cyclin D1 to the endocrine oncogenesis was emphasised by the interaction of cyclin D1 regulatory mechanisms via promoter activity suppression and beta-catenin, an important component of E-cadherin pathway (Iguchi et al., 2007). Extracellular-regulated kinase ERK and p38/ mitogen-activated protein kinase MAPK pathways were also involved both in regulation of cyclin D1 levels (Guo et al., 2003) and down-regulation of E-cadherin within EMT (Gui et al., 2012). Abnormal levels of E-cadherin, in turn, in the present study were shown to be associated with several important processes in PETs,

namely pT ($\chi^2 = 6.128$; $p = 0.047$), tumour grade ($r_s = -0.680$; $p = 0.007$), invasiveness proved by invasion in small blood vessels ($z = -3.016$; $p = 0.003$) and perineural invasion ($z = -3.016$; $p = 0.003$), expression of p53 ($r_s = 0.655$; $p = 0.011$), p21 ($r_s = 0.541$; $p = 0.046$) and p63 ($r_s = 0.665$; $p = 0.009$) proteins and angiogenesis as reflected by MVD ($r_s = 0.701$; $p = 0.005$). Hypothetically, E-cadherin expression could have higher prognostical value serving as the measure of molecular dysregulation in PETs while frequently expressed cyclin D1 (Capurso et al., 2012) would be too ubiquitous for prognostic information still retaining its virtue as a therapeutic target.

Analysing the expression of p21, p27 and cyclin D1, it is essential to interpret the marker expression in the context of the general pathomorphological findings, patient's clinical history and other diagnostic tests.

Bcl-2. Bcl-2 is anti-apoptotic protein. IHC increased expression of Bcl-2 protein is observed usually after translocation in the *BCL2* gene. The over-expression of Bcl-2 protein can block the chemotherapy-induced cell death (Dabbs, 2014).

There were quite contradictory results about Bcl-2 expression analysis. Firstly, studies described relatively frequent Bcl-2 expression in PDAC (35.6–55.0%) and in PET cases (53.3–53.6%), but in the present study only 1/ 75 (1.3%; 95% CI = 0.2–7.2) positive PDAC case was observed and there was no reactivity in PET cases (Sinicrope et al., 1996; Dong et al., 2005; Proca and Frankel, 2008; Yachida et al., 2012). Secondly, 8/ 73 (11.0%; 95% CI = 5.7–20.2) cases showed Bcl-2 positivity exceeding 5% positive cell threshold in NnPD. In a review by Westphal and Kalthoff, the authors described that PC was characterised with maintained normal or even reduced Bcl-2 expression. The current data were in agreement with this. To explain this controversy, in pancreatic carcinoma, over-expression of another anti-apoptotic protein belonging to Bcl-2 family – Bcl-x_L – is more often observed and has greater relevance (Westphal and Kalthoff, 2003). Thus, cross-reactivity can be responsible for a greater fraction of positive cases but was avoided in the present study. It must be emphasised that positive controls (including internal positive control of lymphocytes) were invariably performed and reactive.

Although in the present study Bcl-2 expression in malignant tissues was negative, nevertheless this anti-apoptotic marker has been evaluated as a prognostic marker in several studies (Dong et al., 2005; ShengMian et al., 2009; Jamieson et al., 2011).

E-cadherin. E-cadherin is an epithelial transmembrane glycoprotein. The key role of E-cadherin is in the support of epithelial layer integrity and polarity (Dabbs, 2014). For example, lost expression of E-cadherin in PETs was characterised by larger tumour size and metastases in LN and in liver compared with tumours with retained E-cadherin expression (Dabbs, 2014).

Decreased cell adhesion has been reported in PDAC (Nakajima et al., 2004; Shimamura et al., 2003; Hong et al., 2011). In the present study, positive expression of E-cadherin was seen in a smaller number of cells in PDAC than in NnPD, but the difference lacked statistical significance: the median value of E-cadherin expressing cells was 69.0% (IQR = 57.0) *versus* 85.0% (IQR = 53.0) of cells, and the mean value was $62.9\% \pm 30.3$ (95% CI = 55.9–69.9) *versus* $69.7\% \pm 31.7$ (95% CI = 61.8–77) of the target cells. Other authors reported a significant difference between the decreased E-cadherin expression in PDAC and the maintained level in NnPD (Shimamura et al., 2003; Nakajima et al., 2004; Hong et al., 2011). The relative number of positive cells below the cut-off value of 10% was detected in 6/ 67 (9.0%; 95% CI = 4.2–18.2) NnPD. This finding, albeit unexpected, can be explained by the presence of inflammatory processes in pancreatic tissues. De Boer *et al.* examined E-cadherin expression in normal bronchial epithelium and in bronchial epithelium of asthmatic persons. The authors concluded that E-cadherin expression was significantly lower in the epithelium of asthmatic patients affected by inflammation (de Boer et al., 2008).

Positive cases of E-cadherin expression were statistically significantly more frequent in PDAC than in PETs, and in NnPD than in NnPI, but the relative numbers of positive cells did not differ.

Along with a disappearance of adhesion molecules in PDAC, extinction of glandular structures starts in the tumour mass leading to single cell invasion that defines a higher grade. It also increases the possibility for the cells to migrate and develop lymphogenous and haematogenous metastases which considerably alters the patient's prognosis (Shimamura et al., 2003; Li et al., 2003; Masugi et al., 2010; Hong et al., 2011). Loss of E-cadherin expression in PDAC leading to expression in fewer than 90% of cells shortens median survival by half, i.e., 23.2 *versus* 12 months (Shimamura et al., 2003). PDAC patients with E-cadherin positive expression in less than 5% of cells had a median survival of 5.5 months and 5-year survival was 0% (Hong et al., 2011). Cases exhibiting E-cadherin reactivity in 5% of cells were also observed in the present study. Taking into account all the results, the prognostic value of E-cadherin loss cannot be

denied. From a practical point of view, routine evaluation would disclose the cases of very low reactivity. Careful assessment is a mainstay for identifying the tumour fraction losing E-cadherin expression as this fraction can be small (10%) but still significant.

In the present study, no correlations were found in PDAC which would characterise a higher possibility for the E-cadherin-losing tumour to spread but such strong correlations were found after assessment of E-cadherin expression in PET. Decreased expression of E-cadherin was associated with more frequent tumour invasion in blood vessels ($z = -3.016$; $p = 0.003$), perineural growth ($z = -3.016$; $p = 0.003$), higher pT ($r_s = -0.655$; $p = 0.011$) and also grade ($r_s = -0.680$; $p = 0.007$).

To ensure cell migration, it is necessary to undergo not only loss of adhesion molecules but also the epithelial-mesenchymal transition, resulting in vimentin expression in the cell (Javle et al., 2007; Thiery et al., 2009).

CD44. CD44 is transmembrane adhesion molecule participating in cell-to-cell as well as cell-to-matrix adhesion and in lymphocyte-homing activity (Ringel et al., 2001; Dabbs, 2014). At the same time, CD44 is one of the markers for cancer stem-like cells (Hou et al., 2014). These are self-renewing cells possessing the ability to promote clonogenicity, cell growth and migration as well as metastatic spread (Zhu et al., 2014). Higher resistance to chemotherapy and radiation therapy is an additional negative feature of these cells (Hong et al., 2009; Li et al., 2009; Tajima et al., 2012; Lloyd et al., 2013). Zhang *et al.* revealed that a correlation exists between stem-like cells and EMT (Zhang et al., 2012). Interestingly, Vazquez-Martin *et al.* reported that it was possible to reduce CD44 (positive) CD24 (negative/ low) breast cancer stem cell immunophenotype with anti-diabetic drug metformin affecting EMT pathway (Vazquez-Martin et al., 2010). Analogous findings were published regarding metformin in chemoprevention of PC assuming an increased risk for PC in diabetic patients; the protective ability was shown both by epidemiology in human patients and by animal studies. The explanation of metformin efficacy was associated with CD44 pathway and the observed decrease in PDAC incidence (by 55–60%) in an animal model (Mohammed et al., 2013) was in accordance with the results of the present study identifying CD44 expression in 45.2% (95% CI = 34.3–56.6) of all PDAC. In contrast, interferon-alpha can up-regulate CD44 expression, inhibiting tumour growth but promoting development of metastasis in the early stage of tumour growth (Zhu et al., 2014).

Widely variable proportions of CD44 positive cases have been reported by different authors but mostly its expression was observed in more than half of the PDAC cases (Gansauge et al., 1995; Immervoll et al., 2011; Tajima et al., 2012). In the present study, CD44 positive cases comprised 45.2% (95% CI = 34.3–56.6) of all PDAC. Compared to other target structures such as NnPD and PET, there was no statistically significant difference regarding number of positive cases and mean amount of positive cells. The expression was somewhat lower in NnPI. Only one case of NnPI had a number of positive cells that exceeded the cut-off value of 30%, but CD44 expression was seen in 12/ 14 (92.3%; 95% CI = 66.7–98.6) NnPI cases with a median extent of 10.5% (IQR = 19.0) of cells. Imam *et al.* concluded that normal pancreatic islet cells are negative by CD44 (Imam et al., 2000), and the general trend in the present study was consistent with this.

The uncertainties in CD44 expression were seen because CD44 can be expressed in both normal cells of pancreatic ducts and atrophic, inflammation affected cells, as well as in malignant epithelial cells. Immervoll *et al.* studied CD44 expression in the situations mentioned above and analysed CD44 deposition in cell structures (membrane and/ or cytoplasm) and polarity (basal, lateral or apical), as well as CD44 prognostic value. The researchers concluded that there was no correlation between CD44 expression and survival, but correlation with LN status existed (Immervoll et al., 2011), confirmed also in the present study ($z = -2.577$; $p = 0.010$). The same association was found by Chaudhry *et al.* in PET (Chaudhry et al., 1994). Other researchers have identified co-expression of stem cell markers as an independent prognostic factor for disease-free survival (Hou et al., 2014).

The results of the present study indicated that CD44 positive PDAC cells were characterised with fundamental changes, as CD44 expression was more frequent in poorly differentiated PDAC ($r_s = 0.266$; $p = 0.023$), in PDAC possessing spread to LN ($z = -2.577$; $p = 0.010$) and perineural invasion ($z = -2.781$; $p = 0.005$). The fundamental role was further supported by the evidence that CD44 was significantly associated with survival in PDAC patients, which was also confirmed using multivariate analysis by Cox proportional hazards test. The median survival was longer in the cases lacking CD44 expression than in CD44-positive cases (log-rank, $p = 0.018$): 15 (95% CI = 9.6–20.4) months *versus* 8 (95% CI = 6.0–10.0) months.

According to the literature, CD44 is important as a predictive factor. In particular, the resistance to chemotherapy can be expected in CD44-positive cases indicating higher importance of surgical treatment.

CK. Few articles have been devoted to the prognostic value of CK and CDX2. Among the spectrum of CK (CK 7, CK 19, CK 20, CK 5/6, CK 34 β E12), CDX2 prognostic value was determined only for CK 20 in PDAC and for CK 19 in PET cases. The expression of CK 20 and CK 19 was associated with shorter survival (Deshpande et al., 2004; Matros et al., 2006; Schmitt et al., 2007; Zhang et al., 2009; Han et al., 2013).

CK 7 and CK 19. CK 7 and CK 19 are typical markers of pancreatic ductal epithelium (Dabbs, 2014), which are invariably expressed in normal epithelia with a mean positive cell count of 98.0% (95% CI = 96.8–99.2) and 95.7% (95% CI = 94.0–97.4), respectively, in the present study. At the same time, CK 7 was negative in normal islet cells in accordance with the published data (Dabbs, 2014). Both markers retained the expression in malignant ductal epithelium, but appearance of CK 19 expression in PET was characterised by increased malignant potential (Lin and Prichard, 2011). CK 7 expression was also observed in PETs (Lin and Prichard, 2011).

The frequency of CK 7 positive cases and the extent of CK 7 expression by positive cell amount in PDAC in the present study was similar to previously published data (Bayrak et al., 2012; Liu et al., 2012). Goldstein *and* Bassi and Schüssler *et al.* showed that all PDAC cases were positive (Schüssler et al., 1992; Goldstein and Bassi, 2001). A slightly lower amount of positive CK 7 cases was detected by Chu *et al.* (92% of cases) and Hamidov *et al.* (85.2% of cases), but in the present study CK 7 positive cases constituted 98.6% (95% CI = 92.7–99.8) of PDAC (Chu et al., 2000; Hamidov et al., 2011). The median amount of CK 19 positive cells in PDAC was slightly lower than of CK7 expressing cells, namely 91.0% (IQR = 16.0) *versus* 97.0% (IQR = 12.0).

Schüssler *et al.* described that the expression levels of both CK 7 and CK 19 decreased in the areas of squamous differentiation within PDAC. However, in the present study CK 7 and also CK 19 showed weak and moderate positive correlations with squamous marker CK 34 β E12 expression (Schüssler et al., 1992). Negative correlation between CK 7 and both endocrine markers (ChrA and CD56), indicates that part of CK7 negative cells are endocrine cells which normally do not stain with CK7.

In pancreatic non-neoplastic islets, CK7 and CK 19 was observed neither in this study, nor in other studies, but both markers can appear in PET. In the present study, 2/ 14 (14.3%; 95% CI = 4.0–39.9) PETs were classified as CK 7 positive but 4/ 14

(28.6%; 95% CI = 11.7–54.7) expressed CK 19. Other authors described CK 7 expression in half of PET cases and CK 19 in more than 60% of PETs (Schüssler et al., 1992; Cai et al., 2001; Zhang et al., 2009; Liu et al., 2012; Han et al., 2013).

CK 19 was the only CK that has a reported prognostic value in pancreatic tumours, limited to PETs. Thus, the 5-year survival in the case of CK 19 negative and positive expression was 100% and 68.4% respectively, p value <0.001 (Zhang et al., 2009). At present, CK 19 is considered promising but still a controversial prognostic indicator in PETs (Jonkers et al., 2006; Reid et al., 2014); the present study would support its informativity as CK 19 was associated with aggressive characteristics in PET cases. In PETs, CK 19 was associated with pT ($\chi^2 = 8.068$; $p = 0.018$), pR ($z = -2.234$; $p = 0.025$), grade ($r_s = 0.744$; $p = 0.002$), invasivity as demonstrated by invasion in small blood vessels ($z = -2.335$; $p = 0.020$) along with lymphatic and perineural invasion, proliferative activity by Ki-67 ($r_s = 0.572$; $p = 0.033$), CK 7 ($r_s = 0.607$; $p = 0.021$), epithelial-mesenchymal transition by vimentin ($r_s = 0.551$; $p = 0.041$) and angiogenesis as reflected by MVD ($r_s = -0.639$; $p = 0.014$). It had a trend towards an association with tumour size with 2 cm cut-off ($z = -1.930$; $p = 0.054$) and a trend towards an association with squamous differentiation by p63 ($r_s = -0.516$; $p = 0.059$). Otherwise, CK 7 and CK 19 have been used as markers of histogenesis for differential diagnostic of metastatic tumours (Dabbs, 2014).

CK 20. CK 20 expression was negative in normal but also in reactive ductal epithelium and in normal islet cells (Lin and Prichard, 2011; Dabbs, 2014). Typically it is positive in intestinal epithelium, but less intense and just focal expression of CK 20 can be observed in PDAC (Dabbs, 2014).

In the present study, CK 20 was absent both from normal pancreatic ducts and islets in agreement with Liu *et al.* (Liu et al., 2012). In PDAC, the amount of CK 20 positive cases considerably varied. Similar results to the present study (22.7%; 95% CI = 14.7–33.3 of PDAC cases) were reported also by Liu *et al.* (15%), Goldstein and Bassi (30%) and Bayrak *et al.* (22%) studies (Goldstein and Bassi, 2001; Bayrak et al., 2012; Liu et al., 2012).

In the present study, a negative correlation was found between CK 20 expression and a higher number of metastatic LN ($r_s = -0.290$; $p = 0.041$). Bayrak *et al.* did not disclose the correlation between CK 20 expression and status of LN (Bayrak et al., 2012). In present study, CK 20 also showed a correlation with CDX2 ($r_s = 0.343$; $p = 0.003$) that is normally expressed in intestinal epithelium. Thus, the

immunophenotype disclosed intestinal differentiation in a subset of pancreatic tumours. Matros *et al.* presented the idea that PC has several subtypes because the tumours, in which CK20 expression was not observed, were associated with PanIN lesions lacking CK 20 expression, but those PDAC cases that showed CK 20 expression in the invasive component, were associated with CK 20 expressing PanIN, indicating that the mutation develops early before the invasive process (Matros et al., 2006).

Although CK 20 expression in PETs was not observed in the present study, expression of CK20 has been reported in PETs, for example, in Cai *et al.* it was observed in 33% of PET cases (Cai et al., 2001).

CDX2. CDX2 is a caudal-related homeobox transcription factor. It is expressed in intestinal epithelium. Focal CDX2 expression was also found in less than 10% of NnPD cases (Lin and Prichard, 2011). CDX2 positive expression showed the intestinal differentiation of cells regardless of cell behaviour (non-neoplastic or malignant). The labelling in pancreatic tissues is usually lighter and more variegated than in colorectal carcinoma (Dabbs, 2014).

In the present study, CDX2 protein was detected both in PDAC and NnPD. Lacking a significant difference in number of positive cases and in mean number of positive cells, the results of this study were in accordance with Bayrak *et al.* reporting that 16% of PDAC expressed CDX2 (Bayrak et al., 2012). Several other studies showed slightly less frequent CDX2 expression in PDAC (0 – 5% of cases) and also in NnPD (10% of cases), while elsewhere even more frequent CDX2 expression was identified in PDAC (22% of cases). The results of the present study classifying 12.0% (95% CI = 6.4–21.3) of PDAC cases and 19.4% (95% CI = 12.0–30.0) of NnPD cases as positive were consistent with the reported interval (Chu et al., 2005; Park et al., 2007; Liu et al., 2012).

In the present study, CDX2 expression was not found in either PET or NnPI. Also Liu *et al.* and Saqi *et al.* confirmed the fact that CDX2 was not expressed in NnPI, but Denby *et al.* and Saqi *et al.* found CDX2 expression in PET (Saqi et al., 2005; Denby et al., 2012; Liu et al., 2012).

Although only CK 20 was presented as a prognostic factor by Jamieson *et al.* (Jamieson et al., 2011), in the present study CK 20 and CDX2 did not show an impact on survival. However, both markers were related to lymphogenic spreading, i.e., CK 20 negatively correlated with the number of LN with metastases ($r_s = -0.290$; $p = 0.041$), but CDX2 expression was observed in more cells in tumours presenting invasion into

lymph vessels ($z = -2.477$; $p = 0.013$). However, there was no statistically significant impact according to ROC analysis regarding CDX2 ($p = 0.162$).

Practically, considering that both intestinal markers can show immunoreactivity in PDAC, these findings must be implemented in the diagnostic IHC evaluation of metastatic tissue sample. The present study confirmed that PDAC can be positive by CK 20 and CDX2 thus mimicking colorectal cancer. According to the literature, colorectal cancer can occasionally be positive for CK 7 despite a usual (78% of cases) negative appearance (Bayrak et al., 2012).

CK 5/6. CK 5/6 positive expression is found in cells exhibiting squamous differentiation (Dabbs, 2014). CK 5/6 expression was observed only in PDAC, without any expression in NnPD, PET and NnPI. In Latvian PDAC patients, CK 5/6 expression was observed in 10/ 75 (13.3%; 95% CI = 7.4–22.8) cases, but 5% positive cell cut-off was exceeded only in 5 (6.7%; 95% CI = 2.9–14.7) cases, while Chu *et al.* reported expression in 38% of PDAC (Chu and Weiss, 2002). Although the number of CK 5/6 positive cases was small, CK 5/6 expression showed a statistically significant association between number of positive cells and tumour localisation in pancreas ($\chi^2 = 10.183$; $p = 0.037$), i.e., higher expression in pancreatic head tumours and predominant location (7/ 10) of CK 5/6 positive PDACs in the head of pancreas. Despite the usual ominous prognosis of pancreatic carcinoma possessing adenosquamous differentiation (Simone et al., 2013), there was no evidence of the prognostic value of CK 5/6 expression, possibly attributable to the low number of positive cases.

CK 34 β E12. Among the squamous markers, CK 34 β E12 is a remarkable protein. CK 34 β E12 showed positive expression in both PDAC and NnPD. The number of positive cases and the mean number of positive cells between both target structures lacked statistically significant differences. Compared to p63 expression, the number of CK 34 β E12 positive cases was considerably higher in PDAC, i.e., 96.0% (95% CI = 88.9–98.6) *versus* 24.0% (95% CI = 15.7–34.8), but in NnPD p63 expression was absent. The endocrine pancreatic tissues and corresponding tumours (PET, NnPI) lacked CK 34 β E12 expression.

Higher CK 34 β E12 expression was observed in PDAC with necrosis ($z = -2.642$; $p = 0.008$), a prognostically significant parameter regarding survival (log-rank, $p = 0.001$).

p63. p63 molecule is a member of the p53 family. However, similarly as CK 5/6, it is a marker of squamous differentiation (Dabbs et al., 2014). Evaluating incidence of squamous differentiation in PDAC with p63 protein, in the present study its expression was observed in 28/ 75 (37.3%; 95% CI = 27.3–48.7) cases, but only 18/ 75 (24.0%; 95% CI = 15.8–34.8) cases were characterised by reactivity in more than 10% of cells. Ito *et al.* observed the squamous change in tumour cells significantly more frequently, i.e., in 68.2% of PDAC cases (Ito et al., 2001). To the best of my knowledge, there were no previous published studies evaluating squamous differentiation in PET by p63 protein expression. In the present study, p63 expression was observed in 4/ 14 (28.6%; 95% CI = 11.7–54.7) cases, but it did not reach the cut-off level of 10% positive cells in any case. Both in PDAC, p63 showed a correlation with p21 ($r_s = 0.242$; $p = 0.039$) and in PET p63 showed a trend towards a correlation with p21 ($r_s = 0.521$; $p = 0.056$). At the same time PDAC showing higher p63 expression also displayed higher CD44 expression ($r_s = 0.334$; $p = 0.004$).

In the studied group, no prognostic value of p63 expression was identified in PDAC or PET. There was also no literature regarding this direction. More studies are necessary with more extensive patient groups. The concept of poor prognosis could be presented in Simone *et al.*'s study, where p63 expression was analysed in pancreatic adenosquamous carcinoma. The median OS was 8.2 months with a range of 3–25 months for patients with pancreatic adenosquamous carcinoma (Simone et al., 2013).

Unfortunately, just a few studies were found concerning the evaluation of markers of squamous differentiation in pancreatic tissues, hindering the interpretation of data in the present study. However, the obtained data disclosed the invasiveness of cells expressing squamous markers. These cells have increased ability to invade blood vessels and nerves, for example, increased CK 5/6 expression in PDAC was associated with intraneural invasion ($z = -1.975$; $p = 0.048$), higher CK 34 β E12 expression showed associations with invasion in blood vessels ($z = -2.062$; 0.039), perineural and intraneural invasion ($z = -2.234$; $p = 0.025$ and $z = -2.269$; $p = 0.023$). p63 expression was higher in PETs invading blood vessels ($z = -2.256$; $p = 0.024$). Although all above listed features indicated tumour spread, however, CK 5/6, CK 34 β E12 and p63 expression were not associated with patient survival.

Unequal expression of all three squamous markers (CK 5/6; CK 34 β E12, p63) in PDAC creates a confusing situation, because if the squamous differentiation is recognised in more than 25% of malignant cells, this tumour is classified as

adenosquamous carcinoma, which is a rare and specific type of PDAC (Klöppel et al., 2000; Dabbs, 2014). In the present study in PDACs the proportion of CK 5/6 expressing cells ranged from 2% to 17% with a median value of 7.0 (IQR = 11.0), for CK 34 β E12 from 5% to 99% of cells with a median value of 54.0 (IQR = 52) and for p63 from 1% to 75% of cells with a median value of 21.5 (IQR = 34). This shows how important it is to find the most appropriate markers for pancreatic tissue, including the markers of squamous differentiation. Larger survival studies would be desirable to select the most meaningful marker and cut-off level. Next, this variability proves that IHC is applicable only as a supplementary method along with examination of tissues stained by haematoxylin and eosin. In a broader view, any data interpretation must be made in a morphological and clinical context.

ChrA. Within the scope of neuroendocrine markers, comprising chromogranin, synaptophysin, cytosolic enzyme neuron-specific enolase, and CD56, ChrA possess the highest specificity (Dabbs, 2014). The spectrum of endocrine cells, ranging from the pancreatic islets to the malignant neuroendocrine tumours, showed strong ChrA expression with perivascular accentuation – more marked reactivity in the perivascular zones (Dabbs, 2014). Malignant ductal epithelium was negative by ChrA, but in 30% of PDAC cases the cells with reactivity of neuroendocrine markers were found. It is considered that these cells are non-neoplastic neuroendocrine cells which are closely adherent to the malignant epithelial cells (Dabbs, 2014).

CD56 is a neural cell adhesion molecule. It is expressed in neural structures. Unfortunately, CD56 is also expressed in non-neuroendocrine cells and tumours. Due to insufficient specificity, CD56 is not widely used as a specific marker in pancreatic pathology (Dabbs, 2014).

Regarding both neuroendocrine markers (ChrA and CD56), the frequency of positive cases and positive cell relative count did not significantly differ in PDAC. Results in the present study indicated that both markers can be used in the assessment of neuroendocrine cells in PDAC. It is a practically important finding because if the neuroendocrine cells comprise 30–50% of the malignant cell population, this tumour is classified as mixed ductal-endocrine carcinoma (Klöppel et al., 2000). The expression of both markers in NnPD was different in the present study. The proportion of cases exhibiting ChrA expression in NnPD was statistically significantly lower than CD56 expressing NnPD cases, i.e., 30% (95% CI = 19.9–42.5) *versus* 90% (95% CI = 79.9–95.3) positive cases. There was an important difference in the relative quantity of

positive cells as well. ChrA was expressed in 1%, IQR = 0.0 of cells, but CD56 was 22.5%, IQR = 33.0. Considering Fujisawa *et al.*'s finding that the quantity of CD56 positive luminal cells increased in chronic pancreatitis (Fujisawa *et al.*, 2003), ChrA should be used in order to detect neuroendocrine cells in NnPD. As CD56 expression was increased in reactive ducts, but decreased in invasive PDAC, CD56 could be used as an adjunct in differential diagnostics between both structures (Fujisawa *et al.*, 2003; Naito *et al.*, 2006). Since conflicting results exist regarding the presence of endocrine cells in the invasive areas, the neuroendocrine differentiation should be evaluated in tissue material from the middle of mass lesion.

The prognostic value of neuroendocrine differentiation within PDAC is controversial. Tezel *et al.* and Pour *et al.* described a positive effect on patients' survival with a higher amount of endocrine cells in PDAC, but in Linder *et al.* and the present study such an association was not found (Pour *et al.*, 1993; Tezel *et al.*, 2000; Linder *et al.*, 2006).

In PETs, neuroendocrine markers are the primary diagnostic tools. As both ChrA and CD56 expression were seen in all PET cases and also in all NnPI cases with the same median relative amount of positive cells, both markers can be equally used in PET diagnostic. Similar results were seen in Yachida *et al.* who found preserved CD56 reactivity in 79% of PET cases (Yachida *et al.*, 2012). The early reports classified immunohistochemical investigation as a sufficiently effective auxiliary technology relying on the primary finding that 92% of PETs expressed at least one of three neuroendocrine markers, including ChrA, synaptophysin and neuron specific enolase (Lam and Lo, 1997). Significantly better diagnostic value has been achieved now with sensitivity and specificity values reaching 100% at the diagnostic cut-off (Klöppel *et al.*, 2000).

Vimentin. Vimentin is a major intermediate filament in the mesenchymal cells. The main role is to control cellular motility, directional migration and cell signalling (Eriksson *et al.*, 2009). Malignant pancreatic ductal epithelial cells during epithelial-mesenchymal transition acquire vimentin expression and loose E-cadherin expression; in addition, the tumour gains invasiveness, ability to metastasise, resistance to chemotherapy and generation of cancer stem cells (Krantz *et al.*, 2012).

In this study, the proportion of PDAC cases presenting with EMT (16.2%; 95% CI = 9.5–26.2) was lower than observed by Handra-Luca *et al.*, namely, 45% of cases (Handra-Luca *et al.*, 2011). Although EMT was more frequently associated with

cancer progression, however, in the present study a small amount of vimentin positive cells was also found in NnPD. The median value of vimentin expressing cell proportion was 3.5; IQR = 6.0. It could be explained with another EMT pathogenesis, namely, fibrosis due to chronic inflammation. It has been proven that an increase in fibrotic tissues also increases the amount of myofibroblasts but these myofibroblasts arise from the conversion of epithelial cells through the EMT process (Thiery et al., 2009). Since in pancreatic islets fibrotic processes are not typical even in inflammation, EMT is absent from islets. In PETs, characterised by tumour progression, EMT was observed in 35.7% (95% CI = 16.3–61.2) of PET cases with an average of 37.7% (95% CI = 8.1–67.3) of cells in the present study.

In the present study, a correlation was found between vimentin expression in PDAC and development of distant metastases ($z = -2.538$; $p = 0.011$) with a very strong impact (AUC = 0.915; 95% CI = 0.832–0.999; $p = 0.046$). In PETs, vimentin expression has a trend towards a correlation with tumour size. Previously it was mentioned that in PETs larger tumour sizes were characterised by more frequent development of metastases. In PETs vimentin showed a positive correlation with CK 19 ($r_s = 0.551$; $p = 0.041$), which was also associated with increased invasiveness in PETs. Thus, it can be concluded that particularly aggressive PET is characterised by increased expression of 3 markers, i.e, Ki-67, CK 19 and vimentin.

Similarly to E-cadherin, vimentin is a significant prognostic marker. The prognostic association between vimentin expression and survival was proven in the present study and in several other studies (Javle et al., 2007; Handra-Luca et al., 2011), i.e., PDAC patients lacking vimentin expression in the tumour had a median survival of 14 (95% CI = 10.7–17.3) months, but with positive expression had 4 (95% CI = 1.7–6.3) months in the present study. The difference was statistically highly significant.

EMT possibility in pancreatic adenocarcinomas and endocrine tumours should be considered by performing immunohistochemical diagnostic examination to determine histogenesis of metastatic cancer.

MVD (CD34). In order to objectively assess MVD, the results of this and other studies were recalculated to detect the number of blood vessels per 1 mm^2 (Table 4.2.) instead of very different data per HPF.

Table 4.2.

Comparison of the reported MVD in PDAC and PETs

Nr.	Investigated tissue	Mean MVD in original article	Recalculated MVD per 1.00 mm ²	Reference
1.	PDAC	48.7 vessels/ 0.65mm ²	74.9	Present study
2.	PDAC	58.6 vessels/ 0.785mm ²	74.7	(Ikeda et al., 1999)
3.	PDAC	50.8 vessels/ 0.128mm ²	396.9	(Giannopoulos et al., 2008)
4.	PDAC	101.06 vessels/ 0.949mm ²	106.5	(Esposito et al., 2004)
5.	PDAC	*120.8 vessels/ 0.64mm ²	188.75	(Yamahatsu et al., 2012)
6.	PET	70.4 vessels/ 0.65mm ²	108.3	Present study
7.	PET	311 vessels/ 0.442mm ²	703.6	(Couvelard et al., 2005)
8.	PET	288.3 vessels/ 0.95mm ²	303.5	(Takahashi et al., 2007)

* The specified MVD is the median number of vessels per field

Abbreviations in the table: MVD, microvascular density; PDAC, pancreatic ductal adenocarcinoma; PET, pancreatic endocrine tumour.

In MVD analysis, it was discovered that stroma of PDAC was statistically significantly less vascular than normal pancreatic parenchyma, i.e., median number of vessels was 44.5 (IQR = 30) *versus* 81.0 (IQR = 29) vessels in the present study. In contrast, vascular density was not statistically significant different between PET and NnPI, respectively, 75.5 (IQR = 43) *versus* 81.0 (IQR = 45) vessels. Several authors reported that MVD lacks prognostic significance. However, rare studies indicated the importance of vascularisation regarding survival in both PDAC and PET cases (Ikeda et al., 1999; Esposito et al., 2004; Couvelard et al., 2005; Takahashi et al., 2007; Yamahatsu et al., 2012; Zorgetto et al., 2013). In the present study, the cut-off level of MVD was found (55 blood vessels per field, at magnification 400x, area 0.65 mm²/field) in which patients' survival was different. It is possible that vascular density could be a useful prognostic factor under strict condition that the MVD assessment is carried out in standardised conditions and the other CD34 positive spindle fibroblast-type cells are excluded from the count. In the immunohistochemical angiogenesis studies, a significant association between tumour hypoxia due to decreased tumour blood flow and reduced progression-free survival has been reported (Haldorsen et al., 2014). In PETs, the tumour blood flow reflects microvascular proliferation (d'Assignies et al., 2009).

COX-2. Cyclooxygenase 2 is the rate-limiting enzyme in the production of prostaglandins from arachidonic acid (Dabbs, 2014). Among gastrointestinal tumours, COX-2 expression was observed in gastric, colorectal and pancreatic carcinomas

(Dabbs, 2014). Several studies confirmed that tumours with increased COX-2 expression have poor clinical outcomes, whereas if the patients undergo long-term non-steroidal anti-inflammatory drugs therapy, the risk of cancer mortality was reduced (Crosby and DuBois, 2003).

Wide variability of COX-2 expression has been described in PDAC. The reported fraction of positive cases varied from 21.5 % of PDAC cases with the cut-off level of 5%, up to 80.5% of cases with a cut-off value of 20% (Juuti et al., 2006; Matsubayashi et al., 2007; Hermanova et al., 2010; Lozano-Leon et al., 2011). The COX-2 expression frequency in the studied Latvian patients was evaluated as an average with positivity in 44.6% (95% CI = 33.8–55.9) of PDAC cases. In PETs, positive COX-2 expression exceeding the cut-off level was less frequent, i.e., 1/ 14 (7.1%; 95% CI = 1.3–31.5) case, but generally the expression of COX-2 in the present study was observed in 5/ 14 (35.7%; 95% CI = 16.3–61.2) cases. Regarding PETs, the published data also varied greatly, describing expression in 42.9–100% of PET cases.

Since COX-2 in oncogenesis affects the cell proliferation and angiogenesis, several authors have suggested that PDAC patients presenting COX-2 expression have shorter survival. In contrast, knowing that COX-2 inhibitors inhibit proliferation and induce apoptosis in pancreatic cell culture, there is a possibility to include such medications in the treatment of PC (Masferrer et al., 2000; Stratton and Albert, 2002; Matsubayashi et al., 2007). Considering that in the present study in the PDAC tissues COX-2 positive expression was observed on average only in 23.8% (95% CI = 17.7–29.9) of tumour cells and in PET cases even less frequently (6.2%; 95% CI = 0.0–13.0 cells), COX-2 inhibitors would be useful only as part of complex therapy. Bergmann *et al.* emphasised that COX-2 could be useful as an additional target of chemotherapy in PET treatment (Bergmann et al., 2009).

In the present study, COX-2 positively correlated with CK 5/6 expression ($r_s = 0.238$; $p = 0.041$) in PDAC. Within this combined CK, CK 6 was characterised by expression in proliferative squamous epithelium (Moll et al., 1982). In the present study, the PDAC cases possessing increased COX-2 expression also more frequently developed LN metastases ($z = -2.073$; $p = 0.038$). The pN1 parameter was more commonly observed in the cases with increased count of CD44 positive cells ($z = -2.577$; $p = 0.010$) and CD44 showed a positive correlation with COX-2 positive cell count ($r_s = 0.249$; $p = 0.035$). This fact was highlighted by the finding that PET cases with COX-2 expression were characterised by more frequent tumour invasion in

lymph vessels ($z = -2.024$; $p = 0.043$). The inverse situation was observed by Juuti *et al.* They discovered that COX-2 expression was absent in PDAC possessing distant metastases (Juuti *et al.*, 2006). In contrast, in the present study all patients with distant metastasis of PDAC showed COX-2 expression in tumour cells. Although the present study did not prove direct prognostic value for PDAC and PET, it still showed a significant negative impact of COX-2 on tumour behaviour. Lozano-Leon *et al.* agreed with COX-2 expression having an impact on carcinogenesis due to inhibition of apoptosis and increasing the invasiveness of cancer cells (Lozano-Leon *et al.*, 2011).

BRCA1. BRCA1 protein has many functions in nuclear processes such as transcription, chromatin remodelling and silencing. It takes part in DNA repair (Starita and Parvin, 2003). The cytoplasmic translocation of BRCA1 is closely related in the regulation of cell death after DNA damage by activation of apoptosis (Wang *et al.*, 2010; Wang *et al.*, 2013). Mutations of *BRCA1* gene are characterised by increased relative risk (2.26) of PC (Thompson and Easton, 2002; Beger *et al.*, 2004)

By IHC, positive expression of BRCA1 protein was invariably observed in normal exocrine pancreas (at cut-off of 50%) and in all cases of chronic pancreatitis. However, the relative number of positive cells in inflamed tissues was statistically lower than in normal pancreas. Even fewer positive cells or completely lost expression was observed in pancreatic adenocarcinoma. PDAC with decreased (less than 50% of cells) expression of BRCA1 protein was characterised by shorter survival compared with the group with maintained protein expression: 1 year survival was 40.0% versus 76.0% respectively (Beger *et al.*, 2004). The data are promising. However, this is the single study devoted to IHC assessment of BRCA 1 protein expression in pancreas.

Several authors, who evaluated BRCA1 expression in different tumours, used a cut-off value of BRCA1 expression at the 10% level (Gachechiladze *et al.*, 2013; Garg *et al.*, 2013; Markowska *et al.*, 2013) which was also selected for the present study. Interestingly, Chen *et al.* showed that BRCA1 nuclear expression was observed in normal cell nuclei, but the expression became aberrantly localised in cytoplasm of ovarian and breast tumour cells (Chen *et al.*, 1995).

In the present study, only few NnPD cases exhibited nuclear expression of BRCA1 protein. The nuclear expression in PDAC and PET was negative. In contrast, Beger *et al.* described nuclear expression of BRCA1 in half of pancreatic adenocarcinoma cases at cut-off value of 50% (Beger *et al.*, 2004). In the present study, BRCA1 expression was observed more frequently in cell cytoplasm than in nuclei but

without a statistically significant difference. It was detected in all target cells (PDAC, NnPD, PET, NnPI), but the number of positive cases varied from 1 to 4 cases, without a statistically significant difference among target structures. The highest median relative cell count was observed in NnPI, i.e., 56.0% (IQR = 23.0). Despite the infrequent occurrence, BRCA1 expression was associated with the presence of distant metastases ($z = -2.275$; $p = 0.023$) in PDAC and with tumour size ($r_s = -0.592$; $p = 0.020$) in PETs. More extensive studies would be necessary to reach any conclusions about the significance of BRCA1 expression and IHC evaluation should be performed in parallel with other methods to detect *BRCA1* mutation.

5. CONCLUSIONS

1. The median survival of the studied patients after potentially radical surgical treatment of PDAC was 11.0 months. The survival data, including perioperative mortality, corresponded with worldwide experience. The demographic and surgical background conformed to global findings and practice. However, the gender composition can be influenced by the population age and gender structure.
2. PETs resulted in statistically and biologically significantly more favourable prognosis; by the end of the follow-up 14.3% of patients had died.
3. In PDAC, the median survival showed a significant correlation with tumour grade, invasion in large blood vessels and tumour necrosis. There was a trend towards survival impact according to LN status, tumour stage, RM status and perineural cancer invasion.
4. Regarding the molecular profile, survival was significantly lower in PDAC patients exhibiting vimentin expression, presence of CD44 or loss of p27. A trend towards a shorter survival was identified in association with low angiogenesis.
5. The clinical and morphological characteristics of PDAC in the local population disclosed a high rate of hazardous findings, including marked local spread even in cases amenable to surgical treatment, frequent involvement of extrapancreatic organs and tissues, frequent presence of metastases, high grade and stage. Perineural growth ensured the main threat of high pT and pR1.
6. Regarding PDAC, the constellation of molecular parameters as moderately active proliferation, expression of p53 and CD44, low angiogenesis and frequent EMT limited the possibilities of systemic treatment suggesting that surgical intervention is the most important treatment measure at the present time.
7. In PDAC, cell cycle regulators p21, p27, cyclin D1 as well as CK 19, epithelial-mesenchymal transition by vimentin expression, COX-2 and CD44 were important molecular mechanisms, involved in multiple molecular loops, significantly related to the cardinal features of carcinogenesis including cell proliferation, invasiveness and metastasis and to major clinical characteristics representing tumour size, grade and presence of LN and distant metastasis.
8. Regarding PETs, E-cadherin and CK19 were important molecular targets significantly associated with tumour proliferation, invasivity, EMT by vimentin and

angiogenesis as well as with important clinical parameters including tumour local spread and size, grade and RM status.

9. Subsets of PDAC can show intestinal and squamous differentiation. Such immunophenotypes have diagnostic importance as well as specific associations with tumour course.
10. In PDAC, angiogenesis had an impact on survival and was associated with endocrine differentiation and CD44. In PETs, MVD was associated with invasion in small blood vessels, mitotic count as well as p53, cyclin D1, E-cadherin, CK 19 and p63 levels.

6. PRACTICAL RECOMMENDATIONS

1. Performing morphologic investigation of pancreatic surgical material by the elaborated diagnostic protocol is recommended. Along with appropriate identification of pTNM parameters, evaluation of pancreas in sequential sections by 5 mm interval, careful search for all LN and complete examination of all resection lines in conjunction with gross inking of peripancreatic resection line are the essential requirements.
2. Taking into account the frequent occurrence of positive RM and the relation between R1, perineural invasion and tumour size exceeding 2 cm, and considering the increased number of pN1 cases detected by evaluation of more LN, surgeons must pay attention to wider resection of PC and peripancreatic fatty tissues, if possible. The recommendation is particularly important regarding tumours larger than 2 cm.
3. Being aware of the immunophenotype of PDAC when examining tissues of metastatic carcinoma is recommended. PDAC can show squamous and intestinal differentiation. The immunophenotype of PDAC is characteristic but not specific.
4. If there are clinical or morphological diagnostic difficulties, involving PETs as a differential diagnosis, immunohistochemical investigation for neuroendocrine markers can be recommended. It is expected to be effective due to diffuse expression of these markers in neuroendocrine tumours contrasting with focal expression in PDAC.
5. To estimate the prognosis more exactly in accordance with the requirements of personalised medicine, searching in pancreatic tumours for the following prognostically important morphologic parameters is recommended: PDAC vs. PET, in the case of PDAC – tumour grade and stage with particular emphasis on pN, invasion in large blood vessels, tumour necrosis, pR and perineural invasion, because these factors still maintain their importance in the prognosis of the patient.
6. To estimate the prognosis more exactly in accordance with the requirements of personalised medicine, searching in pancreatic tumours for the following prognostically important immunohistochemical parameters is recommended: neuroendocrine differentiation confirming the diagnosis of PET; vimentin, CD44, p27, MVD in the case of PDAC. In addition, Ki-67, E-cadherin and CK19 can be recommended for analysis in PETs.

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