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PROGNOSTIC FACTORS OF SURGICALLY TREATED PANCREATIC CANCER

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Speciality – Pathology

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LIST OF ABBREVIATIONS

AJCC – American Joint Committee on Cancer

AUC – area under curve

BRCA1 – breast cancer 1 gene

BRCA1 – breast cancer 1 protein

CD – cluster of differentiation

CDK – cyclin dependent kinase

ChrA – chromogranin A

CI – confidence interval

CIA – Confidence Interval Analysis (software)

CK – cytokeratin

COX – cyclooxygenase

DNA – deoxyribonucleic acid

EMT – epithelial-mesenchymal transition

ERK – extracellular-regulated kinase

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)

IHC – immunohistochemistry

IQR – interquartile range

LN – lymph node

M – Mitosis (a phase of the cell cycle)

MAPK – mitogen-activated protein kinase

MVD – microvascular density

NA – not applicable

NnPD – non-neoplastic pancreatic ducts

NnPI – non-neoplastic pancreatic islets

PanIN – pancreatic intraepithelial neoplasia

PC – pancreatic cancer

PDAC – pancreatic ductal adenocarcinoma

PDEC – poorly differentiated endocrine carcinoma

PET – pancreatic endocrine tumours, 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

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

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)

UICC – Union for International Cancer Control

USA – United States of America

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 (39; 124). 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 (112). Endocrine tumours have also attracted marked attention due to the growing incidence (103).

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 (102). 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-fluorourocil, cisplatin) is low because the tumour cells are resistant to this treatment (6; 54; 91; 136). 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 certain cell-produced compounds. However, published reports are contradictory (19; 50; 64; 91; 133; 136; 141). Therefore, to enhance and improve PC treatment options it is necessary to explore cancer-specific biological mechanisms (91). 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 (115) 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 (98), tumour suppressor p53 (89), DNA repair protein – breast cancer 1 protein (BRCA1) (2; 8), cell cycle regulators p21 (89), p27 (72) and cyclin D1 (45), anti-apoptotic protein Bcl-2 (137), cell

adhesion protein E-cadherin (53) and cancer stem cell marker CD44 (55; 100; 144). 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 (31; 48; 65; 96; 112; 119; 142). Neuroendocrine differentiation deserves detailed practical evaluation as well as in-depth studies of the relations with other morphological and immunohistochemical features (103; 130). Epithelial-mesenchymal transition by mesenchymal marker vimentin expression (49), cyclooxygenase 2 (COX-2) activation (67; 89), as well as angiogenesis by CD34 visualisation and microvascular density (MVD) measurements (138) 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 discloses 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. MATERIALS AND METHODS

1.1. 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. This study design was appropriate for profound retrospective evaluation of morphological factors and expression of immunohistochemical markers 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.

1.2. 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.

1.3. 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 1.1.

1.4. Surgical pathology evaluation

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

1.4.1. 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 resection margin, tumour size by the largest diameter, assessment of the tumour colour, tumour consistency by density description and tumour margins (rounded, pushing *versus* infiltrative).

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.

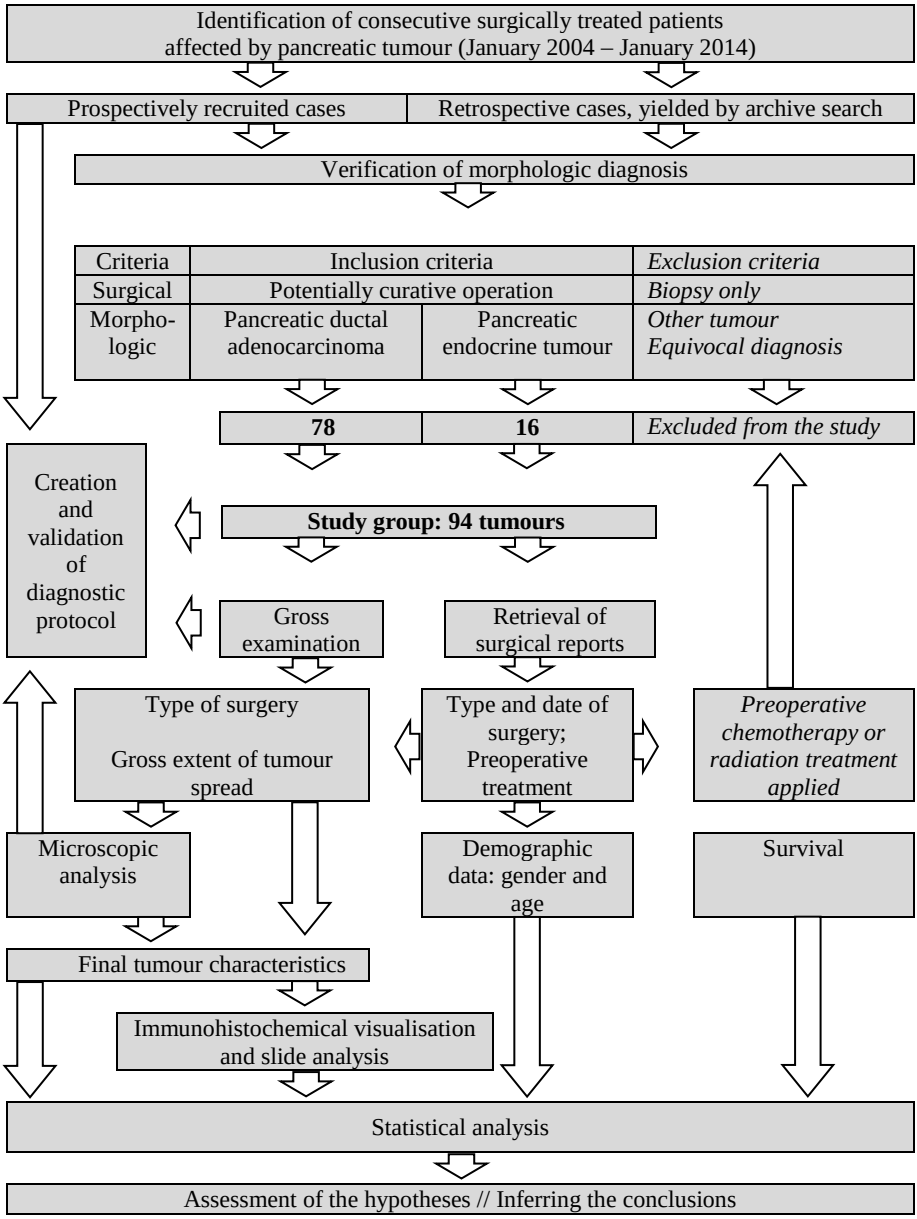

Figure 1.1. The algorithm of data processing

1.4.2. 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 resection margins (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.

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.

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 (52; 75). The pTNM staging was performed in accordance with the Seventh Edition of the AJCC Cancer Staging Handbook (33).

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 1.1. (46).

Table 1.1.

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

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 (52). 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), 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 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.

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 mm^2 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).

1.5. 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 (33; 52; 75; 134).

1.6. 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 containing viable formalin-fixed paraffin-embedded tumour tissue was selected for immunohistochemical visualisation. The block was considered representative if the tissue architecture and atypia corresponded with the predominant tumour grade. Immunohistochemical visualisation data were not collected from sections containing completely necrotic tumour tissue or in the case of tumour disappearance in the particular section. Pancreatic tissues from tumour-free resection lines were used to detect the immunophenotype of non-neoplastic control tissues.

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 1.2. 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.

Expression of immunohistochemical markers was evaluated by light microscopy using high-power magnification 400x (i.e., 40x objective and 10x ocular lenses; 0.65 mm^2 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.

Table 1.2.

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
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

Table 1.2. (continued)

Antigen	Antibody characteristics	Clone	Dilution	Incubation, min.
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	34βE12	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.

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 values were the following: cut-off value of 1% as well as 10% and nuclear expression was used for Ki-67 protein (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) (17; 60; 70; 86; 117). Cut-off value of 1% and cytoplasmic (granular staining pattern) was used for chromogranin A, but cytoplasmic/ membranous for CD56 (14; 130). Cut-off value of 5% and nuclear expression was used for p53, $p21^{WAF1/Cip1}$, $p27^{Kip1}$ proteins and CDX2 (7; 71; 87; 89; 99; 135). Cut-off value of 5% and cytoplasmic expression was used for Bcl-2 oncoprotein, CK 7, CK 19, CK 20, CK 5/6, CK 34βE12 and COX-2 (7; 22; 32; 62; 67; 87; 89). 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 CKs, and

1% for CD56, analogous to chromogranin A. Cut-off value of 10% and nuclear expression was used for cyclin D1, p63 protein and BRCA1 (28; 41; 69; 78; 93; 105). Cut-off value of 10% and cytoplasmic expression was used for vimentin and BRCA1. Cut-off value of 10% and membranous cellular surface expression was used for E-cadherin (92; 121). Cut-off value of 30% and membranous cellular surface expression was used for CD44 (128).

To detect the MVD, endothelial differentiation was highlighted by CD34 expression. 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 mm^2 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 mm^2 was further calculated.

1.7. 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 (20) as well as MVD. The survival analysis was performed separately for PDAC and PET.

1.8. 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 (3).

Before statistical analysis the assumption check of normality using Shapiro-Wilk test was performed. The descriptive data were expressed as mean ± 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 methods such as 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.

2. RESULTS

2.1. Patients and surgical approach

2.1.1. 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 mean age ± standard deviation (SD) was 63.4 ± 11.0 (95% CI = 60.9–65.9) years, the median was 67.0 (IQR = 18.3) years. Women's mean age ± 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 ± SD was 60.5 ± 11.6 (95% CI = 56.6–64.4) years; median age was 59.0 (IQR = 17.5) years.

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

PDAC was localised mostly in the head of the pancreas, respectively, in 65/ 78 (83.3%; 95% CI = 73.5–90.0) cases. Other localisations were in the head and body of pancreas, just in the body, body and tail or in tail of pancreas.

2.1.2. 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 mean ± SD age was 59.4 ± 9.2 (95% CI = 54.5–64.3) years and the median was 56.5 (IQR = 16) years. Women's mean age ± SD was 59.7 ± 10.2 (95% CI = 53.2–66.2), median was 56.5 (IQR = 18) years. Men's mean age ± SD was 58.5 ± 6.0 (95% CI = 49.0–68.0), median was 57.0 (IQR = 11) years.

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. Distant metastases were found and simultaneously removed in 2/ 16 (12.5%; 95% CI = 3.5–36.0) cases.

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.

2.2. Morphology

2.2.1. Characteristics of PDAC cases

The data on the largest tumour diameter were available for 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 ± 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). Two cases lacked information about the tumour size as well as tumour invasion in surrounding extrapancreatic tissues, so the pT parameter in these cases was classified as pTx.

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 ± SD was 19.4 ± 15.1 (95% CI = 16.0–22.8) and the median value was 15.0 (IQR = 24.0) of all 77 cases. The amount of LN affected by cancer metastases ranged from 1 to 13 LN, the mean amount ± SD was 3.6 ± 3.2 (95% CI = 2.7–4.5), and 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.

Among the RM, peripancreatic RM has been identified as the area of significantly highest positivity risk, followed by pancreatic RM (Table 2.1.).

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. The complete results of pTNM parameters, tumour grade and status of RM are shown in Table 2.1.

After complete evaluation of pTNM parameters, the tumour stages were as follows: 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.

Table 2.1.

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 *Vater* or sphincter of *Oddi*	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

Table 2.1. (continued)

Variable	Proportion, %	95% CI for proportion
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 (33; 46).

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 ± SD was 3.7 ± 2.5 (95% CI = 3.1–4.3) mitoses and median value was 3.0 (IQR = 3) mitoses.

Intravascular invasion 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 2.1.). The lymph vessels were invaded (Figure 2.2.) in 29/ 78 (37.2%; 95% CI = 27.3–48.3) cases. Tumour necrosis 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 ± SD was 10.4 ± 9.2 (95% CI = 6.2–14.6); the median value was 10.0 (IQR = 15.3).

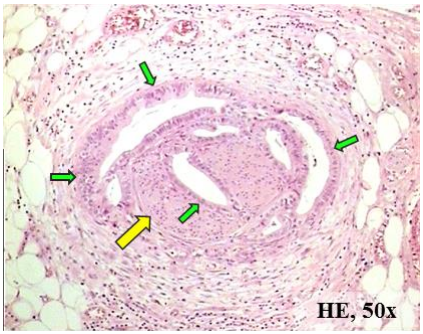

Figure 2.1. Perineural and intraneural invasion of pancreatic ductal adenocarcinoma. Green arrows, pancreatic ductal adenocarcinoma; yellow arrow, nerve. Haematoxylin and eosin (HE), original magnification 50x.

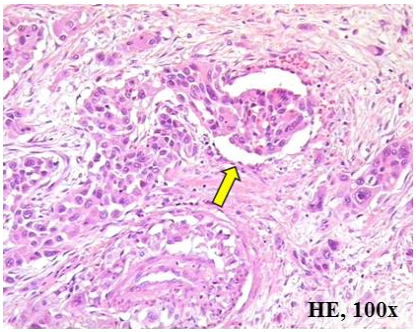

Figure 2.2. Invasion of pancreatic ductal adenocarcinoma in the lymph vessel (arrow). Haematoxylin and eosin (HE), original magnification 100x.

2.2.2. Characteristics of PET cases

The PET size ranged between 1.2 and 12.5 cm. The mean tumour size ± 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 2.2).

The number of assessed LN ranged from 1 to 33. The mean count ± SD was 6.2 ± 10.0 (95% CI = 0.0–13.6) LN, with a median of 1.5 (IQR = 6) LN. The number of LN affected by metastases 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, in 5/ 16 (31.3%; 95% CI = 14.2–55.6) cases the pRx status was issued. The summary of pTNM parameters, tumour grade and status of RM in PETs is represented in Table 2.2.

Table 2.2.

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 *Vater* or sphincter of *Oddi*	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 (33; 52).

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 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 ± 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.

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.

2.3. Immunohistochemical profile of PDAC, PETs, non-neoplastic pancreatic ducts and islets

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 34βE12, p63, E-cadherin, CD44, chromogranin A, CD56, vimentin, COX-2 and BRCA1, were assessed in PDACs, in non-neoplastic pancreatic ducts 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 non-neoplastic pancreatic ducts (NnPD) or normal and neoplastic endocrine cells. In addition, PETs showed higher proliferative activity than non-neoplastic pancreatic islets (NnPI) as evident in Table 2.3.

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. Regarding any level of positive p53 expression, 73.0% (95% CI = 61.9–81.8) was found in 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 2.3.).

The expression of p27 was common in all types of analysed tissues (Table 2.3.). 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 2.3.)

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 2.3. and in Figure 2.3.

Table 2.3.

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

Variable	PDAC	NnPD	PET	NnPI
Ki-67				
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
Mean of Ki-67 expressing cells ± SD (%); 95% CI	23.2 ± 15.0 19.7–26.7	1.8 ± 0.9 1.3–2.3	3.4 ± 3.4 1.5–5.3	0 ± 0 0.0–0.0
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

Table 2.3. (continued)

Variable	PDAC	NnPD	PET	NnPI
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
p53				
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
Mean of p53 expressing cells ± SD (%); 95% CI	46.8 ± 28.6 39.0–54.6	4.4 ± 3.5 1.7–7.1	34.5 ± 26.1 7.0–76.0	4.5 ± 4.9 0.0–48.5
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
p21				
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
Mean of p21 expressing cells ± SD (%); 95% CI	26.2 ± 17.1 22.1–30.3	4.3 ± 3.7 2.6–6.0	8.1 ± 9.5 1.3–14.9	11.2 ± 9.0 0.0–22.4
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
p27				
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
Mean of p27 expressing cells ± SD (%); 95% CI	28.2 ± 17.4 24.2–32.2	42.3 ± 13.5 39.0–45.6	62.6 ± 27.7 46.6–78.6	75.3 ± 17.9 64.5–86.1
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
Cyclin D1				
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
Mean of cyclin D1 expressing cells ± SD (%); 95% CI	19.3 ± 11.7 16.4–22.2	2.6 ± 1.4 1.3–3.9	30.9 ± 32.3 6.1–55.7	20.4 ± 13.9 11.1–29.7
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
Bcl-2				
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

Table 2.3. (continued)

Variable	PDAC	NnPD	PET	NnPI
Mean of Bcl-2 expressing cells ±SD (%); 95% CI	NA	17.5 ± 13.6 6.1–28.9	0 ± 0 0.0–0.0	0 ± 0 0.0–0.0
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

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.

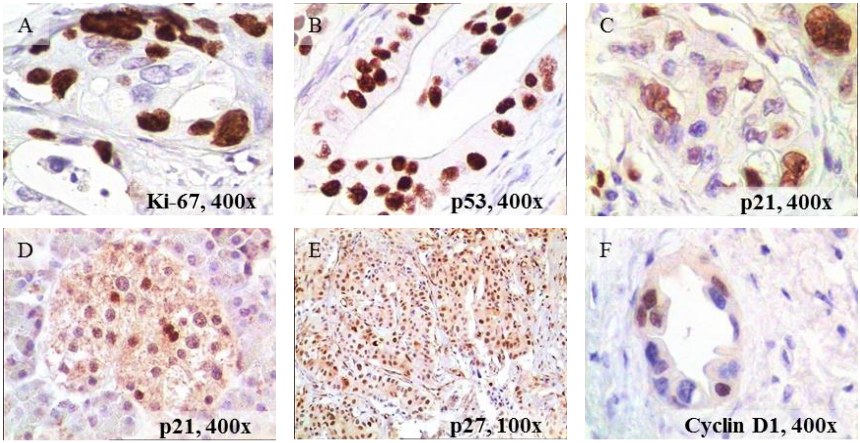

Figure 2.3. Immunohistochemical visualisation of cell cycle markers in pancreatic ductal adenocarcinoma (PDAC), non-neoplastic pancreatic ducts (NnPD), pancreatic endocrine tumour (PET) and non-neoplastic pancreatic islets (NnPI). A, nuclear expression of Ki-67 in PDAC; B, nuclear expression of p53 in PDAC; C, nuclear expression of p21 in PDAC; D, nuclear expression of p21 in NnPI, E, nuclear expression of p27 in PET; F, nuclear expression of cyclin D1 in PDAC. Immunoperoxidase, A, anti-Ki-67; B, anti-p53; C–D, anti-p21; E, anti-p27; F, anti-cyclin D1. Original magnification 100x (E) and 400x (A–D, F).

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 2.4., and findings of the immunohistochemical visualisation are shown in Figure 2.4.

Table 2.4.

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

Variable	PDAC	NnPD	PET	NnPI
E-cadherin				
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
Mean of E-cadherin expressing cells ± SD (%); 95% CI	62.9 ± 30.3 55.9–69.9	69.7 ± 31.7 61.8–77.6	49.6 ± 34.7 28.6–70.6	54.9 ± 27.2 34.0–75.8
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
CD44				
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
Mean of CD44 expressing cells ± SD (%); 95% CI	37.2 ± 23.6 31.3–43.1	39.9 ± 22.2 34.5–45.3	48.8 ± 33.6 20.7–76.9	13.8 ± 10.7 7.0–20.6
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

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.

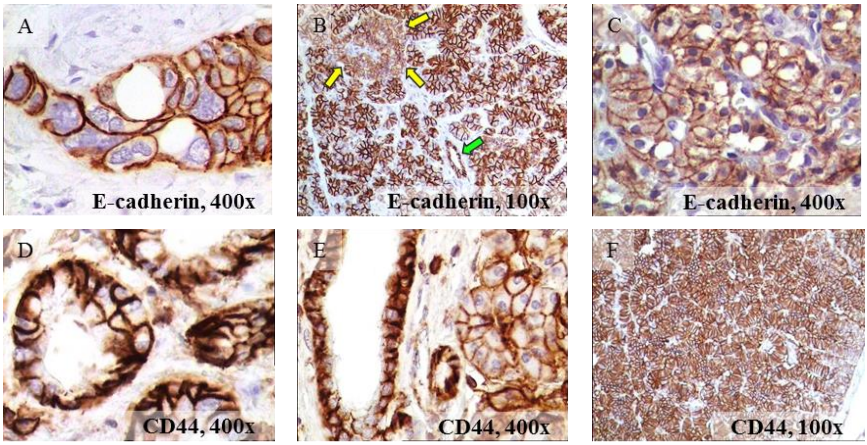

Figure 2.4. Immunohistochemical visualisation of cell adhesion markers in pancreatic ductal adenocarcinoma (PDAC), non-neoplastic pancreatic ducts (NnPD), pancreatic endocrine tumour (PET) and non-neoplastic pancreatic islets (NnPI). A–C, membranous expression of E-cadherin: A, in PDAC; B, in NnPD (green arrow) and in NnPI (yellow arrows); C, in PET; D–F, membranous expression of CD44: D, in PDAC; E, in NnPD; F, in PET. Immunoperoxidase, A–C, anti-E-cadherin; D–F, anti-CD44. Original magnification 100x (B) and 400x (A, C–F).

CK spectrum as a characteristic of tumour histogenesis and differentiation was examined along with CDX2 and marker of squamous differentiation p63 (Table 2.5.). 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 significantly 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 2.5. and in Figure 2.5.

Table 2.5.

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

Variable	PDAC	NnPD	PET	NnPI
CK 7				
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
Mean of CK 7 expressing cells ± SD (%); 95% CI	91.5 ± 14.7 88.1–94.9	98.0 ± 5.0 96.8–99.2	15.5 ± 3.5 0.0–47.0	0 ± 0 0.0–0.0
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
CK 19				
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
Mean of CK 19 expressing cells ± SD (%); 95% CI	86.8 ± 13.5 83.7–89.9	95.7 ± 7.2 94.0–97.4	22.8 ± 34.5 0.0–59.0	0 ± 0 0.0–0.0
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
CK 20				
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
Mean of CK 20 expressing cells ± SD (%); 95% CI	13.7 ± 15.5 7.6–19.8	0 ± 0 0.0–0.0	0 ± 0 0.0–0.0	0 ± 0 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

Table 2.5. (continued)

Variable	PDAC	NnPD	PET	NnPI
CDX2				
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
Mean of CDX2 expressing cells ± SD (%); 95% CI	16.1 ± 7.5 10.3–21.9	18.1 ± 9.6 12.8–23.4	0 ± 0 0.0–0.0	0 ± 0 0.0–0.0
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
CK 5/6				
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
Mean of CK 5/6 expressing cells ± SD (%); 95% CI	8.7 ± 5.8 4.6–12.8	0 ± 0 0.0–0.0	0 ± 0 0.0–0.0	0 ± 0 0.0–0.0
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
CK 34βE12				
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
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
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
p63				
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
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
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

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.

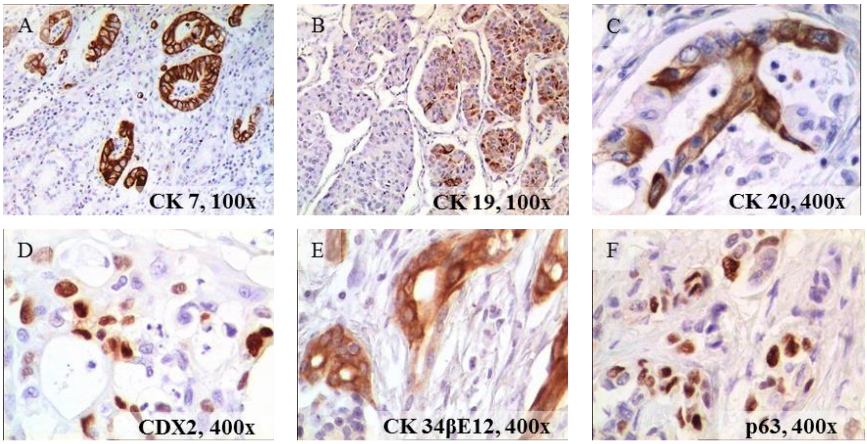

Figure 2.5. Immunohistochemical visualisation of cytokeratin spectrum, CDX2 and squamous differentiation markers in pancreatic ductal adenocarcinoma (PDAC) and in pancreatic endocrine tumour (PET). A, cytoplasmic expression of CK 7 in PDAC; B, cytoplasmic expression of CK 19 in PET; C, cytoplasmic expression of CK 20 in PDAC; D, nuclear expression of CDX2 in PDAC; E, cytoplasmic expression of CK 34βE12 in PDAC; F, nuclear expression of p63 in PDAC. Immunoperoxidase, A, anti-CK 7; B, anti-CK 19; C, anti-CK 20; D, anti-CDX2; E, anti-CK 34βE12; F, anti-p63. Original magnification 100x (A,B) and 400x (C–F).

Chromogranin A 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 chromogranin A 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 chromogranin A expressing cells also showed a significant difference between exocrine and endocrine structures (Table 2.6.). PDAC possessed a significantly higher mean number of endocrine cells 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 chromogranin A in the differential diagnosis of PDAC and PET by WHO criteria, the sensitivity was 100% (95% CI = 79.6–100) and specificity also 100% (95% CI = 94.0–100). The positive and negative predictive values were 100% (95% CI = 79.6–100) and 100% (95% CI = 94.0–100), respectively.

In contrast to chromogranin A, 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) in NnPD but 4.3% (95% CI = 2.1–6.5) in PDAC. In addition, significantly higher number of NnPD cells expressed CD56 than chromogranin A.

The full results characterising neuroendocrine differentiation in pancreatic tissues and neoplasms are represented in Table 2.6. and Figure 2.6.

Table 2.6.

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

Variable	PDAC	NnPD	PET	NnPI
Chromogranin A				
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
Mean of chromogranin A expressing cells ± SD (%); 95% CI	7.6 ± 8.4 4.1–11.1	1.2 ± 0.4 1.0–1.4	97.9 ± 2.2 96.7–99.1	99.4 ± 0.7 99.0–99.8
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
CD56				
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
Mean of CD56 expressing cells ± SD (%); 95% CI	4.3 ± 5.2 2.1–6.5	26.6 ± 17.3 21.9–31.3	97.7 ± 3.2 95.9–99.5	99.1 ± 0.8 98.6–99.6
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

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 2.7. and shown in Figure 2.6.

Table 2.7.

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

Variable	PDAC	NnPD	PET	NnPI
Vimentin				
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
Mean of vimentin expressing cells ± SD (%); 95% CI	33.1 ± 33.1 17.6–48.6	5.2 ± 3.5 2.7–7.7	37.7 ± 28.2 8.1–67.3	0 ± 0 0.0–0.0
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
COX-2				
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
Mean of COX-2 expressing cells ± SD (%); 95% CI	23.8 ± 18.8 17.7–29.9	0 ± 0 0.0–0.0	6.2 ± 5.5 0.0–13.0	0 ± 0 0.0–0.0
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
BRCA1 (nuclear expression)				
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

Table 2.7. (continued)

Variable	PDAC	NnPD	PET	NnPI
Mean of BRCA1 expressing cells ± SD (%); 95% CI	0 ± 0 0.0–0.0	NA	0 ± 0 0.0–0.0	0 ± 0 0.0–0.0
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
BRCA1 (cytoplasmic expression)				
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
Mean of BRCA1 expressing cells ± SD (%); 95% CI	10.8 ± 7.5 0.0–22.7	NA	17.5 ± 19.1 0.0–100	51.5 ± 13.3 30.3–72.7
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

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.

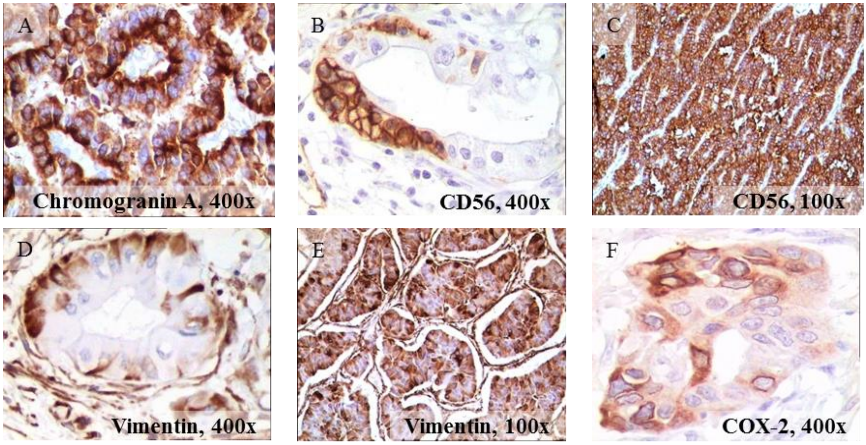

Figure 2.6. Immunohistochemical visualisation of neuroendocrine, mesenchymal, angiogenesis-related markers in pancreatic ductal adenocarcinoma (PDAC), and in pancreatic endocrine tumour (PET). A, cytoplasmic expression (granular staining pattern) of chromogranin A in PET; B and C, membranous and cytoplasmic CD56 expression: B, in PDAC; C, in PET; D and E, cytoplasmic expression of vimentin: D, in PDAC; E, in PET; F, cytoplasmic expression of cyclooxygenase 2 (COX-2) in PDAC. Immunoperoxidase, A, anti-chromogranin A; B–C, anti-CD56; D–E, anti-vimentin; F, anti-COX-2. Original magnification 100x (C, E) and 400x (A, B, D, F).

The MVD was determined following immunohistochemical visualisation of CD34 to highlight the endothelial cells. In PDACs, the MVD (Figure 2.7. and 2.8.) ranged from 11 to 144 vessels per one high power field. The mean MVD ± 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.

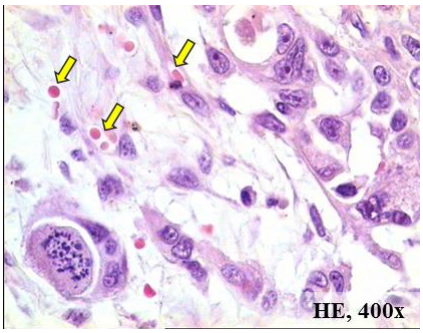

Figure 2.7. The blood vessels (arrows) in pancreatic ductal adenocarcinoma. Haematoxylin and eosin (HE), original magnification 400x.

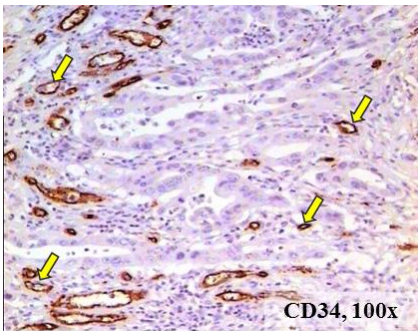

Figure 2.8. Immunohistochemical visualisation of vessels (arrows) in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CD34, original magnification 100x.

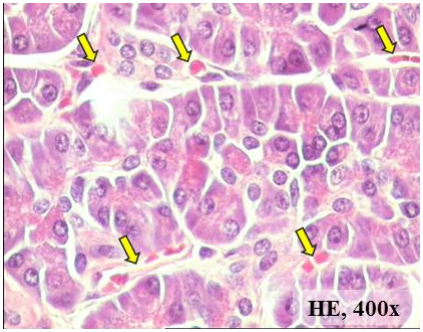

Figure 2.9. The blood vessels (arrows) in normal pancreatic parenchyma. Haematoxylin and eosin (HE), original magnification 400x.

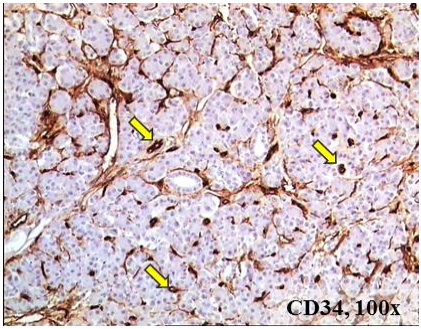

Figure 2.10. Immunohistochemical visualisation of vessels (arrows) in normal pancreatic parenchyma. Immunoperoxidase, anti-CD34, original magnification 100x.

The normal pancreatic parenchyma was characterised by the following MVD (Figures 2.9. and 2.10.): the range was 14–163 vessels, close to the characteristics of PDAC. However, the mean MVD ± 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.

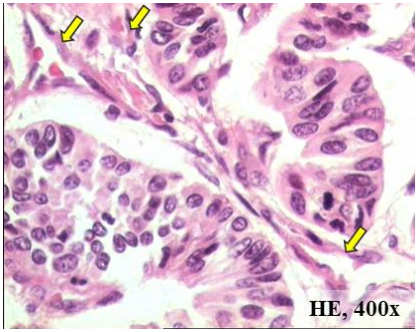

Figure 2.11. The blood vessels (arrows) in pancreatic endocrine tumour. Haematoxylin and eosin (HE), original magnification 400x.

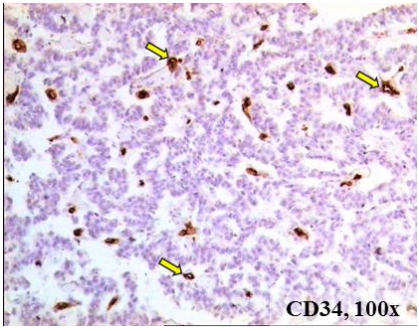

Figure 2.12. Immunohistochemical visualisation of vessels (arrows) in pancreatic endocrine tumour. Immunoperoxidase, anti-CD34, original magnification 100x.

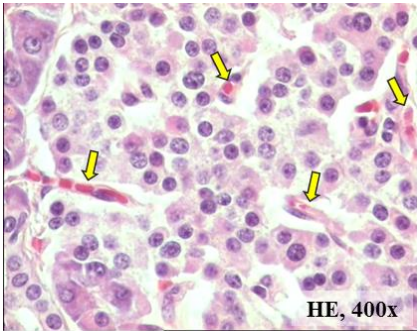

Figure 2.13. The blood vessels (arrows) in non-neoplastic pancreatic islet. Haematoxylin and eosin (HE), original magnification 400x.

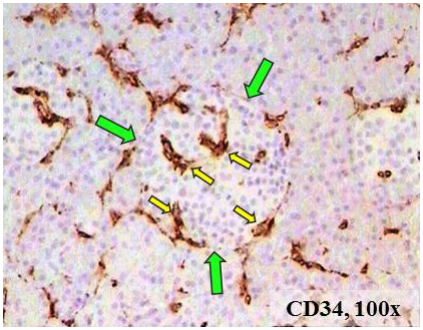

Figure 2.14. Immunohistochemical visualisation of vessels (yellow arrows) in non-neoplastic pancreatic islet (green arrows). Immunoperoxidase, anti-CD34, original magnification 100x.

PETs were characterised by 21–128 vessels per one high power field (Figures 2.11. and 2.12.). The mean MVD ± 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, MVD ranged from 28 to 168 vessels per one high power field. The mean MVD ± SD in NnPI (Figure 2.13. and 2.14.) was 79.5 ± 31.1 (95% CI = 60.7–98.3) and the median was 81.0 (IQR = 45) vessels per one high power field.

2.4. Association between clinical and morphological findings and immunohistochemical variables

2.4.1. 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 2.15. 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.

Tumours greater than 2 cm were characterised by a trend towards more frequent pN1 ($\chi^2 = 3.564$; $p = 0.059$) and a convincing association with more frequent pR1 ($\chi^2 = 9.421$; $p = 0.002$).

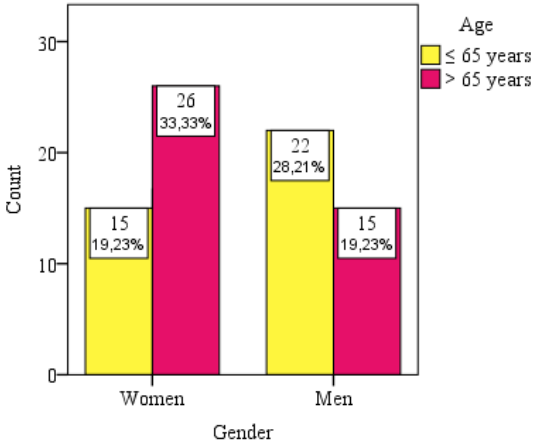

Figure 2.15. The distribution of women and men in age groups ≤ 65 and > 65 years

More pN1 cases were found, when 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 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 ($\chi^2 = 4.919$; $p = 0.027$).

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 was associated 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 a trend with intraneural invasion ($\chi^2 = 3.589$; $p = 0.058$).

A lower degree of tumour differentiation showed an association with invasion in small blood vessels ($\chi^2 = 8.432$; $p = 0.015$), intravascular invasion ($\chi^2 = 10.266$; $p = 0.006$) and perineural invasion ($\chi^2 = 8.021$; $p = 0.018$).

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 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 with number of LN with metastases ($r_s = 0.322$; $p = 0.023$).

Higher p53 expression according to Mann-Whitney U test was identified in lower differentiated tumours (χ^2 = 8.673; p = 0.013). The post-hoc analysis with Bonferroni correction was used to determine differences between grades as presented in Figure 2.16.

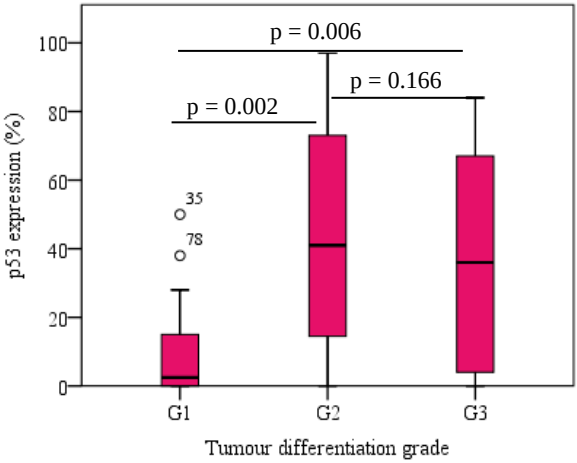

Figure 2.16. The difference of p53 expression (%) by mean ranks between various tumour differentiation grades in pancreatic ductal adenocarcinoma
Abbreviations in the figure: G1, well differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma.

Patients with lower p53 expression had more frequent 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), but significantly higher p53 expression in tumours with perineural invasion (z = −2.021; p = 0.043) according to Mann-Whitney U test with a moderate effect (AUC = 0.707; 95% CI = 0.583–0.830; p = 0.045).

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 2.17.

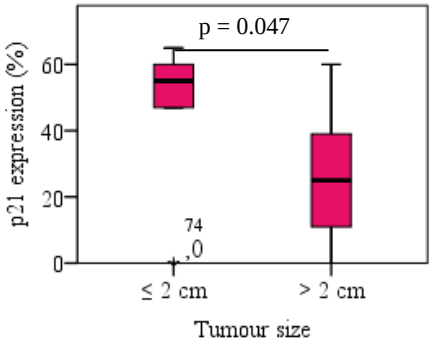

Figure 2.17. The difference of p21 expression (%) by mean rank analysis between patients affected by pancreatic ductal adenocarcinoma ≤ 2cm *versus* > 2cm

Expression of p27 had no correlations with certain clinical and morphological findings.

Patients with lower cyclin D1 had more frequent 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; $p = 0.048$).

The mean ranks of CD44 expression were higher in pN1 patients than in pN0 group ($z = -2.577$; $p = 0.010$) with weak impact (AUC = 0.689; 95% CI = 0.557–0.821; $p = 0.010$) and in tumours with perineural invasion compared with the group lacking perineural invasion ($z = -2.781$; $p = 0.005$). Weak, positive correlations of CD44 were found with tumour grade ($r_s = 0.266$; $p = 0.023$). Regarding mutual immunophenotype analysis, CD44 showed a trend towards a negative correlation with MVD ($r_s = -0.234$; $p = 0.054$) as well as a correlation with Ki-67 ($r_s = 0.281$; $p = 0.016$).

The analysis of CK spectrum comprised evaluation of CK 7, CK 19, CK 20, CK 5/6 and CK 34βE12. Along with the CK spectrum, squamous differentiation was also 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.

Expression of CK 19 did not correlate with any clinical or morphological findings, but CK 19 correlated with CK 34βE12 expression ($r_s = 0.429$; $p < 0.001$), 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.

The CK 34βE12 expression was higher in mean ranks for the following groups: with 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 perineural invasion ($z = -2.234$; $p = 0.025$) with a moderate effect (AUC = 0.731; 95% CI = 0.566–0.895; $p = 0.026$) and with intraneural invasion ($z = -2.269$; $p = 0.023$) with a weak effect (AUC = 0.659; 95% CI = 0.525–0.792; $p = 0.023$).

The difference in mean ranks of CK 5/6 expression was found in tumours with/ without intraneural invasion ($z = -1.975$; $p = 0.048$), with no impact according to ROC curve analysis ($p = 0.265$).

Expression of chromogranin A as well as expression of CD56 (Figure 2.18.) showed a moderate correlation with vascular density with the following values: $r_s = 0.490$; $p = 0.033$ and $r_s = 0.522$; $p = 0.022$. There was also a strong correlation between both endocrine markers ($r_s = 0.985$; $p < 0.001$). Chromogranin A negatively correlated with tumour stage ($r_s = -0.445$; $p = 0.049$). Both chromogranin A and CD56 had a negative correlation with CK 7 ($r_s = -0.507$; $p = 0.022$ and $r_s = -0.483$; $p = 0.031$).

Patients with distant metastasis had convincingly higher mean ranks of vimentin expression (Figure 2.19.) than M0 patients ($z = -2.538$; $p = 0.011$) with a very strong impact (AUC = 0.915; 95% CI = 0.832–0.999; $p = 0.046$).

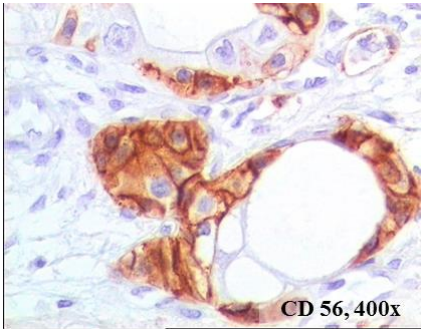

Figure 2.18. Membranous and cytoplasmic expression of CD56 in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-CD56, original magnification 400x.

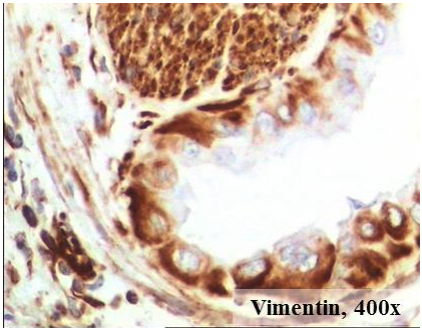

Figure 2.19. Cytoplasmic expression of vimentin in pancreatic ductal adenocarcinoma. Immunoperoxidase, anti-vimentin, original magnification 400x.

COX-2 expression was higher in pN1 patients than in N0 (z = −2.073; p = 0.038) with a weak impact (AUC = 0.644; 95% CI = 0.512–0.776; p = 0.049). All three PDAC which possessed distant metastases also showed COX-2 positive expression with a percentage of positive cells from 11.0–25.0%. A trend towards a difference in mean ranks of COX-2 expression was found according to tumour grade (χ^2 = 5.805; p = 0.055).

2.4.2. Characteristics of PET cases

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 2.20.

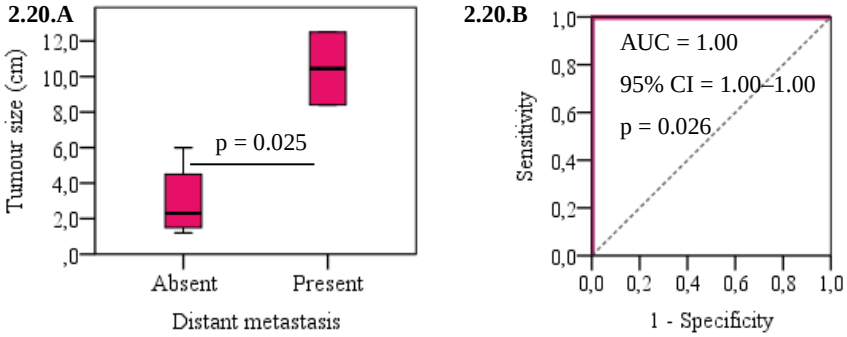

Figure 2.20. The pancreatic endocrine tumour size difference (by mean rank analysis) in accordance with the presence of distant metastasis. 2.20.A, boxplot with p value; 2.20.B, ROC curve analysis

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% CI = 0.55–1.00; p = 0.046). There was an association between tumour size with a 2 cm cut-off (i.e., ≤ 2/ > 2 cm) and presence or absence of tumour invasion in blood vessels (χ^2 = 6.349; p = 0.012).

The pT parameter showed an association with tumour grade (χ^2 = 18.667; p = 0.005) and invasion in blood vessels (χ^2 = 10.667; p = 0.005).

Tumour invasion in blood vessels (absent/ present) showed an association with tumour grade (χ^2 = 9.778; p = 0.021).

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 trended towards a difference between tumours with/ without distant metastases (z = −1.922; p = 0.055). Ki-67 expression showed a moderate correlation 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).

A difference in E-cadherin expression (Figure 2.23.) by mean ranks was observed between tumour pT characteristics (χ^2 = 6.128; p = 0.047) and intravascular invasion (z = −3.016; p = 0.003) with very strong impact (AUC = 0.980; 95% CI = 0.917–1.000; p = 0.003) as shown in Figures 2.21. and 2.22.

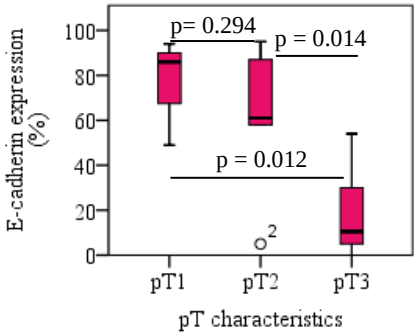

Figure 2.21. The difference of E-cadherin expression (%) by mean ranks between variable pT characteristics in pancreatic endocrine tumour.

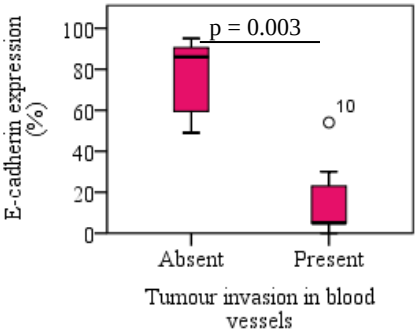

Figure 2.22. The difference of E-cadherin expression (%) by mean ranks between PETs with absent and present tumour invasion in small blood vessels.

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.

A difference regarding E-cadherin expression was also found between groups with/ without tumour perineural invasion ($z = -3.0.16$; $p = 0.003$). 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 ($r_s = 0.701$; $p = 0.005$). Regarding CD44 expression (Figure 2.24.), there was a trend towards a negative correlation with proliferation activity ($r_s = -0.531$; $p = 0.051$), as described above.

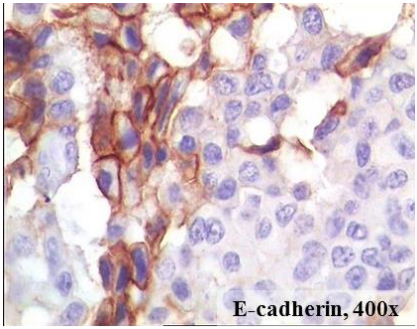

Figure 2.23. Membranous expression of E-cadherin in pancreatic endocrine tumour. Immunoperoxidase, anti-E-cadherin, original magnification 400x.

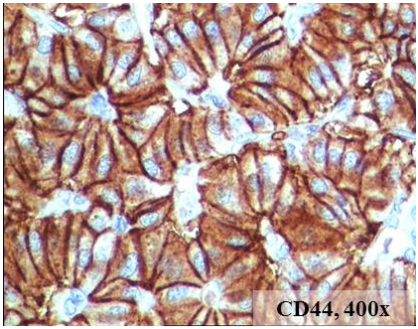

Figure 2.24. Membranous expression of CD44 in pancreatic endocrine tumour. Immunoperoxidase, anti-CD44, original magnification 400x.

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

A statistically significantly different distribution of mean ranks of CK 19 was found for the following groups: tumour pT characteristics ($\chi^2 = 8.068$; $p = 0.018$) with 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. CK 19 expression was higher in tumours invading blood vessels ($z = -2.335$; $p = 0.020$) with a strong effect (AUC = 0.837; 95% CI = 0.604–1.000; $p = 0.035$) and tumours with perineural invasion compared with the group lacking perineural invasion ($z = -2.224$; $p = 0.026$) with a very strong impact (AUC = 0.958; 95% CI = 0.847–1.000;

p = 0.045) and in tumours with size exceeding 2 cm (z = −1.930; p = 0.054) and lower tumour differentiation by grade assessment (χ^2 = 7.803; p = 0.050). 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).

Using Spearman's correlation analysis, expression of CK 19 was correlated with the following findings: 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 Ki-67 (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). Vimentin expression showed a trend towards a correlation with tumour size (r_s = 0.531; p = 0.051) as well as a correlation with proliferation activity (r_s = 0.543; p = 0.045) and CK 19 (r_s = 0.551; p = 0.041).

Cases featuring intravascular invasion had lower mean ranks of MVD (z = −2.113; p = 0.035) with a strong impact (AUC = 0.837; 95% CI = 0.610–1.000; p = 0.035) as shown in Figure 2.25.

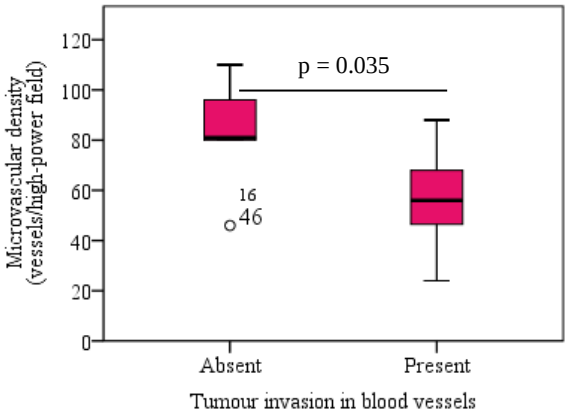

Figure 2.25. The difference in microvascular density by mean ranks among pancreatic endocrine tumours with respect to the invasion in blood vessels

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).

PETs showing either 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) or invasion in lymph vessels (z = −2.024; p = 0.043) but without a significant impact (p = 0.083) had higher COX-2 expression than groups without corresponding invasion.

2.5. Survival

2.5.1. 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 2.26.

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.

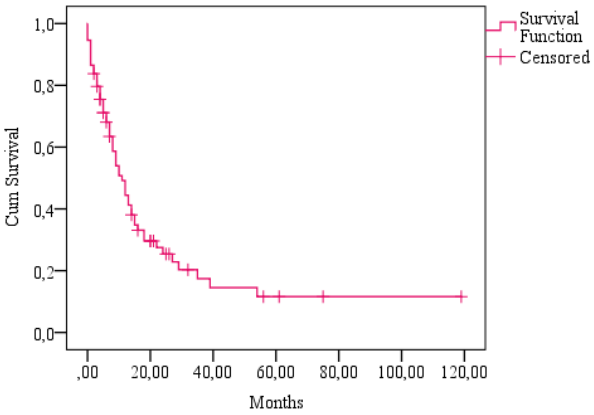

Figure 2.26. Kaplan-Meier survival line of patients affected by pancreatic ductal adenocarcinoma

2.5.2. 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 2.27.

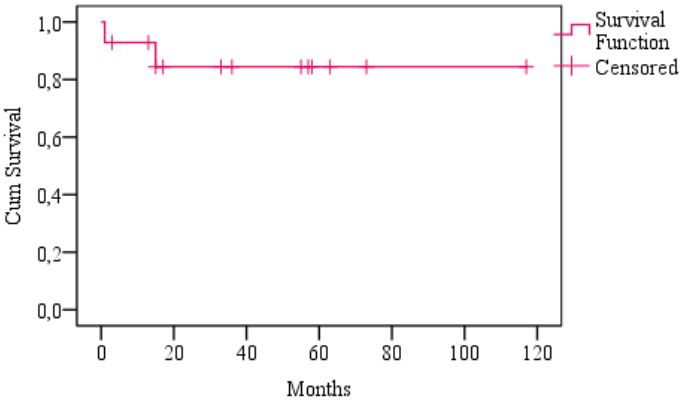

Figure 2.27. Kaplan-Meier survival line of patients affected by pancreatic endocrine tumour

2.6. Associations between survival and clinical and morphological prognostic findings

2.6.1. Prognostic characteristics of PDAC cases

A trend towards a difference in median 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 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 an 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.

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 the median OS was 4.0 (95% CI = 1.4–6.6) months in patients exhibiting tumour necrosis.

Several tests between median OS and clinical or morphological findings showed statistically non-significant differences, but after assessing 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 2.28. and 2.29.

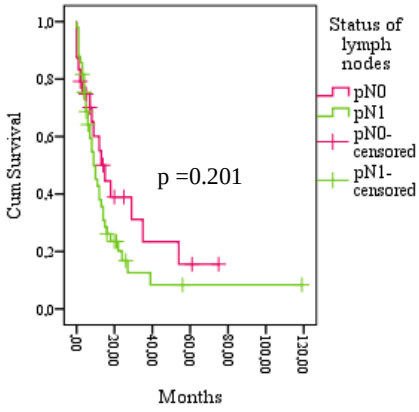

Figure 2.28. Kaplan-Meier survival lines by status of lymph nodes in cases of pancreatic ductal adenocarcinoma. Abbreviations in the figure: pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis is present in at least 1 node.

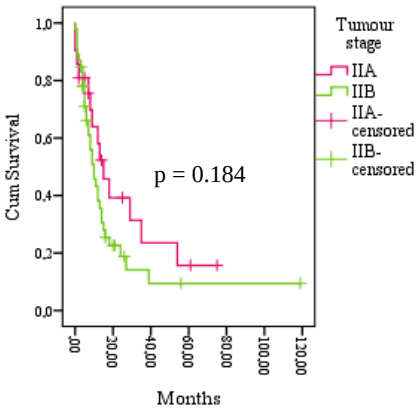

Figure 2.29. Kaplan-Meier survival lines by tumour stages IIA and IIB in cases of pancreatic ductal adenocarcinoma. Abbreviations in the figure: IIA, pT3N0M0; IIB, pT1-3N1M0.

Regarding RM status, the median OS was 14.0 (95% CI = 8.9–19.1) months for patients with a 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 2.30. and 2.31.

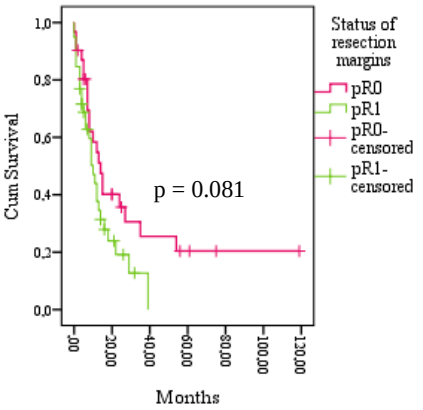

Figure 2.30. Kaplan-Meier survival lines by status of resection margins in cases of pancreatic ductal adenocarcinoma.

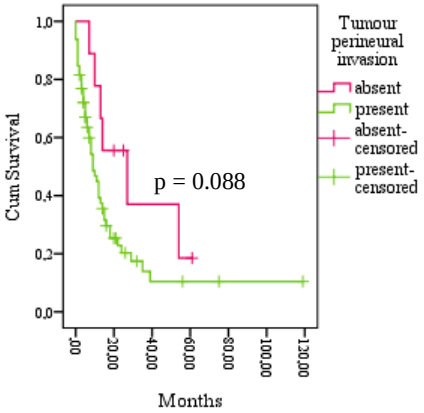

Figure 2.31. Kaplan-Meier survival lines by tumour perineural invasion in cases of pancreatic ductal adenocarcinoma.

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

The parameters with statistically non-significant median survival differences included the following: gender (p = 0.151), patient age ≤ 65/ > 65 years (p = 0.424), type of operation (p = 0.603), tumour localisation (p = 0.075), tumour size ≤ 2/ > 2 cm (p = 0.226), tumour invasion in small blood vessels – present/ absent (p = 0.628), intravascular invasion – present/ absent (p = 0.127), intraneural invasion – present/absent (p = 0.072), invasion in lymph vessels – present/ absent (p = 0.553).

2.6.2. Prognostic characteristics of PET cases

A difference in median OS was not found between clinical and morphological findings: gender (p = 0.284), patient age ≤ 65/ > 65 years (p = 0.431), tumour localisation (p = 0.640), tumour size ≤ 2/ > 2 cm (p = 0.208), pT characteristics (p = 0.216), pN0/ pN1 (p = 0.486), pM0/ pM1 (p = 0.606), tumour stage (p = 0.080), pR0/ pR1 (p = 0.520), tumour grade (p = 0.314), invasion in blood vessels – present/ absent (p = 0.138), perineural

invasion – present/ absent (p = 0.195), invasion in lymph vessels – present/ absent (p = 0.389).

Follow-up time for patients with distant metastases was 13 and 15 months and both patients were alive at the time of renewing survival data.

2.7. Association between survival and immunohistochemical variables

2.7.1. Immunohistochemical prognostic profile of PDAC

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 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 according to 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 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, median OS was 12.0 (95% CI = 7.9–16.1) months in patients with positive p27 expression. However, a statistically significant survival difference was not found between groups with negative or positive expression of Ki-67 by cut-off value of 10% (p = 0.350).

A multivariate analysis using the Cox proportional hazards model to assess the four morphological and immunohistochemical factors which showed an impact on survival in univariate analysis revealed the variable models of tumour invasion in large blood vessels and necrosis as well as positive vimentin and CD44 and negative p27 expression had a higher risk for shorter survival (Table 3.30). The impact for 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.

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 2.8.

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

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 survival difference was found between patients 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 a 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.

A statistically insignificant survival difference was not found ($p \geq 0.05$) regarding negative/ positive expression of other IHC markers: p53, p21, Cyclin D1, E-cadherin, CK 20, CDX2, CK 5/6, p63, Chromogranin A, CD56, COX-2, BRCA1.

2.7.2. Prognostic evaluation of the immunohistochemical profile in PETs

A statistically significant difference in median overall survival was not found ($p \geq 0.05$) between positive/ negative groups regarding any IHC marker expression: p53, p21, Cyclin D1, E-cadherin, CD44, COX-2, BRCA1.

2.8. 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) of 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 non-protocol approach. Less than 12 LN were examined in 52.0% (95% CI = 38.5–65.2) of cases with a median of 10 (IQR = 10) examined LN by free approach versus 7.4% (95% CI = 2.1–23.4) of 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 2.32. and 2.33.)

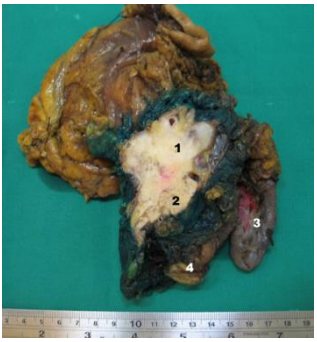

Figure 2.32. Operation material of pancreatoduodenectomy with stained peripancreatic resection margin
1 – PDAC, 2 – pancreatic tissue,
3 – gall bladder, 4 – duodenum

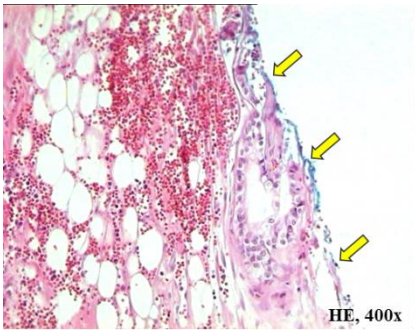

Figure 2.33. Invasion of pancreatic ductal adenocarcinoma in fat tissue and in stained (Alcian-blue) resection margin (arrows). Haematoxylin and eosin (HE), original magnification 400x.

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.

3. DISCUSSION

PC does not rank among the tumours with the highest incidence, but, nevertheless, PC is the fourth leading cause of cancer mortality (39; 124). 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-fluorourocil, cisplatin) is low because the tumour cells are resistant to this treatment (6; 54; 91; 136). 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 certain cell produced compounds. However, contradictory results exist (19; 50; 64; 91; 133; 136; 141). Therefore, to enhance and improve PC treatment options it is necessary to explore cancer-specific biological mechanisms (91). Various methods are used in these types of studies, including immunoblots, *in situ* hybridization or polymerase chain reaction. IHC has also been widely used for these studies (115) 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.

3.1. 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 months which did not differ from other studies that analysed patients from the United Kingdom, Sweden, Canada and China (4; 24; 71). The three-, two- and one-year survival rates in different studies mostly lacked statistically significant differences (4; 83; 101). The 1-month mortality, which is considered to be perioperative mortality, of the target population in this study after 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 by both approaches: including and excluding the perioperative mortality, and reached agreement that there was no difference between results (83).

In comparison to PDAC patients, patients diagnosed with PET have statistically significantly more favourable survival rates. In the present study, during the observation time, 73.0% of PDAC patients had died, but only 14.3% of PET patients. However, the Canadian study reported a mean OS for PET was 225.3 months (101). 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 was two, three or even five times longer than in the studied group of PDAC. Even a ten-year survival rate was presented as 24% (57).

3.2. 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 (4; 71; 49; 83; 95; 140). Only Andrén-Sandberg *et al*. reported that within the younger age group, PDAC was more commonly observed in males (5). Such an association was also found in the present study in the age group ≤ 65 or > 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 for the present study began (27). Within this study, PC was invariably stated as the reason for death, but it would be appropriate to consider also the general processes of aging and other diseases which can affect the patient's overall condition with increasing age.

Age. The median age of PDAC patients was similar between this and several other studies (24; 49). In this study the median age was not significantly different between PDAC and PET patients.

Surgical treatment. No statistically significant survival difference was found between the groups of PDAC patients depending on the type of surgery performed (83). Slight differences were observed in the frequency of selection between operation types. For example, in this study pancreatoduodenectomy was performed in 79.5% 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 (24; 140). In some cohorts, e.g., in the study performed by Yoon *et al*., total pancreatectomy was performed more frequently: 15.9% *versus* 7.7% in the present study (140).

3.3. Morphological findings

Tumour size. The median size of PDAC was similar to other studies (49; 83). Kawesha *et al*. study and the present study indicated that there was no association between survival and tumour size (71). However, a 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 (24; 49; 67; 83; 95; 99).

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 ($p = 0.002$). In PETs, the tumour size was smaller according to median size as well as frequency of tumours exceeding 2 cm in the largest diameter than in PDAC cases. A 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 resulting in an 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 ± SD was 23.2% of cells *versus* 3.4% of cells respectively.

pT parameter. The PDAC cases most often were detected and treated surgically as pT3, which means that the cancer had invaded extrapancreatic tissues (33). pT3 cancers constituted 82% and 94% of cases in Lim *et al*. and Handra-Luca *et al*.'s studies, respectively, but in the present study was 98.7% (95% CI = 92.9–99.8) of cases (49; 83). The peripancreatic adipose tissues represented the highest target for pT3 PDAC invasion (75; 115). In this study tumour invasion in adipose tissues was detected in all pT3 cases. Within this study, PDAC was characterised by statistically less common pT2 and more frequent pT3 parameter findings than PET cases.

The present study has provided more pathogenetically based explanations disclosing significant association between pT and tumour grade ($p = 0.005$). Thus, one reason for increasing pT parameters 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 ($p = 0.005$).

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 (49; 95). In contrast, pN1 finding was statistically significantly less frequent in PET cases (20.0%) than in PDAC cases (67.5%) in the present study in accordance with other observations (10; 20; 51; 57; 73; 107; 113; 129).

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) was high. The mean OS in N1 cases was approximately twice shorter 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% (4; 49; 51; 71; 95; 106; 107; 118). 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.

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 (10; 31; 35; 67; 118; 142). In this study PDAC patient median survival was 12.0 months and 3.0 months without and with tumour invasion in large type blood vessels respectively ($p = 0.013$). Survival rate results indicated that patients' survival decreased dramatically in M1 cases and two-year survival was 0% (67). This conclusion was also confirmed by the present study.

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%) than in Bilimoria *et al.*'s study (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 (12).

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 (113).

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% cases, but it has not been demonstrated as a prognostically important factor (49; 95; 127). Although some studies failed to demonstrate significance of RM condition in survival rate, there were however large scale studies which have disclosed such a correlation (71; 95; 127).

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 ($p < 0.001$). Overall, perineural invasion was one of the most typical features of PDAC. Several studies demonstrated an association or trend for patients with perineural invasion to have shorter survival. There was also a higher probability that the determining factor of survival influence was positive RM not the perineural invasion directly (4; 49; 77; 95; 99; 127). There was also an association between R1 and tumour size exceeding 2 cm ($\chi^2 = 9.421$; $p < 0.002$).

Regarding PETs, both in the present study and literature data, R0 can be found in 68–87.1% of patients (51; 109). In the present study, the rate of R0 (72.7%) exceeded the frequency of R1 (27.3) although the difference was not statistically significant.

Grade of tumour differentiation. One of the parameters showing a relatively close correlation with both morphological and IHC parameters was tumour differentiation grade. The G parameter distribution was similar in this and other published studies. PDAC were mostly moderately differentiated (up to 51.3% according to the literature; 46.2% in the present study), followed by poorly differentiated (up to 44.1% in the literature; 35.9% in the present study) (32; 49; 83).

Poorly differentiated PDAC have 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 (49; 95; 99). In the present study, a trend towards a different survival (log-rank; $p = 0.050$) between patients with varying tumour differentiation grades was also observed.

Nevertheless, the fact that poorly differentiated tumours more frequently invaded blood vessels ($p = 0.006$) and grew perineurally ($p = 0.018$), 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 ($p = 0.021$).

For grade distribution in PETs, according to several authors, well differentiated endocrine carcinomas predominated and comprised 47.2–66% of all PETs, followed by well differentiated endocrine tumours (10; 34; 113; 118).

Necrosis. Tumour necrosis was observed in 26.9% of PDAC cases. There was 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 months, but for patients exhibiting tumour necrosis it was 4.0 months. Thus, significant prognostic value of necrosis have 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.

3.4. 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. Below the most important biomarkers in tumour progression and prognosis are described.

Ki-67. Increase in cell proliferation fraction was a characteristic feature of tumours. Determination of proliferation fraction of the relative amount of Ki-67 positive cells is essential for grading of PET (29). In PDAC increased proliferation fraction was described as a negative prognostic factor (62; 70; 139).

In PDAC this feature was reported in almost all cases and a relative amount of proliferative active cells could be as high as 97% (28; 56). In this study the highest proliferation fraction among PDAC cases was 65.0% and the median value of the relative amount of positive cells was comparatively low, i.e., 18.0% (IQR = 21.0), but in another study it was 29.7% (86). However, in the present study, an association was found between higher Ki-67 expression and higher number of LN metastases in PDAC ($p = 0.023$), perineural invasion

(p = 0.004) indicating increased aggressiveness of malignant cells. Hu *et al*. also found an association of Ki-67 expression with increased N1 cases (56).

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 (60; 108; 118). 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*. (16; 31; 74). Regarding elevated Ki-67 expression in PET, the most convincing correlation was described by more frequent metastatic spread (66; 104; 139), which was also demonstrated in the present study as increasd development of distant metastases (p = 0.055) additionally with a moderately strong association with increased tumour size (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 (p = 0.045) and CK 19 expression (p = 0.033). Both markers were not observed in NnPI cells and were characterised by adverse behaviour (see below) including larger tumour size.

p53. Normal p53 protein is found within cells in small quantities due to very short half-life (29), so in normal pancreatic ducts p53 expression mostly was negative. Very weak reactivity in some cells has been reported (84). Gene *TP53* mutations can result in the 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 proved by anti-p53 antibodies as intensive, diffuse p53 expression.

In the evaluated group, p53 protein expression was observed in 67.6% of PDAC cases in compliance with the report of 60% by Liu *et al*. (87). Some studies showed lower frequency of p53-expressing cases: 41% and 50% (71; 89). Although p53 positive expression is considered to be a sign of malignant change, in pancreatic tissues it starts the expression mostly in high-grade PanINs (PanIN-3 lesion). 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.

In the present study, PDAC with decreased p53 expression was a characteristic feature of more frequent invasion in large blood vessels (p = 0.043). Kawesha *et al*. found that 90% of tumours larger than 5 cm lacked p53 expression (71). 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 between p53 expression and survival.

Among PET cases, contradictory results were observed between the present study and the research work of Yachida *et al*. which 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 (137). It is possible that well differentiated endocrine tumours with uncertain behaviour are malignant in nature but not yet invasive growth, similarly to PanIN-3 lesion in PDAC.

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 (15; 23).

In the present study, an association between decreased expression of p21 and larger PDAC size (exceeding 2 cm) was identified ($p = 0.047$). This could be interpreted as confirmation of the theory advanced by Cazzalini *et al*. and el-Deiry *et al*. stating that cell proliferation increases if p21 is decreased (15; 36). However, a positive correlation between p21 and Ki-67 was shown in PDAC ($p = 0.016$) as well. Thus, p21 positive cases proliferate 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 would also protect tumour cells from chemotherapy. Unfortunately, the expression of p21 showed a correlation with stem cell marker CD44 ($p = 0.024$), known to be a negative prognostic factor associated with treatment resistance. The p21 protein expression correlated also with EMT by vimentin ($p = 0.010$), identified in the present study as a negative prognostic factor regarding overall survival ($p = 0.002$).

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 (76; 122). 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 (21; 76).

In the present study, p27 positive PDAC cases were statistically significantly more frequent (91.9%) than in other studies (41–49% of cases) at equal cut-off value (40; 99). In this study, the mean relative amount of positive cells was significantly lower in PDAC (28.2% of cells) than in NnPD (42.3% of cells), indicating that p27 inhibition is one of the carcinogenetic pathways in PDAC. The p27 could be one of the most important prognostic markers, because many PDAC and PET studies including the present study (log-rank, $p = 0.003$) showed that patients with retained high p27 expression have considerably longer survival than patients with decreased p27 expression (16; 40; 68; 72; 90).

Cyclin D1. As mentioned previously, the transcriptional regulator protein cyclin D1 forms a complex with cyclin dependent kinase. This complex sustains the regulation of G_1 phase to the S phase of the cell cycle (29). In malignant cells, cyclin D1 stability is changed. Thus, the level of cyclin D1 can grow due to affected degradation mechanism, resulting in cell cycle progression (1).

In general, the fraction of cyclin D1 positive PDAC cases was similar in this (64.9%) and other studies (11; 71). From the obtained data, no direct prognostic value of cyclin D1 was found regarding survival in accordance with other researchers (71). Culhaci *et al*. and Lebe *et al*. found that cyclin D1 in PDAC correlated with both tumour size and perineural invasion (28; 80). In the present study, results were contrary to expectations, i.e., that decreased cyclin D1 expression is characteristic in tumours with invasion in the large blood vessels ($p = 0.047$). It could be explained by the fact that tumours which are 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 (70). Thus, decreased expression of cyclin D1 cannot be routinely considered as a favourable sign in tumours. Although cyclin D1 does not show prognostic value, in several studies it was regarded as an important predictive factor, because the investigation of treatment was based on the possibility of cyclin D1 degradation (1). The relevance of cyclin D1 to the endocrine oncogenesis is emphasised by the interaction of cyclin D1 regulatory mechanisms via promoter activity suppression and beta-catenin, an important component of E-cadherin pathway (58). Extracellular-regulated kinase ERK and p38/ mitogen-activated protein kinase MAPK pathways were also involved

both in regulation of cyclin D1 levels (45) and down-regulation of E-cadherin within EMT (44).

Bcl-2. Bcl-2 is an anti-apoptotic protein. Immunohistochemically 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 (29).

There were quite contradictory results about Bcl-2 expression analysis. 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%) positive PDAC case was observed and there was no reactivity in PET cases (32; 111; 125; 137). 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 – was more often observed and has greater relevance (133).

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 (29). 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 (53; 82; 95; 123).

In the present study, 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. Correlations were not found in PDAC which would characterise the described mechanisms 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 ($p = 0.003$), perineural growth ($p = 0.003$), higher pT ($p = 0.011$), grade ($p = 0.007$), and angiogenesis as reflected by MVD ($p = 0.005$).

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 (63; 132).

CD44. CD44 is transmembrane adhesion molecule participating in cell-to-cell as well as cell-to-matrix adhesion and in lymphocyte-homing activity

(29; 114). The uncertainties in CD44 expression are 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 (61). At the same time, CD44 is one of the markers for cancer stem-like cells (55). These cells are self-renewing cells possessing the ability to promote clonogenicity, cell growth and migration as well as metastatic spread, higher resistance to chemotherapy and radiation therapy (54; 81; 88; 128; 144), which indicates a higher importance of surgical treatment. Zhang *et al*. revealed that a correlation exists between stem-like cells and EMT (143).

In the present study, CD44 positive cases comprised 45.2% of all PDAC but according to different authors the frequency of positive CD44 cases as reported as more than half of the PDAC cases (42; 61; 128). CD44 positive PDAC cells were characterised by fundamental changes, as CD44 expression was more frequent in poorly differentiated PDAC ($p = 0.023$), in PDAC possessing spread to LN ($p = 0.010$) and perineural invasion ($p = 0.005$), as also shown in other studies (18; 61). The fundamental role was further supported by the evidence that CD44 was significantly associated with survival in PDAC patients, which was also confirmed by multivariate analysis using 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 months *versus* 8 months.

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 34βE12) and 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 (31; 48; 96; 119; 142).

CK 7 and CK 19. CK 7 and CK 19 are typical markers of pancreatic ductal epithelium (29), which are invariably expressed in normal epithelia with a mean positive cell count of 98.0% and 95.7%, respectively, in the present study. At the same time, CK 7 was negative in normal islet cells in accordance with the published data (29). Both markers retained the expression in malignant ductal epithelium, but appearance of CK 19 expression in PET was characterised by increased malignant potential (84). Schüssler *et al*. described that the expression levels of both CK 7 and CK 19 decreased in the areas of squamous differentiation within PDAC (120). A negative correlation between CK 7 and both endocrine markers (chromogranin A and CD56) indicated that part of CK7 negative cells were endocrine cells which normally do not stain

with CK7. Otherwise, CK 7 and CK 19 have been used as markers of histogenesis for differential diagnostic of metastatic tumours (29).

In pancreatic non-neoplastic islets, CK 19 was observed neither in this study, nor in other studies, but it can appear in PET. In the present study 4/ 14 (28.6%) cases expressed CK 19. Other authors described CK 19 expressed in more than 60% of PETs (13; 48; 87; 120; 142). CK 19 was the only CK that had 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 (142). At present, CK 19 is considered promising but still a controversial prognostic indicator in PETs (65; 112); the present study would support its informativity as CK 19 was associated with aggressive characteristics in PET cases: with high pT ($p = 0.018$), high grade ($p = 0.002$), invasivity as demonstrated by invasion in small blood vessels ($p = 0.020$) along with lymphatic and perineural invasion, proliferative activity by Ki-67 ($p = 0.033$), epithelial-mesenchymal transition by vimentin ($p = 0.041$) and angiogenesis as reflected by MVD ($p = 0.014$). It had a trend towards an association with tumour size with a 2 cm cut-off ($p = 0.054$) and a trend towards associations with squamous differentiation by p63 ($p = 0.059$).

CK 20. Typically it is positive in intestinal epithelium, but less intense and just focal expression of CK 20 can be observed in PDAC (29).

In the present study, CK 20 was absent both from normal pancreatic ducts and islets in agreement with Liu *et al.* (87). In PDAC, the amount of CK 20 positive cases considerably varied. Similar results to the present study (22.7% of PDAC cases) were also reported in other studies – from 15% to 30% of PDAC vases (7; 43; 87). In present study, CK 20 showed a correlation with CDX2 ($p = 0.003$) that is also normally expressed in intestinal epithelium.

CDX2. This is a caudal-related homeobox transcription factor (29). Focal CDX2 expression was also found in less than 10% of NnPD cases (84). 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 (7).

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 developed early before the invasive process (96).

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 (7).

CK 5/6, CK 34βE12 and **p63** positive expression was found in cells exhibiting squamous differentiation (29). All three markers showed positivity in PDAC, but in NnPD just CK 34βE12 was observed. The endocrine pancreatic tissues and corresponding tumours (PET, NnPI) lacked CK 34βE12 expression. 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%) cases, but it did not reach the cut-off level of 10% positive cells in any case.

Although there were many features indicating tumour spread by squamous differentiation in the present study, CK 5/6, CK 34βE12 and p63 expression was not associated with patient survival.

Chromogranin A and **CD56**. Within the scope of neuroendocrine markers, comprising chromogranin, synaptophysin, cytosolic enzyme neuron-specific enolase, and CD56, chromogranin A possessed the highest specificity (29). CD56 is neural cell adhesion molecule. It is expressed in neural structures. Unfortunately, CD56 is also expressed in non-neuroendocrine cells and tumours (29). Malignant ductal epithelium was negative for chromogranin A, but in 30% of PDAC cases the cells with reactivity of neuroendocrine markers were found. These cells are considered non-neoplastic neuroendocrine cells which are closely adherent to the malignant epithelial cells (29).

Regarding both neuroendocrine markers (ChrA and CD56), the frequency of positive cases and positive cell relative count was not significantly different 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 (75). 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 the mass lesion (116). 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 higher amount of endocrine cells in PDAC, but in Linder *et al*. and the present study such an association was not found (85; 110; 131).

In PETs, neuroendocrine markers are the primary diagnostic tools. As both ChrA and CD56 expression was 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*. (29; 137).

Vimentin. Vimentin is a major intermediate filament in the mesenchymal cells. The main role is to control cellular motility, directional migration and cell signalling (37). Malignant pancreatic ductal epithelial cells during EMT acquire vimentin expression and loose E-cadherin expression; in addition, the tumour gains invasiveness, ability to metastasis, resistance to chemotherapy and generation of cancer stem cells (79).

In this study the proportion of PDAC cases presenting EMT (16.2%) was lower than observed by Handra-Luca *et al*., namely, 45% of cases (49). EMT was absent from non-neoplastic islets. In PETs, characterised by tumour progression, EMT was observed in 35.7% of PET cases in an average of 37.7% of cells in the present study.

In the present study, a correlation was found between vimentin expression in PDAC and development of distant metastases ($p = 0.011$) with a very strong impact (AUC = 0.915; 95% CI = 0.832–0.999; $p = 0.046$). In PETs, vimentin expression had a trend towards a correlation with tumour size, which correlated more frequently with development of metastases, respectively. In PETs, vimentin showed a positive correlation with CK 19 ($p = 0.041$), 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.

Vimentin is a significant prognostic marker, as proven in the present study and in several other studies (49; 63), i.e., PDAC patients lacking vimentin expression in the tumour had a median survival of 14 months, but with positive expression had 4 months in the present study. The difference was highly statistically significant.

EMT possibility in pancreatic adenocarcinomas and endocrine tumours should also be considered when performing immunohistochemical diagnostic examination to determine histogenesis of metastatic cancer.

MVD (CD34). 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 the survival in both PDAC and PET cases (25; 38; 59; 129; 138; 145). In the present study, the cut-off level of MVD was found (55 blood vessels per field, at magnification 400x, area 0.65 mm^2/field), in which patients' survival was different. It is possible that vascular density could be a useful prognostic factor under the 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 (47). In PETs, the tumour blood flow reflects microvascular proliferation (30).

COX-2. Cyclooxygenase 2 is the rate-limiting enzyme in the production of prostaglandins from arachidonic acid (29). Among gastrointestinal tumours, COX-2 expression is observed in gastric, colorectal and pancreatic carcinomas (29). 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 is reduced (26).

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 (94; 97; 126). Considering that in the present study in the PDAC tissues COX-2 positive expression was observed on average only in 23.8% of tumour cells and in PET cases even less frequently (6.2%), 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 (9).

In the present study, the PDAC and PET cases possessing increased COX-2 expression also had more frequently lymphogenic invasion and spreading. The inverse situation was observed by Juuti *et al*. They discovered that COX-2 expression was absent in PDAC possessing distant metastases (67). 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 that COX-2 expression had an impact on carcinogenesis due to inhibition of apoptosis and increasing the invasiveness of cancer cells (89).

4. CONCLUSIONS

1. The median survival time 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 died.
3. In PDAC, the median survival showed significant correlations with tumour grade, invasion in large blood vessels and tumour necrosis. There was a trend for 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 pancreatic ductal adenocarcinoma in the local population disclosed high rates 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.

5. PRACTICAL RECOMMENDATIONS

1. Performing the 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 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 prognostic assessment.
6. To estimate the prognosis more exactly in accordance with the requirements of personalised medicine, searching 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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7. PUBLICATIONS

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