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Original paper

Morphological and immunohistochemical profile of pancreatic neuroendocrine neoplasms

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The study represents a comprehensive retrospective morphological and immunohistochemical profiling of pancreatic neuroendocrine neoplasms (PNENs) in order to reveal the associations between morphological and molecular parameters. The local tumour spread (T), presence of metastases in regional lymph nodes (N) and distant organs (M), tumour grade (G) and resection line status (R) by pathology findings (pTNMGR), mitotic activity, perineural, vascular and lymphatic invasion were assessed in 16 surgically resected PNENs. By immunohistochemistry, expression of Ki-67, p53, p27, p21, cyclin D1, Bcl-2, E-cadherin, CD44, vimentin, cyclooxygenase 2 (COX-2), microvascular density, and cytokeratin (CK) spectrum, along with neuroendocrine, intestinal and squamous markers were detected. Descriptive statistics, Chi-square test, Spearman's rank correlation, Mann–Whitney and Kruskal–Wallis methods were applied; $p < 0.05$ was considered significant. Ki-67, CK19, p63, vimentin and COX-2 were significantly up-regulated in PNENs in comparison to benign pancreatic islets. A complex network of morphological and molecular associations was identified. Ki-67 correlated with PNEN size ($p = 0.022$), the World Health Organization 2004 and 2010 classification grades ($p = 0.021$ and $p = 0.002$), stage ($p = 0.028$) and mitotic count ($p = 0.007$) but among molecular markers – with CK19 ($p = 0.033$) and vimentin ($p = 0.045$). CK19 was significantly up-regulated in PNENs, having higher pT ($p = 0.018$), pR ($p = 0.025$), vascular ($p = 0.020$), perineural ($p = 0.026$) and lymphatic invasion ($p = 0.043$). In conclusion, proliferation activity (by Ki-67), E-cadherin, vimentin and CK19 are important molecular characteristics of PNENs due to significant associations with morphological tumour characteristics, pTNMGR and invasive growth.

Key words: pancreatic neuroendocrine neoplasm, immunohistochemistry, cytokeratin 19, E-cadherin, vimentin.

Introduction

Pancreatic neuroendocrine neoplasms (PNENs) represent a rare entity that nevertheless has a high clinical importance due to the growing incidence and distinct biological properties of neuroendocrine tumours [1, 2]. Although PNENs have significantly better prognosis than does the more common pancreatic ductal adenocarcinoma, novel therapeutic strategies are still needed [3], therefore the cornerstones of

PNEN carcinogenesis must be evaluated. Here, we present an integrated molecular and morphological study of PNENs. The proteins that hypothetically can be involved in PNEN development include proliferation markers, notably Ki-67 [4], tumour suppressor p53 [5], cell cycle regulators p21, p27 and cyclin D1 [6-8], anti-apoptotic protein Bcl-2 [5], cell adhesion molecule E-cadherin [9] and cancer stem cell marker CD44 [10, 11]. The cytokeratin (CK) spectrum, in addition to intestinal and squamous markers, is widely used in the assessment of tumour histogenesis; however, cytokeratin-associated cell plasticity or differentiation can confer a pathogenetic role that should be evaluated as well [2, 12, 13]. Epithelial-mesenchymal transition [14], cyclooxygenase 2 (COX-2) induction [3], and angiogenesis [15] also represent reasonable research targets. However, the present data are still controversial. In addition, most of the studies have been devoted to few isolated proteins. We hypothesised that the molecular profile is associated with certain morphological parameters and these correlations can identify target proteins that are significantly involved in the development of PNENs. Thus, the aim of this research is to detect the morphological and immunohistochemical profiles of pancreatic neuroendocrine neoplasms in order to reveal the associations between these parameters.

Material and methods

The study quintessence, ethical principles and case selection

A retrospective morphological and immunohistochemical investigation of PNENs was carried out in accordance with the Declaration of Helsinki and was approved by the Committee of Ethics of Riga Stradins University (E-9(2), issued on October 06, 2011). Consecutive cases were identified using an archive search in a single university hospital (2004-2014). The inclusion criteria comprised a verified unequivocal morphological diagnosis of PNEN in the tissue material submitted after the potentially curative operation. Patients that had a tumour of different histogenesis or of equivocal origin, as well as those that underwent biopsy only or received preoperative chemotherapy or radiotherapy were excluded from the study.

Surgical pathology evaluation

The pathology data were obtained via uniform, protocol-based gross and microscopic examinations of the pancreatic surgery materials. Grossly, tumour localisation and largest diameter were detected, among other findings. Consecutive sections of the whole pancreas were obtained at a distance of 5 mm. The lymph nodes (LN) were entirely submitted for microscopic investigation by anatomic compartments. After specimen inking, the resection margins (RM) were completely submitted for microscopic investigation in accordance with the Leeds Pathology Protocol [16, 17]. In short, proximal and distal transection margins of the stomach and duodenum, respectively, as well as the circumferential (anterior, posterior and superior mesenteric vein groove) RM of the pancreas and transection margins of pancreatic neck and distal bile duct were investigated. The samples were routinely fixed in neutral buffered 10% formalin (Sigma-Aldrich, Saint Louis, USA), processed and stained with haematoxylin and eosin. The slides were examined under light microscopy (Leica DM500, Wetzlar, Germany) to detect histological tumour type and grade [1, 18], the local tumour spread (T), presence of metastases in regional lymph nodes (N) and distant organs (M), summarised into pTNM [19], status of RM [16, 17], mitotic count and vascular, and peri- and intra-neural, as well as lymphatic vessel invasion. To link the study to both the previous and forthcoming publications, the grade was assessed using both the World Health Organization (WHO) clinico-pathological classification of tumour differentiation and behaviour, issued in 2004 (further designated as grade ^{2004}) and the WHO grading, issued in 2010 (further designated as grade ^{2010}), as described in the literature [1, 18]. To detect the mitotic activity, mitoses were counted in 40 consecutive high-power fields (HPFs) within the mitotically most active areas, applying 400× magnification (0.20 mm^2 per field corresponding to 2 mm^2 per 10 HPFs) and recalculating to 10 HPFs/2 mm^2 [1]. The peri- and intra-neural growth, as well as vascular and lymphatic invasion, was evaluated as categorical variables.

Immunohistochemical visualisation and assessment

Immunohistochemical visualisation (IHC) was performed on representative blocks of tumour and non-neoplastic pancreatic tissues. For IHC, 3-micrometre-thick sections were cut using an electronic rotary microtome Microm HM 360 on electrostatic glass slides (Histobond, Marienfeld, Germany). After deparaffinisation, antigen retrieval was performed in a microwave oven (3 × 5 min) using a basic TEG (pH 9.0) buffer, followed by blocking of endogenous peroxidase (Sigma-Aldrich). The sections were incubated with primary antibodies (Table I) at room temperature in the magnetic incubation tray. Bound antibodies were detected by the enzyme-conjugated polymeric visualisation system EnVision, linked with horseradish peroxidase using 3,3'-diaminobenzidine as the chromogen. All IHC reagents were produced by DAKO, Glostrup, Denmark. Positive and negative quality controls were invariably performed and reacted appropriately. The IHC reactivity was clas-

Table I. Characteristics and evaluation of immunohistochemical panel

Antigen	Antibody	Clone	Dilution	Time, min.	Pattern	Cut-off, %
Ki-67 protein	MMAH	MIB-1	1 : 100	60	Nu	None
p53 protein	MMAH	DO-7	1 : 400	60	Nu	5
p21WAF1/Cip1 protein	MMAH	SX118	1 : 25	60	Nu	5
p27Kip1 protein	MMAH	SX53G8	1 : 50	60	Nu	5
Cyclin D1	MRAH	EP12	1 : 500	60	Nu	10
Bcl-2 oncoprotein	MMAH	124	1 : 800	60	Ct	5
E-cadherin	MMAH	NCH-38	1 : 50	60	M	10
CD44	MMAH	DF1485	1 : 50	60	M	30
Cytokeratin 7	MMAH	OV-TL 12/30	1 : 800	60	Ct	5
Cytokeratin 19	MMAH	RCK108	1 : 200	60	Ct	5
Cytokeratin 20	MMAH	Ks 20.8	1 : 200	60	Ct	5
CDX2	MMAH	DAK-CDX2	1 : 50	60	Nu	5
Cytokeratin 5/6	MMAH	D5/16 B4	1 : 100	60	Ct	5
Cytokeratin, HMW	MMAH	34βE12	1 : 400	60	Ct	5
p63 protein	MMAH	DAK-p63	1 : 200	60	Nu	10
Chromogranin A	MMAH	DAK-A3	1 : 1000	60	Gra	20
CD56	MMAH	123C3	1 : 100	60	M	20
Vimentin	MM	V9	1 : 200	60	Ct	10
CD34 class II	MMAH	QBEnd10	1 : 1	30	M	None
COX-2	MMAH	CX-294	1 : 200	60	Ct	5

min – minutes; CD – cluster of differentiation; HMW – high molecular weight; COX-2 – cyclooxygenase 2; MMAH – monoclonal mouse antibody against human antigen; MRAH – monoclonal rabbit antibody against human antigen; MM – monoclonal mouse antibody; Nu – nuclear; Ct – cytoplasmic; M – membranous; Gra – granular cytoplasmic

sified into three intensity levels: low, moderate and high. Only moderate or high intensity expression was considered positive, and the relative amount of positive cells was detected as the percentage within 400 sequential tumour or islet cells. If less than 400 cells were available in the tissue section, the case was excluded from evaluation of the particular antigen expression. For a case to be considered positive, the relative amount of positive cells had to reach the cut-off value [20-28], as shown in Table I. To detect the microvascular density (MVD), the endothelial layer was highlighted by membranous CD34 expression. Four hot spots of the target tissue were identified by whole slide scanning at low power (100×) and assessed on HPF. The MVD was calculated as the mean amount of microvessels per HPF in a hot spot, counting only vessels with a clearly defined lumen or well-defined linear shape.

Statistical analysis

The statistical analysis was performed using the IBM SPSS Statistics Version 20.0 statistical software package (International Business Machines Corp., Armonk, New York, United States of America). The confidence interval was calculated using the Confidence Interval Analysis software [29]. The assumption check of normality was performed using a Shapiro-Wilk test. The descriptive data were expressed as mean ± standard deviation (SD), median with interquartile range (IQR) or relative frequency with 95% confidence interval (CI). Descriptive statistical methods, such as descriptive and cross tabulation with Pearson's χ^2, bivariate correlation as Spearman's rank correlation coefficient, and non-parametric methods, such as Mann–Whitney U test and Kruskal–Wallis one-way analysis of variance by ranks, were used. The *post-hoc* analysis with Bonferroni correction was applied to determine differences between three or more groups. The p-values of < 0.05 were considered statistically significant [30].

Results

Clinical and morphological characteristics

During the study period, surgical treatment for PNENs was performed in 16 patients. The mean ± SD age was 59.4 ±9.2 years (range, 47-78) and

Table II. Clinical and morphological profile of pancreatic neuroendocrine neoplasms

Variable	Count	Proportion, %	95% CI
Gender			
Female	12/16	75.0	50.5-89.8
Male	4/16	25.0	10.2-49.5
Tumour localisation			
Head of the pancreas	4/16	25.0	10.2-49.5
Pancreatic body	4/16	25.0	10.2-49.5
Tail of the pancreas	4/16	25.0	10.2-49.5
Wide involvement of the body and tail	4/16	25.0	10.2-49.5
Surgical treatment			
Pancreatoduodenectomy	2/16	12.5	3.5-36.0
Total pancreatectomy	1/16	6.3	1.1-28.3
Distal pancreatectomy	7/16	43.7	23.1-66.8
Tumour enucleation	6/16	37.5	18.5-61.4
Non-anatomic liver resection for metastases	2/16	12.5	3.5-36.0
Tumour size			
> 2 cm	9/16	56.3	33.2-76.9
≤ 2 cm	7/16	43.7	23.1-66.8
pT characteristics			
pT1	4/16	25.0	10.2-49.5
pT2	6/16	37.5	18.5-61.4
pT3	6 /16	37.5	18.5-61.4
The invaded structure in pT3 cases			
Peripancreatic fat tissue	5/6	83.3	43.7-97.0
Duodenum	2/6	33.3	9.7-70.0
Ampulla of Vater or sphincter of Oddi	1/6	16.7	3.0-56.4
Stomach and spleen	1/6	16.7	3.0-56.4
pN characteristics			
pN0	8/10	80.0	49.0-94.3
pN1	2/10	20.0	5.7-51.0
pM characteristics			
pM0	14/16	87.5	64.0-96.5
pM1	2*/16	12.5	3.5-36.0
Resection margins			
Negative	8/11	72.7	43.4-90.3
Positive	3/11	27.3	9.8-56.6
Location of the invaded resection margin			
Pancreatic transection margin	2/3	66.7	20.8-93.9
Circumferential resection margin	1/3	33.3	6.2-79.2
Transection margin of stomach	1/3	33.3	6.2-79.2
Stage			
IA	2/10	20.0	5.7-51.0
IB	3/10	30.0	10.8-60.3
IIA	2/10	20.0	5.7-51.0
IIB	1/10	10.0	1.8-40.4
IV	2/10	20.0	5.7-51.0
Invasive growth			
Vascular invasion	8/16	50.0	28.0-72.0
Perineural invasion	2/16	12.5	3.5-36.0
Intraneural invasion	0/16	0.0	0.0-22.7
Lymphatic invasion	2/16	12.5	3.5-36.0
Tumour grade (WHO 2004)			
Well differentiated endocrine tumour, benign behaviour	1/16	6.3	1.1-28.3
Well differentiated endocrine tumour, unclear behaviour	9/16	56.3	33.2-76.9
Well differentiated endocrine carcinoma	5/16	31.1	14.2-55.6
Poorly differentiated endocrine carcinoma	1/16	6.3	1.1-28.3
Tumour grade (WHO 2010)			
Neuroendocrine neoplasm, grade 1	8/16	50.0	25.5-74.5
Neuroendocrine neoplasm, grade 2	8/16	50.0	25.5-74.5
Neuroendocrine carcinoma, grade 3	0/16	0.0	0.0-22.7

** Both metastases were located in liver.*
CI – confidence interval; pT1 – tumour limited to the pancreas, 2 cm 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 coeliac axis or the superior mesenteric artery; pN0 – no regional lymph node metastasis; pN1 – metastasis present in at least one regional lymph node; pM0 – distant metastases are absent; pM1 – distant metastases are present by pathology examination; WHO – World Health Organization

the median value was 56.5 years (IQR = 16). The study group was characterised by a female predominance (Table II). The clinical data, as well as the morphological tumour profile, including the local tumour spread (T), presence of metastases in regional lymph nodes (N) and distant organs (M), tumour grade (G) and resection line status (R) by pathology findings (pTNMGR), stage and the frequency of such manifestations of invasive growth as vascular, perineural and lymphatic invasion, are represented in Table II. The mean tumour size ± SD was 3.9 ±3.2 cm (range, 1.2-12.5) and the median value was 2.9 cm (IQR = 4.3). The mean LN count ±SD was 6.2 ±10.0 (range, 1-33) and the median was 1.5

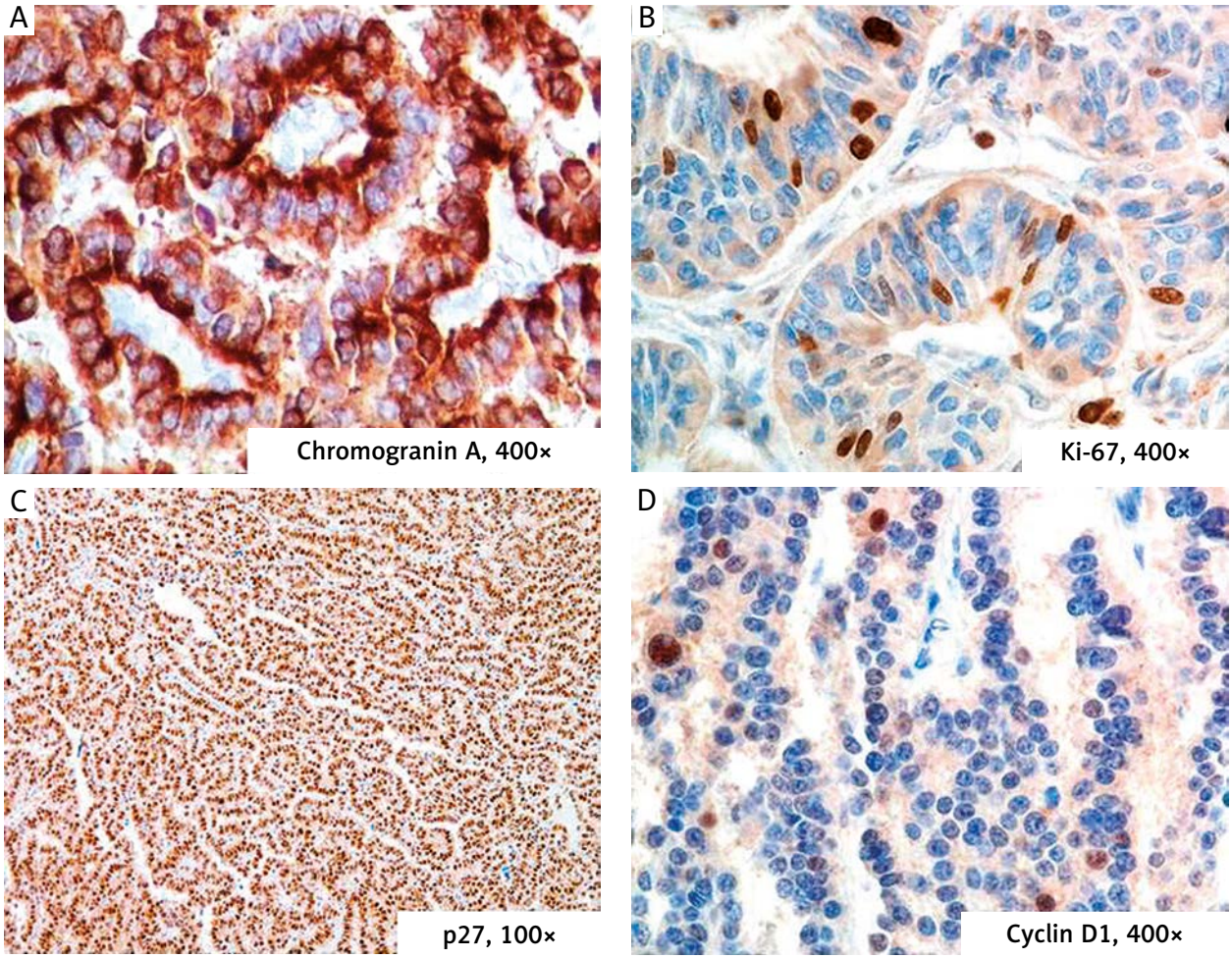

Fig. 1. Diagnostic neuroendocrine features and cell cycle regulation in pancreatic neuroendocrine neoplasms (PNENs). A) Chromogranin A, immunoperoxidase (IP), original magnification 400×. B) Proliferation activity (by Ki-67), IP, original magnification 400×. C) Nuclear expression of p27, IP, original magnification 100×. D) Cyclin D1, IP, original magnification 400×

(IQR = 6) LN. Mitoses were found in 62.5% (95% CI: 38.6–81.5) of the cases, ranging in number from 1 to 13 per 10 HPFs. The mean mitotic count ± SD reached 2.5 ±3.6 (range, 0-13), and the median value was 1.0 (IQR = 3.8) mitoses per 10 HPFs.

Immunohistochemical profile

The expression of immunohistochemical markers was assessed in the PNENs and non-neoplastic islets of the pancreas (Table III). Significant up-regulation of Ki-67, CK19, p63, chromogranin A, vimentin and COX-2 expression was disclosed in the PNENs. The tumours invariably showed proliferative activity, in contrast to the pancreatic islets. Expression of aberrant p53 protein was uncommon. All p53 positive cases were well-differentiated PNENs with unclear behaviour (by WHO 2004 classification), pT1-2, measuring 1.2-3.0 cm. Using the WHO grade [2010], both G1 and G2 tumours were found in the p53 positive group. The levels of p21 and p27 proteins, as well as cyclin D1, did not differ among normal and neoplastic neuroendocrine tissues. Both target tissues lacked any expression of anti-apoptotic protein Bcl-2, intestinal markers CK20 and CDX2, and two squamous markers, CK5/6 and CK34βE12. Epithelial-mesenchymal transition (EMT) by vimentin expression was evident in a significant fraction of PNENs, contrasting with constantly negative islets. COX-2 positive cells also were found only in tumour tissue in low mean number (Table III). In addition, PNENs were characterised by marked IHC heterogeneity (Table IV).

The associations between the studied variables

The statistically significant associations between morphological and molecular variables are shown in Table V. In addition, CK19 was significantly up-regulated in PNENs having higher pT ($p = 0.018$) with significant differences between pT1 and pT3 ($p = 0.022$), as well as between pT2 and pT3 ($p = 0.004$). No associations with clinical, morphological or IHC findings were found regarding p27 expression.

Table III. Immunophenotype of pancreatic neuroendocrine neoplasms and islets

Variable	PNENs	Islets
Ki-67	**p < 0.001***	
Evaluated cases	16	16
F of any level of expression, % [95% CI]	100 [79.6-100]	0 [0.0-20.4]
Range of Ki-67 expressing cells, %	1-12	0
Mean of Ki-67 expressing cells ±SD, %	3.4 ±3.4;	0 ±0
Median of Ki-67 expressing cells, %; IQR	2.0; 3.0	0
p53	**p = 0.285***	
Evaluated cases	15	15
F of any level of expression, % [95% CI]	26.7 [10.9-52.0]	13.3 [3.7-37.9]
Range of p53 expressing cells, %	2-58	1-8
Mean of p53 expressing cells ±SD, %	34.5 ±26.1	4.5 ±4.9
Median of p53 expressing cells, %; IQR	39.0; 49.0	4.5; 3.5
F of positive status, % [95% CI]	20.0 [7.1-45.2]	6.7 [1.2-29.8]
p21	**p = 0.242***	
Evaluated cases	14	13
F of any level of expression, % [95% CI]	71.4 [45.4-88.3]	38.5 [17.7-64.5]
Range of p21 expressing cells, %	4-30	1-23
Mean of p21 expressing cells ±SD, %	8.1 ±9.5	11.2 ±9.0
Median of p21 expressing cells, %; IQR	3.5; 12.5	8.0; 17.0
F of positive status, % [95% CI]	28.6 [11.7-54.7]	30.8 [12.7-57.6]
p27	**p = 0.274***	
Evaluated cases	14	13
F of any level of expression, % [95% CI]	100 [78.5-100]	100 [77.2-100]
Range of p27 expressing cells, %	16-98	38-99
Mean of p27 expressing cells ±SD, %	62.6 ±27.7	75.3 ±17.9
Median of p27 expressing cells, %; IQR	66.5; 49.0	79.0; 23.0
F of positive status, % [95% CI]	100 [78.5-100]	100 [77.2-100]
Cyclin D1	**p = 0.935***	
Evaluated cases	12	13
F of any level of expression, % [95% CI]	75.0 [46.8-91.1]	84.6 [57.8-95.7]
Range of cyclin D1 expressing cells, %	2-90	1-39
Mean of cyclin D1 expressing cells ±SD, %	30.9 ±32.3	20.4 ±13.9
Median of cyclin D1 expressing cells, %; IQR	25.0; 57.0	23.0; 31.0
F of positive status, % [95% CI]	41.7 [19.3-68.1]	61.5 [35.5-82.3]
E-cadherin	**p = 0.278***	
Evaluated cases	14	14
F of any level of expression, % [95% CI]	92.9 [68.5-98.7]	64.3 [38.8-83.7]
Range of E-cadherin expressing cells, %	5-95	8-96
Mean of E-cadherin expressing cells ±SD, %	49.6 ±34.7	54.9 ±27.2
Median of E-cadherin expressing cells, %; IQR	54.0; 76.0	56.0; 42.0
F of positive status, % [95% CI]	71.4 [45.4-88.3]	57.1 [32.6-78.6]
CD44	**p = 0.922***	
Evaluated cases	14	13
F of any level of expression, % [95% CI]	57.1 [32.6-78.6]	92.3 [66.7-98.6]
Range of CD44 expressing cells, %	14-98	2-35
Mean of CD44 expressing cells ±SD, %	48.8 ±33.6	13.8 ±10.7
Median of CD44 expressing cells, %; IQR	43.0; 67.0	10.5; 19.0
F of positive status, % [95% CI]	35.7 [16.3-61.2]	7.7 [1.4-33.3]
CK7	**p = 0.165***	
Evaluated cases	14	13
F of any level of expression, % [95% CI]	14.3 [4.0-39.9]	0 [0.0-22.8]
Range of CK 7 expressing cells, %	13-18	0
Mean of CK 7 expressing cells ±SD, %	15.5 ±3.5; 13-18	0 ±0
Median of CK 7 expressing cells, %; IQR	15.5; 2.5	0
F of positive status, % [95% CI]	14.3 [4.0-39.9]	0.0 [0.0-22.8]
CK19	**p = 0.006***	
Evaluated cases	14	15
F of any level of expression, % [95% CI]	42.9 [21.4-67.4]	0 [0.0-20.4]
Range of CK 19 expressing cells, %	1-92	0
Mean of CK 19 expressing cells ±SD, %	22.8 ±34.5	0 ±0
Median of CK 19 expressing cells, %; IQR	10.0; 33.0	0
F of positive status, % [95% CI]	28.6 [11.7-54.7]	0.0 [0.0-20.4]

Table III. Immunophenotype of pancreatic neuroendocrine neoplasms and islets

Variable	PNENs	Islets
p63	**p = 0.034***	
Evaluated cases	14	14
F of any level of expression, % [95% CI]	28.6 [11.7-54.7]	0.0 [0.0-21.5]
Range of p63 expressing cells, %	2–7	0
Mean of p63 expressing cells ±SD, %	3.3 ±2.5	0 ±0
Median of p63 expressing cells, %; IQR	2.0; 4.0	0
F of positive status, % [95% CI]	0.0 [0.0-21.5]	0.0 [0.0-21.5]
Chromogranin A	**p = 0.042***	
Evaluated cases	16	16
F of any level of expression, % [95% CI]	100 [79.6-100]	100 [79.6-100]
Range of chromogranin A expressing cells, %	93-100	98-100
Mean of chromogranin A expressing cells ±SD, %	97.9 ±2.2	99.4 ±0.7
Median of chromogranin A expressing cells, %; IQR	98.0; 4.0	100; 1.0
F of positive status, % [95% CI]	100 [79.6-100]	100 [79.6-100]
CD56	**p = 0.428***	
Evaluated cases	16	16
F of any level of expression, % [95% CI]	100 [78.5-100]	100 [78.5-100]
Range of CD56 expressing cells, %	89-100	89-100
Mean of CD56 expressing cells ±SD, %	97.7 ±3.2	99.1 ±0.8
Median of CD56 expressing cells, %; IQR	98.5; 3.3	99.0; 2.0
F of positive status, % [95% CI]	100 [78.5-100]	100 [78.5-100]
Vimentin	**p = 0.006***	
Evaluated cases	14	15
F of any level of expression, % [95% CI]	42.9 [21.4-67.4]	0.0 [0.0-20.4]
Range of vimentin expressing cells, %	10–82	0
Mean of vimentin expressing cells ±SD, %	37.7 ±28.2	0 ±0
Median of vimentin expressing cells, %; IQR	28.0; 53.0	0
F of positive status, % [95% CI]	35.7 [16.3-61.2]	0.0 [0.0-20.4]
COX-2	**p = 0.016***	
Evaluated cases	14	13
F of any level of expression, % [95% CI]	35.7 [16.3-61.2]	0 [0.0-22.8]
Range of COX-2 expressing cells, %	3-16	0
Mean of COX-2 expressing cells ±SD, %	6.2 ±5.5	0 ±0
Median of COX-2 expressing cells, %; IQR	4.0; 8.0	0
F of positive status, % [95% CI]	7.1 [1.3-31.5]	0.0 [0.0-22.8]
Microvascular density (MVD)	p = 0.497*	
Evaluated cases	16	16
Range of MVD, vessels per HPF	21-128	28-168
Mean MVD ±SD, vessels per HPF	70.4 ±24.2	79.5 ±31.1
Median MVD, vessels per HPF; IQR	75.5; 43	81.0; 45

**p value for the mean by Mann-Whitney U test*
PNEN – pancreatic neuroendocrine neoplasm; F – frequency; CI – confidence interval; SD – standard deviation; IQR – interquartile range; CD – cluster of differentiation; CK – cytokeratin; COX-2 – cyclooxygenase 2; MVD – microvascular density; HPF – high-power field

Discussion

Pancreatic neuroendocrine neoplasms are infrequent tumours constituting 1.3-5% of pancreatic malignancies [31, 32]. However, they have recently attracted significant attention due to growing incidence, in parallel with other neuroendocrine neoplasms (NENs). As reported by the Surveillance, Epidemiology and End Results (SEER) registry of the National Cancer Institute, the incidence of gastroenteropancreatic NENs has increased from 1 (1973-1977) to 3.65 (2003-2007) per 100,000. In the same time period, the incidence of PNENs increased from 0.17 to 0.43 per 100,000 [33]. PNENs are associated with significantly better outcomes than is pancreatic ductal adenocarcinoma (PDAC). The median survival of surgically treated non-functional PNENs was 60.4 months and the mean survival of PNEN patients reached 225.3 months [34, 35]. In contrast, the median survival of surgically treated PDAC was 22.8 months [36]. The five-year survival of surgically treated PNEN patients is 84.4%, while of PDAC patients, it is 19-24% [37, 38]. However, the clinical course of PNENs is still difficult to predict [39]. Although knowledge about PNEN pathogenesis and biological potential

Table IV. Immunohistochemical heterogeneity in pancreatic neuroendocrine neoplasms

Antigen	Frequency of heterogeneity		Range of positive cells per HPF, %
	Relative amount of cases, %	95% confidence interval, %	
p27	14.3	4.0-39.9	0-77
COX-2	7.1	1.3-31.5	0-37
CK7	14.3	4.0-39.9	0-34
CK19	28.6	11.7-54.7	0-63
E-cadherin	50.0	26.8-73.2	0-100
CD44	28.6	11.7-54.7	0-61
Vimentin	28.6	11.7-54.7	0-76

HPF – high-power field; COX-2 – cyclooxygenase-2; CK – cytokeratin; CD – cluster of differentiation

Table V. Morphological and molecular associations in pancreatic neuroendocrine neoplasms

Independent variable	Dependent variable	p value
Tumour size	pM	0.025
	Vascular invasion	0.045
	E-cadherin	0.052*
	CK19	0.054*
pT	Grade 2004	0.005
	Vascular invasion	0.005
	E-cadherin	0.047
	CK19	0.018
pN	Age	0.037
pM	Size	0.025
	Grade 2004	0.034
	Mitotic count	0.022
	pR	0.011
pR	pM	0.011
	Mitotic count	0.017
	CK19	0.025
	Grade 2004	0.051*
Grade 2004	pT	0.005
	pM	0.034
	Vascular invasion	0.021
	CK19	0.050
	pR	0.051*
Grade 2010	Vascular invasion	0.046
	Ki-67	0.002
Vascular invasion	**Size**	**0.012**
	pT	0.005
	Grade 2004	0.021
	Grade 2010	0.046
	Mitotic count	0.023
	p53	0.038**
	E-cadherin	0.003**
	CK19	0.020
	p63	0.024
	Microvascular density	0.035**
Perineural invasion	**p53**	**0.038****
	E-cadherin	0.003**
	CK19	0.026
	COX-2	0.011
Lymphatic invasion	CK19	0.043
	COX-2	0.043
Mitotic count	pM	0.022
	pR	0.017
	Vascular invasion	0.023

** statistically insignificant trend*
*** negative association*
pT – the local tumour spread; pN – regional lymph node status regarding the presence of metastases; pM – presence or absence of distant metastases {19}; pR – resection line status (R) by pathology findings {16, 17}; grade 2004 – tumour grade by the World Health Organization (WHO) classification issued on 2004 {18}; grade 2010 – tumour grade by the World Health Organization (WHO) classification issued on 2010 {1}; CK – cytokeratin; COX-2 – cyclooxygenase 2

deepened during the preceding years, the molecular mechanisms responsible for tumour progression and metastasis still remain incompletely understood. Molecular markers are increasingly used to predict patient outcome, and immunohistochemistry has been found to be an appropriate, visually controllable, economically effective and fast surrogate method for evaluating the molecular aberrations on the protein level [40]. Recently, the World Health Organization included the Ki-67 level in the PNEN grading criteria, resulting in improved accuracy of the grading system [4]. Deeper understanding of the molecular pathogenesis of PNENs could help to sharpen the grading system.

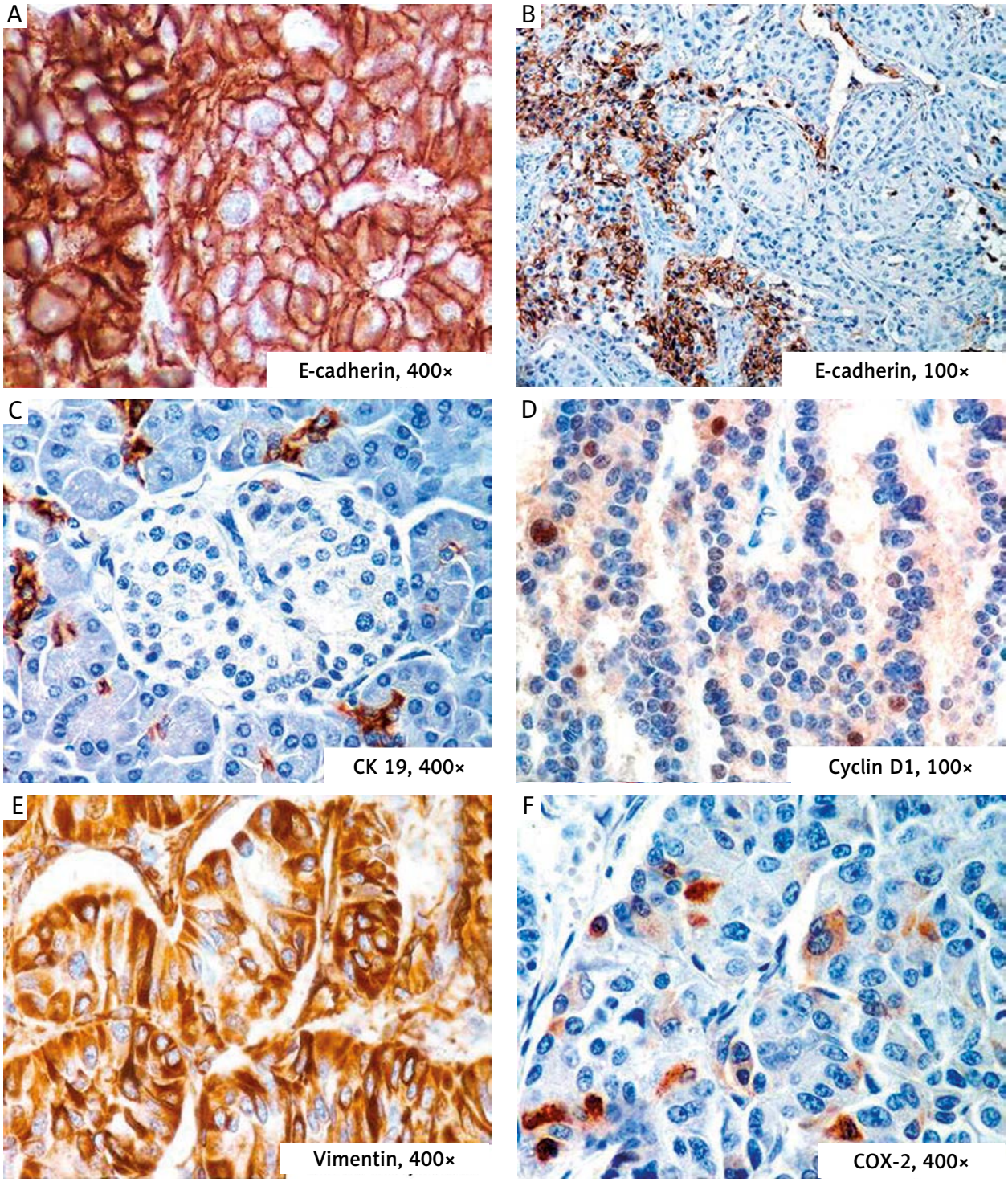

Fig. 2. Cell adhesion, mesenchymal differentiation and COX-2 expression in pancreatic neuroendocrine tumours (PNENs) and islets. A) Intense membranous expression of E-cadherin in an islet, immunoperoxidase (IP), original magnification 400×. B) Loss of E-cadherin in PNEN, IP, original magnification 100×. C) Lack of CK19 in an islet. Note the intense cytoplasmic reactivity in small ducts, IP, original magnification 400×. D) Heterogeneous strong cytoplasmic expression of CK19 in PNEN, IP, original magnification 400×. E) Strong cytoplasmic expression of vimentin in PNEN, IP, original magnification 400×. F) Heterogeneous cytoplasmic expression of COX-2 in PNEN, IP, original magnification 400×

Clinical data

The clinical profile was obtained to characterise the general conformity of the study group to the known PNENs characteristics. The observed mean age of PNEN patients, 59.4 years, was slightly higher than in other studies (48-53 years), possibly due to having an adult group, in contrast with the presence

of adolescent patients in other studies that showed an age range of 14-78 years [34, 41, 42]. Although in this study, women accounted for 75.0% of patients, PNEN incidence is considered equal in both sexes. The observed difference is probably attributable to the relatively small size of the study group [34, 41]. Regarding the tumour location, in this and other similar studies, PNENs were smoothly distributed within the anatomic compartments of the pancreas [41]. The surgical treatment in the present study generally corresponded to the accepted standards [41, 43, 44], providing a reasonable cure to the patient while also yielding representative tissue material.

Tumour size

Within the study, PNEN size ranged from 1.2-12.5 cm, in accordance with the published results [41, 45-47]. Prognostic value has been attributed to the size of PNEN. The five-year survival rate was statistically significantly better (100%) in patients having tumour ≤ 1.5 cm, in contrast to 86%, 71%, 83% and 48% survival rates regarding PNENs sized 1.6-2.0 cm, 2.1-3.0 cm, 3.1-5.0 cm and larger than 5.0 cm. Consequently, observation has even been suggested as the approach for patients having a tumour smaller than 1.5 cm. However, risk of distant metastases increases sharply with a larger PNEN size [48]. This is in agreement with the present study showing an association between tumour size, pM1 ($p = 0.025$), and invasion in the blood vessels ($p = 0.045$), the precursor lesion of distant metastases. In addition, larger PNEN size has been found to correlate with LN metastases [48]. Considering the molecular basis of PNEN growth, the tumour size in the present study showed an association with Ki-67 ($p = 0.022$) and a trend towards association with vimentin expression ($p = 0.051$). PNENs larger than 2 cm showed a trend towards association with E-cadherin ($p = 0.052$) and CK19 ($p = 0.054$) levels. Thus, proliferation, EMT and loss of cell adhesion are among the pathways of increasing tumour mass, paralleling the metastatic spread.

pTNM

The pT frequency distribution between pT1 (25.0%), pT2 (37.5%) and pT3 (37.5%) was even. A similar occurrence of pT3 tumours (32.4%) has been reported [45]. Significant survival differences were previously reported between pT1-2 vs. pT3-4, as well as between T3 and T4 [49]. It could be attributable to the tumour invasion in extrapancreatic tissues and large arteries in the cases of pT3 and pT4. In our study, there was a significant association between pT, tumour grade ^{2004} ($p = 0.005$) and vascular invasion ($p = 0.005$), explaining the association between pT and prognosis through common mechanisms of anaplasia and invasive tumour properties. pN1 was found in 20.0% of PNEN, in accordance with other observations [41, 45-47, 50] and was strongly predicted by older age. Age exceeding 60 years has been shown to be associated with lower survival, even in patients with low-grade (G1) gastroenteropancreatic PNENs [51]. Similarly, non-functioning PNEN patients older than 55 years had a worse survival [39]. Distant metastases were previously reported in 10.8-27.0% of surgically treated PNENs and were found in 12.5% of patients in the present study [12, 49, 52]. The pM1 was associated with higher grade ^{2004} ($p = 0.034$) and increased mitotic count ($p = 0.022$), accentuating cell proliferation.

Resection margins

In order to assess the completeness of surgical treatment, as well as the recurrence risk, surgical RM must be examined carefully. In PNENs, R0 can be reached in 68-87.1% of patients [47, 53]. R0 PNENs still recur in 23.1% of patients, leading to death in 11.1% [46]. However, the five-year survival rate in R0 cases is statistically significantly higher than in the R1: 91.2% vs. 55.0%, respectively [47]. In the present study, pR1 status in PNENs was associated with mitotic count ($p = 0.017$) and had a trend towards the association with the grade ^{2004} ($p = 0.051$). Although shorter survival has been reported in patients with perineural invasion, no association between perineural growth and pR1 has been found in the present study or by other authors [12, 52] that can be explained by limited, mainly intratumoural perineural spread of PNEN, contrasting with pancreatic ductal adenocarcinoma [54].

Ki-67

Proliferation is a characteristic general tumour feature that is also essential in PNEN grading [4]. The observed invariable Ki-67 expression with a median positive cell count of 2% was in agreement with other authors [41, 49]. Regarding elevated Ki-67 expression in PNEN, the most convincing correlation is described with more frequent metastatic spread [55]. In this study, there was strong correlation with tumour stage ($r_s = 0.688$; $p = 0.028$). Along with a higher ability to cell division, PNENs acquired CK19 expression ($p = 0.033$) and EMT by vimentin expression ($p = 0.045$). These markers, similarly to Ki-67, were not observed in islets. Thus, the triad of Ki-67, CK19 and vimentin up-regulation can be used as a tool to identify aggressive behaviour in PNENs. Notably, there was a trend towards negative correlation ($p = 0.051$) between Ki-67 and stem cell phenotype by CD44 expression, consistent with the stem cell nature of slow, steady renewal.

p53

The "genome guard", p53 protein is normally found within cells in small quantities due to a short half-life. *TP53* mutations can result in the synthesis of aberrant p53 proteins that have longer half-lives and thus accumulate in cells and can be detected by IHC. The *TP53* mutation analyses and p53 IHC provide two different levels of molecular examination lacking correlation between gene mutations and aberrant p53 protein expression [56]. The p53 protein expression in PNENs was infrequent in accordance to the previously reported rare occurrence of *TP53* mutations and IHC expression of p53 protein in PNENs [57]. Occasionally, a higher frequency (49%) of p53 protein expression has been described in gastroenteropancreatic NENs due to high expression in gastric neuroendocrine neoplasms [58]. Although p53 expression has been attributed to malignant PNENs [5], in the present study, it was observed in well-differentiated PNENs with unclear behaviour. Hypothetically, these tumours have a malignant nature but do not yet demonstrate invasive growth, similarly to PanIN-3 lesion in pancreatic ductal adenocarcinoma. Using the WHO 2010 classification, p53 expression was not limited to any particular grade in accordance with previous publications [58]. In PNENs, no association has been observed between immunohistochemically detected p53 protein expression and survival [59]. It has been hypothesised that p53 is usually not directly altered in gastroenteropancreatic NENs, but the other molecules involved in the whole p53 pathway are inactivated. Accordingly, we did not observe significant differences regarding p53 expression in PNENs and benign endocrine precursors. Analogous considerations are expressed regarding retinoblastoma tumour suppressor protein (Rb) pathway [60] favouring cyclin D1 as one of the key factors for cell cycle dysregulation in NENs, in contrast to epithelial carcinomas [61].

p21

The p21 protein is a cell cycle inhibitor, which controls cell cycle progression, apoptosis and transcription. It has a dual function, including proliferation inhibition and positive modulation, and can play anti- and pro-apoptotic function, depending on the nature of the apoptotic stimulus [62]. Although strong expression of p21 in gastroenteropancreatic neuroendocrine neoplasms has been previously associated with poor outcome [6], in our study, positive p21 expression showed limited correlations, possibly due to this duality. *In vitro*, induction of p21 has been associated with aspirin-induced inhibition of neuroendocrine tumour cell viability [63], further emphasising the controversies in the interpretation of p21 presence. Regarding other endocrine neoplasms, no difference of p21 levels in thyroid follicular adenomas and carcinomas has been found [64]. In the present study, a significant correlation with E-cadherin was observed, providing evidence for the link between cell cycle regulation and cell adhesion and invasion.

p27

The p27 is a cyclin-cyclin dependent kinase inhibitor and tumour suppressor. It inhibits cell cycle progression, mediating G_1 arrest [7]. In cancer, IHC expression of p27 is decreased due to impaired synthesis or accelerated degradation of the relevant protein [65]. In a mouse model, lack of p27 led to neuroendocrine hyperplasia. However, contact inhibition of cell proliferation remained unchanged, signifying necessity for other molecular alterations to complete the tumorigenesis [66]. Consequently, we did not observe significant differences between p27 levels in PNENs and islets. Alternatively, p27 alterations can be late event in NEN development, related to high tumour grade. Loss of p27 in gastroenteropancreatic NENs has been associated with worse prognosis in some [7, 67], but not all [6], studies. Disappearance of p27 was characteristic in poorly differentiated neuroendocrine carcinomas and metastatic well-differentiated endocrine carcinomas (by 2004 WHO classification), as Grabowski *et al.* [67] reported. Such tumours were infrequent in the present study group. This could explain why no morphological associations were identified in the present study, contrasting with Kim *et al.* [7].

Cyclin D1

The transcriptional regulator cyclin D1 forms a complex with cyclin dependent kinases 4 and 6 that phosphorylate and thus inactivate the tumour suppressor protein Rb, resulting in the cell cycle progress from the G_1 to S phase. It is among the main proliferation control mechanisms [61]. In malignant cells, the level of cyclin D1 can increase due to gene rearrangement, amplification, transcriptional up-regulation [61] or affected degradation [68]. *Rb* gene is infrequently affected in NENs, favouring involvement of other molecules within the Rb pathway [69].

In the present study, there was no significant difference between the cyclin D1 expression in islets and PNENs. Interestingly, in the mouse model, induced cyclin D1 expression in pancreatic β-cells resulted in islet hyperplasia that was not associated with hypoglycaemia, diabetes or tumour [70]. In turn, the relevance of cyclin D1 to the neuroendocrine oncogenesis was emphasised by the interaction with beta-catenin, an important component of the E-cadherin pathway [71]. Extracellular-regulated kinase ERK and p38/mitogen-activated protein kinase MAPK pathways are also involved both in the regulation of cyclin D1

levels [8] and the down-regulation of E-cadherin [72]. Cyclin D accumulation can be the downstream effect of beta-catenin accumulation occurring within E-cadherin/beta-catenin function loss [60]. Alternatively, up-regulation of cyclin D1 is considered an early event in the development of neuroendocrine neoplasms [61].

In PNENs, cyclin D1 possibly should be analysed in conjunction with other proteins to distinguish between hyperplastic and neoplastic pathways. Such complex analysis could include E-cadherin, but should not be limited to it. In the INK4a-ART tumour suppressive network, p21 and p27 loss can have a similar effect as cyclin D1 hyperexpression [60]. Hypothetically, E-cadherin expression could have a higher prognostic value, serving as the limiting measure of molecular deregulation in PNENs, while cyclin D1 expression [73] would be too ubiquitous for prognostic estimates. Other research groups have also failed to identify an association between cyclin D1 expression and survival or clinical and pathological variables [58].

Bcl-2

Bcl-2 is an anti-apoptotic protein. Translocation in the *BCL2* gene yields increased expression of the Bcl-2 protein [56]. It is considered that PNEN development necessitates both derangement of cell proliferation and apoptosis [60]. The estimates of Bcl-2 expression are contradictory, ranging from relatively frequent (53.3-53.6%) to no reactivity in PNENs [5, 74]. The hypothetical explanation of this controversy is as follows. In pancreatic carcinoma, over-expression of another anti-apoptotic protein belonging to the Bcl-2 family – Bcl-x_L is more common and has a greater relevance [75]. Thus, cross-reactivity can be responsible for most of the positive cases, but was avoided in the present study. It must be emphasised that positive controls (including internal positive control of lymphocytes) were invariably performed and were reactive.

E-cadherin

E-cadherin is an epithelial transmembrane glycoprotein supporting epithelial layer integrity and polarity [56]. Loss of E-cadherin or displacement of it, apart from the cell membrane, has been observed in advanced malignant tumours. The resulting functional derangement of E-cadherin weakens cell-cell adhesion and facilitates cell migration and invasion. Lower E-cadherin levels are associated with a higher incidence of cancer metastasis [76]. In the present study, we confirmed a significant association between E-cadherin loss and advanced tumours, characterised by a higher pT ($p = 0.011$) in parallel with previously reported association between larger tumour size and the loss of E-cadherin in pancreatic and pulmonary NENs [56, 77]. Both in pulmonary neuroendocrine tumours and in PNENs, reduced E-cadherin expression or displacement of this protein from the cell membrane to the cytoplasm has been associated with the presence of LN metastases [56, 77]. Loss of E-cadherin in PNENs is characterised by more frequent liver metastases [56]. In the present study, decreased E-cadherin levels were associated with more frequent vascular and perineural invasion (both $p = 0.003$), showing a higher spreading capacity of the E-cadherin-losing tumour. E-cadherin significantly correlated with p53 status ($p = 0.011$). Wild type p53 proteins can suppress EMT that involves up-regulation of vimentin and loss of E-cadherin; recently, the mechanism has been studied in regard to microRNA regulation. The miR-154-mediated mechanism has been shown in the prostate cancer cell line [78]. Here, we provide *in vivo* evidence of the p53 and E-cadherin relationship in PNENs.

Molecularly, we also identified a significant association between E-cadherin and the cell cycle regulator p21 ($p = 0.046$). In hepatocellular carcinoma, miR-148b was recently shown to regulate p21 levels, as well as to influence the Wnt pathway, finally up-regulating E-cadherin expression [79]. Here, we provide *in vivo* evidence of analogous p21 and E-cadherin association in PNENs. E-cadherin levels in PNENs showed also a strong positive correlation with angiogenesis, as reflected by MVD ($p = 0.005$). In turn, high vascularity is associated with a lower NEN grade [80]. In the present study, decreased E-cadherin levels were associated with grade ^{2004} ($p = 0.007$). Similarly, in pulmonary NENs, functional derangement of E-cadherin complex was associated with a higher proliferation activity, the hallmark of PNEN grading [77].

CD44

CD44 is a transmembrane adhesion molecule that participates in the interaction between different cells or between cells and the matrix, as well as in cell migration [81]. It is also characteristic for stem-like cancer cells, representing self-renewing cells, able to promote clonogenicity, cell growth and migration, metastatic spread and resistance to chemotherapy [11, 27].

The identified trend towards a negative correlation between CD44 and Ki-67 in PNENs ($p = 0.051$) was typical for the slow, continuous regeneration predicted for stem cells. CD44 expression has been shown in mesenchymal stromal cells that were able to differentiate into islet cells [82]. In accordance with this, a small subpopulation of CD44-positive cells was almost invariably evident in non-neoplastic islets in the present study. However, tissue stem cells

exhibit similarities with cancer stem cells, but also show differences [83].

In pulmonary carcinoids, low levels of CD44 mRNA and absence of the CD44 protein are associated with a low 20-year survival [84]. In a combined group of pulmonary and gastrointestinal neuroendocrine neoplasms originally described as carcinoids, the patients with lymph node or distant metastasis lacked CD44 in the tumours significantly more frequently [85]. Thus, the prognostic role of CD44 expression in NENs is different from the negative findings in many carcinomas, including pancreatic ductal adenocarcinoma, gastric adenocarcinoma, clear cell renal carcinoma or non-small cell lung carcinoma, among others [11, 86-88]. In our study, there also was no association between CD44 and such morphological parameters, such as high grade and stage, which are indicative of a poor prognosis.

Thus, in neuroendocrine neoplasms, CD44 exhibits mainly tumour suppressor functions, promoting cell adhesion, limiting metastatic spread and activating apoptosis in accordance with the reported tumorigenicity of CD44-null fibroblasts in nude mice or tumour apoptosis studies [84]. The dual functions of CD44 are pathogenetically important. In practice, pathologists must be aware of the diverse prognostic role of CD44 in tumours of different histogenesis.

Cytokeratins

Although few articles have been devoted to the prognostic value of CKs, the expression of CK19 has been associated with shorter survival in some [12, 13, 52], but not all, studies [32, 39]. In the present study, CK19 was associated with aggressive characteristics, including higher pT ($p = 0.011$), stage ($p = 0.025$), grade ^{2004} ($p = 0.002$), invasive capacity confirmed by vascular ($p = 0.020$), lymphatic ($p = 0.043$) and perineural ($p = 0.026$) invasion, proliferative activity by mitotic count ($p = 0.008$) and Ki-67 ($p = 0.033$), EMT ($p = 0.041$) and suppressed angiogenesis ($p = = 0.014$). The findings are in accordance with previous studies reporting increased tumour size, higher mitotic activity, lymphatic, vascular and perineural invasion, higher grade and higher TNM parameters in CK19-expressing PNENs [13, 32]. Our findings suggest that CK19 confers increased plasticity to tumour cells, facilitating local invasion and metastatic spread similar to pancreatic ductal adenocarcinoma, which typically expresses CK19 [89] and is known for its fulminant course.

Alternative explanations for CK19 role exist as well. CK19 is present in benign pancreatic ducts [32]. As ductal epithelia can up-regulate the endocrine cells [90], self-stimulation can be provided by CK19-positive PNEN cells. Some authors have hypothesised that PNENs develop from pluripotential ductal stem cells [91], analogous to the unitarian theory regarding colonic carcinoma and endocrine tumours [92]. This hypothesis is supported by the occasional coexistence of PNEN and intraductal papillary mucinous pancreatic neoplasms [93, 94]. If so, CK19 can be the precursor marker of pristine PNENs, retaining some features of the pluripotential primitive ancestor cell. This is in accordance with the embryonic development of the pancreas characterised by CK19 expression in the endocrine component during the early stages, while later it is retained in ducts, but absent from islets [13, 32]. The observed statistically significant ($p = 0.006$) up-regulation of CK19 in 28.6% of PNENs thus recapitulates early developmental stages.

CK20 is absent from normal pancreatic islets [26, 56]. Although CK20 expression in PNENs has been reported in up to 33% of cases [95], it was not observed in the present study. The published data also vary widely – only 5% of gastroenteropancreatic tumours expressed CK20 in Knosel *et al.* [96] study.

CDX2

CDX2 is a caudal-related homeobox transcription factor, expressed in the intestinal epithelium. It discloses intestinal differentiation, both in non-neoplastic or malignant cells. The labelling in pancreatic tissues is usually lighter and more variegated than in colorectal carcinoma [56]. Although expression of CDX2 has been reported in a small fraction of PNENs [97, 98], it was absent both from PNENs and islets in the present study.

Squamous differentiation

The endocrine pancreatic tissues and corresponding tumours lacked CK5/6 and CK34βE12 proteins that were found in cells exhibiting squamous differentiation [56]. To the best of our knowledge, there are no previously published reports evaluating the trend towards squamous differentiation in PNENs by p63 expression. In the present study, it was observed in 28.6% of cases. Although the expression did not reach the cut-off level of 10% positive cells, the up-regulation was significant in contrast to non-neoplastic endocrine cells in the pancreatic islets ($p = 0.034$). Thus, association with tumorigenesis could be suspected rather than the squamous differentiation. The pathogenetic role of p63 in a fraction of PNENs is further supported by the trend towards correlation with cell cycle regulator p21 ($p = 0.056$) and by the invasiveness of p63-expressing PNENs, as p63 expression was significantly higher in tumours that invaded blood vessels ($p = 0.024$). The association with p53 ($p < 0.001$) and E-cadherin ($p = = 0.009$) was also indirect evidence of aggressive features. Previously, expression of p63 was reported in pulmonary non-small cell neuroendocrine carcino-

mas [99] and neuroendocrine carcinomas of the head and neck [100] in studies of diagnostic value.

Vimentin

Vimentin is a major mesenchymal intermediate filament, controlling cellular motility, signalling and directional migration [101]. Malignant pancreatic epithelial cells acquire vimentin and lose E-cadherin during EMT, resulting in tumour invasiveness, metastatic capacity, resistance to chemotherapy and the generation of cancer stem cells. Although EMT has been associated with cancer progression, it has also been attributed to fibrosis due to chronic inflammation. Fibrosis is associated with increased numbers of myofibroblasts arising from epithelial cells through EMT [102]. Since pancreatic islets lack fibrosis even in inflammation, EMT is absent from islets. In PNENs, vimentin was significantly up-regulated in comparison with islets ($p = 0.006$), suggesting a significant role in neuroendocrine tumorigenesis. Vimentin also showed a positive correlation with CK19 ($p = 0.041$), which is associated with increased invasiveness in PNENs. Thus, particularly aggressive PNEN could be characterised by an up-regulated triad, i.e., Ki-67, CK19 and vimentin. In pulmonary NENs, vimentin has been significantly associated with higher tumour grade [77]. Appendicular NENs, having favourable clinicopathological profiles, mostly lack vimentin [51].

Alternatively, vimentin could mark a separate molecular subtype of neuroendocrine neoplasms, as evidenced by relatively frequent occurrence and the reported association with low biological potential [51].

The observed frequency of EMT in PNENs in this study is within the reported range (11–58%) in gastroenteropancreatic neuroendocrine neoplasms and/or rectal carcinoids [51, 103]. Coexpression of vimentin and neuroendocrine markers has also been reported in selected peripheral and visceral glomus tumours that show clear cell morphology [104, 105], PNEN associated with tuberous sclerosis [106], and in 80% of renal NENs [107].

Microvascular density

Although rich vascularity is typical in NENs, the differences between these tumours and the precursors are less known. In the present study, PNENs retained the same MVD as islets. In an experimental model of digestive NENs, angiogenesis was associated with well-differentiated cell lines and tumours, and lacked correlations with tumour progression, as well as invasive and metastatic properties [80]. Analogous clinical observations have been published [108]. Low vascularity is characteristic for poorly differentiated neuroendocrine carcinomas [109] and can be quantified by non-invasive xenon-inhalation computed tomography [110]. Pathogenetically, hypoxia-inducible factors are associated with malignant progression of well-differentiated neuroendocrine tumours of the ileum [111].

COX-2

COX-2 is the rate-limiting enzyme in the production of prostaglandins from arachidonic acid. It is expressed in gastric, colorectal and pancreatic carcinomas [56], and has been associated with poor outcome, whereas the risk of cancer mortality is reduced by long-term non-steroidal anti-inflammatory drug therapy. COX-2 selectively promotes the expression and activity of the neuroendocrine marker chromogranin A, and therefore it is a promising research direction in NENs [112]. COX-2 expression has been explored in neuroendocrine tumours, including studies of the biological potential and therapeutic possibilities. It was widely expressed in up to 82% of medullary thyroid carcinoma, showing statistically significant up-regulation in comparison with benign thyroid tissue [113]. In phaeochromocytoma, COX-2 is associated with malignant behaviour and, along with lack of nm-23 and presence of galectin-3, can be applied to predict malignancy [114]. Immunohistochemical expression of the COX-2 protein has been identified in 54-75% of gastroenteropancreatic [115, 116] and 65% of pancreatic NENs [117]. In our study, we showed statistically significant up-regulation of COX-2 expression in PNENs, contrasting with invariably negative islets ($p = 0.016$) and providing *in vivo* evidence that COX-2 is involved in the pancreatic neuroendocrine tumorigenesis.

The prognostic role of COX-2 in PNENs has been addressed in a few studies. COX-2 overexpression in gastroenteropancreatic NENs was significantly associated with poor survival. However, only 10/247 NENs in this cohort originated in the pancreas [116]. In a cohort of mid-gut carcinoids comprising NENs of the distal duodenum, jejunum, ileum and proximal part of the colon, a higher COX-2 histoscore was significantly associated with worse survival within the COX-2 positive group [118]. The correlation between COX-2 and proliferation activity by Ki-67 could at least partially explain the negative prognostic meaning of COX-2 [116]. COX-2 expression in gastroenteropancreatic NENs correlated significantly with grade, but not primary location [116]. In contrast, Bergmann et al. concluded that COX-2 expression was independent of the malignant potential of pancreatic endocrine tumours [3]. In the present study, COX-2 expressing PNENs more frequently invaded lymphatic vessels ($p = 0.043$), suggesting an undesirable course. The association between COX-2 overexpression and lymphatic invasion in gastroenteropancreatic NENs was reported by Ince *et al*. [115] and Kim *et al*. [116]. Higher invasive capac-

ity of COX-2-expressing PNEN represents a pathogenetic mechanism for worse survival and is further substantiated by the observed association between COX-2 and the cytokeratin profile, influencing the tumour cell plasticity.

COX-2 inhibitors have been studied in regard of NEN treatment [3]. *In vitro* NEN studies have been encouraging, revealing the induction of apoptosis [119] and the potentiation of chemotherapy [120] in the medullary thyroid cancer cell line. Enhanced apoptosis has also been observed in PNEN cell lines [3]. The mechanisms of COX-2 inhibitor activity include reduction of the expression of multidrug resistance 1 gene *MDR1*, resulting in lower levels of the corresponding transmembrane glycoprotein, that prevents intracellular accumulation of cytotoxic medications [120]. Cell proliferation is also decreased due to cell cycle arrest at the G_0/G_1 transition [119]. However, at present, significant controversies exist regarding the dynamics of COX-2 up-regulation in neuroendocrine neoplasms. In PNENs, COX-2 was more frequently found in the primary tumour than in the metastasis [3]. Cadden *et al.* [118] reported contrary findings. Considering the focality and heterogeneity of COX-2 expression in this and other studies [121], as well as the beneficial influence of COX-2 inhibition on tumour chemosensitivity, COX-2 inhibitors would be useful only as part of complex therapy.

Neuroendocrine markers

Neuroendocrine markers are the primary diagnostic tools in PNENs. As both chromogranin A and CD56 are invariably expressed in PNENs and islets, both markers can be equally used in PNEN diagnostics. Interestingly, despite the high level of chromogranin A expression, the reactivity is still up-regulated in PNENs at a statistically significant level ($p = 0.042$). The mechanisms of carcinogenesis can explain these changes, e.g., selective and functionally important up-regulation of chromogranin A by COX-2 [112].

Our report has some limitations, especially with the small size of the study group, despite enrolment of all PNENs within a 10-year long period. We restricted the time period to 10 years in order to avoid loss of immunohistochemical reactivity [122]. Appropriate methods of statistical analysis were selected [30]. The single-hospital approach ensured a homogeneous study group. The strength of our study includes a simultaneous analysis of 20 immunohistochemical markers and 12 gross and microscopic parameters in the same study group using non-neoplastic islets for comparison. The integrated approach allowed us to reveal multiple significant morphologic and molecular associations including the up-regulated triad of Ki-67, vimentin and CK19 as a tool to detect aggressive behaviour of PNEN. Loss of E-cadherin can also be used in the complex with these markers. We also were able to show a link between tumour cell adhesion, invasion and cell cycle regulation as disclosed by the associations between Ki-67, p21, p53, CK19 and E-cadherin. The expression and role of p63 in PNENs has been reported for the first time.

Conclusions

Significant up-regulation of Ki-67, CK19, p63, chromogranin A, vimentin and COX-2 expression was found in PNENs in comparison with non-neoplastic pancreatic islets.

In PNENs, proliferation activity (by Ki-67), E-cadherin, vimentin and CK19 are important molecular traits due to widespread associations with such important oncological parameters, like pTNMGR, tumour size and invasive growth. The triad of Ki-67, CK19 and vimentin up-regulation in association with E-cadherin loss can be used as a tool to identify aggressive behaviour in PNENs.

CD44 expression shows limited associations, contrasting with the negative role in epithelial non-neuroendocrine neoplasms.

The correlations between CK19 and Ki-67, as well as between E-cadherin and p21, provide evidence of the links between cell plasticity, adhesion and proliferation.

To the best of our knowledge, we reported p63 expression in PNENs for the first time. The associations with p53 and E-cadherin suggest that p63 in PNENs has a tumorigenetic role, rather than serving as evidence of squamous differentiation.

PNENs retain the same microvascular density characteristic for islets. The microvascular density shows a negative correlation with mitotic count, thus lower vascularity can be expected in high-grade NENs. MVD is associated with p53, cyclin D1, E-cadherin, p63 and CK19 molecular pathways.

Notably, the correlations between the molecular features of PNENs and tumour grades using the 2004 and 2010 WHO classifications differ remarkably.

The authors declare no conflict of interest.

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