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Utility of pVHL, maspin, IMP3, S100P and Ki67 in the distinction of autoimmune pancreatitis from pancreatic ductal adenocarcinoma

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ABSTRACT

Morphology plays an important role in the distinction of autoimmune pancreatitis (AIP) from pancreatic ductal adenocarcinoma (PDAC). However, we aimed to determine the utility of immunohistochemical tumor markers to contribute in the distinction of these entities. In surgical specimens with AIP (n = 20), PDAC (n = 20) and normal pancreas (n = 20), the expression of pVHL, maspin, IMP3, S100P and Ki67 was examined. We evaluated intralobular reactive ducts / acinoductal metaplasia (ILDs) and extralobular ducts (ELDs) in AIP, neoplastic glands in PDAC, and ductal epithelium in the normal pancreas, using a five-tiered scoring system. The Ki67 hot spot index (Ki67-HSPI) was determined manually and using automated digital imaging analysis of virtual double stains of Ki67 and CK8. Besides, sequential dual-immunohistochemical staining of maspin/pVHL, maspin/IMP3 and Ki67/maspin was performed in a subset of the specimens. Strong overexpression of IMP3, maspin, S100P and Ki67 and loss of pVHL was observed in PDAC compared to AIP and normal pancreas. In AIP however, focal and weak aberrant expression was observed with the following proportions in ILDs/ELDs: pVHL in 45 %/85 %, maspin in 30 %/70 %, IMP3 in 55 %/5%, S100P in 10 %/35 % and Ki67-HSPI > 20 % in 15 %/70 %. At least two markers were aberrantly expressed in ILDs/ELDs in 45 %/60 %. The aberrant expression was more pronounced in type 2 AIP compared to type 1. In conclusion, our data indicate that pVHL, maspin, IMP3, S100P and Ki67 can be focal and weak aberrantly expressed in AIP. However, when used as a panel, these markers seem to be useful for the differentiation of AIP from PC.

1. Introduction

Autoimmune pancreatitis (AIP) is an established entity of chronic pancreatitis that can be difficult to distinguish from pancreatic ductal adenocarcinoma (PDAC) because it often forms a tumorlike mass and thus the two conditions may have similar symptoms and imaging features [1–3]. Even though the diagnosis of type 1 AIP can often be established based on imaging together with serum IgG4, the sensitivity of serum IgG4 for AIP is relatively low in certain western countries, and a serological marker for type 2 AIP is currently lacking. These are important factors limiting the opportunity for a non-invasive diagnosis in AIP and emphasizing the role of laparoscopic or endoscopic-ultrasound (EUS)-guided pancreatic biopsies [4–7].

AIP is histologically characterized by lymphoplasmacytic inflammation, storiform fibrosis and obliterative phlebitis, but is subdivided into type 1 and type 2 based on additional microscopic features [7–10]. Type 1 AIP is characterized by strong infiltration with IgG4-positive plasma cells and represents one of the main manifestations of IgG4-related disease (IgG4-RD), while type 2 AIP typically is IgG4-negative or only shows few IgG4-positive cells, but instead granulocytic epithelial lesions (GELs) in the pancreatic ducts. These histological changes form the basis of the histological International Consensus Diagnostic Criteria (ICDC), published in 2011 [3].

Correct differentiation between AIP and PDAC is utmost important since AIP is rather easily medically treated with steroids and should not undergo surgery or antineoplastic treatment like PDAC. To avoid

Abbreviations: AIP, autoimmune pancreatitis; CP, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; ILDs, intralobular reactive ducts; ELDs, extralobular ducts; Ki67-HSPI, Ki67 hot spot index

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Table 1
List of antibodies, antigen retrieval, incubation time and dilutions used for immunohistochemistry.

Antibody	Species and clonality	Clone	Company	Product ID	Epitope retrieval	Incubation	Dilution
CK8	Rabbit mAb	EP17	Epitomics	AC-0007	HIER: CC1_32_100	32 min/36 °C	1:100
IMP3	Mouse mAb	69.1	DAKO	M3626	HIER: CC1_48_100	16 min/36 °C	1:25
Ki67	Rabbit mAb	30-9	Ventana Medical System	790-4286	HIER: CC1_48_100	12 min/36 °C	RTU
Maspin	Mouse mAb	G167-70	Pharmingen	554292	HIER: CC1_32_100	32 min/36 °C	1:100
pVHL	Rabbit pAb	FL-181	Santa Cruz	Sc-5575	Non-HIER: Protease1 – 4 min	32 min/36 °C	1:400
S100P	Mouse mAb	16/f5	Cell Marque	376M-96	Non-HIER: Protease 1–8 min	16 min/36 °C	1:1000

CC1: cell conditioning solution 1 (pH 8,5, Ventana Medical Systems), *CC1_X_X*: CC1_minutes incubated_degrees Celcius, *CK8*: cytokeratine 8, *HIER*: heat induced epitope retrival, *mAb*: monoclonal antibody, *pAb*: polyclonal antibody *RTU*: ready to use.

Table 2
Sequential dual-immunohistochemistry (IHC): List of markers, sequence of antibodies, antigen retrieval and detection systems.

Markers	First sequence IHC			Antibody denaturation	Second sequence IHC		
	Antibody	Epitope retrieval	Detection system		Antibody	Epitope retrieval	Detection system
Maspin / IMP3	Maspin	CC1_32_100	OptiView-DAB	4 min. at 90 °C	IMP3	None	UltraView-RED with amplification
Maspin / pVHL	Maspin	CC1_32_100	OptiView-DAB	4 min. at 90 °C	pVHL	Protease 3–4 min	UltraView-RED with amplification
Ki67 / Maspin	Ki67	CC1_48_100	OptiView-DAB	4 min. at 90 °C	Maspin	None	UltraView-RED with amplification

Dilution and incubation times for all antibodies are identical to the data in Table 1, except for IMP3 where 32 min incubation time was used. *CC1*: cell conditioning solution 1 (pH 8,5, Ventana Medical Systems), *CC1_X_X*: CC1_minutes incubated_degrees Celcius, *OptiView-DAB:* HRP-based detection system (Ventana Medical Systems), *UltraView-RED with amplification:* AP-based detection system (Ventana Medical System).

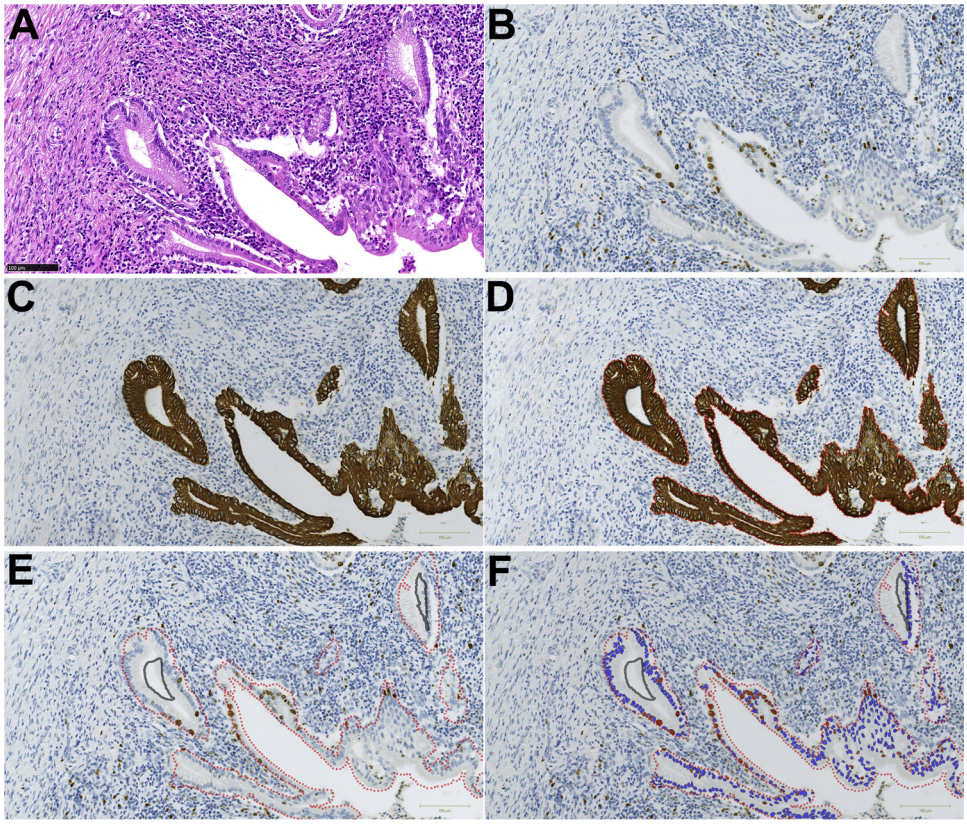

Fig. 1. The virtual double staining (VDS) principle, demonstrated using a case with autoimmune pancreatitis (AIP) type 2. (A) Periductal lymphoplasmacytic inflammation is shown (H&E, scale bar: 100 µm). (B) Ki67 staining shows proliferative activity in inflammatory cells and epithelial cells. (C) CK8-positive epithelial cells. (D) CK8 stained slide where the automated image analysis detects positive areas (epithelial cells) and marks these (red line) as the region of interest (ROI). (E) Ki67-stained slide with marked ROI. (F) Ki67 stained slide with cell profiles classified as either positive (red nuclei) or negative (blue nuclei) in the ROI. Scattered weakly stained nuclei are unclassified. Stromal areas are excluded from the analysis.

unnecessary invasive treatment, histological pancreatic biopsies are used increasingly at many centers [5,11–13]. However, as in other types of chronic pancreatitis, the histological distinction between AIP and PDAC can be a challenge when the biopsy specimen is small [14]. Particularly the intralobular reactive ducts (ILDs) and acinoductal metaplasia (also called acinoductal transdifferentiation) that are often found at the periphery of pancreatic lobules in AIP can imitate the pattern of well differentiated PDAC and challenge the conclusiveness of pancreatic biopsy for the diagnosis of AIP in these cases [14]. Besides, the ductocentric inflammation typical of AIP can lead to atypical changes in pancreatic ducts that can imitate a dysplastic process at microscopy. A number of immunohistochemical markers have been tested to distinguish between PDAC and non-neoplastic pancreatic changes, such as those that may be found in chronic pancreatitis. Traditionally, the proliferation marker Ki67 has been used as an adjunct for the distinction of reactive changes in chronic pancreatitis from well differentiated PDAC [15,16]. Besides, a panel of four markers, namely pVHL, maspin, S100P and IMP3, has been recommended for the

Table 3
Immunohistochemical expression of pVHL, maspin, IMP3 and S100P and Ki67 HSPI in the normal pancreas, autoimmune pancreatitis (AIP) and pancreatic ductal adenocarcinoma (PDAC).

pVHL	Score				
	0	1	2	3	4
Normal pancreas	19 (95 %)	1 (5%)	0	0	0
AIP – Intralobular reactive ducts	11 (55 %)	8 (40 %)	1 (5%)	0	0
Type 1	8 (66.6 %)	4 (33.4 %)	0	0	0
Type 2	3 (37.5 %)	4 (50.0 %)	1 (12.5 %)	0	0
AIP - Extralobular ducts	3 (15 %)	10 (50 %)	5 (25 %)	2 (10 %)	0
Type 1	3 (25.0 %)	8 (66.7 %)	0	1 (8.3 %)	0
Type 2	0	2 (25.0 %)	5 (62.5 %)	1 (12.5 %)	0
PDAC	0	0	0	0	20 (100 %)

Maspin	Score				
	0	1	2	3	4
Normal pancreas	18 (90 %)	2 (10 %)	0	0	0
AIP - Intralobular reactive ducts	14 (70 %)	4 (20 %)	2 (10 %)	0	0
Type 1	10 (83.4 %)	1 (8.3 %)	1 (8.3 %)	0	0
Type 2	4 (50.0 %)	3 (37.5 %)	1 (12.5 %)	0	0
AIP - Extralobular ducts	6 (30 %)	10 (50 %)	4 (20 %)	0	0
Type 1	6 (50.0 %)	5 (41.7 %)	1 (8.3 %)	0	0
Type 2	0	5 (62.5 %)	3 (37.5 %)	0	0
PDAC	0	0	1 (5%)	1 (5%)	18 (90 %)

IMP3	Score				
	0	1	2	3	4
Normal pancreas	18 (90 %)	2 (10 %)	0	0	0
AIP - Intralobular reactive ducts	9 (45 %)	11 (55 %)	0	0	0
Type 1	5 (41.7 %)	7 (58.3 %)	0	0	0
Type 2	4 (50 %)	4 (50 %)	0	0	0
AIP - Extralobular ducts	20 (100 %)	0	0	0	0
Type 1	12 (100 %)	0	0	0	0
Type 2	8 (100 %)	0	0	0	0
PDAC	4 (20 %)	1 (5%)	0	3 (15 %)	12 (60 %)

S100P	Score				
	0	1	2	3	4
Normal pancreas	20 (100 %)	0	0	0	0
AIP - Intralobular reactive ducts	18 (90 %)	2 (10 %)	0	0	0
Type 1	11 (91.7 %)	1 (8.3 %)	0	0	0
Type 2	7 (87.5 %)	1 (12.5 %)	0	0	0
AIP - Extralobular ducts	13 (65 %)	5 (25 %)	2 (10 %)	0	0
Type 1	8 (66.7 %)	4 (33.3 %)	0	0	0
Type 2	5 (62.5 %)	1 (12.5 %)	2 (25.0 %)	0	0
PDAC	0	2 (10 %)	3 (15 %)	2 (10 %)	13 (65 %)

Ki67 HSPI (manual counting)	Median (range)
Normal pancreas	0 (0–3)
AIP - Intralobular reactive ducts	8.5 (2–44)
Type 1	8.5 (3–44)
Type 2	8 (2–19)
AIP - Extralobular ducts	33 (3–95)
Type 1	20 (3–65)
Type 2	45.5 (35–95)
PDAC	92 (58–100)

Data are given for the normal pancreas (ducts, n = 20), AIP (all cases (n = 20) and separated into type 1 (n = 12) and type 2 (n = 8)) and PDAC (n = 20). Ki67 HSPI; Ki67 hot spot index.

differentiation of PDAC from normal pancreas, but the utility of this panel has not been tested for the distinction of PDAC from AIP [17].

The aim of this study was to evaluate the utility of an immunohistochemical panel consisting of five markers for the differentiation of AIP from PDAC. We examined the expression of the markers pVHL, maspin, S100P, IMP3 and Ki67 in the normal pancreas, AIP and PDAC. The Ki67 hot spot index was calculated using two different methods – manual counting and automated digital imaging analysis of virtual double stains of Ki67 and CK8.

2. Material and methods

This study was approved by the Ethics Committee of the Region of Southern Denmark (project-ID S-20150087) and by the Danish Data

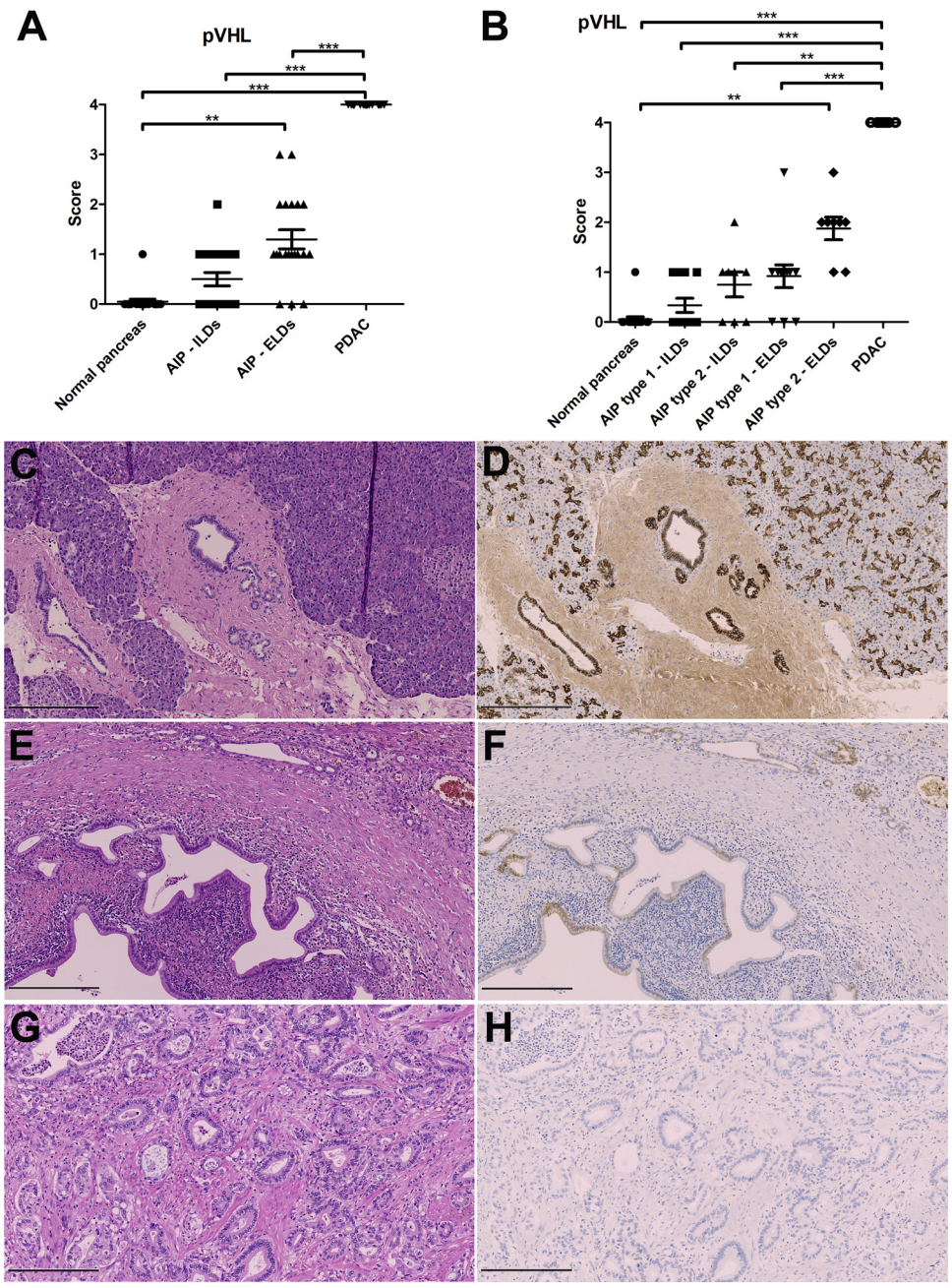

Fig. 2. Immunohistochemical expression of pVHL in the normal pancreas, autoimmune pancreatitis (AIP) and pancreatic ductal adenocarcinoma (PDAC). (A) Semiquantitative scores for pVHL in the normal pancreas, intralobular reactive ducts / acinoductal metaplasia (ILDs) and extralobular ducts (ELDs) in AIP and in PDAC (** $P < 0.01$, *** $P < 0.001$). (B) Semiquantitative scores for pVHL in normal pancreas, ILDs and ELDs in type 1 AIP and type 2 AIP, and PDAC (** $P < 0.01$, *** $P < 0.001$). (C) Acini and ELDs in the normal pancreas (H&E). (D) Strong pVHL expression in ductal epithelial cells in the normal pancreas. (E) Periductal (lower left) and intralobular (upper right) lymphoplasmacytic inflammation in AIP type 2 (H&E). (F) Loss of pVHL expression in epithelial cells in the area shown in Fig. 2E. (G) PDAC, showing infiltrating gland-like structures (H&E). (H) Complete loss of pVHL expression in the neoplastic glands. Scale bars: 250 μm.

Protection Agency (project-ID 15/22496). Besides, we ensured that patients had not advocated against the use of their tissue in the Danish registry for the use of tissue in research ('Vævsanvendelsesregisteret').

2.1. Tissues

Formalin-fixed and paraffin-embedded (FFPE) tissue blocks with AIP were obtained from 20 surgical specimens (11 men and 9 women; mean age at surgery 58.6 years (range 33–78 years)). The International Consensus Diagnostic Criteria (ICDC) for AIP were fulfilled in all cases (twelve patients had type 1 and eight patients type 2 AIP) [3]. The patients had undergone pancreatic head resection (n = 15) or left-sided pancreatic resection (n = 5). Surgery had been performed at Odense University Hospital, Denmark (n = 8), Aalborg University Hospital, Denmark (n = 5), Rigshospitalet, Copenhagen, Denmark (n = 4) or the Technical University of Munich, Germany (n = 3). From each case, the tissue block showing the most severe intralobular inflammation with reactive ducts and the most pronounced inflammation of extralobular pancreatic ducts was selected. FFPE tissue blocks with PDAC grade 2-3 were obtained from 20 surgical specimens from patients who underwent surgery at Odense University Hospital, Odense, Denmark. Moreover, 20 cases showing normal pancreatic tissue without inflammation or malignancy were included as controls (5 tissue blocks and 15 cores with a diameter of 4 mm included in tissue micro arrays). These cases were obtained from surgical specimens from patients operated for serous cystic neoplasms, grade 1 neuroendocrine tumors or ductal adenocarcinoma. One slide from all included FFPE blocks was stained with hematoxylin and eosin.

2.2. Immunohistochemistry

Four μm sections were cut on a microtome and mounted on FLEX IHC slides (Dako, Glostrup, Denmark). The immunohistochemical staining procedures for all antigens was automated, including

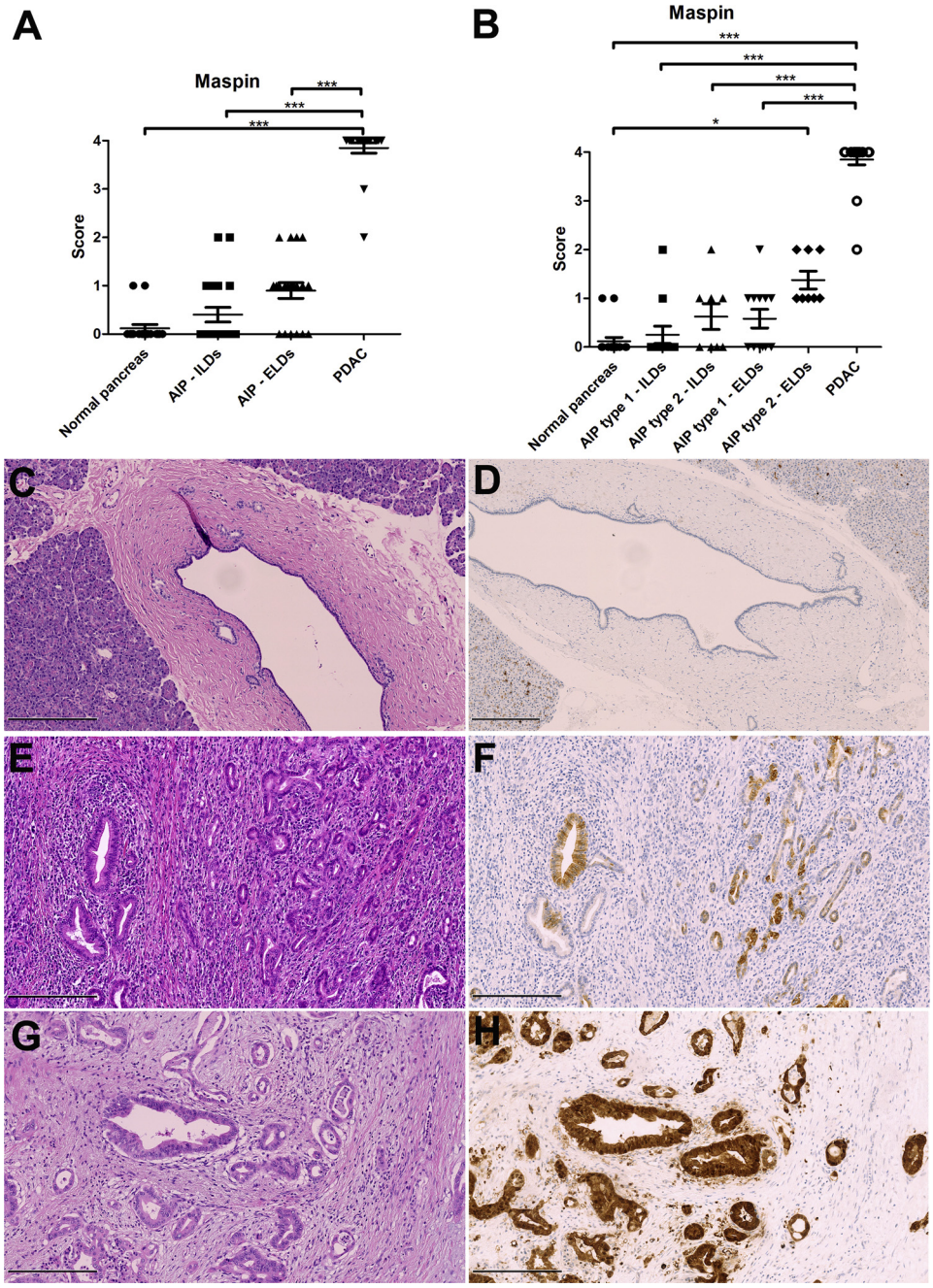

Fig. 3. Immunohistochemical expression of maspin in the normal pancreas, autoimmune pancreatitis (AIP) and pancreatic ductal adenocarcinoma (PDAC). (A) Semiquantitative scores for maspin in the normal pancreas, intralobular reactive ducts / acinoductal metaplasia (ILDs) and extralobular ducts (ELDs) in AIP and in PDAC (*** $P < 0.001$). (B) Semiquantitative scores for maspin in normal pancreas, ILDs and ELDs in type 1 AIP and type 2 AIP, and PDAC (* $P < 0.05$, *** $P < 0.001$). (C) ELD in the normal pancreas (H&E). (D) Lack of maspin expression in ductal epithelial cells in the normal pancreas. (E) Diffuse lymphoplasmacytic inflammation in AIP type 1 (H&E). (F) Moderate maspin expression in inflamed and reactive ductal structures, in the area shown in Fig. 3E. (G) PDAC, showing infiltrating gland-like structures (H&E). (H) Strong maspin expression in the neoplastic glands. Scale bars: Fig. 3C-D 250 μm; Fig. 3E-H 100 μm.

deparaffinization, epitope retrieval and blocking of endogenous peroxidase activity, using the BenchMark Ultra Immunostainer (Ventana Medical Systems, Tucson, AZ), with the OptiView-DAB detection kit (Ventana Medical Systems, Tucson, AZ). Heat-induced epitope retrieval (HIER) as well as non-HIER protocols were tested for antigen retrieval to obtain the highest signal-to-noise ratio. Table 1 presents the details on primary antibodies, dilutions, incubation times and epitope retrieval procedures. Sequential Dual-IHC staining was performed on selected cases and was also automated at the BenchMark Ultra Immunostainer using the OptiView-DAB detection for the first sequence and the UltraView-RED (Ventana Medical Systems, Tucson, AZ) for the second sequence. Primary antibodies, dilutions, incubation times, sequence of antibodies and epitope retrieval procedures are specified in Tables 1 and 2. Nuclear counter staining was performed with the BenchMark Ultra Immunostainer, using Hematoxylin II (Ventana Medical Systems, Tucson, AZ). Slides were washed, dehydrated, and mounted with coverslips using the Tissue-Tek Film coverslipper (Sakura, Alphen aan den Rijn, The Netherlands). Tissue microarrays with a variety of normal and malignant tissues were used as controls.

2.3. Scanning, viewing software and image analysis platform

All slides were scanned using a Hamamatsu NanoZoomer XR (Hamamatsu Photonics, Japan), applying the x20 scanning resolution mode (corresponding to a magnification of 200 times). The images of the scanned slides were evaluated using the NDP.view software, version 2.3.13 (Hamamatsu Photonics, Japan). Moreover, for virtual double staining analysis of CK8 and Ki67, the scanned H&E stained slides and the slides stained for CK8 and Ki67 were transferred to the image analysis platform VIS (Visiopharm Integrator System, Oncotopics with the Tissue Alignment Module, Visiopharm, Denmark).

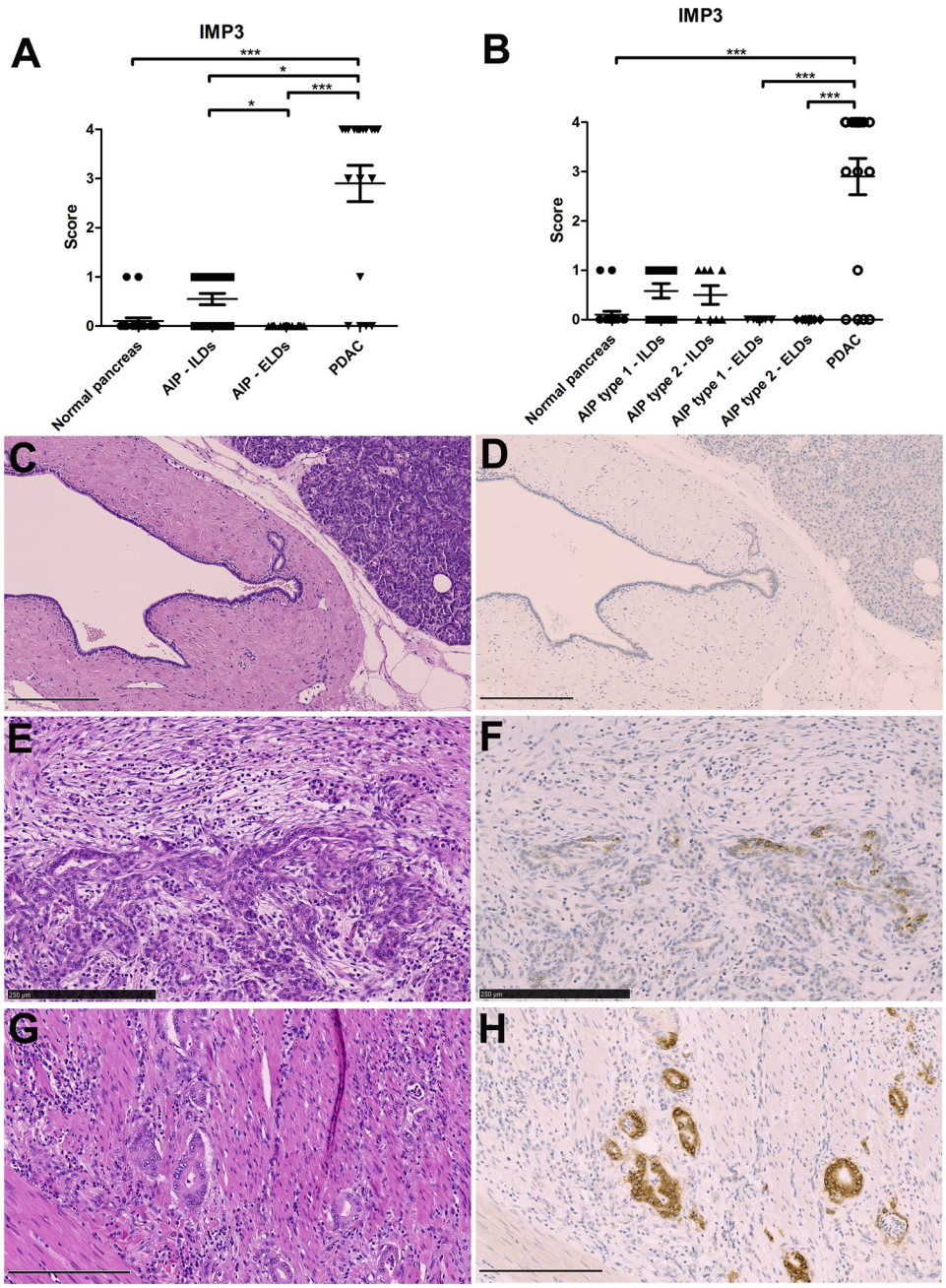

Fig. 4. Immunohistochemical expression of IMP3 in the normal pancreas, autoimmune pancreatitis (AIP) and pancreatic ductal adenocarcinoma (PDAC). (A) Semiquantitative scores for IMP3 in the normal pancreas, intralobular reactive ducts / acinoductal metaplasia (ILDs) and extralobular ducts (ELDs) in AIP and in PDAC (* $P < 0.05$, *** $P < 0.001$). (B) Semiquantitative scores for IMP3 in normal pancreas, ILDs and ELDs in type 1 AIP and type 2 AIP, and PDAC (*** $P < 0.001$). (C) ELD in the normal pancreas (H&E). (D) Lack of IMP3 expression in ductal epithelial cells in the normal pancreas. (E) Diffuse lymphoplasmacytic inflammation in AIP type 1 (H&E). (F) Focal, weak IMP3 expression in ILDs (area shown in Fig. 4E). (G) PDAC, showing infiltrating gland-like structures (H&E). (H) Strong IMP3 expression in the neoplastic glands. Scale bars: Fig. 4C-D 250 μm; Fig. 4E-H 100 μm.

2.4. Evaluation of immunohistochemical expression of pVHL, maspin, IMP3 and S100P

In the AIP specimens, the immunohistochemical expression of IMP3, maspin, pVHL and S100P was evaluated separately in intralobular reactive ducts and acinoductal metaplasia (ILDs) and in extralobular ducts (ELDs). In PDAC, the expression was evaluated in the neoplastic glands. In the normal pancreas, the expression was evaluated in intralobular and interlobular duct epithelium. For maspin and S100P, either nuclear staining or nuclear and cytoplasmic staining was regarded positive. For IMP3 and pVHL, cytoplasmic and / or membranous staining with or without nuclear staining was regarded positive. A 5-tiered scoring system was used, according to Liu and coworkers [17]. For IMP3, maspin and S100P, score 0 indicated that no or ≤5% of the cells were stained, score 1 that 6 %–25 %, score 2 that 26%–50%, score 3 that 51%–75% and score 4 that > 75 % were stained. For pVHL, score 0 indicated loss of staining in no or ≤5% of the cells, score 1 that 6 %–25 %, score 2 that 26%–50%, score 3 that 51%–75% and score 4 that > 75 % showed loss of staining. All cases were evaluated by a trained pathology resident and a pathologist with special interest in pancreatic pathology. In case of disagreement, consensus was reached by joint evaluation of the respective case.

2.5. Manual counting of the Ki67 index

The Ki67 hot spot proliferation index (Ki67 HSPI) was assessed manually by a pathologist with special interest in pancreatic pathology. The Ki67 HSPI was defined as the area with the highest number of Ki67-positive cells out of 100 cells in the respective area. For each case of AIP, the Ki67 HSPI was determined separately in ILDs and ELDs. In PDAC, the expression was evaluated in the neoplastic glands. In the normal pancreas, the expression was evaluated in epithelial cells of intralobular and perilobular ducts.

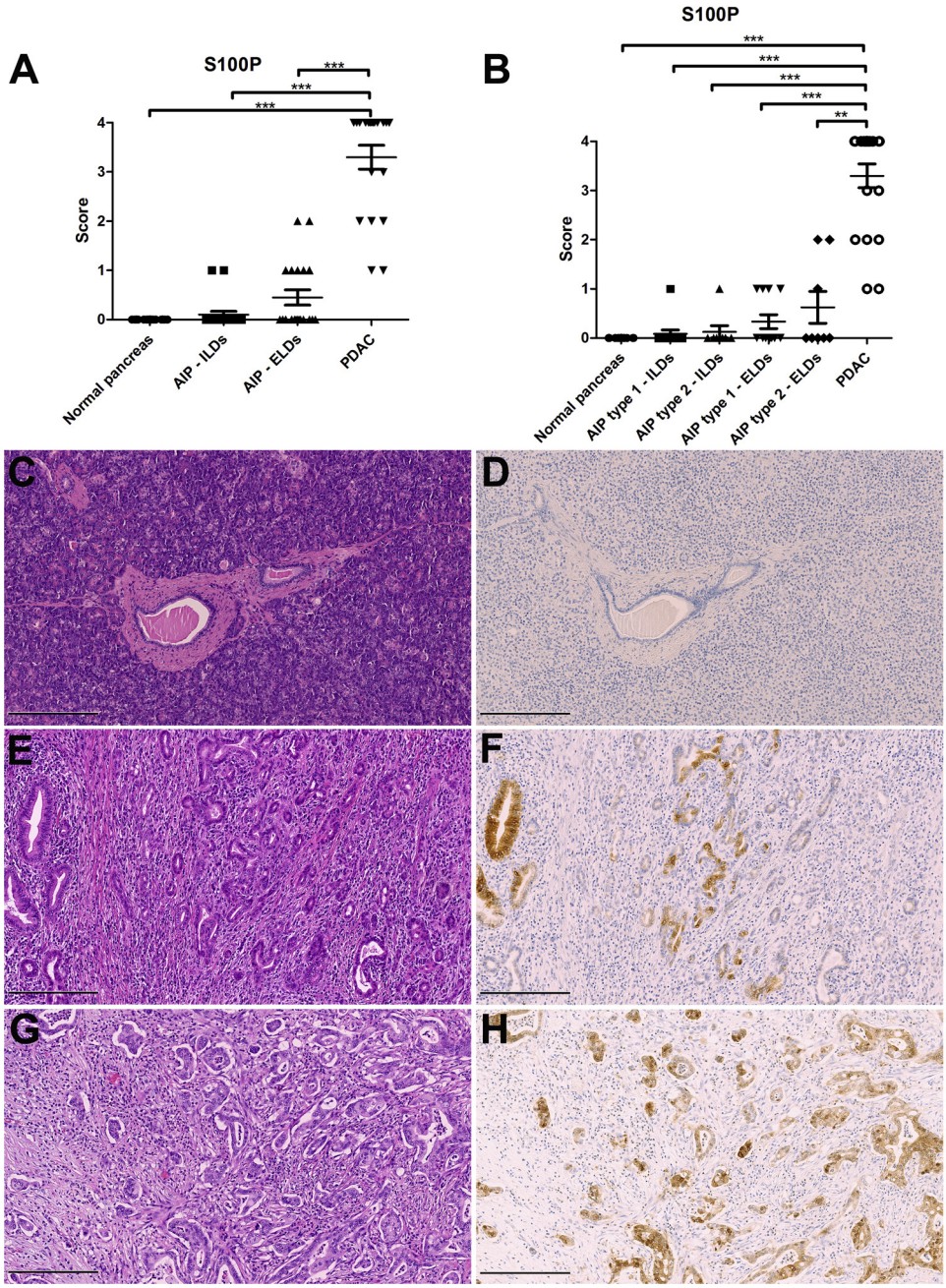

Fig. 5. Immunohistochemical expression of S100P in the normal pancreas, autoimmune pancreatitis (AIP) and pancreatic ductal adenocarcinoma (PDAC). (A) Semiquantitative scores for S100P in the normal pancreas, intralobular reactive ducts / acinoductal metaplasia (ILDs) and extralobular ducts (ELDs) in AIP and in PDAC (*** $P < 0.001$). (B) Semiquantitative scores for S100P in normal pancreas, ILDs and ELDs in type 1 AIP and type 2 AIP, and PDAC (** $P < 0.01$, *** $P < 0.001$). (C) Acini and ILDs in the normal pancreas (H&E). (D) Lack of S100P expression in ductal epithelial cells in the normal pancreas. (E) Diffuse lymphoplasmacytic inflammation in AIP type 1 (H&E). (F) Focal, moderate S100P expression in inflamed and reactive ductal structures, in the area shown in Fig. 5E. (G) PDAC, showing infiltrating gland- and bud-like structures (H&E). (H) Strong S100P expression in the neoplastic glands. Scale bars: 250 μm.

Table 4
Number and proportion of cases showing focal and weak abberantly expressed markers (pVHL, maspin, IMP3 and S100P) in autoimmune pancreatitis (AIP) (n = 20).

	Intralobular reactive ducts (ILDs), AIP (n = 20)	Extralobular ducts (ELDs), AIP (n = 20)
At least 1 abberantly expressed marker	15 (75 %)	20 (100 %)
At least 2 abberantly expressed markers	9 (45 %)	12 (60 %)
At least 3 abberantly expressed markers	4 (20 %)	6 (30 %)
All 4 markers abberantly expressed	0	1 (5%)

2.6. Virtual double staining, CK8 and Ki67 analysis

Virtual double staining (VDS) is based on the digital alignment of scanned images of two or more parallel slides and their fusion to one image [18]. Digitalized slides stained for H&E, CK8 and Ki67 (the slides had been cut as precise serial sections) were loaded into the "TMA Workflow" module of VIS. The "Image Alignment" module of VIS was then used to create one virtual slide, containing the information from the single slides (Fig. 1). In the next step, the "Image Analysis" module of VIS was used to highlight the region of interest (ROI). For the AIP cases, the virtual Ki67 analysis was performed in two independent steps – first, the ROI consisted of areas with ILDs and next, the ROI consisted of ELDs. For the cases with PDAC, the ROI consisted of areas with neoplastic glands. In the last step, CK8-positive areas were enhanced using filtering of the RGB pixel values and segmented in "tumor cell area" (for PDAC cases) and "epithelial cells" (for AIP cases) and

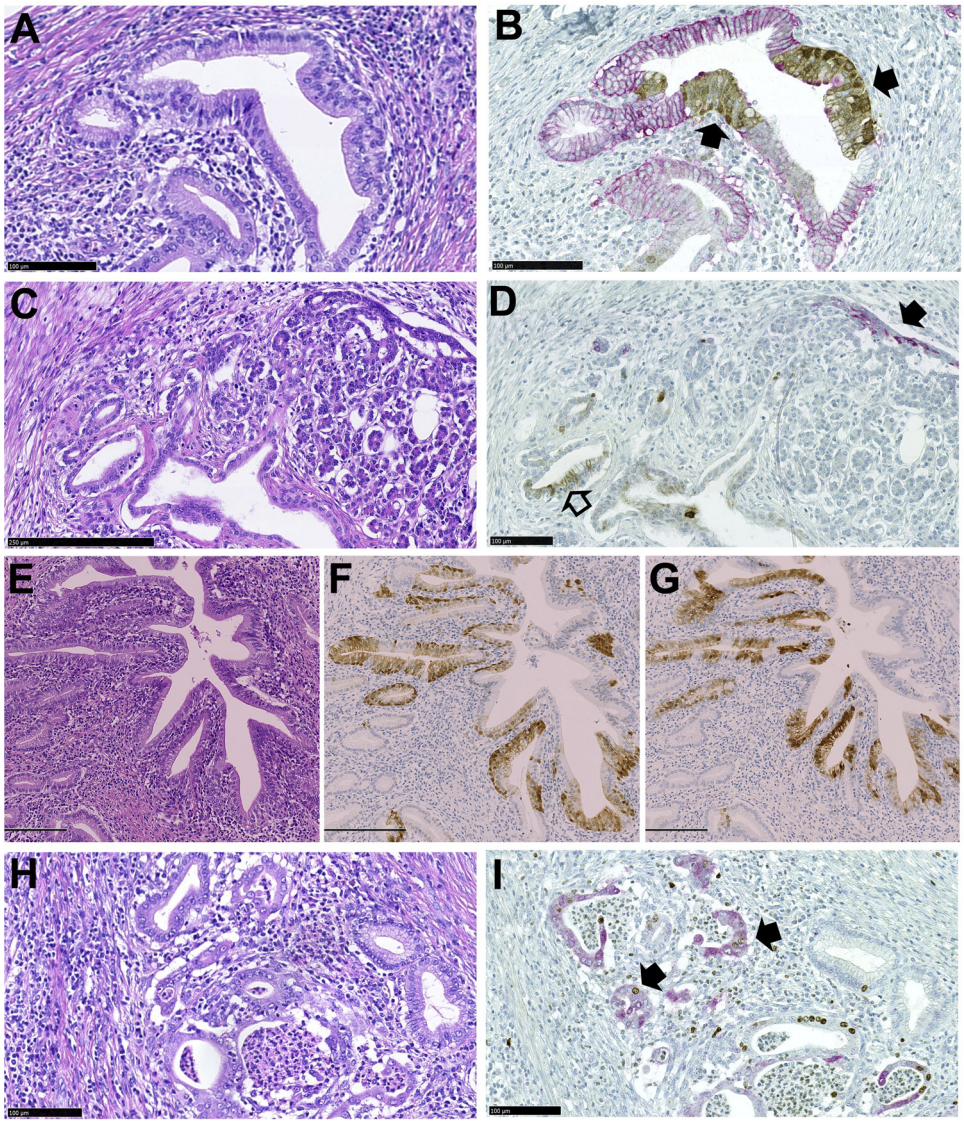

Fig. 6. Expression of the immunohistochemical (IHC) markers pVHL, maspin, IMP3, S100P and Ki67 in AIP. (A-B) Periductal inflammation in type 2 AIP. (A) H&E. (B) Reduced expression of pVHL (red) and overexpression of maspin (brown), occurring in the same epithelial cells (arrows) (Dual-IHC of pVHL (red) and maspin (brown)). (C-D) Lobular inflammation in type 2 AIP. (C) H&E. (D) Overexpression of maspin (brown, open arrows) and IMP3 (red, filled arrows) in small ducts and acinoductal metaplasia, but in different areas (Dual-IHC of maspin (brown) and IMP3 (red)). (E-G) Serial sections, showing periductal inflammation in type 2 AIP. (E) H&E. Overexpression of maspin (F) and S100P (G) in the same ductal epithelial cells. (H-I). Several small ducts with granulocytic epithelial lesions (GELs) in type 2 AIP. (H) H&E. (I) Overexpression of Ki67 (brown) and maspin (red), focally occurring in the same epithelial cells (arrows) (Dual-IHC of Ki67 (brown) and maspin (red)). Scale bars: Fig. 6A-B, D & H-I 100 μm; Fig. 6C & E-G 250 μm.

"stroma" (for PDAC as well as AIP), using a Bayesian classifier, based on in-program stored predefined values. These areas were then transferred to the image of the Ki67-stained slide for further analysis, where nuclear profiles were detected based on form and size, and segmented as either Ki67-positive or –negative, based on pixel-color intensity cut-points. Before analysis, several slides with AIP and PDAC had been examined by visual inspection in order to select the best cut-point between Ki67-positive and –negative cells. Finally, the numbers of positive and negative cells in hot spots within the ROI and the calculated Ki67 HSPI were exported from the programme.

2.7. Statistics

A nonparametric test was selected after evaluating the data with the Shapiro-Wilk normality test. Scatter plots and statistical analyses were created in GraphPad Prism, ver. 5.01 (GraphPad Software, La Jolla, CA, USA), illustrating the immunoscores with standard errors of the mean (SEM). Ordinal data were compared using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test. In the graphs, * corresponds to $P < 0.05$, ** to $P < 0.01$, and *** to $P < 0.001$. For the correlation between estimation of the Ki67 HSPI using manual counting and automated digital imaging analysis of virtual double stains (DIA-VDS), Spearman correlation coefficients were calculated using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA). Level of statistical significance was set as $P < 0.05$.

3. Results

3.1. Evaluation of immunohistochemical expression of pVHL, maspin, IMP3 and S100P

The results of the evaluation of the immunohistochemical expression of pVHL, maspin, IMP3 and S100P in the normal pancreas, AIP and PDAC are given in Table 3 and in Figs. 2–5. There was a clear and statistically highly significant difference in the expression of these markers in the normal pancreas compared to PDAC. When looking at the entire cohort of AIP cases, there was also a statistically significant difference in the expression of pVHL, maspin, IMP3 and S100P in ILDs and ELDs compared to PDAC. However, when comparing each subtype of AIP separately with PDAC, there were the following exceptions, where no statistically significant difference between AIP and PDAC was found: pVHL (Fig. 2B) and maspin (Fig. 3B) in ELDs of type 2 AIP and IMP3 (Fig. 4B) in ILDs of type 1 and type 2 AIP.

When comparing the expression of these markers in ductal epithelium in the normal pancreas with ILDs and ELDs in AIP, the aberrant expression reached statistical significance for pVHL and maspin in ELDs of type 2 AIP (Figs. 2B and 3 B). However, the IHC score did in most cases not exceed score 1 or score 2 (Figs. 2–5). The aberrant expression

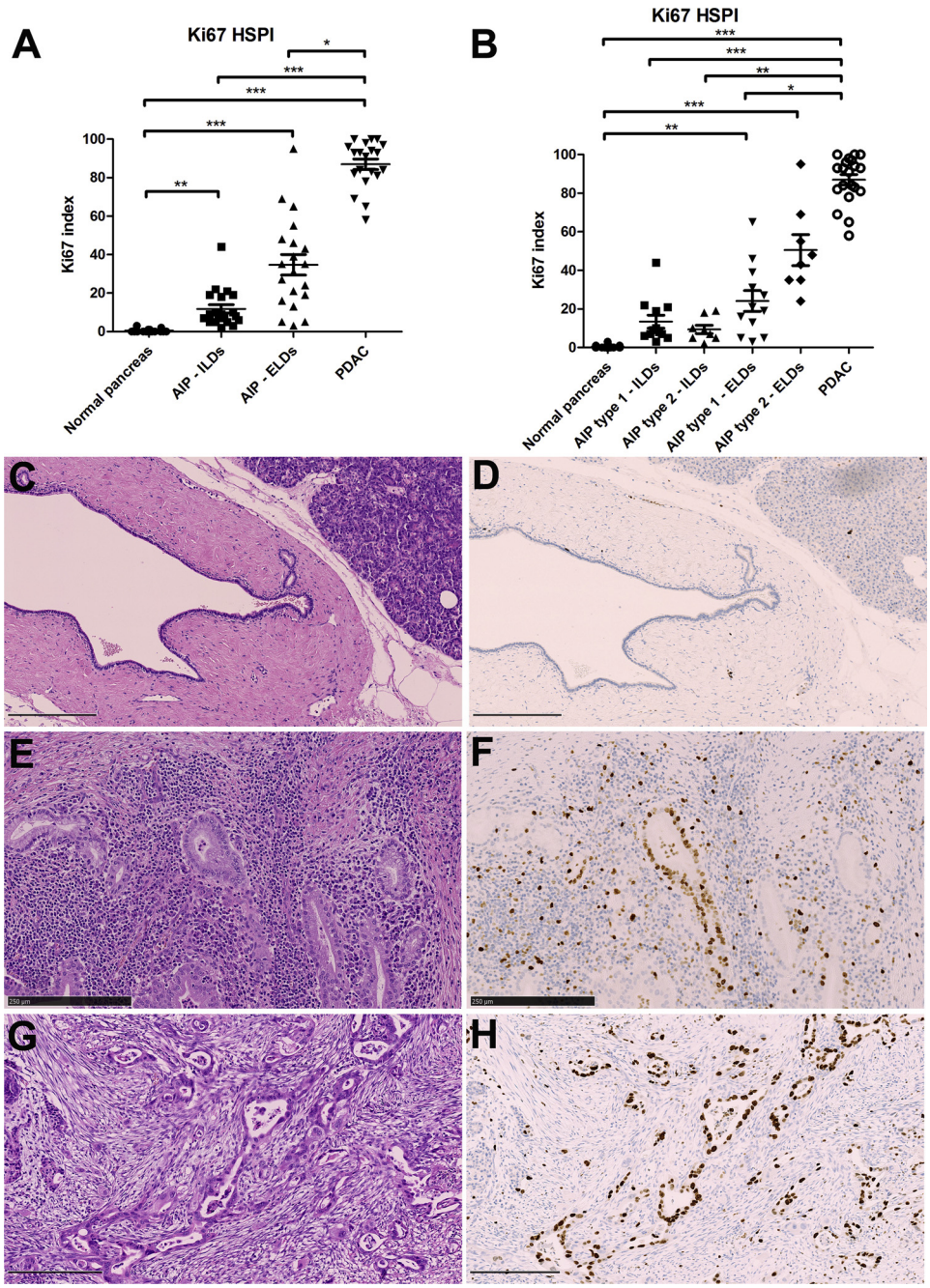

Fig. 7. Immunohistochemical expression of Ki67 in the normal pancreas, autoimmune pancreatitis (AIP) and pancreatic ductal adenocarcinoma (PDAC). (A) Manual Ki67 hot spot indices (Ki67 HSPI) in the normal pancreas, extralobular ducts (ELDs) and intralobular reactive ducts / acinoductal metaplasia (ILDs) in AIP and in PDAC (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (B) Ki67 HSPI in normal pancreas, ILDs and ELDs in type 1 AIP and type 2 AIP, and PDAC (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (C) ELD in the normal pancreas (H&E). (D) Lack of Ki67 expression in ductal epithelial cells in the normal pancreas. (E) Lymphoplasmacytic inflammation in AIP type 2 (H&E). (F) Ki67 overexpression in epithelial cells of inflamed ducts in AIP type 2. (G) PDAC, showing infiltrating gland- and bud-like structures (H&E). (H) Strong Ki67 overexpression in the neoplastic glands. Scale bars: Fig. 7C-F 250 μm; Fig. 7G-H 100 μm.

of pVHL and maspin was most pronounced in ELDs of type 2 AIP (Table 3, Figs. 2B, 3 B and 5 B). Overexpression of IMP3 in AIP was only observed in ILDs, not in ELDs ($P < 0.05$) (Fig. 4B). Table 4 shows the number AIP cases that showed either one, two, three or even four focal and weak aberrantly expressed (score 1 or more) markers. In ILDs and ELDs in AIP, at least two markers were aberrantly expressed in 45 % and 60 % (Table 4). Sometimes, aberrant expression of different markers in the same structures and even in the same epithelial cells was observed, for example of maspin together with pVHL, S100P or Ki67, but not of maspin with IMP3 (Fig. 6).

3.2. Ki67 hot spot proliferation index in autoimmune pancreatitis, pancreatic ductal adenocarcinoma and normal pancreas

The Ki67 HSPI in PDAC cases was high, with a median of 92 % and range of 58%–100% (Table 3, Figs. 6H–I and 7). In the normal pancreas, the median was 0% (range 0-3%). The differences in Ki67 HSPI between PDAC and the normal pancreas were statistically highly significant ($P < 0.001$). When looking at the entire AIP cohort, there was also a statistically significant difference between Ki67 HSPI in PDAC and ILDs and ELDs in AIP ($P < 0.05$ and $P < 0.001$) (Fig. 7A). However, in some cases with AIP, focally increased Ki67 HSPI values compared to the normal pancreas were found, in ILDs ($P < 0.01$) and in ELDs ($P < 0.001$). The median Ki67 HSPI in ILDs and ELDs in AIP was 8.5 % (range 2–44%) and 33 % (range 3–95%). The increased Ki67 HSPI in AIP was most pronounced in ELDs in type 2 AIP, and there was no statistically significant difference between ELDs in this subtype of AIP and PDAC (Table 3, Fig. 7B).

3.3. Correlation of Ki67 hot spot proliferation index by manual counting with automated DIA-VDS

The correlation of Ki67 HSPI assessed by manual counting compared to automated DIA-VDS was good. The best correlation was found in ILDs (Pearson's correlation coefficient 0.88), followed by ELDs (0.83) and PDAC (0.77) (data not shown).

4. Discussion

Our study shows that the expression of the immunohistochemical markers pVHL, maspin, IMP3, S100P and Ki67 is significantly different in PDAC compared to epithelial cells in normal pancreatic ducts, in accord with previous studies [17,19]. We also found a statistically significant difference in the expression of these markers in PDAC compared to AIP. However, focal weak to moderate aberrant expression of pVHL, maspin, IMP3, S100P, and Ki67 was observed in several of the AIP cases. In ILDs and ELDs in AIP, at least two markers were focal and weak aberrantly expressed in 45 % and 60 %. The focal weak aberrant expression was more pronounced in type 2 AIP compared to type 1 AIP and in ELDs compared to ILDs, with the exception of IMP3, that only was overexpressed in ILDs and newer in ELDs. Hence, our data indicate that this panel of immunohistochemical markers is useful for the distinction of AIP from PDAC, but that they should be used in combination, as a panel, particularly in type 2 AIP.

The diagnosis of AIP, particularly the diagnosis of type 2 AIP that is more frequent in Western countries as compared to East Asia, relies often on histology, because a serological marker for type 2 AIP is currently lacking [3]. Moreover, the value of serum IgG4 for the diagnosis of type 1 AIP is limited in some patients, particularly in some Western countries [4,20,21]. As the differential diagnosis of ILDs secondary to pancreatitis versus low-grade pancreatic adenocarcioma can be challenging when using EUS-guided histological fine-needlbe biopsies where the amount of tissue often is limited, it is sometimes important to use immunohistochemical markers as ancillary tools [14,17]. To our knowledge, this is the first report on the expression of pVHL, maspin, IMP3, S100P and Ki67 in AIP. We found one study that had examined the expression of IMP3 in PDAC and AIP, reporting a focal and weak IMP3 staining in 33 % of biopsies and 11 % of resection specimens with sclerosing pancreatitis, with an expression pattern in inflamed areas containing ILDs, similar to our data [22]. In our study, S100P was the most robust of the examined markers, as it only showed aberrant expression in ILDs in 10 % of our AIP cases.

In the present study, KI67 HSPIs were significantly higher in PDAC compared to the normal pancreas and AIP, but a considerable (focal) upregulation of Ki67 in both ILDs and ELDs of AIP was noted. In some cases, the KI67 HSPI was surprisingly > 50 %, particularly in ELDs of type 2 AIP. Manual estimates of KI67 showed a good correlation with automated DIA-VDS, and thus virtual double stains may be considered a valid method to estimate the KI67 index in PDAC and AIP. It is important to note that the inflammation in AIP often is severe and centered around small and medium-sized as well as large ducts. In this regard, it has to be emphasized that we evaluated the Ki67 index in hot spots of 100 cells, unlike most of the studies that examined the Ki67 index in pancreatitis other than AIP. Unfortunately, we were not able to identify previous studies on the expression of Ki67 in AIP in the English-language literature.

Several recent studies on the utility of EUS-guided pancreatic fine needle biopsy showed a high sensitivity and specificity, including for the diagnosis of AIP [5,11,23]. When using the examined panel of immunohistochemical markers together with the well-established morphological features of AIP, they should be able to contribute in the distinction of this entity from PDAC, particularly when combined with immunostains for IgG4 and PD-L1. IgG4 is a well-established (even though not perfect) marker for type 1 AIP, and recently, it was reported that Programmed death-ligand 1 (PD-L1) is overexpressed in type 2 AIP, but virtually absent in ductal structures of type 1 AIP, obstructive chronic pancreatitis and peritumoral pancreatic tissue in PDAC [24].

In conclusion, our data demonstrate that the markers pVHL, maspin, IMP3, S100P, and Ki67 are useful adjuncts to differentiate PDAC from AIP, in addition to the microscopic features. However, as weak and focal aberrant expression may be found in AIP, these markers should be used in a panel.

Authors contribution

Study conception and design: SD and GHJ. Acquisition and analysis of data: All authors. Supervision: SD. Drafting of the manuscript: SD, GHJ. Critical revision: All authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] S.T. Chari, T.C. Smyrk, M.J. Levy, M.D. Topazian, N. Takahashi, L. Zhang, et al., Diagnosis of autoimmune pancreatitis: the Mayo Clinic experience, Clin. Gastroenterol. Hepatol. 4 (2006) 1010–1016.
[2] M.J. Levy, M.J. Wiersema, S.T. Chari, Chronic pancreatitis: focal pancreatitis or cancer? Is there a role for FNA/biopsy? Autoimmune pancreatitis, Endoscopy 38 (Suppl. 1) (2006) S30–S35.
[3] T. Shimosegawa, S.T. Chari, L. Frulloni, T. Kamisawa, S. Kawa, M. Mino-Kenudson, et al., International consensus diagnostic criteria for autoimmune pancreatitis: guidelines of the International Association of Pancreatology, Pancreas 40 (2011) 352–358.
[4] S. Detlefsen, J.D. de Vos, J.T. Tanassi, N.H.H. Heegaard, C. Fristrup, O.B. Schaffalitzky de Muckadell, Value of anti-plasminogen binding peptide, anti-carbonic anhydrase II, immunoglobulin G4, and other serological markers for the differentiation of autoimmune pancreatitis and pancreatic cancer, Medicine (Baltimore) 97 (2018) e11641.
[5] S. Detlefsen, M.T. Joergensen, M.B. Mortensen, Microscopic findings in EUS-guided fine needle (SharkCore) biopsies with type 1 and type 2 autoimmune pancreatitis, Pathol. Int. 67 (2017) 514–520.
[6] S. Detlefsen, M.B. Mortensen, T.K. Pless, A.S. Cribe, O.B. de Muckadell, Laparoscopic and percutaneous core needle biopsy plays a central role for the diagnosis of autoimmune pancreatitis in a single-center study from Denmark, Pancreas 44 (2015) 845–858.
[7] F. Maire, B.Y. Le, V. Rebours, M.P. Vullierme, A. Couvelard, H. Voitot, et al., Outcome of patients with type 1 or 2 autoimmune pancreatitis, Am. J. Gastroenterol. 106 (2011) 151–156.
[8] S. Detlefsen, G. Zamboni, L. Frulloni, B. Feyerabend, F. Braun, O. Gerke, et al., Clinical features and relapse rates after surgery in type 1 autoimmune pancreatitis differ from type 2: a study of 114 surgically treated European patients, Pancreatology 12 (2012) 276–283.
[9] R.P. Sah, S.T. Chari, R. Pannala, A. Sugumar, J.E. Clain, M.J. Levy, et al., Differences in clinical profile and relapse rate of type 1 versus type 2 autoimmune pancreatitis, Gastroenterology 139 (2010) 140–148.
[10] S. Detlefsen, IgG4-related disease: a systemic condition with characteristic microscopic features, Histol. Histopathol. 28 (2013) 565–584.
[11] M.H. Larsen, C.W. Fristrup, S. Detlefsen, M.B. Mortensen, Prospective evaluation of EUS-guided fine needle biopsy in pancreatic mass lesions, Endosc. Int. Open 6 (2018) E242–e248.
[12] C.J. DiMaio, J.M. Kolb, P.C. Benias, H. Shah, S. Shah, O. Haluszka, et al., Initial experience with a novel EUS-guided core biopsy needle (SharkCore): results of a large North American multicenter study, Endosc. Int. Open 4 (2016) E974–979.
[13] D.G. Adler, B. Witt, B. Chadwick, J. Wells, L.J. Taylor, C. Dimaio, et al., Pathologic evaluation of a new endoscopic ultrasound needle designed to obtain core tissue samples: a pilot study, Endosc. Ultrasound 5 (2016) 178–183.
[14] G. Klöppel, N.V. Adsay, Chronic pancreatitis and the differential diagnosis versus pancreatic cancer, Arch. Pathol. Lab. Med. 133 (2009) 382–387.
[15] S.D. Slater, R.C. Williamson, C.S. Foster, Proliferation of parenchymal epithelial

cells enhanced in chronic pancreatitis, J. Pathol. 186 (1998) 104–108.
[16] A.C. Buck, H.H. Schirrmeister, C.A. Guhlmann, C.G. Diederichs, C. Shen, I. Buchmann, et al., Ki-67 immunostaining in pancreatic cancer and chronic active pancreatitis: does in vivo FDG uptake correlate with proliferative activity? J. Nucl. Med. 42 (2001) 721–725.
[17] H. Liu, J. Shi, V. Anandan, H.L. Wang, D. Diehl, J. Blansfield, G. Gerhard, F. Lin, Reevaluation and identification of the best immunohistochemical panel (pVHL, Maspin, S100P, IMP-3) for ductal adenocarcinoma of the pancreas, Arch. Pathol. Lab Med. 136 (2012) 601–609.
[18] R. Røge, R. Riber-Hansen, S. Nielsen, M. Vyberg, Proliferation assessment in breast carcinomas using digital image analysis based on virtual Ki67/cytokeratin double staining, Breast Cancer Res. Treat. 158 (2016) 11–19.
[19] S. Jeong, D.H. Lee, J.I. Lee, J.W. Lee, K.S. Kwon, P.S. Kim, et al., Expression of Ki-67, p53, and K-ras in chronic pancreatitis and pancreatic ductal adenocarcinoma, World J. Gastroenterol. 11 (2005) 6765–6769.
[20] T. Kamisawa, K. Takuma, T. Tabata, Y. Inaba, N. Egawa, K. Tsuruta, et al., Serum IgG4-negative autoimmune pancreatitis, J. Gastroenterol. 46 (2011) 108–116.
[21] W.H. Paik, J.K. Ryu, J.M. Park, B.J. Song, J.K. Park, Y.T. Kim, et al., Clinical and pathological differences between serum immunoglobulin G4-positive and -negative type 1 autoimmune pancreatitis, World J. Gastroenterol. 19 (2013) 4031–4038.
[22] D.L. Wachter, A. Schlabrakowski, J. Hoegel, G. Kristiansen, A. Hartmann, M.O. Riener, Diagnostic value of immunohistochemical IMP3 expression in core needle biopsies of pancreatic ductal adenocarcinoma, Am. J. Surg. Pathol. 35 (2011) 873–877.
[23] C.J. DiMaio, J.M. Kolb, P.C. Benias, H. Shah, S. Shah, O. Haluszka, et al., Initial experience with a novel EUS-guided core biopsy needle (SharkCore): results of a large North American multicenter study, Endosc. Int. Open 4 (2016) E974–E979.
[24] R. Gupta, A. Neyaz, A. Chougule, M. Akita, Y. Zen, D. Forcione, et al., Autoimmune pancreatitis type 2: diagnostic utility of PD-L1 immunohistochemistry, Am. J. Surg. Pathol. 43 (2019) 898–906.