Diploma thesis

Impact of invasion type of ductal adenocarcinoma of the pancreas on local recurrence and distant metastasis

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Graz, am 27.07.2018 *Marc Steffen Hoyler eh.*

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Abstract

Background

Pancreatic ductal adenocarcinoma (PDAC) is characterised by unfavourable long-term survival. Perineural, lymphovascular, and microvascular invasion (PNI, LVI and MVI) as well as the number of lymph node metastases and resection margin status are known risk factors. However, the impact of the exact number of such invasion events on disease-free and overall survival is not known. In this study we therefore aimed to assess the role of the number of perineural, lymphovascular and microvascular invasions, and the distance of the carcinoma to the resection margins and compare the prognostic utility of these and other histological and clinical factors for the prediction of disease-free and overall survival.

Methods

A retrospective detailed histopathological evaluation of 88 PDAC cases with completely sampled pancreatic head after classical or pylorus-preserving Whipple procedure was conducted. Each case was evaluated for the exact number of perineural, lymphovascular and microvascular invasions, the number of lymph node metastases and the distance of the carcinoma from the resection margins was measured. In addition, demographic data, comorbidities, patient management characteristics and histopathological features were analysed for their impact on disease-free survival and overall survival.

Results

Kaplan-Meier analysis showed that cases with ≥ 30 perineural invasions, as well as lymphovascular and microvascular invasion were associated with shorter disease-free and overall survival as compared to patients without these morphological features, whereas no differences were found with respect to a distance from the circumferential resection margin of more or less than 1mm.

For disease-free survival univariate Cox regression identified tumour differentiation grade, >30 PNI, LVI, MVI, presence of lymph node metastasis, ratio of total to cancer-infested lymph nodes (LNR) as histological and preoperative symptoms and intraoperative treatment with inotropics as clinical parameters associated with

shorter DFS. Multivariate Cox regression revealed grade, LNR and preoperative symptoms (weight loss, pancreatitis) as independent predictors of DFS.

For overall survival the same histological and similar clinical parameters as for DFS and Charlson Comorbidity Index were found to be associated with OAS on univariate Cox regression. However, only MVI and LNR were identified as histological and preoperative symptoms as clinical independent predictors of outcome.

Conclusion

DFS and OAS is best predicted by a combination of histological and clinical factors. Complete sampling of the pancreatic head in cases with PDAC enables efficient detection of morphological features of invasion of which tumour differentiation grade, PNI>30 and MVI emerged as potent predictors of outcome.

Zusammenfassung

Hintergrund

Das Pankreaskarzinom stellt einen hochaggressiven Tumor mit nach wie vor schlechtem Langzeitüberleben dar. Nervenscheiden-, Lymphgefäß- und Blutgefäßeinbrüche sowie die Anzahl der tumorbefallenen Lymphknoten und der Status des Resektatrands sind als prognostische Faktoren beschrieben. Es ist jedoch nicht bekannt welche exakte Anzahl der zuvor genannten Invasionen einen Einfluss auf das tumorfreie Überleben sowie das Gesamtüberleben hat. Das Ziel dieser Studie war es daher festzustellen, welchen Einfluss die Anzahl an Nervenscheiden-, Lymphgefäß- und Blutgefäßeinbrüche sowie die Tumorentfernung zum Resektatrand auf das tumorfreie Überleben und Gesamtüberleben hat. Zudem sollte der prognostische Wert von diesen und weiteren histologischen und klinischen Faktoren für das tumorfreie Überleben und Gesamtüberleben bestimmt werden.

Methoden

Es erfolgte eine retrospektive histopathologische Auswertung von 88 vollständigen Operationspräparaten von Personen mit duktalem Adenokarzinom des Pankreaskopfes, welche nach Whipple (klassisch oder pyloruserhaltend) reseziert worden waren. Für jeden Fall wurden Lymphgefäß- und Blutgefäßeinbrüche, sowie Nervenscheideninfiltrationen und die Distanz des Tumors zu den Resektaträndern ermittelt. Zusätzlich wurde der Einfluss von demografischen Daten, Komorbiditäten, perioperativen Parametern und weiteren histopathologischen Eigenschaften auf das tumorfreie Überleben und Gesamtüberleben evaluiert.

Ergebnisse

Die isolierte Betrachtung der Invasionstypen mit der Kaplan Meier Analyse ergab für ≥ 30 Nervenscheideneinbrüche sowie für Lymphgefäß- und Blutgefäßinvasion ein reduziertes tumorfreies Überleben und Gesamtüberleben, wohingegen ein tumorfreier Resektatrand von mehr oder weniger als 1mm keinen signifikanten Unterschied hinsichtlich des Outcomes ergab.

Für das tumorfreie Gesamtüberleben stellten sich in der univariaten Cox-Regression folgende Parameter als prognostische Faktoren heraus:

Histopathologisches Grading, ≥ 30 Nervenscheideninfiltrationen, Lymphgefäßinvasion, Blutgefäßinvasion, Lymphknotenmetastasen, das Verhältnis von resezierten zu krebsbefallenen Lymphknoten (LNR) sowie präoperative Symptome und intraoperative Vasopressorapplikation. Die multivariate Cox-Regression ermittelte histopathologisches Grading, die LNR und präoperative Symptome (Gewichtsverlust und Pankreatitis) als Prädiktoren für das Auftreten eines Rezidivs.

Für das Gesamtüberleben erwiesen sich dieselben histologischen und ähnliche klinische Parameter sowie der Charlson Comorbidity Index als Prädiktoren. Die multivariate Cox-Regression konnte allerdings lediglich Blutgefäßinvasion, das Lymphknotenverhältnis (>0.2), und präoperative Symptomatik als unabhängige Prädiktoren des Gesamtüberlebens feststellen.

Schlussfolgerung

Die Ergebnisse zeigen, dass sich tumorfreies Überleben und das Gesamtüberleben von Personen mit duktalem Adenokarzinom des Pankreaskopfes am besten durch eine Kombination von histologischen und klinischen Parametern abschätzen lassen. Durch die gründliche Untersuchung des gesamten Operationspräparats kann eine präzise Einschätzung des Krankheitsverlaufs anhand der Prädiktoren histopathologisches Grading, ≥ 30 Nervenscheideninfiltrationen sowie Blutgefäßinvasion erfolgen.

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List of Abbreviations

CA 19-9	Carbohydrate antigen 19-9
CR	Cancer recurrence
CRM	Circumferential resection margin
DFS	Disease-free survival
DM	Diabetes mellitus
ECOG	Eastern Cooperative Oncology Group
HR	Hazard ratio
IPMN	Intraductal papillary mucinous neoplasm
LNR	Lymph node ratio
LVI	Lymphovascular invasion
MCN	Mucinous cystic neoplasm
MVI	Microvascular invasion
NCCN	National Comprehensive Caner Network
OAS	Overall survival
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PNI	Perineural invasion
PPPD	pylorus preserving pancreaticoduodenectomy
95%CI	95% confidence interval

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1 Introduction

1.1 Epidemiology of pancreatic ductal adenocarcinoma

The worldwide incidence of pancreatic ductal adenocarcinoma (PDAC) is increasing. High incidence is reported in developed regions such as Northern America and Western Europe [1]. It is the fourth most frequent cause of cancer-related death in Europe [2]. In Western countries 3 to 10 new cases per 100 000 inhabitants are observed annually and the prevalence is slightly higher in men as compared to women (male-to-female ratio of 1.5:1) [3]. The highest rates of PDAC is found in indigenous people in Oceania and in African Americans [4, 5]. The increased occurrence in African Americans may result from a higher frequency of mutations in the Kras gene, one of the key genetic alterations driving PDAC development. Indians, South-East Asians and Central Africans seem to have the lowest rates of PDAC. However, this could be biased by a high rate of undiagnosed cases [4, 5]. Approximately 80-90% of patients are in an inoperable state of their PDAC at first diagnosis and median survival is only 4 months which results in matching incidence and mortality rates [3, 6]. Most of PDAC develop spontaneously and only 5-10% are the result of genetic disorders [3].

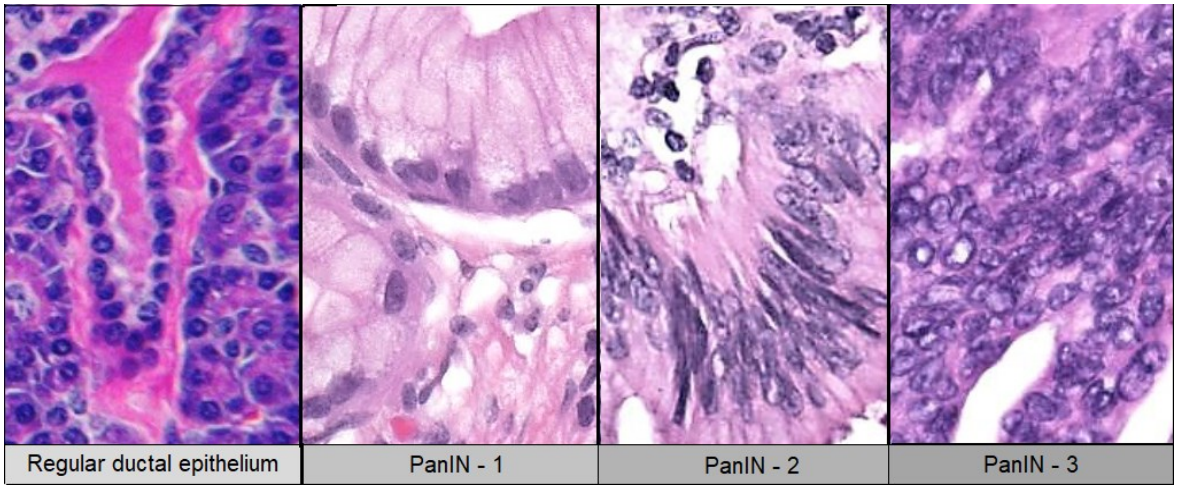
1.2 PDAC pathogenesis and precursor lesions

The precursors of PDAC, so called pancreatic intraepithelial neoplasias (PanIN), are microscopic lesions (<5mm in size) residing within the pancreatic duct and its branches [8]. PanIN is classified into three different grades, according to the severity of cytological and architectural changes (Figure 1; Table 1).

Genetic alterations associated with the development of PanIN types and finally PDAC occur in a sequential manner in proto-oncogenes and tumour suppressor genes [9]. The mutation of proto-oncogenes leads to a gain of function stimulating tumour progression. The mutation of tumour suppressor genes in contrast leads to a loss of function disrupting the induction of apoptosis or their inhibiting effect on the cell cycle [3]. As summarized in a simplified fashion in Figure 2 the progression of PanIN is thought to occur in a step-wise manner. Each step corresponding to a

PanIN type is characterised by certain genetic alterations [10]. However, the speed of progression towards invasive cancer in an individual case remains unknown [9].

Figure 1: Regular ductal epithelium and PanINs



The regular epithelium shows cuboidal cells with round nuclei. The lumen is filled with pancreatic juice. PanIN-1 consists of high-columnar cells with round to oval nuclei placed at the cellular base whereas PanIn-2 shows papillary growth with nuclear variation and altered nuclear location. In PanIN-3 nuclear irregularities and a loss of nuclear polarity can be observed.

Table 1: Histopathological differences of PanIN

Characteristics of regular ducal epithelium and PanINs		
	Architecture and cellular appearance	**Nuclear characteristics**
Regular	flat epithelium, cuboidal or low-columnar	round, basally located
PanIN-1a	flat epithelium, tall-columnar, ample production of mucin	round to oval, basally located, no aberrations
PanIN-1b	papillary or micropapillary epithelium, tall-columnar, ample production of mucin	oval, perpendicular to the base, no aberrations
PanIN-2	flat or papillary epithelium, production of mucin	nuclear size variation, hyperchromatism, altered nuclear location, rarely mitoses
PanIN-3	papillary, flat or cribriform epithelium, cellular budding off, luminal necrosis	loss of nuclear polarity, nuclear irregularities, occasionally abnormal mitoses

based on Hruban R. H. et Fukushima N., Modern Pathology volume 20, pages S61–S70 (2007)

Key genetic alterations in the progression of PDAC include:

Kras is the most frequently altered gene with a prevalence of about 95% in PDAC. As illustrated in Figure 2 mutations often occur early and are thus often already present in PanIN-1 lesions. Kras mutations are associated with inhibition of

apoptosis, promotion of cellular proliferation, enhanced cellular migration, invasion and metastasis [10].

P53 arrests the cell cycle and induces apoptosis in settings of massive cellular injury and genetic damage. P53 mutations usually occur in later stages and accompany the transition from PanIN-3 into invasive carcinoma [10].

P16 is a tumour suppressor gene involved in cell cycle arrest and is altered in about 90% of PDAC [10].

SMAD4 is a tumour suppressor gene inhibiting TGFß signalling and therefore exerting growth inhibiting effect. Mutations cause cell growth as the restricting effect on TGFß ceases [10].

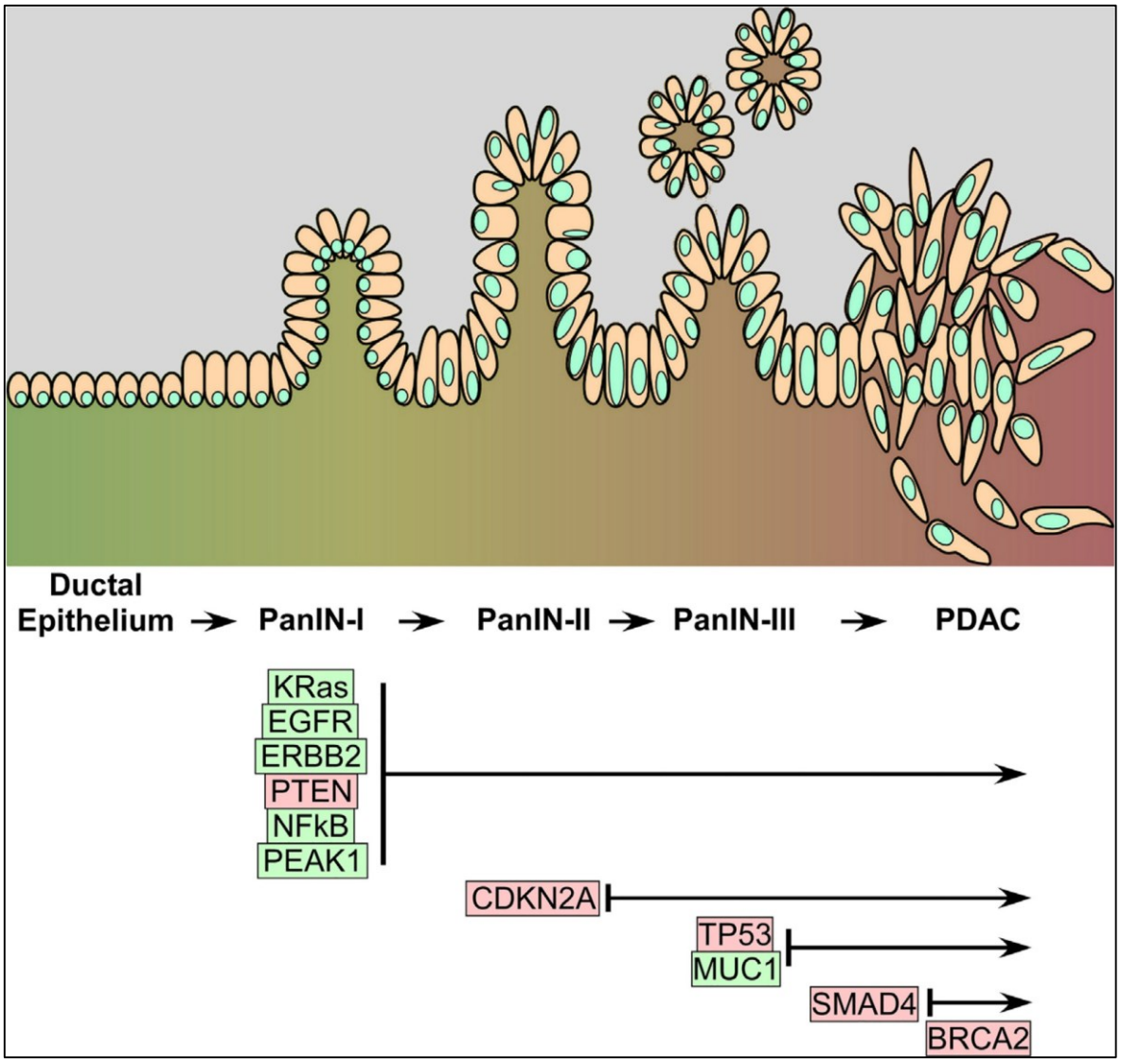
MUC1 is a glycoprotein often overexpressed in PanIN. Tumour growth is accelerated especially when also Kras mutation is prevalent. MUC1 mutation has been shown to increase invasive behaviour and metastasis [10].

EFGR, epidermal growth factor receptor, is a transmembrane receptor activating pathways within the cell which finally promote acinar-ductal metaplasia. A dimerization of EGFR with **ERBB2** supports proliferation and migration. If ERBB2 is aberrant cell growth can be increased uncontrollably [10].

PEAK1 impacts cytoskeleton structure as well as the cellular shape and therefore controls cell migration. Especially in PDAC its overexpression and interaction with growth factors support cell motility and proliferation [10].

Other precursor lesions of PDAC are the intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN) (Figure 3) [3]. MCN occurs in women only and is usually located in the pancreatic tail. These neoplasms consist of a single or multiple mucus filled cyst(s) lined by mucinous epithelium of variable dysplasia [3, 11]. In the wall of the cyst(s) so called cytogenic stroma, histologically and immunohistochemically resembling ovarian stroma is found. MCN with severely dysplastic epithelium can give rise to invasive adenocarcinoma either of classical ductal or more frequently of colloid type. Approximately one-third of MCN will eventually develop into invasive carcinoma [11].

Figure 2: Schematic progression from regular epithelium to PDAC



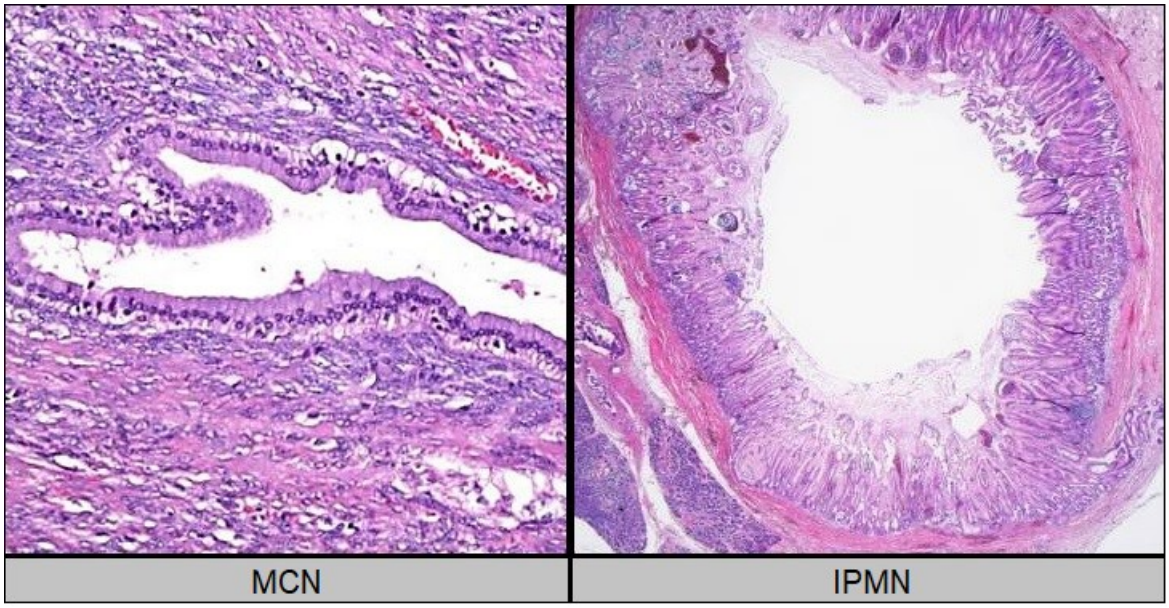
taken from Gharibi A. et al., Acta Histochem. 2016 April; 118(3): 305–316.

Morphologic and genetic alterations in the development of PDAC from PanIN with increasing grades of dysplasia: PanIN-1 is characterised by tall-columnar to papillary formations as compared to the regular cuboid epithelium. PanIN-2 is characterised by nuclear variation both in size and location. In PanIN-3 the grade of cellular atypia is increased and the "budding-off" of smaller cell clusters can be observed. Irrespective of the grade of differentiation all precursor lesions of the PDAC do not penetrate the basal membrane until final PDAC development. PDAC is characterised by pancreatic invasion and remote metastasis. The bottom of the figure depicts the most frequent genetic mutations in precursor lesions according to their stage. The arrows indicate the possibility of an occurrence also later on in PDAC carcinogenesis. The green genes illustrate a gain of function whereas the red genes indicate a loss of function.

IPMN is characterised by intraductal papillary structures covered by epithelial cells of ductal differentiation and various extent of dysplasia. The lesions of IPMN are macroscopically visible and are associated with the production of viscous mucin often obstructing the main pancreatic duct or its branches leading to dilation and elongation of the ducts distal of the obstruction and to atrophy of the drained

segment of the pancreatic parenchyma. IPMN can be subdivided into 4 different types according to the differentiation of the epithelium in a pancreatobiliary, oncocytic, intestinal and gastric type. While the gastric type often occurs in the smaller pancreatic ducts, the other types are mostly detected in the main duct. The pancreatobiliary and oncocytic type more often progress to classical PDAC, whereas the intestinal type can give rise to colloid carcinoma [3, 11]. Low-grade IPMN shows a single layer of columnar cells with nuclei situated at the base of the epithelial cells. The nuclei show minimal atypia. IPMN with moderate dysplasia is characterised by a higher degree of nuclear atypia with nuclear enlargement, pleomorphism and pseudostratification. High-grade IPMN forms pseudopapillary and/or even cribriform structures composed of highly atypical epithelial cells [3, 12].

Figure 3: Microscopic view on MCN and IPMN



MCN is characterised by the epithelial lining of single or multiple cyst(s) producing mucin and subepithelially located ovarian stroma. IPMN is characterised by intraductal, atypical epithelium forming papillary structures and ample production of mucin.

1.3 Risk factors for PDAC

Risk factors for PDAC development include age, gender, type 2 diabetes mellitus (DM), chronic pancreatitis, race, smoking and overweight/obesity.

Age: Main incidence of PDAC is between the age of 65 and 85. Only rarely these tumours occur under the age of 40. Median age at the point of diagnosis is 72 years [3, 7].

Gender: With a distribution of 1.5:1 males are a little more likely to develop PDAC than females [3].

Type 2 DM: Patients suffering from type 2 DM bear a 1.5-2-fold increased risk of developing PDAC [13].

Chronic pancreatitis: The chronic inflammatory process of chronic alcoholic or biliary pancreatitis is said to pave the way for PDAC development via cytokine- and reactive oxygen species-mediated DNA damage [14].

Race: Ethnicity has great impact on the incidence of PDAC. An epidemiologic study conducted in the USA showed that African Americans are at considerably higher risk for PDAC development (15.8 per 100 000) than for example non-Hispanic whites (12.0 per 100 000), Hispanics (10.7 per 100 000) or Asians (9.5 per 100 000) [4, 5, 15].

Smoking: The risk for PDAC development doubles with smoking compared to the non-smoking population [7].

Overweight and obesity: Studies have identified obesity and increased body circumference to be directly linked with an increased risk for PDAC development. Whether physical activity has an impact on the reduction of PDAC incidence is subject of debate [15, 16].

1.4 Genetic disorders leading to PDAC

Around 90-95% of PDAC are caused by the spontaneous genetic alteration of precancerous lesions. However, some hereditary diseases are associated with a disposition for development of PDAC. These disorders are responsible for 5-10% of PDAC cases [3, 17]. Familial pancreatic cancer is defined as the occurrence of PDAC in at least two first-grade relatives [17].

Early activation or insufficient deactivation of trypsinogen in hereditary pancreatitis precipitates an autodigestion-associated destruction of pancreatic parenchyma. Ongoing inflammation triggers a cytokine-supported proliferation of epithelial cells eventually leading to malignant degeneration. A similar mechanism may underlie the association of PDAC and cystic fibrosis, except that for cystic fibrosis the

inflammation is a consequence of a CFTR gene defect eventually leading to obliteration of the pancreatic duct due to thickened secretion [17].

The hereditary breast and ovarian cancer syndrome (HBOC) results from genetic mutation in the genes BRCA1 or BRCA2. BRCA2 shifts are the most frequently observed mutations of hereditary PDAC. In 17-19% of familial pancreatic cancers and in 7.3% of all spontaneous pancreatic cancers alterations of the BRCA2 gene are found [17].

Lynch syndrome, also referred to as hereditary non-polyposis colorectal cancer, is the most frequent hereditary colorectal cancer syndrome. Besides PDAC the cancers of the endometrium, ovary, stomach, small intestine, urinary tract and brain are also contained in the spectrum of Lynch syndrome-associated malignancies. A defect in mismatch repair genes, especially MSH2, MSH6 und MLH1 are accountable for cancer development. Histologically the cancers often are poorly differentiated with a solid or medullary histological pattern [17].

Besides an increased incidence of malignant melanoma and atypical melanocytic naevi in first- and second-grade relatives, patients suffering from familial atypical multiple mole melanoma are at risk of developing PDAC, caused by a mutation in the p14INK4a gene [17].

The autosomal-dominant hereditary Peutz-Jeghers syndrome is associated with a defective STK11 gene usually coding for a tumour suppressor gene. The gene defect leads to gastrointestinal polyps (hamartomas) and mucocutaneous pigmentation. Besides the risk of bleeding from hamartomas the mutation is also associated with an increase in both gastrointestinal and extraintestinal tumours [17].

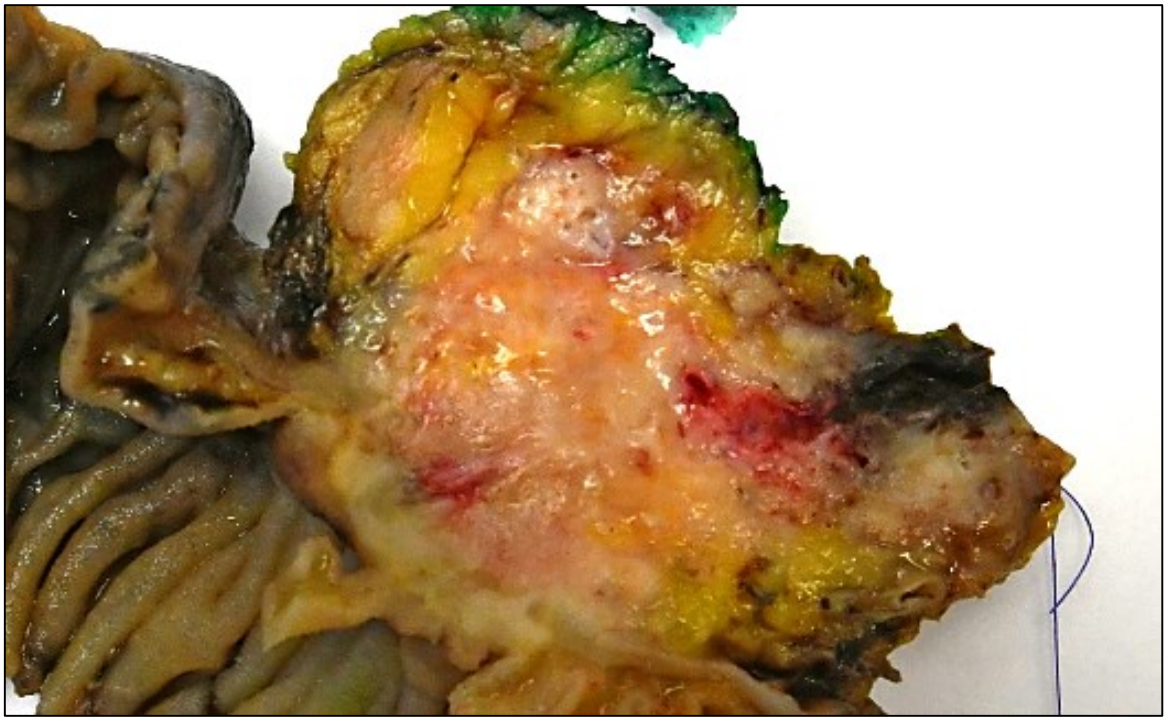
1.5 Pathological features of PDAC

1.5.1 Macroscopic aspects

The PDAC usually imposes as a solid tumour of 1-5cm in diameter located in about 60-70% of cases in the head of the pancreas [8]. Tumours found in the body or tail of the pancreas (20-25%) can become substantially larger bevor causing any symptoms. In approximately 10-20% cases diffuse spread of the carcinoma within the pancreas parenchyma is observed [8]. Depending on tumour stage PDAC can

also involve the duodenal wall and/or the ampulla of Vater [8]. On gross pathology PDAC often presents as a poorly demarcated mass with a grey-white to yellow cut surface and firm consistency (Figure 4). Microcystic areas and necrosis are features of larger tumours. PDAC frequently causes obstruction of the intrapancreatic part of the bile duct as well as the main pancreatic duct. Secondary obstructive pancreatitis is associated with atrophy of the peritumoral parenchyma. [3, 8].

Figure 4: Cross-section of a PDAC



In the inked and thread-marked resectate the PDAC can be identified as grey-white mass with intermingling yellow areas.

1.5.2 Microscopic aspects

The PDAC typically is a tubular adenocarcinoma of ductal differentiation (i.e. ductal adenocarcinoma) featuring mucin producing cancer cells and abundant desmoplastic stroma (Figure 5). Four histological differentiation grades are described (G1-G4) [18]:

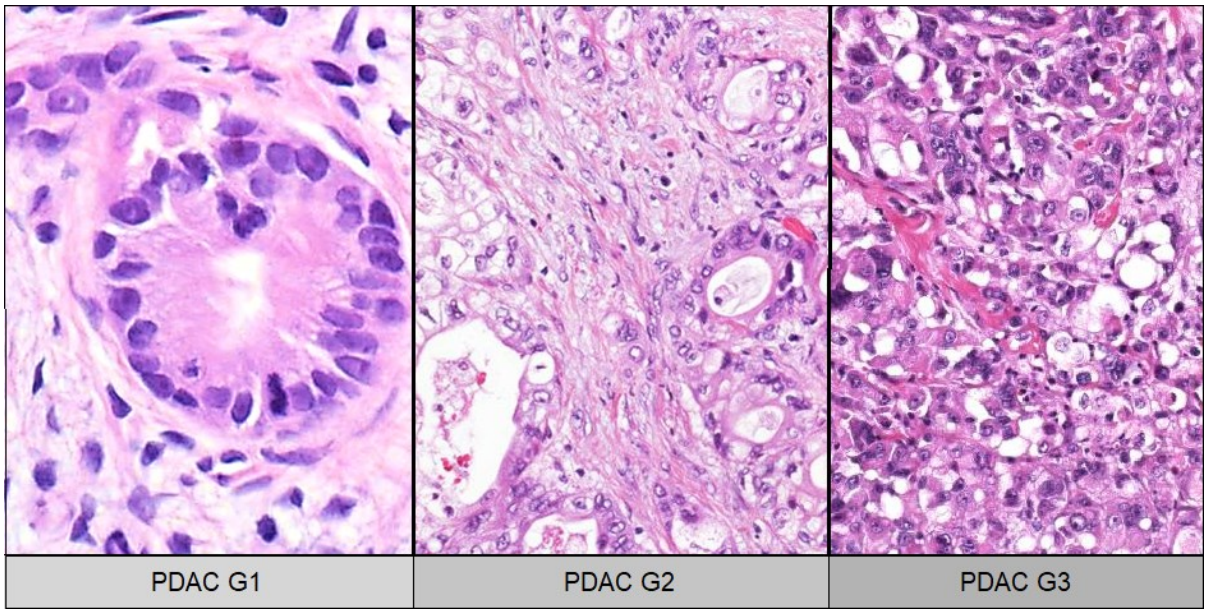
1. **Well-differentiated (G1) PDAC:** This low-grade tumour forms single-layered cuboid to columnar well-formed duct-like structures with low-grade atypia of cancer cells. Mucin production is a frequent and mitosis an infrequent feature. Spreading is observed in pancreatic septa and within the parenchyma with intermingling of adenocarcinoma and non-neoplastic parenchyma [18].

2. **Moderately-differentiated (G2) PDAC:** The duct-like structures of moderately-differentiated PDAC are less well-formed than in G1 PDAC and show higher grade of cytological atypia. While the number of mitotic figures is increased, the production of mucin of the cancer cells is decreased as compared to G1 PDAC. [18].

3. **Poorly-differentiated (G3) PDAC:** Ill-formed ductal structures, solid sheets of cancer cells and/or disseminated single or small groups of highly polymorphic cancer cells are features of high-grade PDAC. Mucin production is much decreased or even lacking. Necrosis or haemorrhages may be found [18].

4. **Undifferentiated cancer (G4):** These malignancies, also called anaplastic carcinoma are characterised by loss of features of epithelial differentiation on H&E histology. Immunohistochemical analyses are often necessary to proof epithelial differentiation. [18].

Figure 5: Microscopic view on PDAC



PDAC G1 is characterised by well-formed duct-like structures with only little atypia. PDAC G2 does show more extensive pleomorphism with only moderately-differentiated duct-like structures. PDAC G3 is characterised by ill-formed glandular structures with necrosis and high-grade cellular atypia.

Several rare histological subtypes have been described. These include adenosquamous carcinoma, colloid carcinoma, hepatoid carcinoma, medullary carcinoma, signet ring carcinoma and carcinomas with mixed differentiation [18].

1.6 Clinical features of PDAC

Most symptoms of PDAC including nausea, loss of appetite, fatigue, diffuse abdominal or back pain, constipation and diarrhoea are non-specific [3, 19, 20]. As a consequence of late diagnosis and advanced tumour stage PDAC of the pancreatic head are often no longer resectable because of upper mesenteric vessel and/or portal vein involvement. [3, 19]. Back pain may indicate retroperitoneal invasion of the splanchnic nerve plexus [21]. Weight loss might be attributable to the consuming nature of the disease itself and/or be attributed to the compression of the pancreatic duct resulting in exocrine insufficiency. Extensive loss of regular endocrine pancreatic tissue through cancer infiltration sometimes is associated with the onset of type 2 DM. So called painless jaundice often accompanied by pruritus is regarded as one of the most characteristic clinical features of PDAC [21].

1.7 Screening

The frequent lack of symptoms in early stage disease is responsible for a high rate of late diagnosis of PDAC. Screening methods are subject to current research. Up to date, no method has been identified to be suited for early diagnosis in general population screening. Many experts agree that endoscopic retrograde cholangiopancreaticography, endoscopic ultrasound, spiral computed tomography and/or fine needle aspiration may be useful for screening in high-risk settings such as familial pancreatic cancer, familial atypical multiple mole melanoma syndrome, hereditary pancreatitis, Peutz-Jeghers syndrome, and p53 or BRCA mutation carriers [22].

Carbohydrate antigen 19-9 (CA 19-9) represents the most frequently used soluble tumour marker in the blood. CA 19-9 serum values before or after tumour resection are useful for the prediction of resectability, overall survival (OAS) and disease-free survival (DFS). However, serum CA 19-9 levels cannot be recommended for general population screening because the positive predictive value is low [23].

1.8 Surgical approach for tumours of the pancreatic head

To date, complete surgical resection is the only curative approach for PDAC [24]. The surgical procedure is dependent on the location of the tumour: Pancreatic masses in the head are resected with classical Kausch-Whipple procedure or pylorus preserving pancreaticoduodenectomy (PPPD) [24]. If intraoperative abdominal exploration reveals peritoneal carcinosis, extrapancreatic disease (e.g. metastasis to the liver) or invasion of the superior mesenteric artery resection is discontinued [25].

The Whipple procedure includes several steps. Resection starts with the mobilisation of the gallbladder. The bile duct is cut above the entry of the cystic duct into the common bile duct. At this point either the distal part of the stomach or the proximal part of the duodenum are cut, depending on if the pylorus can be preserved or not. Then the head of the pancreas is separated from the body and the portal vein. In the next step, the distal part of the duodenum is separated from the jejunum and the resected tissue consisting of pancreatic head, gallbladder, common bile duct, duodenum and potentially the distal part of the stomach are removed en bloc. After resection, the reconstruction is performed via terminoterminal or terminolateral pancreatojejunostomy, a terminolateral biliodigestive anastomosis (hepaticojejunostomy) and a gastroenterostomy [25].

For classification it is recommended to resect at least 10 regional lymph nodes and to indicate the ratio of infested lymph nodes as it is of prognostic value. Extended lymphadenectomy has been shown not to improve survival but to increase perioperative complications [24].

1.9 Risk factors for tumour recurrence after curative PDAC resection

Tumour recurrence after Whipple procedure is frequent and occurs in 80-87% of all initial R0 resections [26, 27]. Types of tumour recurrence include local recurrence, distant metastasis or peritoneal dissemination [28]. Risk factors for tumour recurrence include resection margin status, perineural invasion, blood vessel

invasion, lymphovascular invasion, tumour size and grade and lymph node involvement [29, 30, 31, 32].

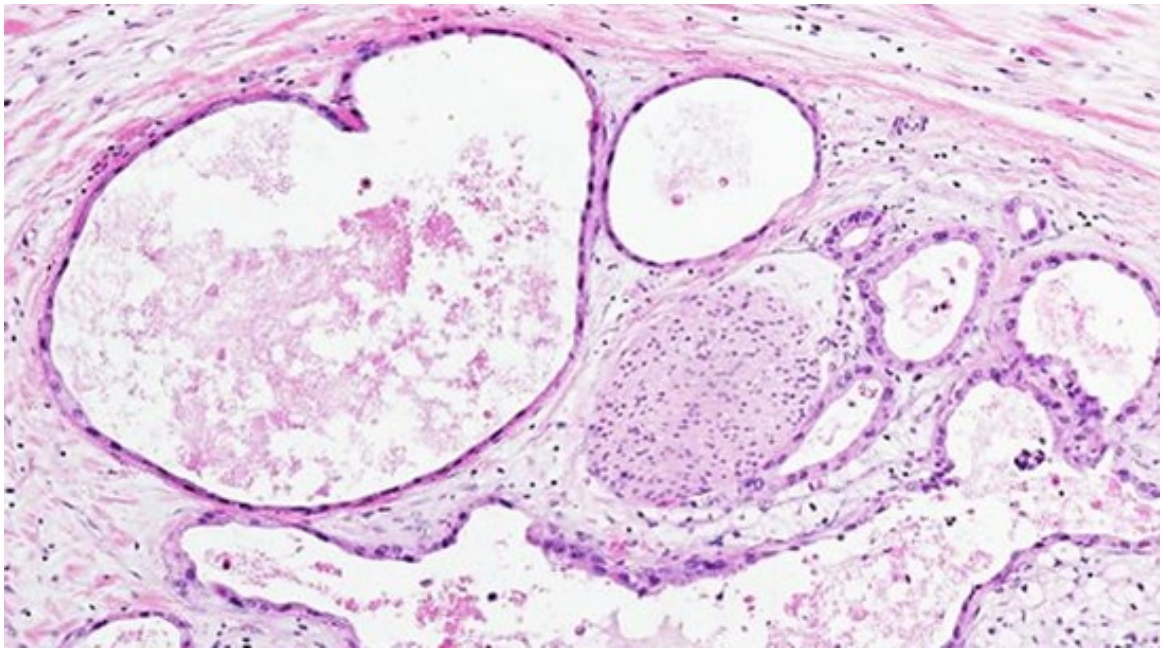
1.9.1 Resection margin status

R0 is defined as complete tumour resection without residual tumour tissue remaining in the patient. R1 is defined as tumour tissue at the resection margin on light microscopy whereas R2 is defined as tumour tissue at the resection margin on gross pathological evaluation [33]. Recently a novel definition was proposed: R0 is defined as cancer-free resection margin of more than 1mm which is associated with better 1-year survival rates than in cases with conventional definition of R0 status [34, 35, 36].

1.9.2 Perineural invasion

Perineural invasion (PNI) is associated with poor prognosis [3, 37]. PNI has been defined as the spreading of tumour cells within any of the nerve layers (epineurium, perineurium, endoneurium) or the circumferential growth along nerves (Figure 6).

Figure 6 : PDAC with PNI



Moderately-differentiated PDAC with a nerve being infiltrated by tumour cells. The duct-like structures of this G2 PDAC appear dilated with intraluminal mucus production

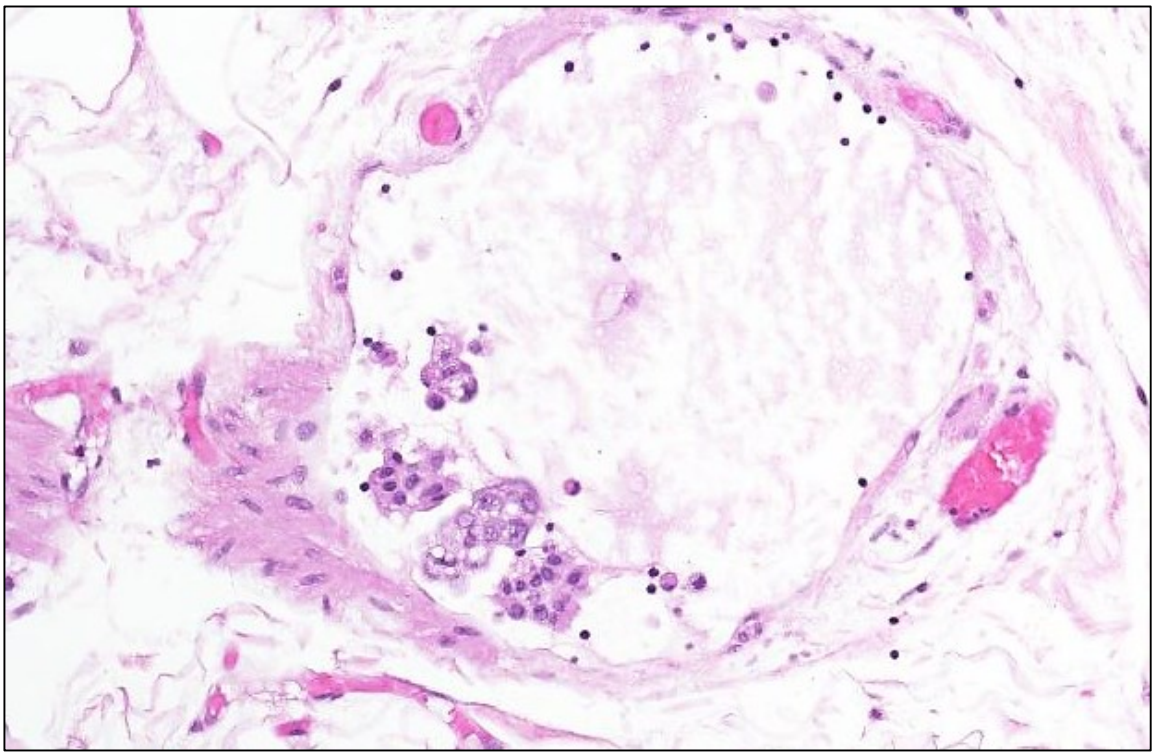
PNI is thought to be mediated by PDAC-produced neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived

neurotrophic factor and neurotrophin 3 [38]. The growth along retroperitoneal nerves is associated with higher risk of local PDAC recurrence [39].

1.9.3 Lymphovascular invasion

Lymphovascular invasion (LVI) is common in PDAC (Figure 7). Tumour growth narrows lymphatic vessels and generates mechanical stress promoting access and infiltration of cancer cells into the lumen of dilated lymphatic vessels at the periphery of the tumour. LVI is among the most important risk factors for metastatic spread into regional lymph nodes and the formation of distant metastases via the blood stream [40].

Figure 7: PDAC with LVI



A lymphatic vessel of a moderately-differentiated PDAC with small intraluminal cell clusters with moderate pleomorphisms.

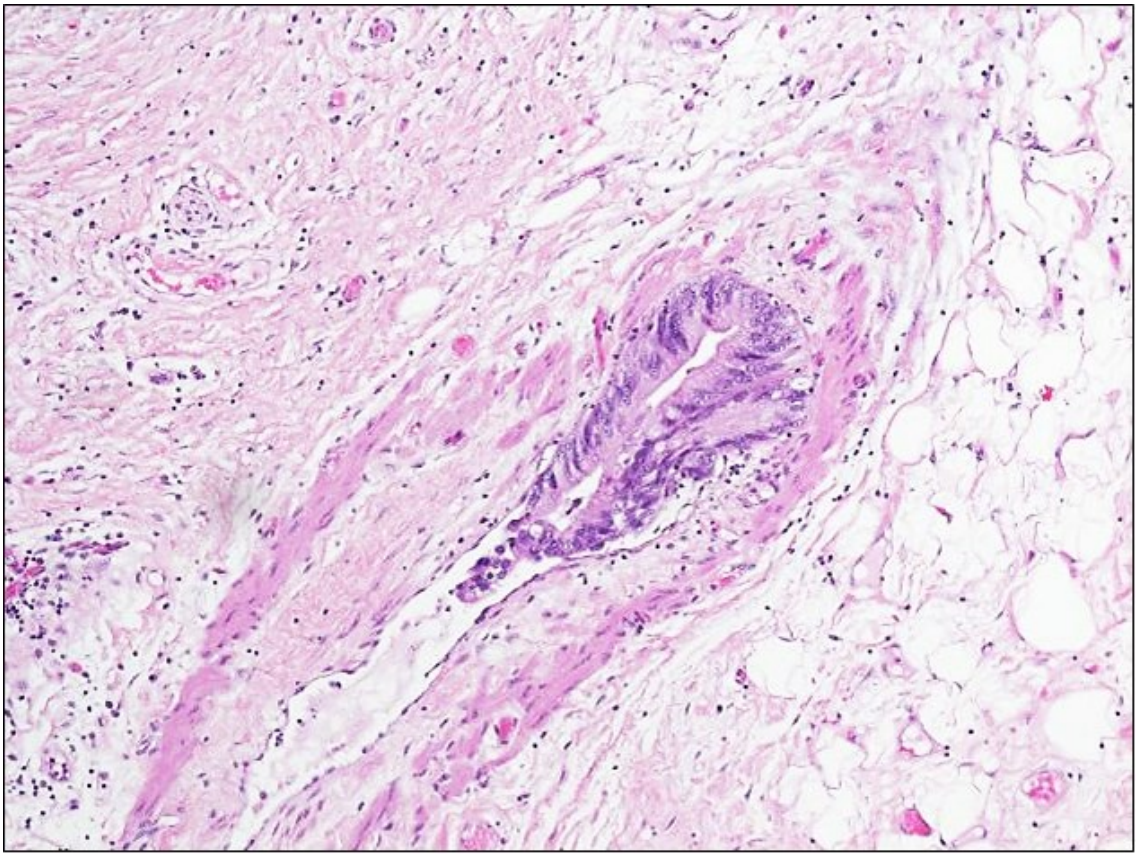
1.9.4 Vascular and microvascular invasion

Invasion of large vessels (vascular invasion) of the confluens venosum is frequently present at initial diagnosis of PDAC (Figure 8) [41]. Extensive vascular invasion is a contraindication to resection. However, in some patients with limited involvement of the superior mesenteric vein, the portal vein or a short part of gastroduodenal

arteries and the superior mesenteric artery Whipple procedure may still be possible. Criteria for resectability in such settings have been defined by the National Comprehensive Caner Network (NCCN) [8].

Besides major vessel involvement also microvascular invasion (MVI) is associated with a high risk of cancer recurrence (CR) and shorter survival rates [42, 43]. Compared to PNI it seems to be less frequently detected in histological sections of PDAC [43].

Figure 8: PDAC with MVI



A moderately-differentiated PDAC with venous vessel invasion. The tumour cells show a wall-adherent growth pattern.

1.9.5 Tumour size and Tumour Node Metastasis classification

Tumour size is not only a prognostic factor for DFS but also a criterion for resectability [44]. A meta-study compared tumour size with median survival after curative resection. On average, tumour sizes less than 2cm were associated with better patient survival compared to larger tumours presumably due to higher probability of lymphatic spreading as well as micro-metastasis in larger PDACs [43].

Prognosis is also reflected by the UICC TNM staging system (Table 2). T status refers to tumour size and has been characterized as follows in its 8th Edition of the staging system: All tumours ≤ 2cm in diameter are defined as T1 and are further subdivided into 3 sub-stages. T1a comprises PDAC with a maximum of 0.5cm in diameter. PDAC corresponding to T1b are >0.5cm but smaller than 1cm and cancers defined as T1c are >1cm to a maximum of 2cm in diameter. T2 includes PDAC with a size of >2cm to a maximum of 4cm. T3 contains all PDAC larger than 4cm in diameter and finally cancers with the involvement of the common hepatic artery, coeliac axis or superior mesenteric artery are defined as T4.

N status describes the involvement of regional lymph nodes. Negative lymph node status is defined as N0. PDAC with 1-3 metastasised lymph nodes are defined as N1 and cancers with 4 or more lymph nodes involved are defined as N2.

M status gives information on distant metastasis status. Whereas M0 describes a locally limited PDAC, M1 is defined as the prevalence of distant metastasis [45].

Table 2: TNM classification of PDAC

TNM classification of the pancreatic ductal adenocarcinoma	
T1	Tumour 2cm or less
T1a	Tumour ≤ 0.5cm
T1b	Tumour >0.5 but ≤ 1cm
T1c	Tumour >1cm but ≤2cm
T2	Tumour >2cm but <4cm
T3	Tumour >4cm
T4	Tumour involves coeliac axis, common hepatic
	artery and/or superior mesenteric artery
N0	No lymph node metastasis
N1	Lymph node metastasis in 1 to 3 nodes
N2	Lymph node metastasis in 4 or more nodes
M0	No distant metastasis
M1	Distant metastasis

based on Brierley J.D., Gospodarowicz M.K., Wittekind C., TNM Classification of Malignant Tumours 8th Edit., 2017

1.9.6 Tumour grade

Tumour grade (G) has been described as an indicator for biological aggressiveness. A meta-analysis from 2008 and a more recent study from 2012 demonstrated higher rates of tumour recurrence in PDAC with worse differentiation (G3+G4) than in low-grade tumours which may be due to a faster growth rate of less differentiated cancers [29, 43].

1.9.7 Lymph node involvement

Both the absolute number of cancer infiltrated (positive) lymph nodes (lymph node involvement) but also the ratio of infiltrated versus non-infiltrated (negative) lymph nodes have been described to correlate with an increased risk of CR. Lymph node ratio (LNR) may even be a better predictor of prognosis than the number of positive lymph nodes alone [43]. A LNR of greater than 0.2 correlates with worse survival as compared to a ratio of less than 0.2 [46].

1.10 Prognosis in patients with distant metastasis

PDAC with distant metastasis is a palliative condition. Management options include chemotherapy, biliary and duodenal stenting and coeliac plexus block for pain control. The therapeutic options depend on the patient's individual physical condition. In 1982, the Eastern Cooperative Oncology Group (ECOG) devised an algorithm for the definition of ECOG Performance Status which can be used to classify patients for individualised management (Figure 9) [8]. Besides mandatory pain management, chemotherapy can be offered to patients with lower ECOG score (0-2) whereas patients with high ECOG scores (3-4) are supported with symptomatic palliation.

Figure 9: ECOG performance status

Grade	ECOG Performace Status
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of walking hours
3	Capable of only limited selfcare; confined to bed or chair more than 50% of waking hours
4	Completely disabled; cannot carry on any selfcare; totally confined to bed or chair
5	Dead

taken from Oken M, Creech R, Tormey D, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol. *1982;5:649-655.*

1.11 Objectives

Over the past decades, risk factors for recurrent disease and OAS after curative resection of PDAC have been described. Although PNI, LVI and MVI have been described as predictors of poor outcome, they have not been quantified in relation to DFS and/or OAS.

1.12 Aims

The aim of this study was to conduct a retrospective histopathological evaluation of 88 patients that underwent curative resection of PDAC of the pancreatic head for whom complete follow-up data were available. Enumeration of the exact number of PNI, LVI and MVI and the exact distance of cancer infiltrates with respect to the circumferential and left-sided resection margin were assessed on H&E histology and evaluated for their correlation with DFS and OAS. The utility of histological and clinical parameters for prediction of DFS and OAS was evaluated with uni- and multivariate Cox regression analysis.

2 Material and Methods

2.1 Study cohort

Consecutive patients who underwent Whipple procedure or PPPD for PDAC of the pancreatic head at the Medical University of Graz during January 2000 and December 2014 were included in this study after ethical approval had been granted (approval number: EK 25-404 ex 12/13). Medical charts of all patients were reviewed and data collection was assisted by the information systems AURAWeb and MEDOCS of the Medical University of Graz.

2.1.1 Inclusion criteria

Patients were selected according to the following criteria:

- Whipple procedure or PPPD for tumour of the pancreatic head
- Completely sampled pancreatic head and histologically confirmed PDAC
- R0 on gross pathological evaluation
- Clearly defined resection margins at the pancreatic head by inking in different colours
- Available medical history and follow-up data

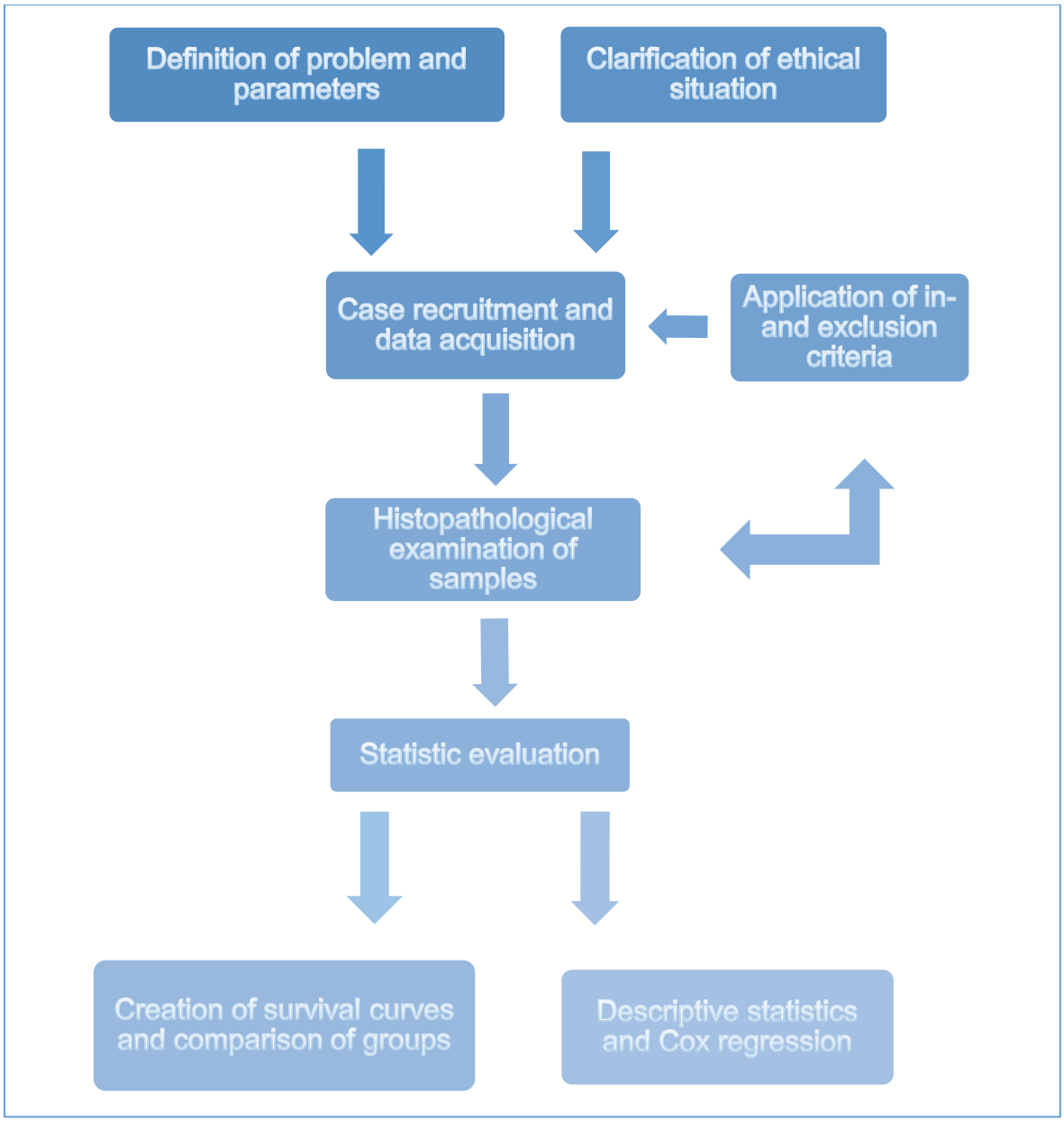
2.1.2 Exclusion criteria

Following factors lead to exclusion from this study:

- Other subtypes of PDAC including adenosquamous carcinoma, colloid carcinoma, hepatoid carcinoma, medullary carcinoma and signet ring carcinoma
- Intra- or extrapancreatic malignant disease other than PDAC
- Patients with IPMN and MCN precursors of PDAC
- Insufficient material for detailed histopathological analysis (in particular incomplete sampling of the pancreatic head)
- PDAC with R2 status of resection margin

The Study design is outlined in Figure 10.

Figure 10: Study design



Patient medical charts were evaluated for demographic data, body mass index and comorbid conditions. Comorbid conditions were classified into cardiovascular (arterial hypertension, peripheral arterial occlusive disease and coronary artery disease), pulmonary (asthma bronchiale or chronic obstructive pulmonary disease) and gastrointestinal comorbidities or conditions (hiatus hernia, gastroesophageal reflux disease or gastritis). In addition, the Charlson Comorbidity Index for prediction of 10-year survival was calculated [47]. Preoperative symptoms including weight loss, emesis, abdominal pain, jaundice, diarrhoea and DM were recorded. Patients

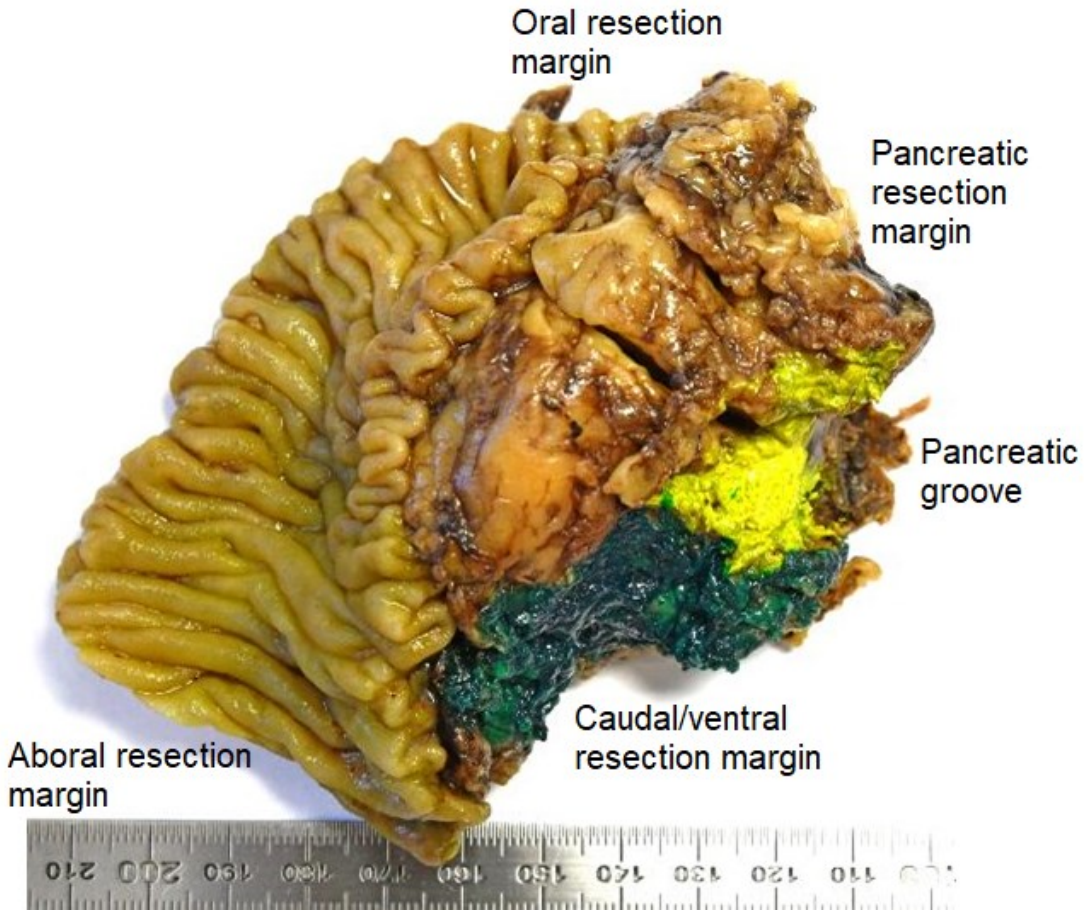
were followed until tumour recurrence and/or death or censoring based on follow-up data collected at the Department of Surgery at the Medical University of Graz.

2.2 Histopathological examination

2.2.1 Gross pathology

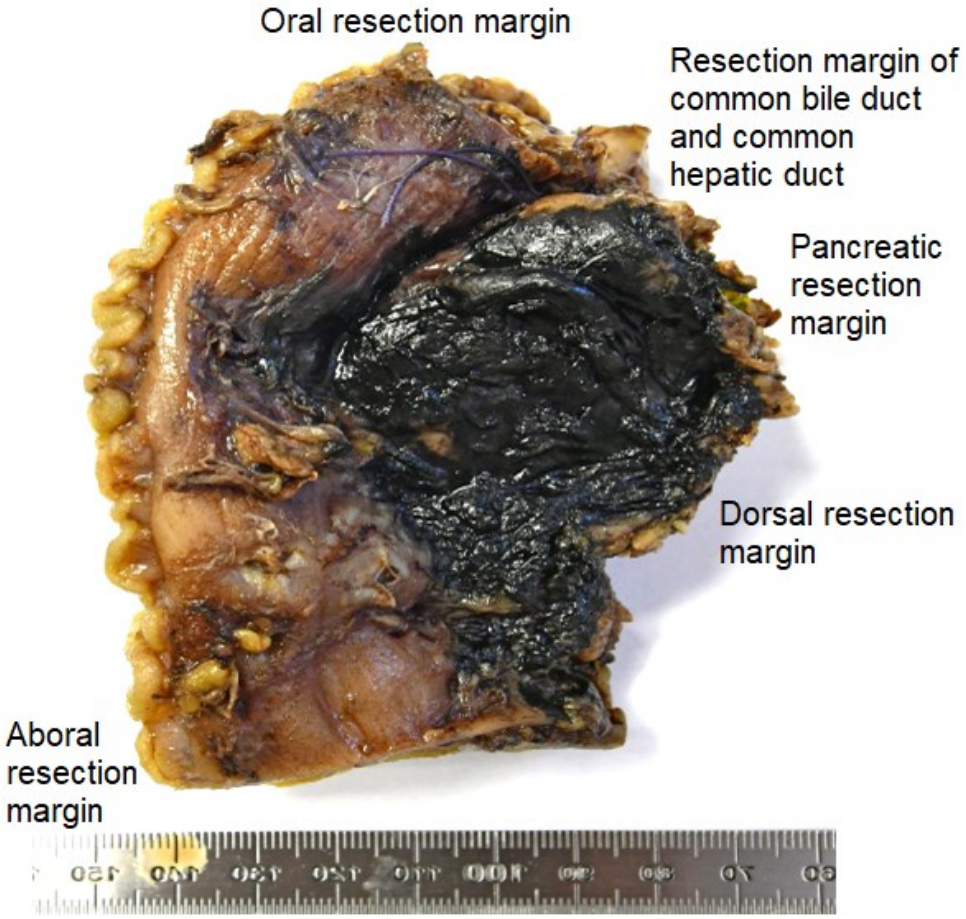
Whipple and PPPD specimen were evaluated according to a standardised protocol as described [48]: The resection margins were marked with ink as shown in Figure 11 and 12.

Figure 11: Inking of resection margins (ventral view)



The resection margin of PPPD specimen are marked with ink in different colours: Pancreatic groove: yellow; caudal margin: green.

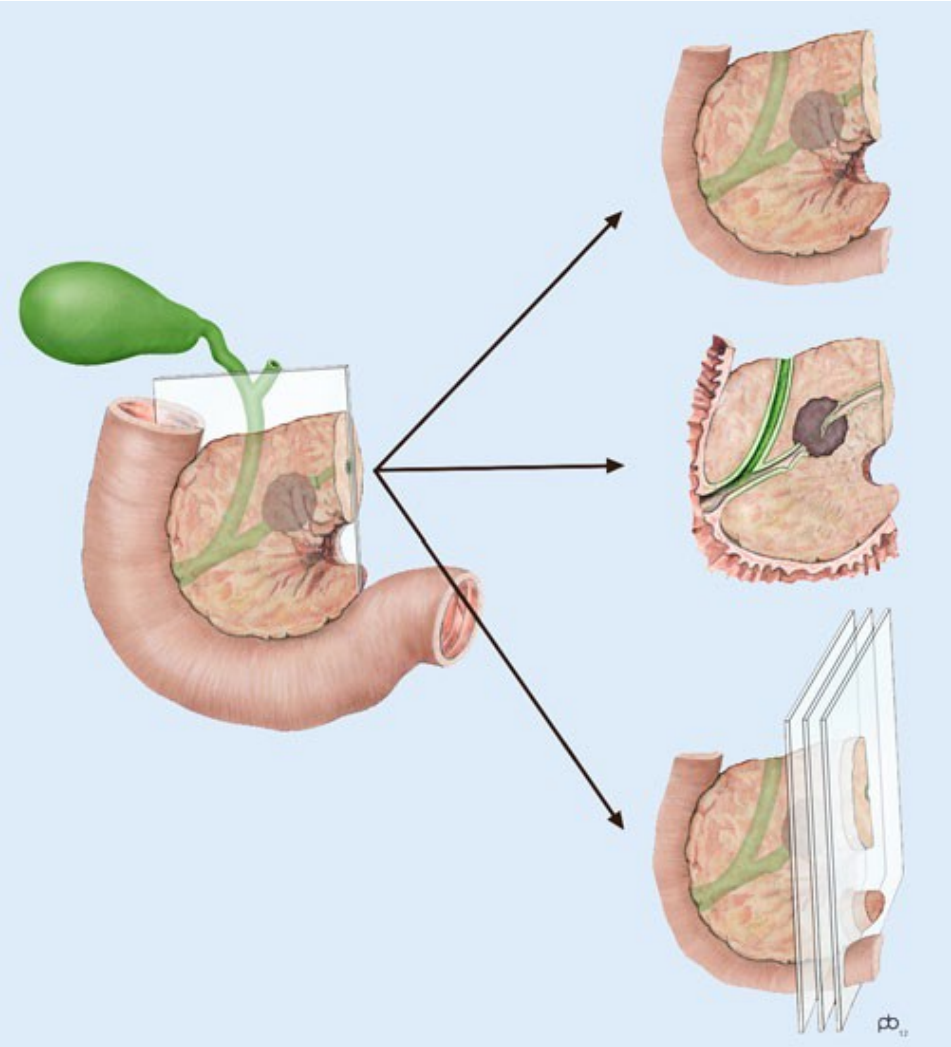
Figure 12: Inking of resection margin (dorsal view)



The dorsal resection margin of the PPPD specimen is inked in black

Thereafter the complete pancreatic head was sampled using the bi- and multivalving technique. In a first step, the main pancreatic duct together with the common bile duct are probed and then the pancreatic head is cut along the plain formed by both ducts resulting in longitudinally opened ducts. This procedure results in a ventral and a dorsal half of the pancreatic head. Both halves are then cut in a perpendicular plain into multiple slices. All slices are sampled for paraffin embedding and histological evaluation (Figure 13) [49].

Figure 13: Bi- and multivalving technique



taken from Verbeke C.S., Resection margin in pancreatic cancer, Pathologe 2013, 34:241-247
The Whipple specimen is first cut along a pain formed by the common bile duct and the main pancreatic duct. Then the two halves are cut in a perpendicular manner to create multiple slices.

2.2.2 Histology

Each case was reviewed by two investigators (MH and CL) in consensus using a multiheaded microscope. All H&E stained tissue sections of the pancreatic head (including the completely sampled PDAC) were evaluated. For each case the following features were counted:

- **Reevaluation** of the histological subtype of PDAC, PDAC grade as well as TNM classification.
- **Evaluation of resection margins:** The minimal distance (in mm) between PDAC and the caudal, groove and dorsal margin (i.e. the circumferential

resection margin; CRM) as well as the distance to the pancreatic-left sided margin was measured.

- **Clear cell features of tumour cells**: Some types of PDAC cells can show clear or lightly stained abundant cytoplasm and pycnotic nuclei (clear cell change). The degree of clear cell change was assessed by application of a numerical score as follows: Score 0: clear cell change absent, score1 and 2: clear cell change in <50 or >50% of tumour cells, respectively.
- **Mucinous component:** A mucinous component, defined by evidence of extracellular mucin and tumour cells with mucinous cytoplasm was evaluated by application of numerical scores such as: Score 0: mucinous component comprises 0-10% of PDAC, score 1 and 2: mucinous component comprises 11-30% or >30% of PDAC, respectively.
- **PNI**: PNI was defined by growth of tumour cells within the nerve sheath (perineural) or within the nerve fascicles (intraneural).
- **LVI:** LVI was defined as the presence of tumour cells within the lymphatic vessel lumen.
- **MVI:** The occurrence of tumour cells within the lumen of an intact blood vessel was defined as MVI.

In addition, the extent of inflammation (predominantly mononuclear cells or neutrophils) within PDAC was evaluated as follows: Predominantly monocytic infiltration: Score 0: none to mild, score 1: severe, and score 2: resembling medullary carcinoma. Predominantly neutrophil infiltration: Score 0: none to mild, score 1: moderate, and score 2: severe.

2.3 Statistical Analysis

Demographic, clinical and histological variables were described using median or mean, minimum and maximum for continuous variables and absolute and relative frequencies for categorical variables.

Cases were stratified according to the prognostic relevant number of PNI, LVI and MVI into PNI-, LVI-, and MVI-invasion types, respectively. Similar analysis was performed to determine the prognostic relevance of the CRM of the PDAC.

In this retrospective study two endpoints were evaluated: DFS and OAS. The effect of variables on DFS and OAS was analyzed by Kaplan-Meier method and compared by log-rank test. Furthermore, univariate Cox regression analysis was performed. Baseline variables that were associated with DFS or OAS in univariate analysis ($p \leq 0.05$) were included as potential covariates in a Cox proportional hazards regression analysis using forward selection.

Statistical analysis was conducted with IBM SPSS v24 on Windows and illustrated with either IBM SPSS v24 or Microsoft Excel 2016.

3 Results

3.1 Clinical characteristics of the patients

Between January 2000 and December 2014, 149 consecutive patients with diagnosis of PDAC underwent Whipple procedure or PPPD for PDAC of the pancreatic head at the Medical University of Graz. Of those, 88 patients fulfilled the inclusion and exclusion criteria. The median follow-up time was 19 months with a range of 0-96 months. Forty (45.5%) individuals were male and 48 (54.5%) were female. Median age was 65 years, ranging from 44 years to 83 years. At the end of the follow-up 67 (76.1%) patients had died of the PDAC, 7 patients (8.0%) were alive with CR and 14 (15.9%) patients did show no signs of PDAC recurrence.

Table 3: Clinical characteristics of the study cohort

Clinical characteristics of the study cohort	
Attribute	N(%), median (range)
Total number of cases	88
Age	65 (45-83)
Female sex	45 (54.5%)
BMI	25 (18-38)
Nicotine abuse	14 (16.3%)
Alcohol abuse	8 (9.1%)
Comorbidity	
Cardiovascular disease*	54 (61.4%)
Diabetes mellitus	23 (26.1%)
Pulmonary disease**	10 (11.5%)
Gastrointestinal disease***	10 (11.4%)
Chronic pancreatitis	7 (8.2%)
Symptoms on admission	
Abdominal pain	47 (53.4%)
Jaundice	37 (42.0%)
Weight loss	28 (32.2%)
Emesis	7 (8.3%)
Diarrhoea	6 (7.1%)
* Arterial hypertension, PAOD,CAD ** Asthma bronchiale, COPD *** Hiatus hernia, gastritis, GERD, chronic pancreatitis	

Comorbidity was frequently present. More than half of the patients had cardiovascular comorbidities such as arterial hypertension, peripheral arterial occlusive disease and coronary artery disease. A minority of patients was diagnosed with gastrointestinal comorbidities including hiatus hernia, gastritis, gastroesophageal reflux disease and chronic pancreatitis. Diabetes mellitus was present in approximately 25% of patients. Another minority suffered from pulmonary comorbidities including asthma bronchiale and chronic obstructive pulmonary disease. BMI was in the mildly overweight range. A minority of patients were nicotine- and/or alcohol abusers.

The most frequent preoperative symptoms were abdominal pain, jaundice and unintentional weight loss. Less frequent symptoms included emesis and diarrhoea. Clinical characteristics of the patient cohort is detailed in Table 3.

3.2 Histopathological characteristics of the study cohort

The majority of patients had PDAC of 2 to 4 cm in diameter, whereas small tumours of < 2 cm or large tumours with > 4 cm were much less frequent. A little more than half of the patients had poorly-differentiated high-grade PDAC (G3). Only slightly less than half of the patients had low-grade PDAC classified as G1 and G2. Regional lymph node metastases were found in most cases. Data on the TNM classification of the study cohort are compiled in Table 4.

As defined in the inclusion criteria the vast majority of cases was of the classical ductal type of pancreatic cancer. However, in some PDAC also a mucinous or clear cell component was found. The component was mild in the majority of cases for mucinous change whereas at least mild clear cell change was present in about 20% of the tumours.

On microscopic assessment the vast majority of PDAC showed PNI. Besides PNI, a minor group of PDAC was also affected by intraneural growth. In comparison to LVI and MVI, PNI was by far the most frequent invasion attribute. However, a count of 30 or more PNI was only observed in a small minority of PDAC. About two-thirds of PDAC showed LVI, whereas MVI was only observed in a minority of cases representing the least frequent invasion feature.

Table 4: TNM classification and grading of the study cohort

TNM classification and grading of the study cohort			
Attribute	Score	Notation	N (%), median(range)
Tumour size			
	0	T1a (≤ 0.5 cm)	2 (2.3%)
	1	T1b (> 0.5 but ≤ 1cm)	0 (0.0%)
	2	T1c (> 1cm but ≤ 2cm)	19 (21.6%)
	3	T2 (> 2cm but < 4cm)	60 (68.2%)
	4	T3 (> 4cm)	4 (4.5%)
	5	T4*	3 (3.4%)
WHO Grading			
	0	Well-differentiated	5 (5.7%)
	1	Moderately-differentiated	37 (42%)
	2	Poorly-differentiated	45 (51.1%)
	3	Undifferentiated	1 (1.1%)
Lymph node metastases			
	0	No lymph node metastases	12 (14.1%)
	1	1-3 lymph node metastases	36 (42.4%)
	2	≥ 4 lymph node metastases	37 (43.5%)
* involvement of the coeliac axis, common hepatic artery and/or SMA			

More than 90% of the study cohort had cancer-free resection margins of 1mm or less. In comparison, however, the majority of PDAC showed a cancer-free resection margin of the common bile duct with more than 1mm. Only in a small number of PDAC a cancer-positive resection margin of the bile duct was verified.

In a predominant number of PDAC no distinct inflammation was detected. Most cases only showed little inflammatory activity. However, a small subset of PDAC did show moderate or even severe inflammation. A medullary character was exceptionally rare.

A detailed presentation of histopathological attributes is listed in Table 5.

Table 5: Histopathological characteristics of the study cohort

Histopathological characteristics of the study cohort			
Attribute	Score	Notation	N (%), median(range)
Mucinous component			
	0	0-10%	85 (96.6%)
	1	11-30%	2 (2.3%)
	2	30-80%	1 (1.1%)
Clear cell score			
	0	no clear cell comp.	58 (65.9%)
	1	clear cell comp. < 50%	17 (19.3%)
	2	clear cell comp. > 50%	13 (14.8%)
PNI			
	0	absent	8 (9.1%)
	1	present	80 (90.9%)
	2	intraneural growth	13 (14.8%)
PNI ≥ 30			
	0	< 30	77 (87.5%)
	1	≥ 30	11 (12.5%)
LVI			
	0	absent	29 (33.0%)
	1	present	59 (67.0%)
MVI			
	0	absent	71 (80.7%)
	1	present	16 (19.3%)
CRM			
	0	≤ 1 mm cancer-free	72 (92.3%)
	1	> 1 mm cancer-free	6 (7.7%)
Resection margin of common bile duct			
	0	> 1mm	76 (88.4%)
	1	< 1mm	5 (5.8%)
	2	R1	5 (5.8%)
Monocytic infiltration			
	0	none to mild	82 (93.2%)
	1	severe	5 (5.7%)
	2	medullary	1 (1.1%)
Neutrophil infiltration			
	0	none to mild	72 (81.8%)
	1	moderate	8 (9.1%)
	2	severe	8 (9.1%)

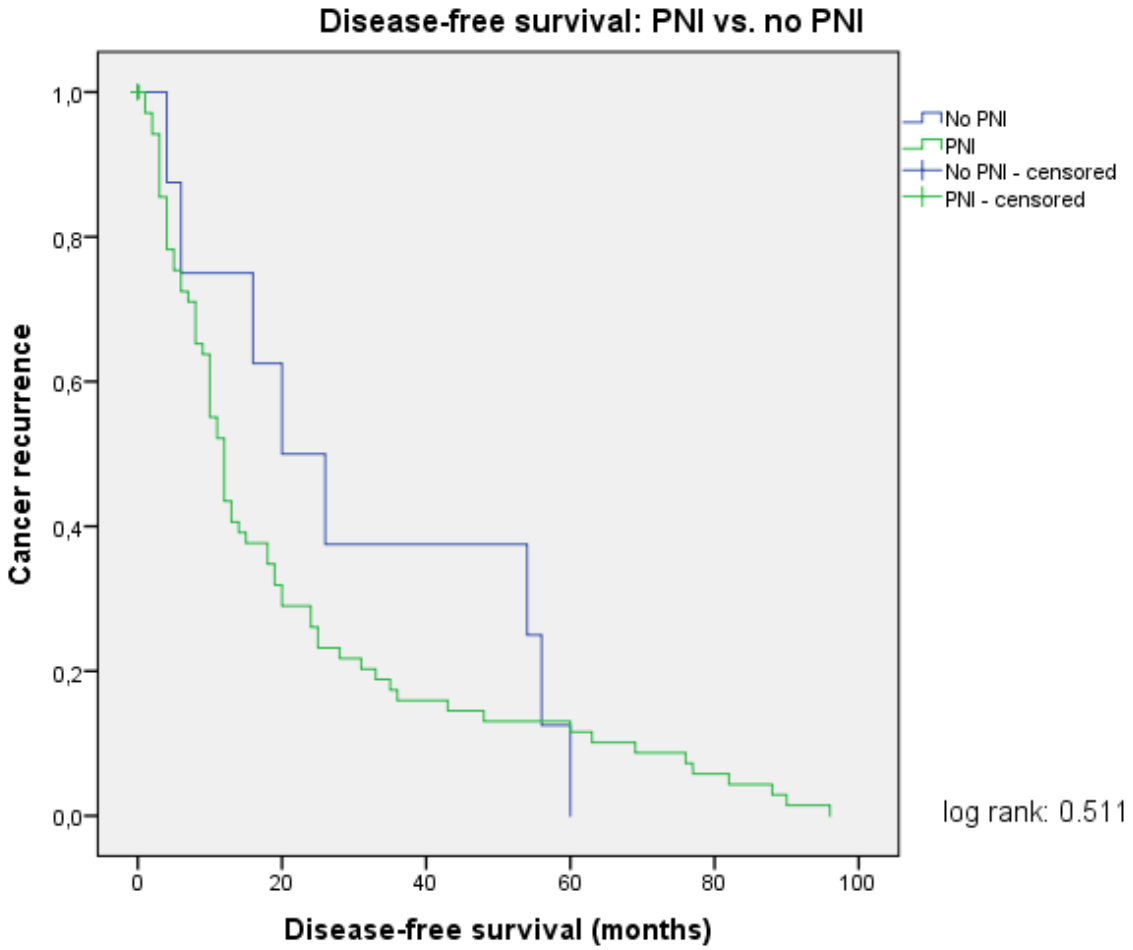
3.3 Survival Analysis

3.3.1 Disease-free survival

Impact of PNI, LVI, MVI and distance of PDAC to the CRM on DFS

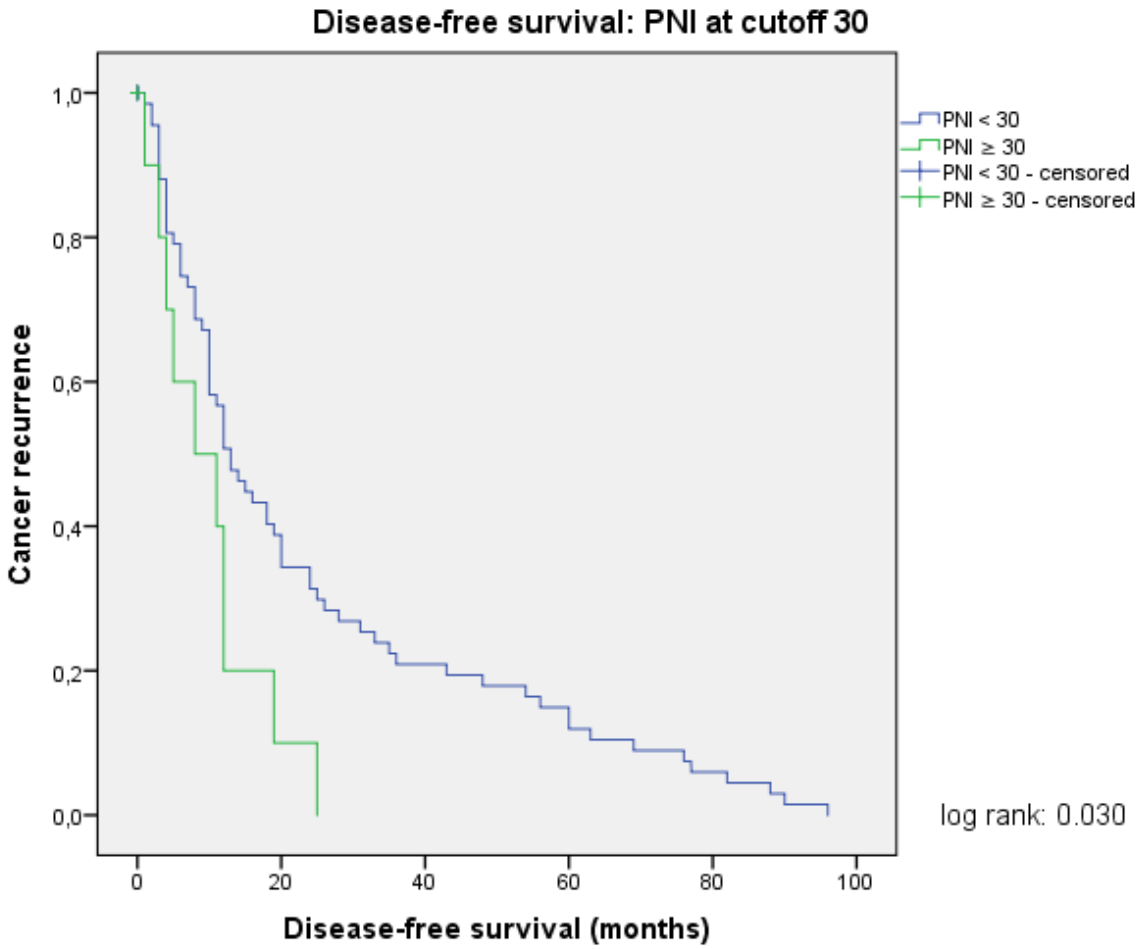
Kaplan Meier analysis revealed that the comparison between the PNI group versus the non-PNI did not result in significant differences concerning DFS (Figure 14). Group size was 69 in the PNI group as compared to 8 in the non-PNI group. Median DFS was 12 months in the PNI group and 20 months in the non-PNI group, respectively. The latest case of CR occurred after 96 months in the PNI group as compared to 60 months in the non-PNI group.

Figure 14: Kaplan Meier analysis on DFS: PNI vs. no PNI



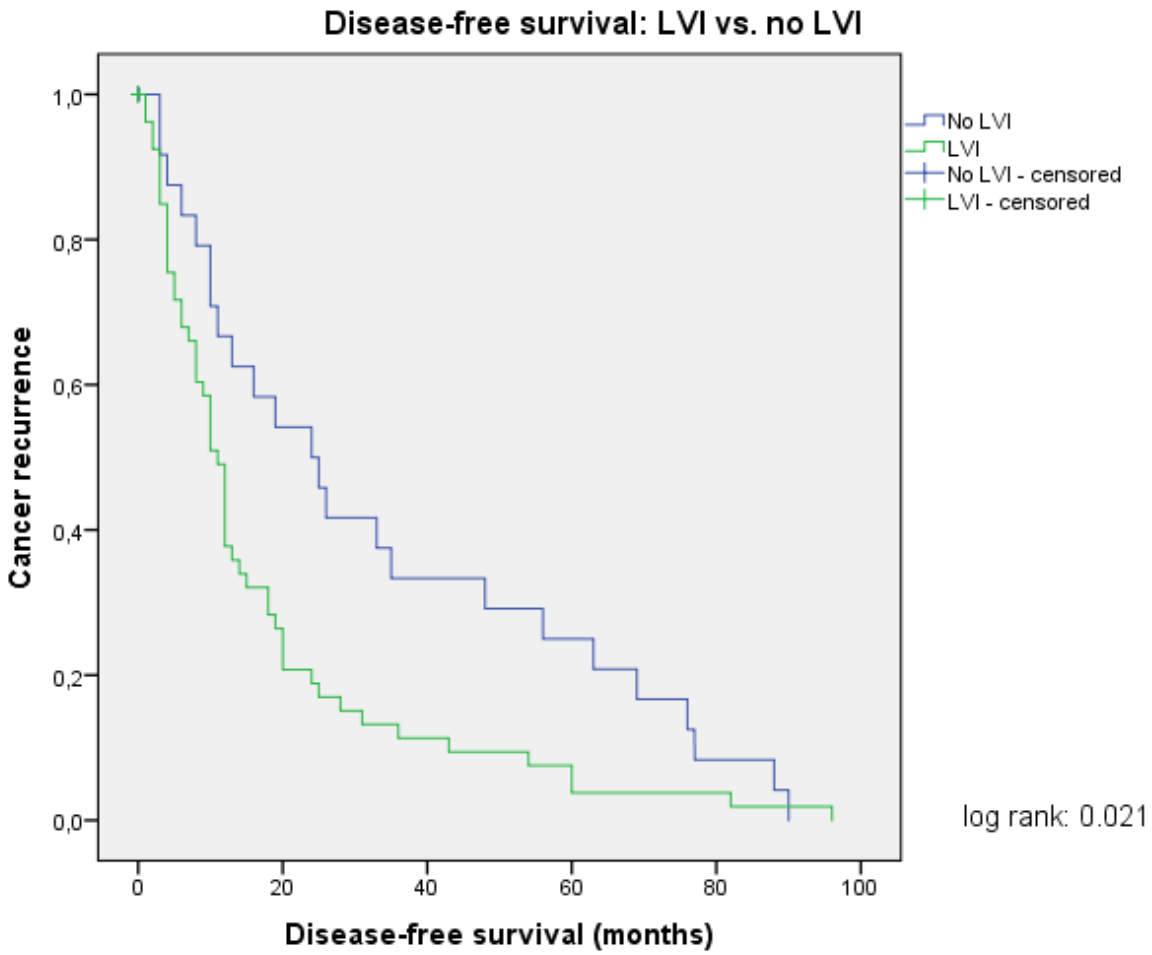
However, as numeric PNI reached from 0 to 102 invasions per case, we further analysed whether a cutoff point resulting in DFS deterioration does exist. Whereas a cutoff set at 5, 10, 15, 20 and 25 PNI did not result in significantly reduced DFS, it did for cases with 30 or more invasions (Figure 15). As Table 6 illustrates ≥ 30 PNI indicate a reduced median DFS of 8 months as compared to 13 months in the group with less than 30 invasions. The group with ≥ 30 PNI consisted of 11 cases as compared to 71 cases in the group with less than 30 PNI. Longest DFS in the group with ≥ 30 PNI was 25 months as compared to 96 months in the group with less than 30 invasions.

Figure 15: Kaplan Meier analysis on DFS: PNI < 30 vs. PNI ≥ 30



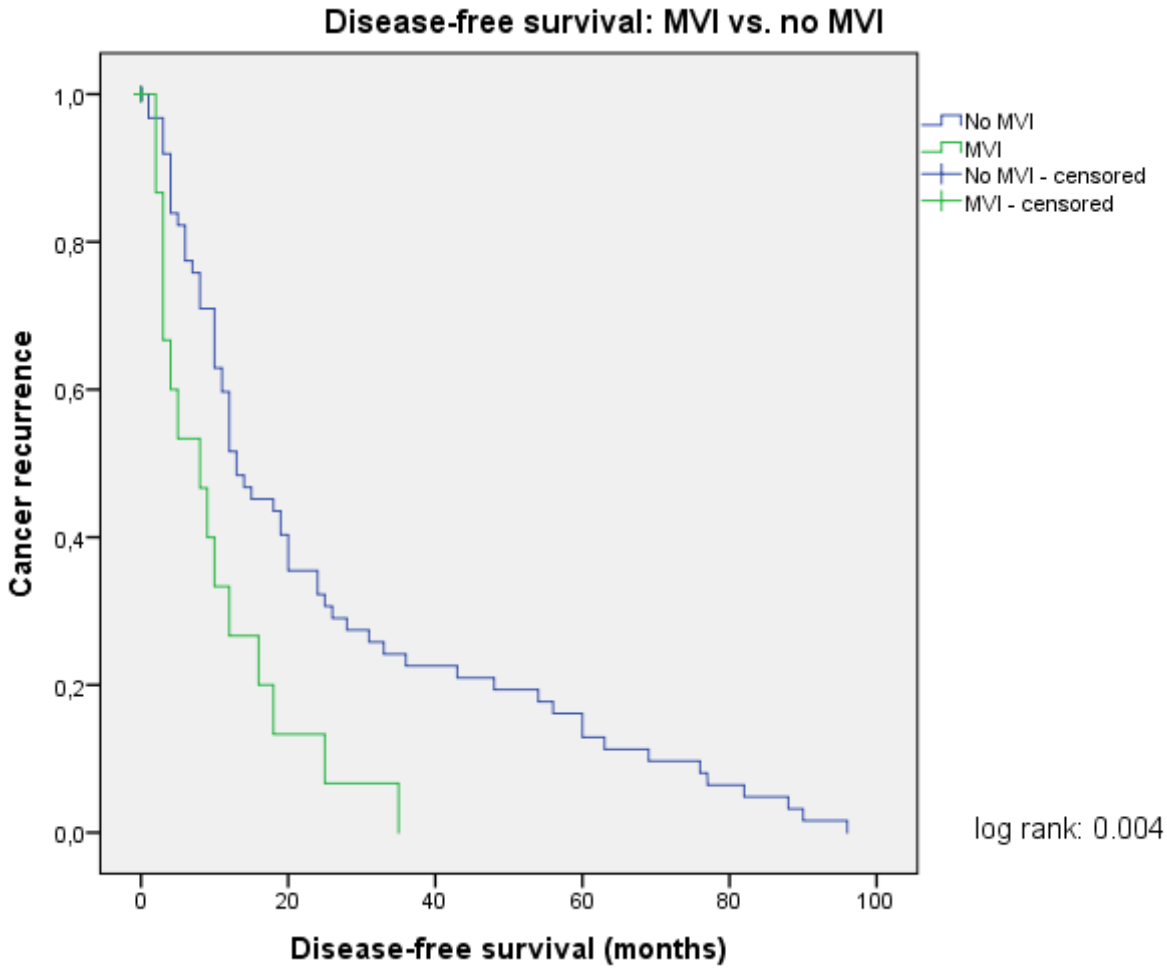
For LVI Kaplan Meier analysis proved a significantly reduced median DFS (Figure 16) for the LVI group as compared to the non-LVI group (LVI group: 11 months; non-LVI group: 24 months). Fifty-five cases were included in the LVI group and compared to 27 cases in the non-LVI group. Latest CR in the LVI group was after 96 months as compared to 90 months in the non-LVI group.

Figure 16: Kaplan Meier analysis on DFS: LVI vs. no LVI



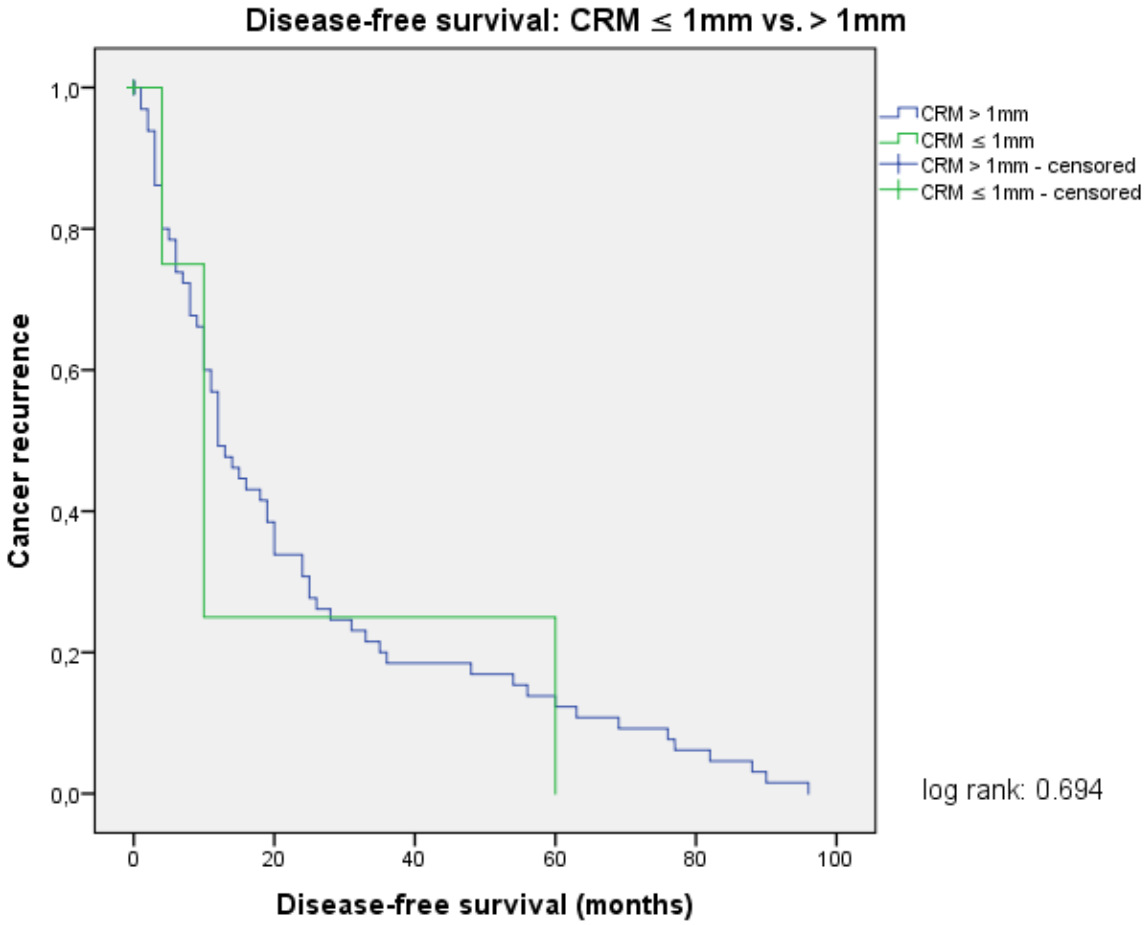
For MVI Kaplan Meier analysis revealed a significant correlation with DFS (Figure 17). The group with MVI contained 16 cases whereas the group without MVI consisted of 66 cases. Median DFS in the MVI group was 8 months as compared to 13 months in the group without MVI. Latest CR in the MVI group occurred after 35 months. In the negative group the latest CR occurred after 96 months.

Figure 17: Kaplan Meier analysis on DFS: MVI vs. no MVI



At a log rank value of 0.694 cancer-free CRM > 1mm did not show significant differences on DFS as compared to the ≤ 1mm group (Figure 18). The results show the frequent proximity of the PDAC of the head to resection margins: whereas the ≤ 1mm group (R1) contained 69 cases, the > 1mm group (R1) consisted of only 5 cases. Median DFS was 12 months in the R1 group as compared to 10 months in the R0 group. The longest DFS in the R1 groups was 96 months as compared to 60 months in the R0 group.

Figure 18: Kaplan Meier analysis on DFS: CRM ≤ 1mm vs. >1 mm



For group comparison the 88 cases were administered to the corresponding invasion group according to the prevalence of PNI, LVI and MVI. It was differentiated between a PNI, LVI and MVI group, and a mixed type. Cases with only one exclusive invasion feature were allocated to either the PNI, LVI or MVI group and all cases with more than one invasion feature were assigned to the mixed group.

The group comparison on DFS did not reveal significant differences between the groups (log rank: 0.727) (Figure 19). The MVI and LVI group consisted of 3 cases each, the PNI group contained 27 cases and the mixed type group consisted of 49 cases. The high count of the mixed type indicates the frequent prevalence of more than one invasive feature in PDAC. Median survival for PNI type, LVI type and mixed type were 12 months as compared to 10 months for the MVI type. The longest DFS for MVI type, LVI type, PNI type and mixed type was 16, 31, 90 and 96 months, respectively.

Figure 19: Kaplan Meier analysis on DFS: group comparison

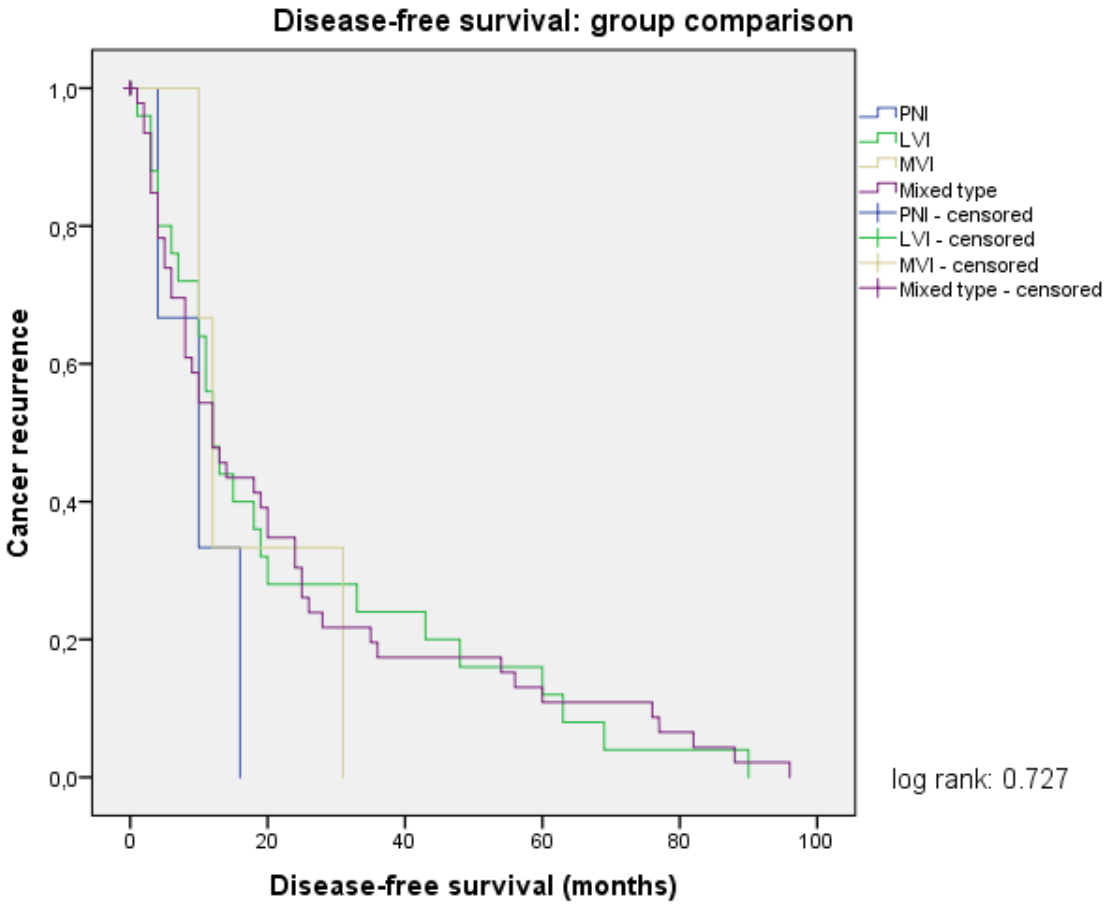


Table 6: Schedular Kaplan Meier analysis on DFS

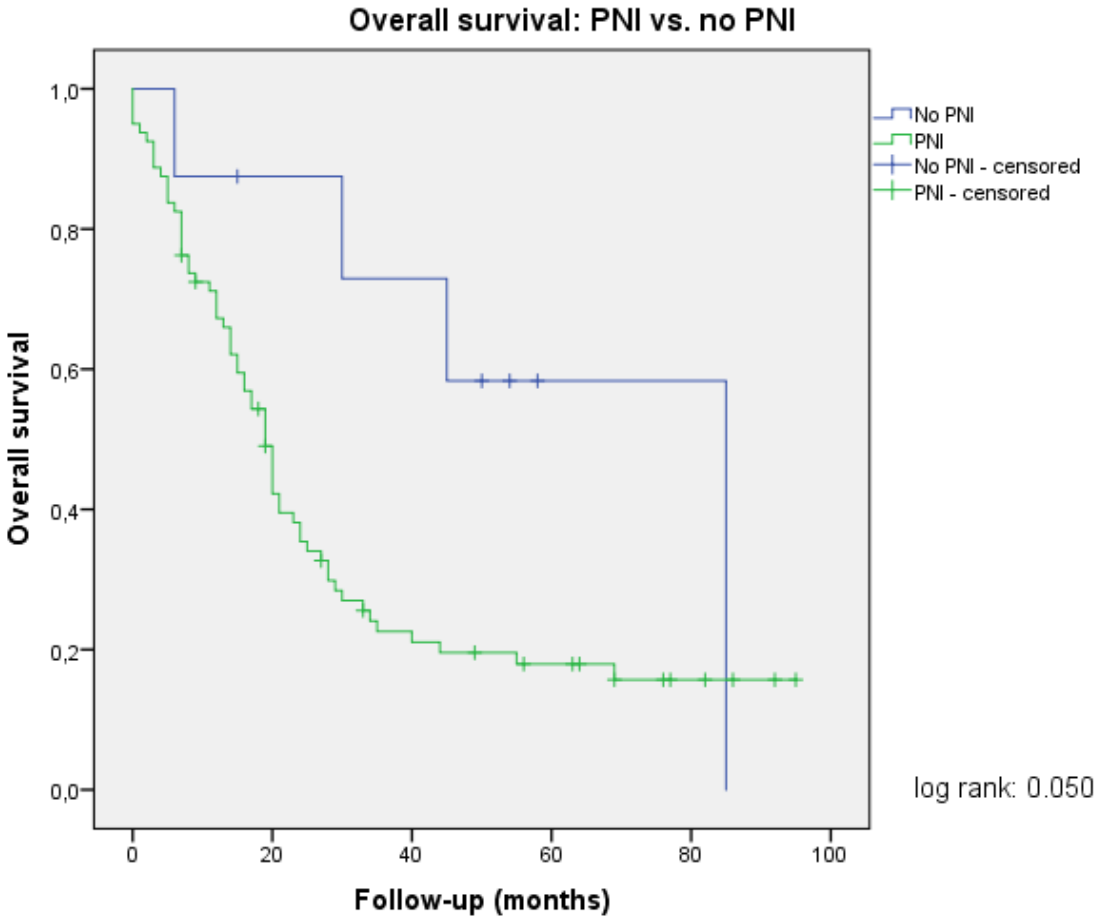
Disease-free survival analysis						
Single factor comparison	Total	Censored		Remaining	Median DFS	log rank
PNI	n	n	%	n	months	
Positive	74	5	6.8	69	12	
Negative	8	0	0.0	8	20	
Overall	82	5	6.1	77		0.511
PNI ≥ 30	n	n	%	n	months	
Positive	**11**	**1**	**9.1**	**10**	**8**	
Negative	**71**	**4**	**5.6**	**67**	**13**	
Overall	**82**	**5**	**6.1**	**77**		**0.030**
LVI	n	n	%	n	months	
Positive	**55**	**2**	**3.6**	**53**	**11**	
Negative	**27**	**3**	**11.1**	**24**	**24**	
Overall	**82**	**5**	**6.1**	**77**		**0.021**
MVI	n	n	%	n	months	
Positive	**16**	**1**	**6.3**	**15**	**8**	
Negative	**66**	**4**	**6.1**	**62**	**13**	
Overall	**82**	**5**	**6.1**	**77**		**0.004**
CRM	n	n	%	n	months	
> 1mm (R0)	5	1	20.0	4	10	
≤ 1mm (R1)	69	4	5.8	65	10	
Overall	74	5	6.8	69		0.694
Group comparison	n	n	%	n	months	
PNI	27	2	7.4	25	12	
LVI	3	0	0.0	3	12	
MVI	3	0	0.0	3	10	
Mixed type	49	3	6.1	46	12	
Overall	82	5	6.1	77		0.727

3.3.2 Overall survival

Impact of PNI, LVI, MVI and distance of PDAC to the CRM on OAS

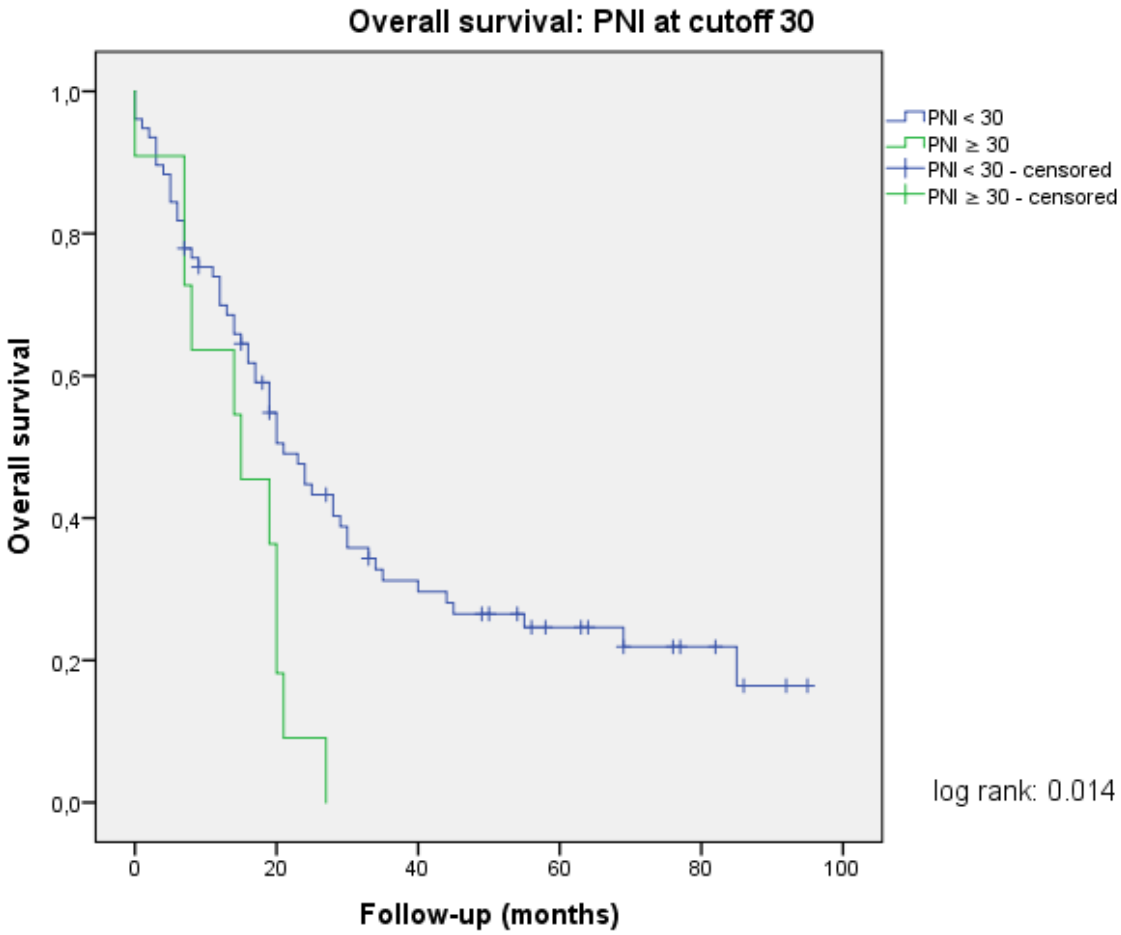
Kaplan Meier analysis revealed significant differences on OAS (p-value: 0.050) for PNI. As most cases showed nerval invasion, the non-PNI group consisted of only 8 cases as compared to 80 cases in the PNI group. Median survival in the non-PNI group was 85 months and 19 months in the PNI group, respectively (Figure 20, Table 7). The last recorded death in the non-PNI group occurred after 85 months as compared to 69 months in the PNI group. 5-year survival for the non-PNI group was 58.3% as compared to 17.9% in the PNI group.

Figure 20: Kaplan Meier analysis on OAS: PNI vs. no PNI



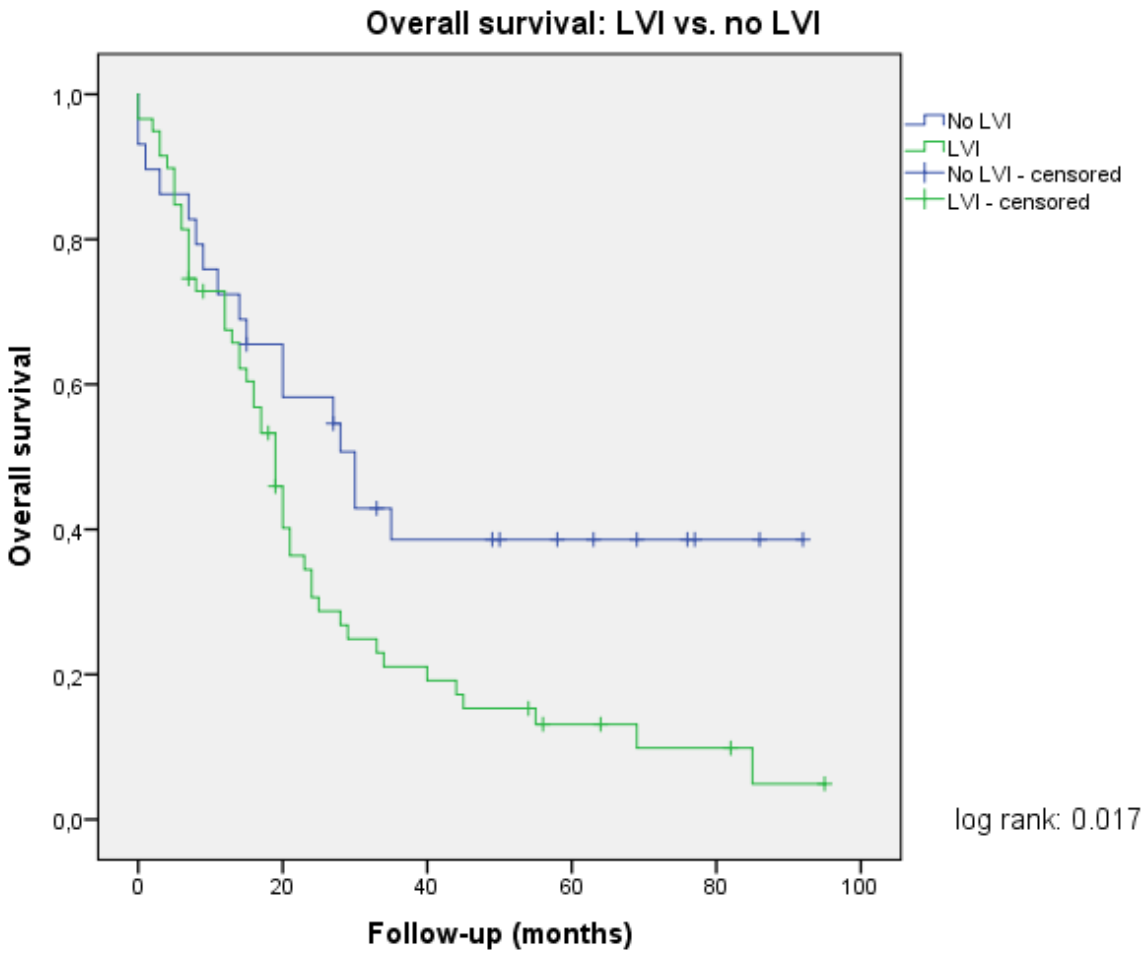
Furthermore, cases with ≥ 30 PNI were associated with a significant deterioration of OAS (p-value: 0.014) when compared to cases with < 30 PNI (Figure 21). The ≥ 30 PNI group consisted of 11 cases, whereas the < 30 PNI group included 77 cases. Median survival for the ≥ 30 PNI group was 15 months as compared to 21 months for the < 30 PNI group. Maximum survival and 5-year survival for the ≥ 30 PNI group was only 27 months and 0% in comparison to 85 months and 24.6% for the < 30 PNI group.

Figure 21: Kaplan Meier analysis on OAS: PNI < 30 vs. PNI ≥ 30



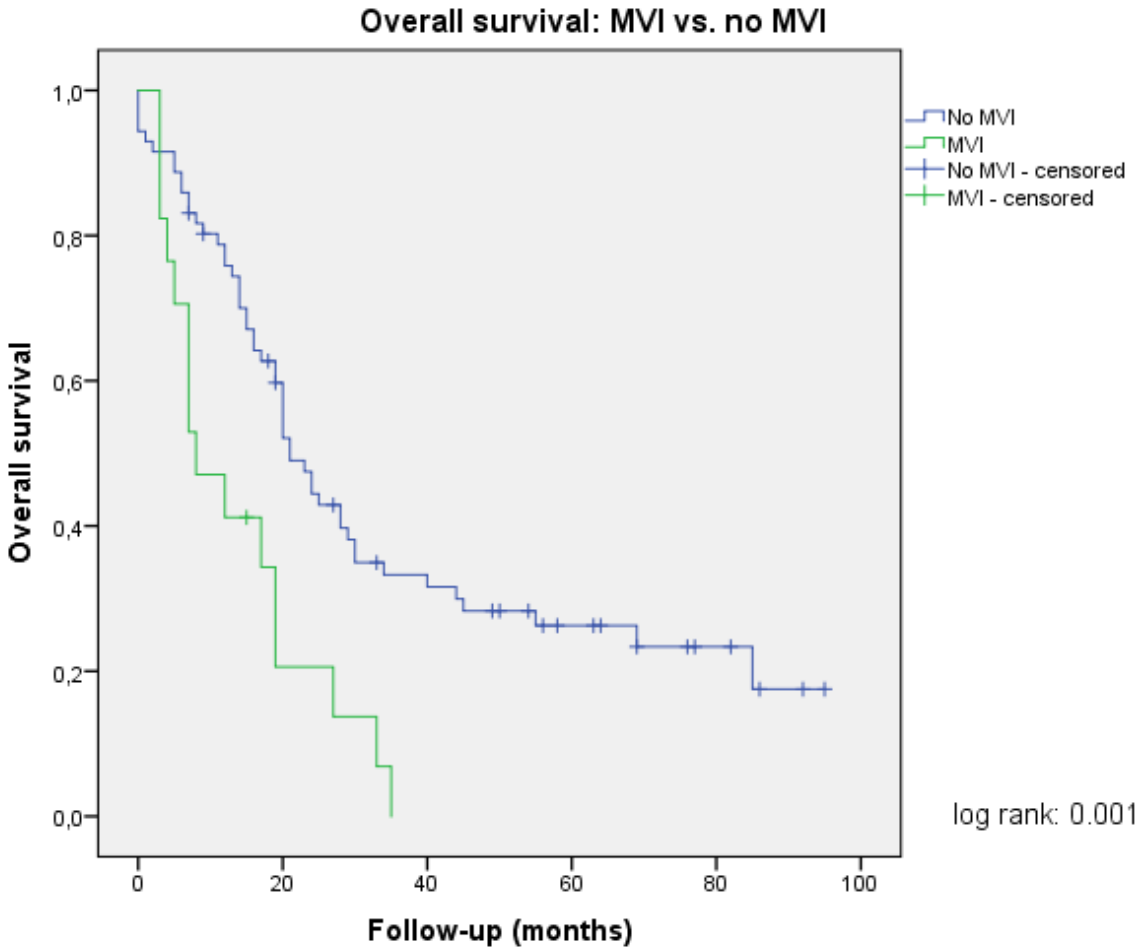
Also LVI did show significant impact on OAS (log rank: 0.017, Figure 22). The LVI group consisted of 59 cases with a median survival of 19 months as compared to 29 cases and 30 months of median survival in the non-LVI group. 5-year survival for the LVI group was 13.1% as compared to 38.6% in cases without LVI. The last registered death occurred after 35 months in the LVI group and after 85 months in the non-LVI group.

Figure 22: Kaplan Meier analysis on OAS: LVI vs. no LVI



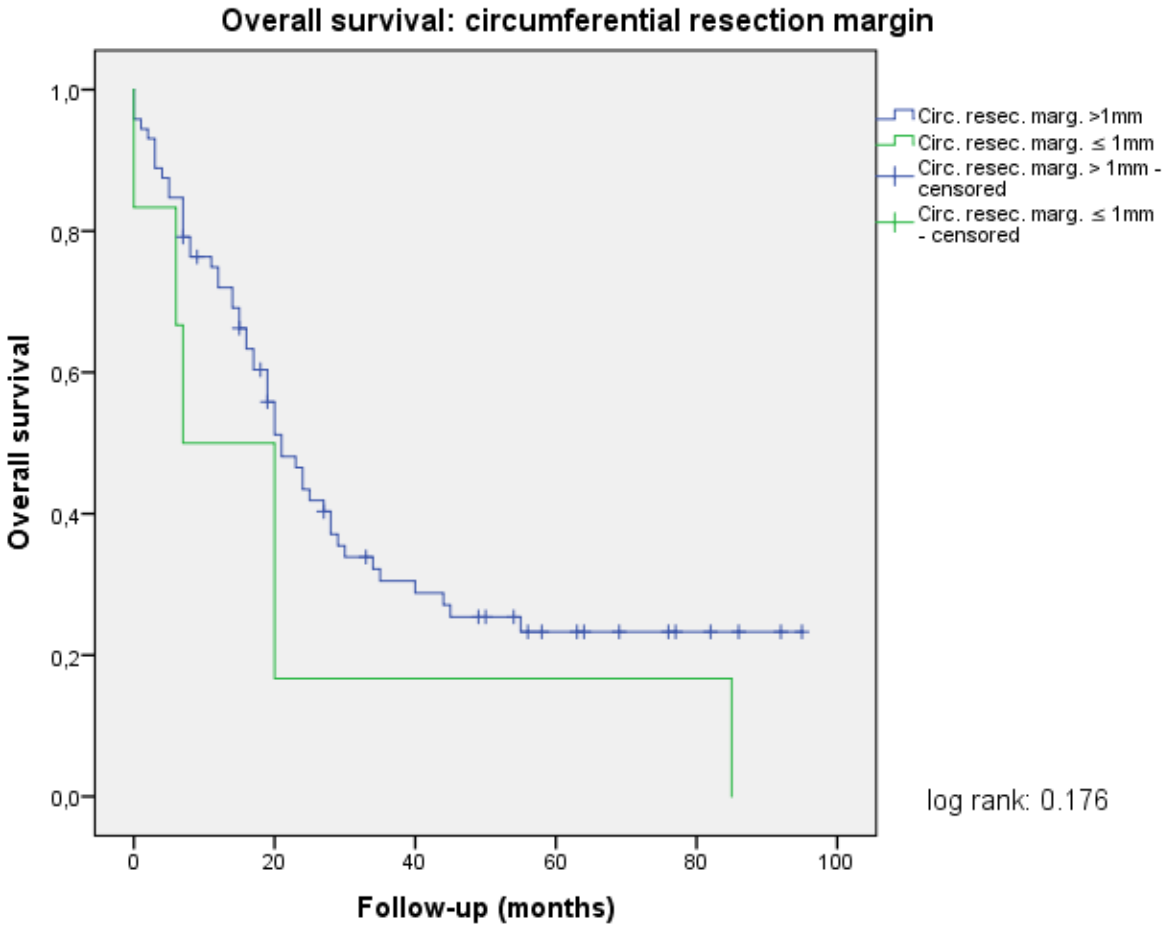
Significant differences concerning OAS (log rank: 0.001) could be determined for the MVI type as compared to cases without MVI. The MVI group contained 17 cases whereas the non-MVI group included 71 cases. The unequal distribution illustrates the comparably seldom occurrence of MVI as compared to PNI or LVI. Maximum survival in the MVI group was 35 months whereas the last recorded death in the non-MVI group occurred after 85 months. Median survival time was 8 months for the MVI group and 21 months for the non-MVI group, respectively (Figure 23, Table 7). 5-year survival for the MVI group and the non-MVI group was 0% and 26.3%, respectively.

Figure 23: Kaplan Meier analysis on OAS: MVI vs. No MVI



In Figure 24 the effect of cancer-free CRM is illustrated. In only 6 cases a cancer-free CRM of more than 1mm (R0) could be achieved. Seventy-two cases showed a resection margin of ≤ 1mm (R1). The two groups did not reveal significant differences (log rank: 0.176) concerning OAS. With only 7 months, median survival for the R0 group was reduced as compared to 21 months in the R1 group. 5-year survival in the R1 group was 23.3% as compared to 16.7% in the R0 group.

Figure 24: Kaplan Meier analysis on OAS: CRM status



The curves depicted in Figure 25 show the comparison between the different invasion types. Group administration was likewise to the Kaplan Meier analysis on DFS. PNI type contained 28 cases, both LVI and MVI type included 3 cases and mixed type formed the largest group with 54 cases. Log rank test did not reveal significant differences in OAS between the groups. For the PNI type, LVI type, MVI type and mixed type median survival was 20, 21, 8 and 23 months, respectively. 5-year survival for PNI type and mixed type was 25.6 months and 21.0 months, respectively. In the LVI group maximum survival was 40 months. For MVI type no maximum survival could be determined due to early censorship after 15 months.

Figure 25: Kaplan Meier analysis on OAS: group comparison

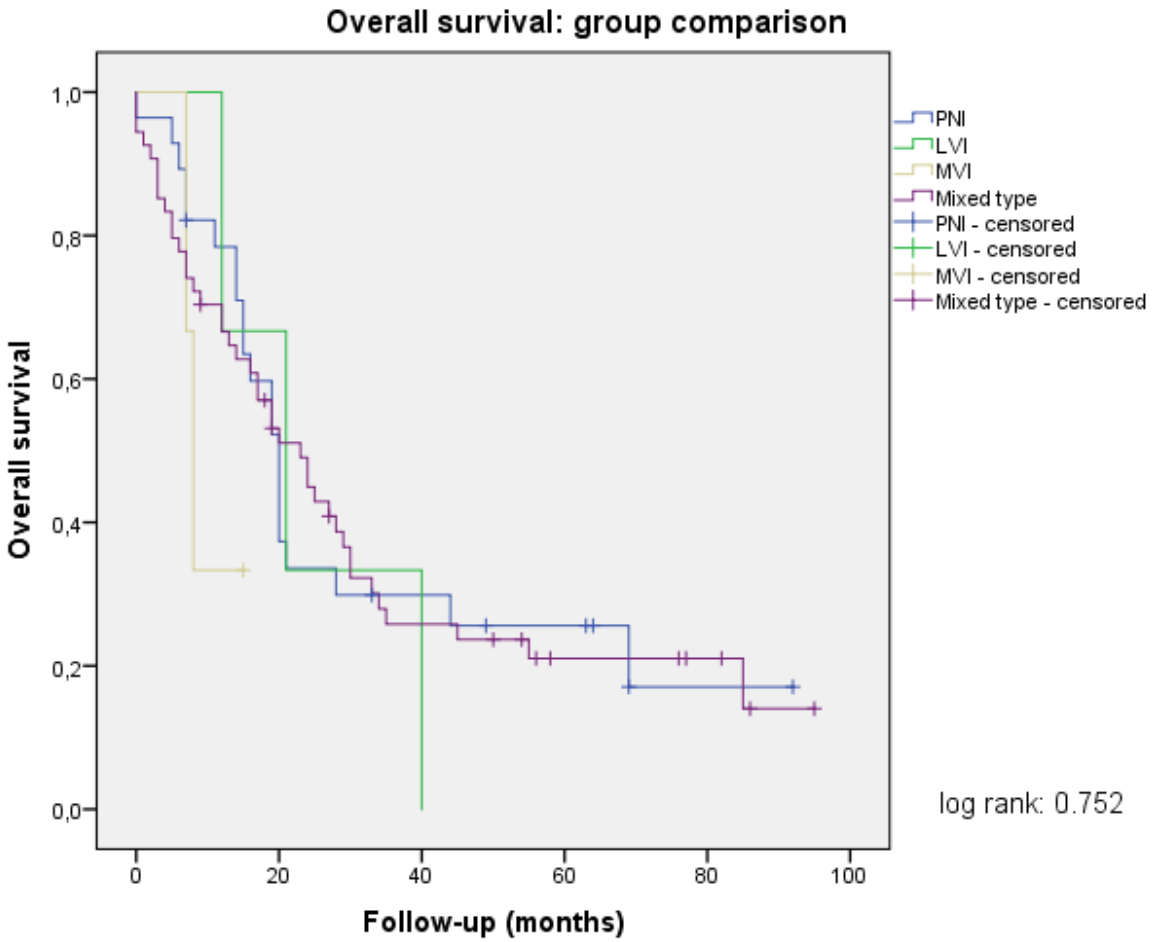


Table 7: Schedular Kaplan Meier analysis on DFS

Overall survival analysis						
Single factor comparison	Total	Censored		Remaining	Median time	log rank
PNI	n	n	%	n	months	
Positive	**80**	**17**	**21.4**	**63**	**19**	
Negative	**8**	**4**	**50.0**	**4**	**85**	
Overall	**88**	**21**	**23.9**	**67**		**0.050**
PNI ≥ 30	n	n	%	n	months	
Positive	**11**	**1**	**9.1**	**10**	**8**	
Negative	**71**	**4**	**5.6**	**67**	**13**	
Overall	**82**	**5**	**6.1**	**77**		**0.030**
LVI	n	n	%	n	months	
Positive	**59**	**9**	**15.3**	**23**	**19**	
Negative	**29**	**12**	**41.4**	**17**	**30**	
Overall	**88**	**21**	**23.9**	**67**		**0.017**
MVI	n	n	%	n	months	
Positive	**17**	**1**	**5.9**	**16**	**8**	
Negative	**71**	**20**	**28.2**	**51**	**21**	
Overall	**88**	**21**	**23.9**	**67**		**0.001**
CRM	n	n	%	n	months	
> 1mm (R0)	6	0	0.0	6	7	
≤ 1mm (R1)	72	21	29.2	51	21	
Overall	78	21	26.9	57		0.176
Group comparison	n	n	%	n	months	
PNI	28	7	25.0	21	20	
LVI	3	0	0.0	3	21	
MVI	3	1	33.3	1	8	
Mixed type	54	13	24.1	54	23	
Overall	88	21	23.9	67		0.752

3.3.3 Impact of clinical and histopathological factors on DFS and OAS

The impact of demographic and clinical variables on DFS and OAS was assessed using univariate Cox regression analysis. The following parameters were investigated: Demographic data included gender, age and BMI. Comorbid conditions included DM, nicotine and alcohol abuse, Charlson Comorbidity Index, preoperative weight loss, preoperative symptoms, preoperative pancreatitis, jaundice, anamnestic extrapancreatic malignancies, as well as pulmonary, gastrointestinal and cardiovascular comorbidities. Histopathologic features included tumour size, grading (G1/2 vs. G3/4), T stage, PNI vs. no PNI, PNI <30 vs. PNI ≥30,

intraneural tumour growth, LVI vs. no LVI, numeric CRM, CRM ≤1 mm vs. > 1mm, pancreatic groove resection margin, dorso-caudal resection margin, positive lymph nodes, LNR (< 0.2 vs > 0.2), mucinous component, clear cell component, as well as monocytic and neutrophil infiltration. The perioperative patient management data included ASA score, preoperative ERCP, type of surgery (Whipple vs. PPPD), skin-to-skin time, intra- and postoperative blood products, overall blood transfusion, chemotherapy and intraoperative inotropic support.

Variables significantly correlated with DFS and/or OAS were further evaluated in multivariate Cox regression to identify independent predictors. The Tables 10-13 illustrate significant findings and the primary parameters analysed in Kaplan Meier analysis.

3.3.3.1 Predictors of DFS

Univariate Cox regression on DFS

In univariate Cox regression PNI ≥ 30, LVI, MVI, grading (G1/2 vs. G3/4), positive lymph nodes and LNR > 0.2 revealed to be significant histopathological features predicting DFS. Also the comorbid factors preoperative weight loss, preoperative pancreatitis, preoperative symptoms as well as the perioperative parameter intraoperative inotropic support proved to be predictors of DFS. Demographic data as well as the CRM status had no impact on DFS (Table 8).

On closer examination MVI was identified as the morphological invasion parameter with the highest risk increase for shortened DFS (HR=2.27), followed by PNI ≥ 30 (HR=2.06) and LVI (HR=1.76). As also reported in previous studies both positive lymph nodes and an increased LNR of > 0.2 impact DFS [46]. With a HR of 2.15 LNR > 0.2 revealed to have greater impact on DFS as the analysis of positive lymph nodes alone. Worse differentiation of PDAC increased the risk for a reduced DFS. The HR of 2.83 identified preoperative pancreatitis to be the predictor with the highest risk increase of all analysed factors.

Table 8: Univariate Cox regression analysis: DFS

Variable	HR	95%CI	p-value
Grading 1/2 vs. 3/4	**2.07**	**1.31 - 3.29**	**0.002**
PNI < 30 vs. PNI ≥ 30	**2.06**	**1.04 - 4.10**	**0.039**
LVI vs. no LVI	**1.76**	**1.07 - 2.89**	**0.025**
MVI vs. no MVI	**2.27**	**1.26 - 4.09**	**0.006**
CRM ≤ 1mm vs. >1 mm	1.22	0.44 – 3.37	0.702
Positive lymph nodes	**1.09**	**1.04 - 1.15**	**0.001**
LNR (< 0.2 vs. > 0.2)	**2.15**	**1.32 - 3.48**	**0.002**
Preoperative weight loss	**1.90**	**1.17 - 3.09**	**0.009**
Preoperative symptoms	**2.16**	**1.25 - 3.74**	**0.006**
Preoperative pancreatitis	**2.83**	**1.11 - 7.22**	**0.030**
Intraoperative inotropics	**2.19**	**1.01 - 4.72**	**0.046**

Variables entered: demographic data, comorbidities, patient management data and histopathological features

Multivariate Cox regression analysis on DFS

Multivariate Cox regression on DFS identified grading, LNR > 0.2, preoperative weight loss and preoperative symptoms to be independent predictors of DFS (Table 9). No significant interaction for PNI ≥ 30, LVI, MVI, CRM, positive lymph nodes, preoperative symptoms and intraoperative inotropic support could be identified.

Table 9: Multivariate Cox proportional hazard analysis: DFS

Variable	HR	95%CI	p-value
Grading 1/2 vs. 3/4	**2.20**	**1.20 - 4.02**	**0.011**
PNI < 30 vs. PNI ≥ 30	1.21	0.51 - 2.87	0.672
LVI vs. no LVI	1.36	0.67 - 2.77	0.397
MVI vs. no MVI	1.92	0.86 - 2.77	0.114
CRM ≤ 1mm vs. >1 mm	1.12	0.35 - 3.59	0.854
LNR (< 0.2 vs. > 0.2)	**3.31**	**1.27 - 8.61**	**0.014**
Preoperative weight loss	**2.08**	**1.05 - 4.10**	**0.035**
Preoperative pancreatitis	**6.71**	**1.67 - 26.96**	**0.007**

Variables entered: grading 1/2 vs. 3/4, PNI < 30 vs. PNI ≥ 30, LVI vs. no LVI, MVI vs. no MVI, CRM ≤ 1mm vs. >1 mm, positive lymph nodes, LNR (<0.2 vs. >0.2), preoperative weight loss, preoperative symptoms, preoperative pancreatitis, intraoperative inotropics

3.3.3.2 Predictors of OAS

Univariate Cox regression on OAS

In univariate Cox regression grading, PNI ≥ 30, LVI, MVI, positive lymph nodes, LNR > 0.2, Charlson Comorbidity Index, preoperative symptoms and preoperative pancreatitis were identified as predictors of increased mortality in univariate Cox regression on OAS (Table 10).

The most severe predictors were MVI (HR=2.53), preoperative pancreatitis (HR=2.47) and LNR > 0.2 (HR=2.36). Charlson Comorbidity Index (HR=1.08) and positive lymph nodes (HR=1.09) revealed only a small risk increase on mortality.

Table 10: Univariate Cox regression analysis: OAS

Variable	HR	95%CI	p-value
Grading 1/2 vs. 3/4	**2.11**	**1.29 - 3.44**	**0.003**
PNI vs. no PNI	2.62	0.95 – 7.24	0.063
PNI < 30 vs. PNI ≥ 30	**2.23**	**1.14 - 4.37**	**0.019**
LVI vs. no LVI	**1.93**	**1.11 - 3.36**	**0.020**
MVI vs. no MVI	**2.53**	**1.42 - 4.52**	**0.002**
CRM ≤ 1mm vs. >1 mm	1.77	0.76 - 4.17	0.189
Positive lymph nodes	**1.09**	**1.03 - 1.14**	**0.002**
LNR (< 0.2 vs. > 0.2)	**2.36**	**1.42 - 3.91**	**0.001**
Charlson Comorbidity Index	**1.08**	**1.01 - 1.15**	**0.027**
Preoperative symptoms	**2.09**	**1.16 - 3.73**	**0.015**
Preoperative pancreatitis	**2.47**	**1.15 - 5.29**	**0.020**

Variables entered: demographic data, comorbidities, patient management data and histopathological features

Multivariate Cox regression analysis on OAS

In multivariate Cox regression analysis MVI, LNR > 0.2 and preoperative symptoms proved to be interacting predictors on OAS. PNI, PNI > 30, LVI, CRM ≤ 1mm, positive lymph nodes, Charlson Comorbidity Index and preoperative pancreatitis did not reveal to be independent predictors on OAS (Table 11).

Table 11: Multivariate Cox proportional hazard analysis: OAS

Variable	HR	95%CI	p-value
Grading 1/2 vs. 3/4	1.70	0.93 - 3.08	0.082
PNI vs. no PNI	2.54	0.75 - 8.60	0.134
PNI < 30 vs. PNI ≥ 30	1.10	0.46 - 2.63	0.839
LVI vs. no LVI	1.96	0.88 - 4.36	0.099
MVI vs. no MVI	**3.24**	**1.53 - 6.86**	**0.002**
CRM ≤ 1mm vs. >1 mm	2.79	0.93 - 8.37	0.068
LNR (< 0.2 vs. > 0.2)	**2.95**	**1.21 - 7.22**	**0.018**
Preoperative symptoms	**2.14**	**1.06 - 4.32**	**0.034**

Variables entered: grading 1/2 vs. 3/4, PNI vs. no PNI, PNI < 30 vs. PNI ≥ 30, LVI vs. no LVI, MVI vs. no MVI, CRM ≤ 1mm vs. >1 mm, positive lymph nodes, LNR (<0.2 vs. >0.2), Charlson Comorbidity Index, preoperative symptoms and preoperative pancreatitis

4 Discussion

The objectives of our study were to evaluate the impact of quantification of the morphological features PNI, LVI, MVI, the distance to CRM, as well as clinical parameters on outcome (DFS and OAS) in patients with PDAC who underwent Whipple procedure or PPPD.

PNI is a well-accepted factor associated with adverse outcome of patients with PDAC [50]. The results of our study are in support of these earlier findings. Intraneural growth was not associated with outcome, neither for DFS nor OAS. The cause of these discrepancies is not known. Differences in study design may play a role.

Data on the impact of MVI are sparse. A recent study showed a correlation of MVI with reduced OAS, but the exact number of MVI associated with adverse outcome was not described [51]. MVI may be an indicator of aggressive tumour behaviour. MVI seems to be less frequently observed in histological sections than PNI or LVI. In our study the mean number of MVI was 0.39 as compared to 14.75 for PNI or 2.07 for LVI, respectively. Nevertheless, when present MVI showed the strongest correlation with DFS and OAS among all morphological features evaluated with Kaplan Meier analysis and also on univariate Cox regression. On multivariate Cox proportional hazard analysis MVI was identified as an independent strong predictor of OAS but not DFS. MVI seems to correlate with the formation of distant metastasis which may be among the major limiting factors for survival, even in patients with local recurrence of PDAC [49].

Data on the impact of LVI on OAS are controversial and not numerous. In 2012, Chatterjee et al. described a correlation of LVI in vessels with muscular layer and DFS as well as OAS, though not for LVI in vessels without muscular layer. Although we made no such differentiation between muscular and non-muscular lymphatic vessels, in our cohort LVI was clearly associated with reduced DFS and OAS compared to those without LVI. Further study is required to elaborate whether subdivision of LVI affects prognosis in patients with PDAC [50]. In contrast to LVI there is broad consensus about the adverse prognostic effect of lymph node metastasis [52, 46]. However, the prognostic utility of LNR may even be superior to

the number of affected lymph nodes alone [46]: Baldwin et al. found reduced DFS and OAS at a LNR of < 0.2. An even worse outcome was reported for a LNR of 0.2-0.4 [46]. Our data are in support of the results of Baldwin et al.. An approximately threefold elevated risk of CR or death was associated with a LNR of > 0.2. Current TNM classification so far only considers the amount of cancer-positive lymph nodes. However, in accordance with other groups our results suggest that outcome prediction may be improved by the use of LNR instead of the number of positive lymph nodes alone.

In general, a strong correlation between adverse DFS and resection margin status (distance of PDAC to the resection margin) was described [34, 49, 53]. However, discrepancies were noted with respect to the exact distance of PDAC to the CRM. Variable numbers for CRM have been found which may be one of the consequences of inconsistent modes of gross pathological evaluation of the Whipple or PPPD resection specimen in different institutes of pathology [34, 49, 53]. In a seminal paper Esposito et al. suggested a standardised examination protocol for PDAC in order to improve interobserver bias [34]. The group proposed to define R0 as a minimal distance of > 1mm of the PDAC invasive front to the inked resection margin as they were able to prove worse outcome in patients whose PDAC were analysed by pathologists without a consistent definition of R0. However, also other distances for a prognostically relevant definition of R0 have been proposed and a consensus on a universally accepted R0 definition has not yet been reached [49, 52]. A similar observation was made in a meta-analysis of 1932 patients in 2016 [54]. The distance of PDAC to CRM based on the definition proposed by Esposito and colleagues was not identified as a prognostic variable for DFS or OAS in our study although a strong trend towards significance for OAS was seen on multivariate Cox regression analysis. The cause for this discrepancy is not known. However, the relatively small number of patients in our study could play a role.

Unintentional weight loss is considered a consequence of the disease-associated high metabolic rate of abnormal cells. It was reported that weight loss of ≥ 10% of initial body weight correlates with increased mortality [55]. However, data on the impact of preoperative weight loss and outcome are limited [56]. In contrast to OAS, uni- and multivariate analysis revealed preoperative weight loss to be a predictor for

DFS in our study suggesting that pronounced weight loss might be an indicator for a more aggressive cancer. This hypothesis requires further study.

In 2010, Wasif et al. described worse OAS for high-grade PDAC (G3, G4) as compared to low-grade PDAC (G1, G2) [57]. Differentiation grade of PDAC emerged as one of the most important prognostic factors in our study. Our univariate analysis on OAS was able to support the finding of Wasif et al.. Furthermore, we were able to demonstrate differentiation grade to be an independent predictor on DFS. As high grade PDAC is associated with less cohesive cells, those might scatter from the main mass of PDAC causing recurrence when remaining after resection.

Our study revealed preoperative symptoms and preoperative pancreatitis to be predictors of both DFS and OAS. Preoperative pancreatitis and weight loss were analysed independent and also as part of preoperative symptoms. Weight loss and preoperative symptoms therefore could be the dominant factors for significance in preoperative symptoms, as jaundice did not prove to be an independent predictor. Other preoperative symptoms like emesis, diarrhoea and abdominal pain were not analysed due to their rarity or their unspecific character.

In our study, also intraoperative inotropic support proved to be a predictor for DFS. Norepinephrine and adrenaline may induce damage to the DNA of leukocytes limiting their function [58]. The effect of inotropic substances (e.g. on cAMP receptor exchange protein) is thought to activate intracellular molecular pathways stimulating cancer progression via the activation of adrenoceptor-related cell stress which might be involved in impaired immunologic response [59]. However, data on the effect of preoperative patient management are controversial and further studies are required to define their impact on patient outcome.

It has to be recognized that the relatively small number of patients in our study is a limiting factor with respect to the robustness of statistical significance. This limitation may be outweighed by the elaborate analysis of patients with a completely sampled pancreatic head. Our results indicate that DFS and OAS are best predicted by a combination of histological and morphological factors. Among the histological factors, poor differentiation grade, MVI and LNR are important predictors, whereas preoperative symptoms are indicators of adverse outcome among the clinical factors evaluated. We suggest that histopathological evaluation of PDAC should be

performed on completely sampled pancreatic head to minimize the risk of missing any of these morphological prognostic factors due to sampling error.

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