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# Host Immune Response and Tumour Microenvironment in Pancreatic cancer

Impact on Patients' Survival

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine and Health Technology
of Tampere University,
for public discussion in the Auditorium
of the Finn-Medi 5, Biokatu 12, Tampere,
on 10th December, at 12 o'clock.

ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Finland

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# ACKNOWLEDGEMENTS

# ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a malignant disease with an increasing incidence. Surgery is the only option to achieve cure, but it can be offered for a minority of patients since most patients present with unresectable disease. Five-year prognosis is only approximately 20 %, even for patients undergoing surgery with a curative aim.

Host immune response and tumour microenvironment have a significant impact on the progression of the disease and on the survival of the patients with PDAC. PDAC is known to develop mechanisms for immune escape. Tumour-node-metastasis (TNM) classification has been the most used method for years when estimating the prognosis of the patients with PDAC. However, it describes only the stage of progression of the disease, not its' biological features or the level of immune response leading to inadequate prognostic accuracy. This thesis aimed to study the prognostic role of immune cell infiltration and other microenvironmental factors in PDAC. The suitability of immune cell infiltration-based immune cell score (ICS) as a prognostic tool in PDAC was also assessed. The thesis comprises four studies based on two cohorts of PDAC patients who had undergone surgery with curative intent.

The first part of the study demonstrates the prognostic value of CD3+ and CD8+ cell -based ICS in a cohort of 108 PDAC patients, operated on in Central Finland Central Hospital between 2000 and 2016. High ICS is shown to be an independent prognostic factor for prolonged survival regardless of TNM stage.

In the second part of the study the prognostic value of ICS is shown in another cohort of 79 patients, operated on in Oulu University Hospital with curative intent between 1993 and 2015. ICS determination was performed using two different techniques in this part of the study. The study shows the superiority of whole tissue section technique over hot-spot technique.

The third part focuses on the impact of immune suppressive protein CD73 on the survival of patients with PDAC. According to the results, high CD73 expression in tumour cells is an independent negative prognostic factor in PDAC. Moreover, high expression of CD73 in tumour infiltrating lymphocytes was associated with lymph node metastasis.

The fourth part of the study shows the prognostic role of stromal hyaluronan accumulation in PDAC. The hyaluronan accumulation in stroma is shown to be associated with poor prognosis and low-level host immune response.

As a conclusion, progression of PDAC is heavily dependent on host immune response and other microenvironmental factors such as overexpression of CD73 and hyaluronan. ICS as an indicator of immune response can be used as a predictor of the survival among patients with PDAC.

# TIIVISTELMÄ

Haiman duktaalinen adenokarsinooma on yleistyvä pahanlaatuinen sairaus. Kirurgia on edelleen ainoa hoitomuoto, johon liittyy pysyvän parantumisen mahdollisuus. Se on kuitenkin tarjolla vain pienelle osalle haimasyöpäpotilaista, koska tauti on yleensä diagnoosivaiheessa edennyt leikkaushoidon ulottumattomiin. Vain noin viidennes leikatuistakin potilaista elää yli viisi vuotta.

Elimistön omalla immuunivasteella ja kasvaimen mikroympäristöllä on todettu olevan merkittävä vaikutus haimasyövän etenemiseen ja sitä kautta potilaiden selviytymiseen. Haimasyövän tiedetään kehittävän immuunivastetta vaimentavia mekanismeja. Haimasyöpäpotilaiden ennusteen arviointiin käytetyin menetelmä on kasvaimen kokoa sekä leviämisastetta kuvaava TNM -luokitus, johon kuitenkin liittyy merkittävää epätarkkuutta. Luokitus ei huomioi kasvaimen biologisia piirteitä eikä immuunivasteen voimakkuutta. Tämä väitöskirjatutkimus pyrki selvittämään kasvaimen mikroympäristön kuten kasvainkudoksen sisältämien immuunisolujen määrän ennusteellista vaikutusta haimasyöäpotilaan ennusteeseen. Lisäksi tutkittiin kasvaimen immuunisolumääriin perustuvan immune cell score (ICS) -luokituksen soveltuvuutta haimasyöpäpotilaiden selviytymisen ennustamiseen. Neljästä osatyöstä koostuva tutkimus perustuu kahden eri sairaalan haimasyöpäleikkauksen läpikäyneiden potilaiden aineistoon.

Ensimmäisessä osatyössä osoitettiin CD3+ ja CD8+ T -solujen määrään perustuvan ICS- luokituksen ennustavan haimasyöpäpotilaiden selviytymistä. Keski-Suomen Keskusssairaalassa leikatun 108:n haimasyöpäpotilaan aineistossa korkea ICS ennusti parempaa selviytymistä riippumatta TNM -luokasta.

Toinen osatyö osoitti ICS:n toimivuuden toisessa, Oulun Yliopistollisessa sairaalassa leikatussa haimasyöpäpotilaiden aineistossa. Tässä työssä ICS -luokitus toteutettiin kahdella eri tekniikalla, käyttäen sekä kasvaimesta tehtyjä kokoleikkeitä että niin sanottua hot-spot -tekniikkaa, joista ensimmäinen osoittautui toimivammaksi.

Kolmannessa osatyössä selvitti immuunivastetta vaimentavan CD73 -proteiinin ennustevaikutusta haimasyövässä. Tulokset osoittivat kasvainsolujen korkean CD73 -ekspression ennustavan huonoa selviytymistä. Lisäksi todettiin

imusolmukemetastasoinnin olevan yleisempää niillä potilailla, joilla kasvainalueen immuunisolut ilmensivät runsaasti CD73 -proteiinia.

Neljännessä osatyössä osoitettiin kasvaimen strooman hyaluronaanin suuren pitoisuuden liittyvän huonompaan ennusteeseen. Tämän todettiin myös olevan yhteydessä heikompiasteiseen immuunireaktioon.

Johtopäätöksenä voidaan todeta haimasyövän etenemisen riippuvan vahvasti elimistön immuunivasteesta ja muista kasvaimen mikroympäristössä vaikuttavista tekijöistä kuten CD73 -proteiinin ja hyaluronaanin ylimäärästä. Immuunivasteen voimakkuutta kuvaava ICS vaikuttaa tämän tutkimuksen perusteella soveltuvan haimasyöpäpotilaiden selviytymisen ennustamiseen.

# CONTENTS

# ABBREVIATIONS

AJCC             American Joint Committee on Cancer
APC              antigen presenting cell
CA 19-9          carbohydrate 19-9 antigen
CAF              cancer-associated fibroblast
CD73             cluster of differentiation 73
CEA              carcinoembryogenic antigen
CFCH             Central Finland Central Hospital
CI               confidence interva
CRP              C-reactive protein
CT               computed tomography
CTLA-4           cytotoxic T lymphocyte-associated antigen 4
DC               dendritic cell
DNA              deoxyribonucleic acid
DSS              disease-specific survival
ECM              extracellular matrix
ER               endoplasmic reticulum
ERCP             endoscopic retrograde cholangiopancreatography
EUS              endoscopic ultrasonography
FNA              fine needle aspiration
FNB              fine needle biopsy
FOLFIRINOX       chemotherapy regimen composed of folinic acid, fluorouracil,
                 irinotecan and oxaliplatin
5-FU             5-fluorouracil
HA               hyaluronan
HARE             hyaluronic acid receptor for endocytosis
HAS              hyaluronan synthase
HMW-HA           high molecular weight hyaluronan
Hyal-1           hyaluronidase-1
ICS              immune cell score
IFN- γ           interferon- γ

| | |
|---|---|
| IHC | immunohistochemistry |
| IL-10 | interleukin-10 |
| IPMN | intraductal papillary mucinous neoplasm |
| LIF | leukaemia inhibitory factor |
| LYVE-1 | lymphatic vessel endothelial hyaluronan receptor 1 |
| MCN | mucinous cystic neoplasm |
| MDSC | myeloid derived suppressor cell |
| miRNA | micro ribonucleic acid |
| MLH-1 | mutL homologue-1 |
| MRCP | magnetic resonance cholangiopancreatography |
| MRI | magnetic resonance imaging |
| MSI | microsatellite instability |
| NAB | nanoparticle albumin-bound |
| OS | overall survival |
| Pan-In | pancreatic intraepithelial neoplasia |
| PDAC | pancreatic ductal adenocarcinoma |
| PD-1 | programmed cell death protein-1 |
| PD-L1 | programmed cell death ligand 1 |
| PET | positron emission tomography |
| PNI | perineural invasion |
| PSC | pancreatic stellate cell |
| RHAMM | receptor for HA-mediated motility |
| ROC | receiver operating characteristic |
| SD | standard deviation |
| SRR | summary related risk |
| TAM | tumour-associated macrophage |
| T-eff | effector T cell |
| TGF-β | tumour-growth factor β |
| Th | helper T cell |
| TIL | tumour infiltrating lymphocytes |
| TMA | tissue micro array |
| TNF | tumour necrosis factor |
| TNM | tumour-node-metastasis classification |
| TOL | toll -like receptor |
| tp53 | tumour protein 53 |
| T-reg | regulatory T cell |

| | |
|---|---|
| TSLP | thymic stromal lymphopoietin |
| TRK | tropomyosin receptor kinase |
| WHO | World Health Organisation |

# ORIGINAL COMMUNICATIONS

Publication I      High immune cell score predicts improved survival in pancreatic cancer. Tahkola K, Mecklin J-P, Wirta E-V, Ahtiainen M, Helminen O, Böhm J*, Kellokumpu I*. Virchows Arch. 2018 Apr; 472(4):653-665.

Publication II     Immune cell score in pancreatic cancer – comparison of hotspot and whole section techniques. Tahkola K, Leppänen J, Ahtiainen M, Väyrynen J, Haapasaari KM, Karttunen T, Kellokumpu I, Helminen O, Böhm J. Virchows Arch. 2019 Jun;474(6):691-699.

Publication III    Prognostic impact of CD73 expression and its relationship to PD-L1 in patients with radically treated pancreatic cancer. Tahkola K, Ahtiainen M, Kellokumpu I, Mecklin JP, Laukkarinen J, Laakkonen J, Kenessey I, Jalkanen S, Salmi M, Böhm J. Virchows Arch. 2021 Feb;478(2):209-217.

Publication IV     Stromal hyaluronan accumulation is associated with low immune response and poor prognosis in pancreatic cancer. Tahkola K, Ahtiainen M, Mecklin J-P, Kellokumpu I, Laukkarinen J, Tammi M, Tammi R, Väyrynen JP, Böhm J. Sci Rep. 2021 Jun 9;11(1):12216.

# AUTHORS CONTRIBUTION

Paper I of the Thesis:

The author of this thesis (KT) conceived the study design together with the supervisors. He contributed to the clinical data collection and review of patient records. He carried out the computer-assisted immune cell count and manual review of the images followed by immune cell score determination. He also contributed to the assessment of MLH1-expression and perineural invasion. He carried out the statistical analyses and contributed to figure design and production, and finally wrote the manuscripts with input from other authors.

Paper II of the Thesis:

KT designed the study in consultation with his supervisors and adjunct professor Helminen. He contributed to the review of the clinical data as well as computer-assisted image analysis and immune cell score determination and statistical analyses. He wrote the manuscript with support from the other authors.

Paper III of the Thesis:

KT conceived the study together with his supervisors and Professor Jalkanen. He contributed to the clinical data collection and review from patient records. He carried out the assessment of CD73 expression together with his supervisors and Dr Kenessey and PD-L1 expression with Dr Ahtiainen. He performed data analyses including statistical analyses. He wrote the manuscript with input from the other authors and contributed to figure production.

Paper IV of the Thesis:

KT contributed to study design and clinical data collection as well as review from patient records. He carried out manual assessment of the hyaluronan expression and contributed to computer -assisted image analysis. He performed data analyses

including statistical analyses. He wrote the manuscript with input from other authors and contributed to figure production.

# 1 INTRODUCTION

Pancreatic cancer is a lethal condition with a dismal prognosis, the worldwide 5-year survival rate being around 6% (McGuigan et al., 2018; Mizrahi et al., 2020). The age adjusted relative 5-year-survival rate in Finland corresponds to worldwide rates being 6.69% (Finnish cancer registry, n.d.). This is mainly because of the asymptomatic nature of the disease leading to diagnosis at late stages. Surgery provides an opportunity for cure for some, but only approximately 20 % of patients survive for five years even after surgery with curative intent.

Tumour microenvironment, including tumour infiltrating lymphocytes (TILs) and the extra-cellular matrix (ECM) have been an object of intensive research in recent decades due to observations of their significant impact on cancer progression. The evidence confirming the prognostic value of efficient host immune response, represented by immune cell infiltration at the tumour site, is increasing in pancreatic ductal adenocarcinoma (PDAC). However, PDAC is known to develop several immune escape mechanisms thereby limiting the host immune response.

This thesis focuses on assessing the prognostic value of TILs in PDAC, finding an optimal method with prognostic value to measure host immune response and investigating the impact of microenvironmental factors such as CD73, PD-L1 and hyaluronan (HA) on the progression of PDAC.

# 2 REVIEW OF THE LITERATURE

## 2.1 Malignant tumours of the pancreas

Several pancreatic neoplasms and tumour-like lesions have been characterized. These neoplasms can be classified based on the lines of cellular differentiation, the gross configuration of the tumour or the degree of dysplasia. Most pancreatic tumours recapitulate one or more epithelial cell lines of the pancreas: ductal, acinar or neuroendocrine. However, mesenchymal and lymphatic pancreatic tumours have also been described, although these are rare. (Klimstra, David S; Adsay, 2015)

**Table 1.**     Malignant tumours of the pancreas classified by the lines of cellular differentiation

| Line of cellular differentiation | Malignancy |
| --- | --- |
| Ductal | Conventional ductal adenocarcinoma |
| | Unusual histologic variants of conventional ductal adenocarcinoma |
| | Other carcinomas of ductal origin, like colloid carcinoma |
| Acinar | Acinar cell carcinoma |
| | Acinar cell cystadenocarcinoma |
| | Pancreatoblastoma |
| Neuroendocrine | Well-differentiated neuroendocrine tumours |
| | Poorly differentiated neuroendocrine carcinoma |
| Lymphatic | Lymphoma |
| Mesenchymal | Soft tissue sarcomas |
| Mixed | Mixed acinar -neuroendocrine carcinoma |
| | Mixed ductal-neuroendocrine carcinoma |
| | Mixed acinar-neuroendocrine-ductal carcinoma |
| | Mixed acinar-ductal carcinoma |

Invasive PDAC accounts for around 90% of pancreatic malignancies. In this thesis, as generally in the literature, the term pancreatic cancer is used synonymously with invasive PDAC.


## 2.2     Epidemiology of pancreatic cancer

PDAC is the sixth deadliest cancer worldwide (Fitzmaurice et al., 2019). Its age-standardized incidence increased from 5.0 in 1990 to 5.7 per 100 000 person-years

in 2017, meaning 448 000 annual cases globally. There was a 2.3-fold increase in the number of deaths during the same period, reflecting both an increase in incidence and growth of the population. The increase in incidence of PDAC is likely to continue in the future as the population ages (Rahib et al., 2014).

The incidence of pancreatic cancer varies significantly across regions and populations. The highest incidence and death rates are observed in affluent north America, western Europe, Asia-Pacific and central Europe and the lowest rates are observed in South-Asia and sub-Saharan Africa. According to the Finnish Cancer Registry the incidence of pancreatic cancer in Finland was around 19.6 per 100,000 in 2019, with a significant increase from the level of 15.09 per 100,000 in 2000 and 11.6 in 1980 (Finnish cancer registry, n.d.). The incidence of pancreatic cancer in Finland between 1953 and 2019 is shown in Fig 1.

**Figure 1.** Figure 1. Incidence of pancreatic cancer in Finland in 1953-2019. Data obtained from the Finnish Cancer Registry (Finnish cancer registry, n.d.).
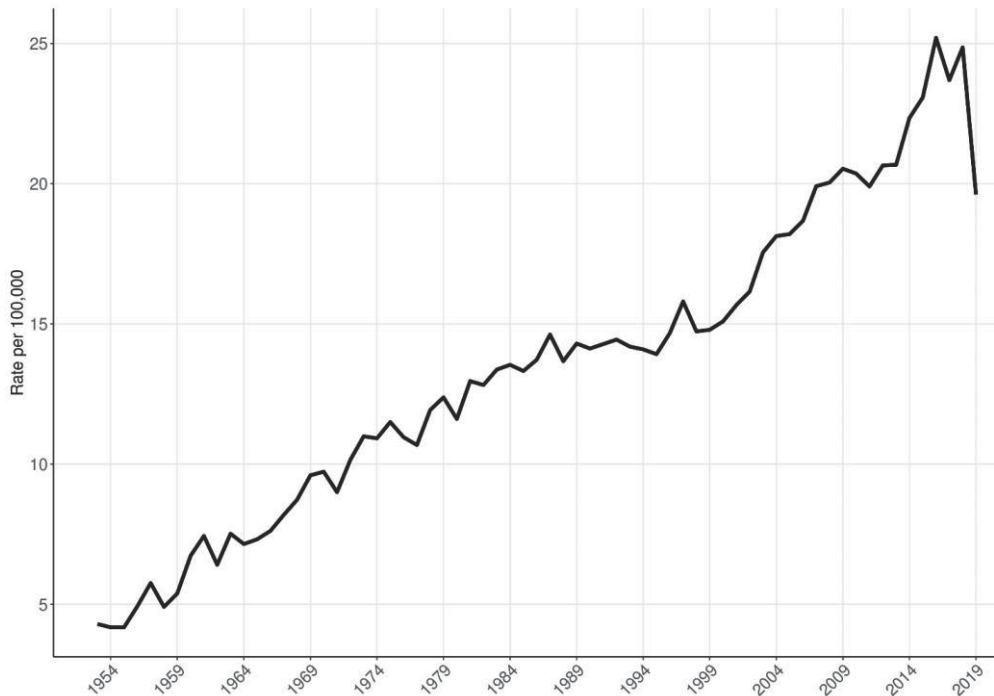


In 2017, 51.9 per cent of total incident cases and 53% of total deaths occurred in males globally. The incidence and death rates are higher among men in almost all regions, with the exception of Andean Latin America and western sub-Saharan Africa (Pourshams, Akram; Sepanlou Sadaf; Ikuta, Kevin; Bisignano, Catherine; Safiri, Saeid; Roshandel, 2019). The reasons for this are not completely understood. Females have less exposure to smoking, which is assumed to explain at least part of the difference between the sexes (Capasso et al., 2018).

## 2.3 Risk factors

According to single studies, several environmental exposures have been suspected of being risk factors for PDAC. However, the strength of the evidence for an association between these and PDAC is poor, except for exposure to chlorinated hydrocarbon solvents and related compounds (SRR 1.4-2.2) and exposure to nickel

(SRR 2.0) (Maisonneuve & Lowenfels, 2015). Daily consumption of ≥30 g of alcohol, or the equivalent of >3 portions of any alcoholic beverage per day is associated with an increased risk of PDAC with an estimated relative risk of 1.2 (Genkinger et al., 2009; Lucenteforte et al., 2012; Michaud et al., 2010; Tramacere et al., 2010). There are also data concerning associations between PDAC risk and several dietary items, for instance red meat, and drugs like metformin, but these associations have not yet been irrefutably confirmed in pooled analysis (Maisonneuve & Lowenfels, 2015).

Tobacco smoking is the most well known risk factor for PDAC, with an estimated relative risk of 1.6-1.7 (Ansary-Moghaddam et al., 2006; Capasso et al., 2018; Iodice et al., 2008; Maisonneuve & Lowenfels, 2015; Pourshams et al., 2019). There is also strong evidence of an association between diabetes, whether recently onset or long term, and PDAC, likewise between pancreatitis and PDAC (Ben et al., 2011; Bosetti et al., 2014; Duell et al., 2012; Elena et al., 2013; Y. Huang et al., 2014; Raimondi et al., 2010; Sasazuki et al., 2013; Starup-Linde et al., 2013). A history of cholecystectomy, gastrectomy, periodontal diseases or hepatitis B infection also appears to be associated with increased risk of PDAC (Bosetti et al., 2013; Fiorino et al., 2013; Fitzpatrick & Katz, 2010; Gong et al., 2012; L. Li et al., 2013; Lin et al., 2012; Luo et al., 2013; Yunxia Wang et al., 2013; Xu et al., 2013). Having a non-O blood group is associated with an increased risk of PDAC, with a 30-40% increased risk (Iodice et al., 2010; Risch et al., 2013; Wolpin et al., 2010). Gastric colonization with Helicobacter Pylori is also associated with greater risk of PDAC (Risch et al., 2010).

Obese, and also tall individuals, have an increased risk of PDAC (Ansary-Moghaddam et al., 2006; Arslan et al., 2010; Aune et al., 2012; Berrington de Gonzalez et al., 2003; Genkinger et al., 2011; Jiao et al., 2010; Larsson et al., 2007). A positive family history of PDAC is clearly associated with an increased risk of this disease with an 80% increased risk (Jacobs et al., 2010; Permuth-Wey & Egan, 2009). Associations between PDAC risk and various germ-line mutations (BRCA1, BRCA2, PALB2, ATM, CDKN2A, APC, MLH1, MSH2, MSH6, PMS2, PRSS1 and STK11) have been reported, often as part of a familial cancer syndrome (Canto et al., 2013; Grover & Syngal, 2010; Solomon et al., 2012).

Conversely, atopic allergy or hay fever seem to reduce the risk of PDAC by 20-30% (Gandini et al., 2005; Olson et al., 2013). There is also some evidence suggesting that occupational physical activity protects against PDAC (Bao & Michaud, 2008; O'Rorke et al., 2010).

## 2.4     Aetiopathogenesis of pancreatic cancer

PDAC has a multifactorial aetiology. Some of the known risk factors, like tobacco, cause direct DNA damage. Others, like Helicobacter Pylori, alcohol, pancreatitis and cholecystectomy are known triggers of inflammation, which is one of the established pathways leading to PDAC carcinogenesis (Greer & Whitcomb, 2009). Another aetiological pathway is linked to insulin resistance, related to risk factors like obesity and diabetes, even though the exact mechanisms behind this pathway are not fully understood. Haemostasis is also one process behind the carcinogenesis of PDAC, linked to risk factors like non-O-blood group (Nakchbandi & Löhr, 2008). These pathways together lead to an accumulation of genetic and epigenetic alterations and notable changes in tumour microenvironment, finally resulting in invasive PDAC.

### 2.4.1    Precursor lesions

PDAC develops following a series of step-wise mutations from normal mucosa to specific non-invasive precursor lesions. There are three different kinds of known precursor lesions: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). PanINs are small, <5mm diameter. These can harbour the same somatic genetic alterations as seen in PDAC. The prevalence of these alterations rises as the extent of cytological and architectural atypia in PanIN increases (Hruban et al., 2000; Hruban et al., 2008). IPMNs are bigger, >5mm diameter and less frequent (Hruban et al., 2004). The malignant potential of these lesions depends on the amount of cytological and architectural neoplasia and their location: Main duct IPMNs have greater malignant potential than IPMNs located in the branch ducts. MCN is composed of mucin-producing epithelial cells and an associated ovarian-type stroma. It occurs predominantly in women (Reddy et al., 2004).

### 2.4.2    Genetic abnormalities

Regardless of the type of precursor lesion, the genetic abnormalities accumulate during carcinogenesis. There are, however, differences between the genetic alteration profiles of precursor lesions. The mutational activation of KRAS oncogene and telomere shortening are the earliest known genetic abnormalities (van Heek et al., 2002). Inactivation of tumour-suppressing genes including CDKN2A, TP53,

SMAD4 and BRCA2 together with widespread chromosomal losses and gene amplifications are the other most frequent genetic alterations developing during the carcinogenesis (Jones et al., 2008; Rozenblum et al., 1997). A quantitative analysis of the timing of the genetic evolution of PDAC indicates that the interval between the occurrence of the initiating mutation and the moment at which the tumour has gained metastatic ability is more than 15 years (Yachida et al., 2010). There are, however, data supporting a view according which PDAC tumourigenesis is not gradual and does not follow a specific mutation order, at least in a subset of cases (Notta et al., 2016).

By means of genomic analysis, PDAC has been divided into four sub-groups based on 32 recurrently mutated genes. These groups, namely squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine, have unique genomic signatures corresponding to histopathological findings and prognosis (Bailey et al., 2016). In addition to the genetic alterations described above, epigenetic changes, such as alterations in DNA methylation and histone modifications, can also alter gene function during carcinogenesis (Omura & Goggins, 2009).

## 2.4.3 Changes in immune microenvironment

In addition to the genetic and epigenetic alterations described in tumoural cells, remarkable changes in immune microenvironment are also seen during the progression of PDAC: Prevalence of immunosuppressive regulatory T cells (T-reg) increases during the progression of premalignant lesions. Conversely, the infiltration of intraepithelial CD8+ T cells as an indicator of immune surveillance simultaneously diminishes, thereby optimizing the circumstances for multistep carcinogenesis of PDAC (Hiraoka et al., 2006).

## 2.4.4 Genetic susceptibility

Between 4 and 10 % of cases of PDAC are familial, depending on the definition of familial pancreatic cancer. PDAC is usually defined as a familial pancreatic cancer when a patient with PDAC has two or more pancreatic cancer patients among first degree relatives and there is no association with known hereditary genetic syndromes. In addition, some inherited syndromes, such as Peutz-Jeghers syndrome, hereditary pancreatitis, familial atypical multiple mole melanoma, hereditary breast-

ovarian cancer, hereditary non-polyposis colorectal cancer , Lynch syndrome, familial adenomatous polyposis and Werner syndrome, are associated with higher risk of PDAC. (Matsubayashi et al., 2017).

In contrast to the inherited syndromes, the genetic basis for familial aggregation has not been identified in most familial PDAC cases. Indeed, the known germline mutations account for less than 20% of familial pancreatic cancer cases (Matsubayashi et al., 2017). ATM, BRCA1 and 2, CHEK2 and PALB2 belong to the known genes responsible for familial pancreatic cancer. Some familial PDAC cases have been shown to be associated with environmental factors (Yeo et al., 2009), confirming the fact that familial pancreatic cancer is not a synonym for inherited pancreatic cancer.

## 2.5 Diagnosis

### 2.5.1 Symptoms and clinical findings

The symptoms of PDAC overlap with those of other benign and malignant diseases. The symptoms often appear in the late stage and may be intermittent. These common symptoms include weight loss, malabsorption, abdominal and back pain, dyspepsia and nausea, without any cardinal symptom. Sometimes an attack of pancreatitis is the first clinical presentation. About 25 % of patients with PDAC have diabetes mellitus at diagnosis and roughly another 40% have impaired glucose tolerance (Chari et al., 2008; Pannala et al., 2008).

### 2.5.2 Laboratory tests

There is currently no reliable diagnostic laboratory test for PDAC, although several potential biomarkers have been evaluated. Carbohydrate antigen 19-9 (CA 19-9) is probably the most commonly used marker, but it has several flaws. Because of its low predictive value of 0.5% - 0.9% it is not appropriate as a screening tool. It may yield false positives in cases of biliary obstruction, infection and inflammation without cancer (Marrelli et al., 2009). Its sensitivity is also suboptimal; CA 19-9 may be negative in patients with advanced PDAC due to the fact that 5-10 % of

population lack the enzyme required for CA 19-9 antigen production (Ballehaninna & Chamberlain, 2011; Zhang et al., 2018). However, its specificity and sensitivity increase when used on symptomatic patients, reaching a sensitivity of 0.80 and a specificity of 80% (Z. Huang & Liu, 2014).

Other typical presenting laboratory abnormalities include high plasma glucose, low albumin and hypersedimentation, but these are common findings and cannot be used as markers.

## 2.5.3    Imaging

The appropriate diagnostic imaging modalities include abdominal ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance cholangiopancreatography (MRCP), endoscopic ultrasound (EUS) and positron emission tomography (PET). Each of these modalities has some advantages but also disadvantages depending on the various aspects of pancreatic imaging: identification of the primary tumour, local tumour resectability, distant metastasis and treatment monitoring (E. S. Lee & Lee, 2014; Shrikhande et al., 2012). The best accuracy is often achieved when using different modalities in combination (Schima et al., 2020).

Due to its wide availability and safety, abdominal US is often the first investigation made in patients with upper abdominal pain, biliary complaints or even non-specific abdominal pain. Unfortunately, the specificity and sensitivity of US are limited regarding identification of pancreatic lesions, mainly because of the retroperitoneal location of the pancreas. It may, however, help to identify some other manifestations of PDAC, such as hepatic metastasis or ascites, leading to further investigations.

Thin-cut intravenous contrast-enhanced multidetector CT is the radiological investigation of choice (Mayo et al., 2009). Its sensitivity for detection of pancreatic cancer ranges 75-100% and specificity 70-100% (Shrikhande et al., 2012), while for tumours >2cm sensitivity may be as high as 98% (Kitano et al., 2004). CT is also the investigative method of choice for staging PDAC because of its ability to provide an assessment of the entire abdominal cavity. CT is the primary imaging modality also when assessing the resectability of the tumour, with accuracy of up to 90% in the diagnosis of vascular invasion (Karmazanovsky et al., 2005).

A PDAC tumour presents on US as a hypoechoic solid mass with ill-defined margins. In CT, a PDAC tumour is most often seen as a hypoattenuating mass (Fig

2.), but it can also be isoattenuating relative to the surrounding pancreatic parenchyma. In MRI, PDAC typically appears hypointense on fat-suppressed, T1-weighted imaging. On T2-weighted images, the appearance is variable. Indirect signs, such as upstream pancreatic and/or common bile duct dilation and atrophy of the pancreas distal to the tumour, are typical and often seen on all these modalities. (E. S. Lee & Lee, 2014).

**Figure 2.** A contrast-enhanced CT scan showing PDAC tumour in the head of the pancreas. The arrows show a dilated common bile duct (left arrow) and the main pancreatic duct (right arrow). Courtesy of Dr. Emilia Pynnönen, CFCH.
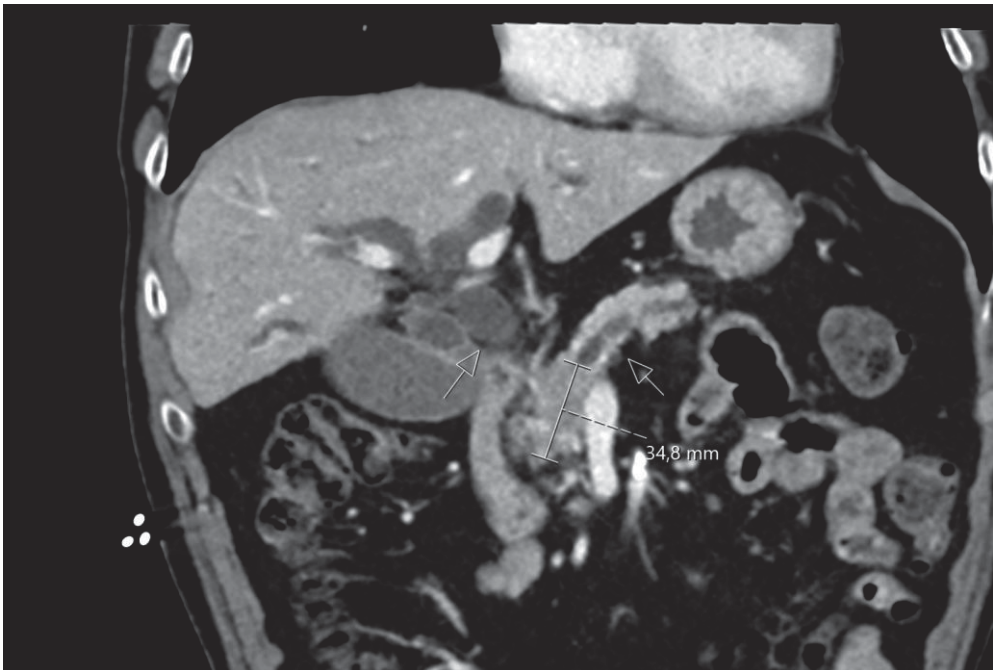


MRI has been reported to have a diagnostic accuracy superior even to that of CT (Trede et al., 1997), but taking account of all studies the accuracy of CT and MRI seems to be comparable with no significant differences between them. The choice between CT or MRI/MRCP as a primary method of investigation in clinical use seems to be determined by availability and technical expertise in reporting them (Shrikhande et al., 2012). The differences lie more in the additional information they provide than in the accuracy of detecting the tumour itself: MRI probably performs better in identifying liver metastases while CT may yield more information about the abdominal cavity as a whole (Litjens et al., 2020).

The role of EUS appears to be as a complementary investigation to CT or MRI/MRCP. It sometimes offers additional information when the lesion assessed is not clearly detected but suspected in CT/MRI/MRCP (Artifon et al., 2009). EUS also affords an opportunity to obtain histological evidence using fine needle aspiration (FNA) (Fisher et al., 2009).

Since PET offers no any additional benefits over CT and/or MRI and is less readily available, its use is limited to specific indications: borderline resectable disease, locally advanced disease and resectable disease with suspected metastases (Shrikhande et al., 2012).

## 2.5.4 Preoperative biopsy

Multiple sampling techniques are used to obtain a cytopathological diagnosis of PDAC. Nevertheless, biopsy of the pancreatic mass is still a challenge mainly due to the anatomical location of the pancreas.

EUS -guided FNA has become a cornerstone of pancreatic mass biopsy. According to large meta-analyses, EUS -FNA has pooled sensitivity of more than 85% to 92% and a pooled specificity of 94% to 100% in the diagnosis of pancreatic lesions (Zhang et al., 2018). Its availability has significantly improved during the last decade and the complication rate is extremely low (Eloubeidi et al., 2006). Fine needle biopsy (FNB) needles have been designated in order to increase the amount of tissue acquisition, but studies comparing FNA and FNB have not shown significant differences between the techniques (Zhang et al., 2018).

Endoscopic retrograde cholangiopancreatography (ERCP) can provide duct brushing cytology. However, ERCP is mainly used for therapeutic intentions due to the unfavourable complication profile of the procedure. In addition, the sensitivity of brush cytology is poor (Pereira et al., 2020). Cholangiopancreatoscopy using SpyGlass system is used increasingly during ERCP in order to directly visualize lesions in bile ducts. It has also been used in the pancreatic duct for the identification of pancreatic cystic lesions and strictures (Ang & Kwek, 2019; Du et al., 2021), yet its role in PDAC diagnosis is so far minor.

Core biopsy can rarely be used to identify pancreatic lesions due to the posterior anatomical location of the pancreas. It is, however, widely used to obtain a tissue biopsy of the site of metastasis when such are present.

## 2.5.5    Histologic criteria

PDAC is microscopically characterized by a proliferation of small tubular structures lined with cuboidal mucinous cells in abundant desmoplastic stroma (J. Chen & Baithun, 1985). The normal lobular arrangement of benign ducts is usually replaced by gland-like structures with disorderly arranged tubules. The cytoplasm of the tumour cells usually contains mucin and may be abundant. Loss of polarity of some of the glands is typical, even though the nuclei may retain a basal orientation in the cells. The nuclei in each gland typically vary in size, shape and intracellular location between cells. Vascular and perineural invasion (PNI) are common features. (Klimstra, David S; Adsay, 2015). The finding of a solitary gland lying individually in adipose tissue (isolated solitary ductal unit) is a specific sign of malignity (Bandyopadhyay et al., 2009). Finding a gland situated adjacent to muscular blood vessels also suggests carcinoma (Adsay et al., 2006). In poorly differentiated PDAC, apparently normal glands are typically seen alongside small clusters or individual cells with marked cytologic atypia. The periphery of PDAC is usually indistinct, and neoplastic glands may be found well beyond the apparent gross extent of the tumour. (Klimstra, David S; Adsay, 2015). PDAC is also associated with fibrosclerotic and inflammatory changes in the adjoining non-neoplastic pancreas.

**Figure 3.**    Photomicrograph images of two punches of pancreatic ductal adenocarcinoma showing abundant desmoplastic stroma (asterisks), perineural invasion (left arrow) and gland-like structures composed of tumour cells (right arrow).
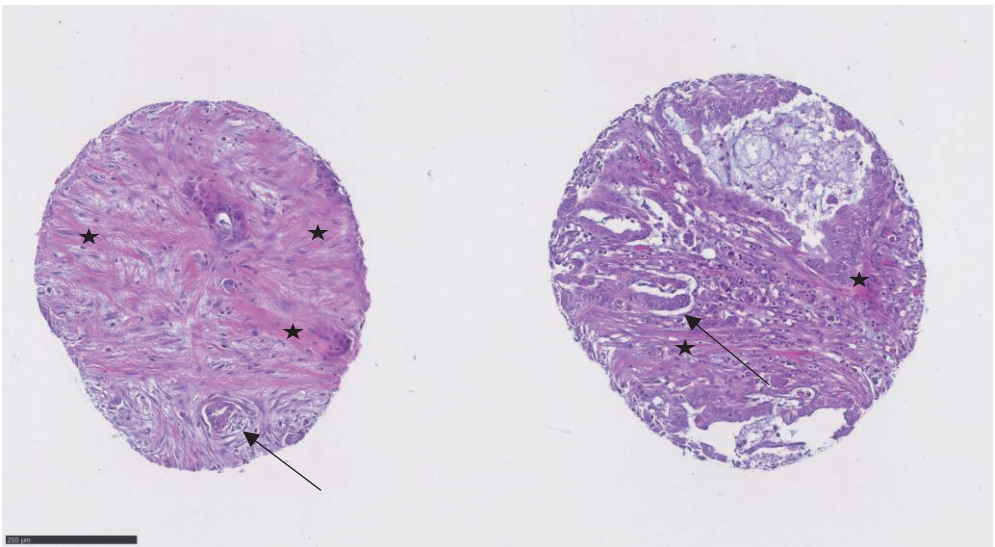
The current World Health Organization (WHO) grading system for PDAC is based on Klöppel's grading system, taking into account glandular differentiation, mucin production, nuclear atypia and mitotic activity. Like many grading systems, this system has the disadvantage of being subjective, not sufficiently reproducible and dependent on the experience of the observer. It is, however, an important independent prognostic factor in PDAC. (Klöppel et al., 1985; Lüttges et al., 2000).

## 2.6   Treatment

Surgery still remains the only treatment offering curative potential in PDAC. Unfortunately, approximately 80-85% of patients have either locally unresectable or metastatic disease at the time of diagnosis (Ducreux et al., 2015). The classification of PDAC regarding resectability into resectable, borderline resectable (BR-PDAC), locally advanced and metastatic disease has been established in clinical practice (Isaji et al., 2018). The main idea behind this classification is to find patients having non-metastatic but, at the time of diagnosis, non-resectable disease, since the oncologic treatments developed offer opportunities to downstage some of these tumours to attempt secondary curative intent surgery.

The anatomic criteria for BR-PDAC tumour include contact with the superior mesenteric artery and/or celiac artery of less than 180° without signs of stenosis or deformity, contact with the common hepatic artery without tumour contact with the proper hepatic artery and/or celiac artery and contact with the superior mesenteric vein and/or portal vein without extending beyond the inferior border of the duodenum. In addition, PDAC tumour should be classified as BR-PDAC even without the anatomic characteristics mentioned above, if the following biological factors are present: clinical findings causing suspicion of distant metastases, regional lymph node metastases diagnosed be PET-CT or biopsy or CA 19-9 level more than 500 units/ml. PDAC is considered to be locally advanced if there is a tumour contact ≥ 180° with the celiac axis or superior mesenteric artery or contact or invasion of the proper hepatic artery, superior mesenteric vein/portal vein bilateral narrowing or occlusion exceeding inferior border of duodenum. (Isaji et al., 2018; Mizrahi et al., 2020).

Resectability status is based on CT imaging and should be determined by a multidisciplinary team. An R0 resection should always be aimed at, since it has been shown to be associated with improved survival compared to R1 -resection(Demir et al., 2018). Local major venous or even arterial involvement is no longer considered

to be a contraindication for surgery, when deemed reconstructable (Loveday et al., 2019). (Mizrahi et al., 2020).

When a patient with PDAC is considered to be a suitable candidate for curative-aiming surgery, two common questions still remain: First, is there a need for biliary draining before surgery? Second, will the patient benefit from neoadjuvant therapy? The literature currently recommends preoperative biliary draining only when there is a strong indication, such as a need for neoadjuvant therapy (P. J. Lee et al., 2018). There is recent evidence favouring neoadjuvant therapy over immediate surgery (Jang et al., 2018; Versteijne et al., 2020). The indications for neoadjuvant therapy are still under debate and it is not in routine clinical use. Today, neoadjuvant therapy is commonly reserved for patients with BR-PDAC or locally advanced disease (Ducreux et al., 2015; Khorana et al., 2019; Pentheroudakis, 2019; Tempero, 2019).

## 2.6.1    Surgical treatment

For patients with resectable PDAC, primary surgical resection of the tumour and regional lymph nodes should always be offered when the performance status of the patient does not exclude major abdominal surgery.

PDAC in the pancreatic head is typically resected with pancreaticoduodenectomy, which includes resection of the pancreatic head, duodenum, proximal jejunum, common bile duct, gall bladder and usually a segment of the stomach. (Andrew L Warshaw & Thayer, 2004).

Distal pancreatectomy is the surgical option for PDAC tumours in the distal pancreatic corpus or cauda. This is usually performed with splenectomy. However, the spleen may be preserved without dissecting the splenic vessels free from the pancreatic parenchyma by dividing them at the cutting line of the pancreas and distally close to the pancreatic parenchyma in order to preserve the splenic blood flow via the short gastric vessels (A L Warshaw, 1988).

Total pancreatectomy may be necessary for PDAC tumours involving the pancreatic neck or the proximal body of the pancreas to achieve R0 resection. It may also sometimes be necessary because of the postoperative complications of pancreaticoduodenectomy. It may be performed with a preservation of the spleen (F. Yang et al., 2019). Sometimes vascular resections are needed to achieve negative surgical margins. Arterial resections in particular are associated with increased post-operative morbidity and these should be performed in specialized high-volume centres.

These procedures can be performed using either conventional open, laparoscopic or robotic-assisted approach with comparable surgical and oncological outcomes according the recent evidence (Esposito et al., 2019; Pędziwiatr et al., 2017). However, this evidence is based mainly on observational, not randomized studies. Thus, more trials are needed to confirm the real long-term results.

For patients with borderline resectable PDAC (BR-PDAC) tumour, upfront surgery is not the recommended option. Instead, neoadjuvant therapy should be considered, followed by a complete restaging evaluation and final surgical planning (Isaji et al., 2018; Khorana et al., 2016; Mizrahi et al., 2020).

For patients with locally advanced PDAC, systemic chemotherapy should be offered. Although the majority of these patients have incurable disease, complete restaging evaluation must be done after chemotherapy to identify the patients with significant response and, in case of downstaging, secondary curative-intent surgery should be considered (Mizrahi et al., 2020).

Despite the dramatic decrease in the rate of postoperative complications in recent decades, pancreaticoduodenectomy in particular is still associated with relatively high postoperative morbidity and also significant mortality, with respective rates of around 40% and 3-5% (Adam et al., 2015; Tahkola et al., 2020; Witzigmann et al., 2016). The centralization of pancreatic cancer surgery has been shown to improve outcomes, as surgeons' expertise increases and the risk of failure to rescue critically ill patients decreases (Ahola et al., 2017; Lynch et al., 2009).

## 2.6.2    Oncological treatment

The oncological treatment for PDAC consists of neoadjuvant and adjuvant treatment for patients receiving curative-aiming surgery, and palliative chemotherapy for patients with metastatic or locally unresectable disease.

Two out of three commonly used guidelines for pancreatic cancer treatment (the National Comprehensive Cancer Network Guideline and the European Society of Medical Oncology Clinical Practice Guideline) recommend neoadjuvant therapy only for the patients with borderline resectable PDAC or for patients with high-risk features, and one (Association of Clinical Oncology Clinical Practice Guideline) also for patients with resectable PDAC as an alternative strategy (Ducreux et al., 2015; Khorana et al., 2019; Pentheroudakis, 2019; Tempero, 2019). Neoadjuvant treatment regimen options for consideration include FOLFIRINOX and gemcitabine plus nanoparticle albumin-bound (NAB) paclitaxel. The recent evidence of the benefits

of neoadjuvant chemotherapy in PDAC is based mainly on two randomized trials including single-agent (gemcitabine) chemotherapy regimens (Jang et al., 2018; Versteijne et al., 2020). However, the multi-drug regimen FOLFIRINOX has been shown to be superior to gemcitabine in patients with metastatic PDAC (Conroy et al., 2011), and promising results have also been reported in neoadjuvant settings (Quisette P Janssen et al., 2019). FOLFIRINOX is currently the most commonly used neoadjuvant chemotherapy in observational studies and ongoing phase II trials (Quisette P Janssen et al., 2020). The results of on-going phase III trials comparing FOLFIRINOX to gemcitabine-based chemotherapy in the neoadjuvant setting are not yet available, leaving open the question of the most appropriate neoadjuvant therapy (Q P Janssen et al., 2021).

5-fluorouracil (5-FU) based adjuvant chemotherapy after upfront surgery in PDAC was first shown to be superior to observation alone in 2004 (Neoptolemos et al., 2004). 5-FU as a primary regimen was first replaced by gemcitabine and later by FOLFIRINOX as new evidence became available (Conroy et al., 2018; Oettle et al., 2013). More fragile patients are still recommended to be treated by dual therapy with gemcitabine and cabecitabine since the administration of FOLFIRINOX is associated with increased risk of complications (Ghosn et al., 2016).

Palliative chemotherapy follows the same principles as in adjuvant therapy with regard to the first-line drugs administered. FOLFIRINOX is recommended for patients with favourable comorbidity profile as a first-line option, whereas gemcitabine plus NAB-paclitaxel should be offered to patients with an adequate comorbidity profile. Gemcitabine alone, sometimes with the addition of cabecitabine or erlotinib, is a recommended first-line option for patients with comorbidity profile precluding other treatments. (Sohal et al., 2016, 2018, 2020; Taieb & Abdallah, 2020).

New therapies such as poly polymerase inhibitors, programmed cell death protein-1 (PD-1) checkpoint inhibitor therapy and tropomyosin receptor kinase (TRK) fusion inhibitors have been shown to benefit selected patients with metastatic PDAC (Sohal et al., 2020; Taieb & Abdallah, 2020) and are currently recommended to be offered to patients with PDAC experiencing disease progression or intolerable toxicity with first-line regimens. Early testing for microsatellite instability, BRCA mutations and NTRK gene fusions is recommended to select patients who will benefit from these therapies (Sohal et al., 2020). Regardless of the use of the new therapies mentioned above, the second-line therapy may also include gemcitabine plus NAB-paclitaxel, fluorouracil plus nanoliposomal irinotecan, irinotecan or oxaliplatin, or gemcitabine alone, depending on the first-line therapy used, patient

comorbidity profile and patient preference (Sohal et al., 2020; Taieb & Abdallah, 2020).

## 2.7 Tumour-related prognostic factors

An ideal biomarker should be measured with minimal variability and change promptly and reliably in response to changes in the disease or its therapy (Aronson & Ferner, 2017). The ease of measuring also plays an important role, for instance, in preoperative decision-making. Multiple laboratory and molecular factors have been evaluated to find an ideal biomarker in PDAC, yet the ability of current prognostic biomarkers to predict survival of PDAC patient is far from satisfactory.

On the other hand, biomarker research usually provides information about the pathophysiological phenomena behind the relationship between marker and endpoint.

### 2.7.1 Stage

The tumour-node-metastasis (TNM) staging system published by the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (Union Internationale Contre le Cancer, UICC) has for decades been the basis of tumour staging assessment. It is based on three key components: extent of local tumour (T), dissemination to regional lymph nodes (N) and the presence of metastatic spread at distant sites (M). As the body of knowledge about factors influencing the prognosis of different cancers increases over the years, new editions have been published to achieve as accurate a prognostic value as possible.

For PDAC, no changes were made to the TNM staging system after the publication of the fifth edition in 1997 until the publication of the eighth edition in 2016, with significant changes regarding T and N classifications (Edge & Compton, 2010; van Roessel et al., 2018). T, N, and M categories of the seventh and eighth editions of TNM classifications are presented in Table 1. The eighth edition modestly increased the prognostic accuracy compared to the seventh edition. However, even the revised T stage remains only weakly associated with survival, whereas the revised N stage has proven highly prognostic (van Roessel et al., 2018). The prognostic value of the presence of distant metastasis is well known (Hidalgo, 2010).

**Table 2.** T, N and M categories in PDAC based on the seventh and the eighth editions of the TNM classification.

| Category | Description (7th edition) | Description (8th edition) |
|---|---|---|
| Tumour (T) | | |
| T1 | Tumour limited to the pancreas, ≤2 cm in greatest dimension | Maximum tumour diameter ≤2 cm |
| T2 | Tumour limited to the pancreas, ≥2 cm in greatest dimension | Maximum tumour diameter ≥2 cm and ≤4 cm |
| T3 | Tumour extends beyond the pancreas but without the involvement of the celiac axis or the superior mesenteric artery | Maximum tumour diameter >4cm |
| T4 | Tumour involves the celiac axis or the superior mesenteric artery | Tumour involves the celiac axis or the superior mesenteric artery |
| Regional lymph node metastasis (N) | | |
| N0 | No regional lymph node metastasis | No regional lymph node metastasis |
| N1 | Regional lymph node metastasis | Metastasis in 1-3 regional lymph nodes |
| N2 | Non existent | Metastasis in ≥4 regional lymph nodes |
| Distant Metastases (M) | | |
| M0 | No distant metastases | No distant metastases |
| M1 | Distant metastases | Distant metastases |

**Table 3.** TNM stage grouping in PDAC based on the seventh and the eighth editions of TNM classification.

| Stage | 7th edition | 8th edition |
|---|---|---|
| IA | T1 N0 M0 | T1 N0 M0 |
| IB | T2 N0 M0 | T2 N0 M0 |
| IIA | T3 N0 M0 | T3 N0 M0 |
| IIB | T1-3 N1 M0 | T1-3 N1 M0 |
| III | T4 N0-1 M0 | T1-4 N2 M0 |
| IV | T1-4 N0-1 M1 | T1-4 N0-2 M1 |

However, there are data showing significant variation in the prognosis of patients within the same TNM stage in several cancers associated with other tumour-related factors such as tumour microenvironment and genetic mutational profile (J Galon et al., 2014; Karamitopoulou, 2019). This is reasonable, given that TNM classification focuses solely on the spread of the tumour cells, failing to incorporate

the effects of the host immune response or the mutational profile of the tumour cells.

## 2.7.2    Histopathological grade

Histopathological grading aims to grade tumours on the basis of differentiation and proliferation activity. In PDAC, both the three-degree grading system by WHO and the four-degree grading system by the College of American Pathologists are in clinical use.

According the WHO grading system, well-differentiated PDACs of grade one form well-defined glands, mitoses are neither numerous nor atypical and only minimal nuclear pleomorphism is seen. In moderately differentiated adenocarcinomas, grade two, the gland formation is not so well defined, nuclear pleomorphism is seen and mitoses are more common and often atypical. Poorly differentiated adenocarcinomas form poorly glands, nuclear pleomorphism is prominent, the nuclei large and bizarre and the nucleoli large and multiple (Ralph H Hruban & Fukushima, 2007). The grading classification by College of American Pathologists mainly follows the same logic.

Tumour grade has been shown to be one of the most important prognostic factors in PDAC (Hlavsa et al., 2018). The downsides of the grading include the subjective nature of its assessment and the heterogeneity of tumours, both reducing its reliability. The assessment by two pathologists and checking the different areas of the tumour are recommended to minimize the impact of factors mentioned above.

## 2.7.3    Perineural invasion

According to the current definition, PNI consists of cancer cells in nerves or surrounding or pass-through nerves, tumour cells closely contacting the nerve and surrounding at least 33% of the nerve periphery, or tumour cells invading any of the three layers of the neurolemma structure (Liebig et al., 2009). This is an extremely common feature of PDAC with reported incidences of 43.2% to 100% (Schorn et al., 2017). The wide variation in the published incidence rates is allegedly attributable to the differing slice thicknesses and analysis techniques used during the pathological processing of specimens. The current data supports the conviction that PNI, in some form, is present in almost all PDAC tumours if searched for with thin tissue sections and cases with low severity PNI are also taken into account (Liebl et al., 2014; Schorn

et al., 2017). However, PNI found in routine histopathological analysis, which probably reflects mainly PNI with high severity, seems to be an independent prognostic factor (Schorn et al., 2017).

### 2.7.4    Immune cell infiltration

Due to the emerging awareness of the impact of host immune response on cancer patients' survival, tools to measure this response have been developed. The density of all lymphocytes (CD3+) and CD8 effector cells (CD8+) has been shown to be positively associated with survival of PDAC patients (Ino et al., 2013; Orhan et al., 2020). Various methods have been used to form a perception of the immune cell infiltration within the tumour area and its invasive margin. The immune cell infiltration at the tumour core seems to have the greatest impact on survival in PDAC, but the differences between the different locations are not significant according to recent meta-analysis including 43 studies (Orhan et al., 2020).

Among all the immune cell infiltration -based scores in cancer research, the most standardized one is probably Immunoscore®, developed for use in colorectal cancer (Pages et al., 2018). It takes into account the density of CD3+ and CD8+ lymphocytes at the core of the tumour and on the invasive margin and has been shown to have a prognostic value superior to and independent of AJCC/UICC TNM classification. Attempts to introduce it in clinical practice are currently ongoing (Jérôme Galon & Lanzi, 2020).

### 2.7.5    Microsatellite instability

Microsatellite instability (MSI), the hallmark of Lynch syndrome, results from reduced genome integrity due to missing functional DNA mismatch repair proteins. In general, this condition is associated with high risk for several cancers, such as colorectal, endometrial, urothelial and also bilio-pancreatic cancers. MSI -tumours, in general, are characterized by increased immunogenicity given to higher neo-antigen load due to accumulation of somatic mutations and therefore associated with better survival in several cancers. (Ghidini et al., 2020).

Pancreatic cancer tumours in patients with MSI often have a medullary appearance, and, as described above, prominent immune cell infiltration in tumour microenvironment. The reported rates of MSI tumours vary significantly between studies in PDAC, but the overall rate seems to be as low as 2 % of all PDAC cases.

(Ghidini et al., 2020). MSI has also been reported to be associated with better survival in PDAC (Nakata et al., 2002), but the overall data are limited and controversial with regard to its prognostic value (Lupinacci et al., 2019).

## 2.7.6    Laboratory and molecular factors

CA 19-9 is the most commonly used serum biomarker used for detecting PDAC in patients with symptoms suspicious for PDAC, but it is also used for determining the prognoses of patients with PDAC. High CA 19-9 level has been shown to predict diminished survival, likewise the rate of change between two separate measurements in the preoperative setting (Chang & Kundranda, 2017). However, the predictive value of CA 19-9 is far from perfect (Yizhi Wang et al., 2020). A combination of carcinoembryonic antigen (CEA) and CA 19-9 seems to predict survival better than each of these alone; the best survival is seen in patients with normal levels of both CEA and CA 19-9, and the worst in patients with elevated levels of both biomarkers (Distler et al., 2013).

Noncoding RNA and especially its small-sized subtype miRNA has been an object of extensive research in recent years, revealing inter alia its ability to modulate protein-coding genes involving in the development, progression and metastatic spread of PDAC cells. There are numerous publications on the potential of its expression, assessed in the tissue of resected PDAC, to be used as a prognostic biomarker in PDAC. MiR-21 as an object of the majority of these studies seems to have prognostic significance as a predictor of survival among patients with PDAC. (Previdi et al., 2017).

Unfortunately, this is not the case with circulating miRNAs, which would be much easier to use, requiring only serum analysis. There are data showing that circulating miRNAs are altered in PDAC but at least most of these changes appear late and thus cannot be used for early detection (Franklin et al., 2018). The prognostic significance of these changes is still poorly understood (Gablo et al., 2019).

Several inflammation-related biomarkers have been evaluated. C-reactive protein to albumin ratio (CAR) and neutrophil-lymphocyte ratio (NLR) have been associated with overall survival (OS) in several studies, and these associations were evaluated to be supported by highly convincing evidence in a recent umbrella meta-analysis, unlike other biomarkers included in the meta-analysis (Yizhi Wang et al., 2020). The recently developed C-reactive protein lymphocyte ratio (CLR) has been reported to

have even better prognostic value than CAR or NLR in a single study assessing survival of 997 PDAC patients (Fan et al., 2020).

CD73, also called ecto-5'-nucleotidase, is a glucosylphosphatidylinositol (GPI) - anchored protein, which, in co-operation with CD39 (nucleoside triphosphate diphosphorylase) degrades pro-inflammatory adenosine triphosphate (ATP) into immunosuppressive adenosine (Pellegatti et al., 2008). In addition, it acts as a signal and adhesive molecule thus regulating growth, migration and invasion (Q. Chen et al., 2020). Overexpression of CD73 has been associated with poor survival in many cancers, and seems to be an independent prognostic factor also in PDAC (Zhou et al., 2019b). Although serum CD73 has been shown to be a prognostic factor in melanoma (Turiello et al., 2020), the expression of CD73 in PDAC has been assessed from tumour specimens in published studies showing its prognostic significance, a fact that reduces the suitability for such biomarkers in clinical use.

Several other biomarkers, such as expression of B7H1, CD44 and CD133 as well as circulating tumour cells have also been suggested to be suitable as prognostic tools in PDAC, but none of these has so far come into clinical use.

# 2.8     Microenvironment of the pancreatic cancer tumour site

## 2.8.1    The role of inflammation and tumour microenvironment

To provide a logical framework for understanding the complex nature of neoplastic diseases, six hallmarks of cancer where proposed by Hanahan and Weinberg in 2000 (D Hanahan & Weinberg, 2000). These hallmarks included sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. These hallmarks were revised in 2011 by adding evading immune destruction and reprogramming energy metabolism to the list of emerging hallmarks. In addition, two characteristics underlying the emerging hallmarks were described (Douglas Hanahan & Weinberg, 2011). These enabling hallmarks are genomic instability and inflammation. The significance of the latter has been highlighted along with the improved understanding of the tumour microenvironment.

## 2.8.2    Characteristics of tumour microenvironment in pancreatic cancer

The microenvironment of a pancreatic cancer tumour site comprises an active population of cellular and non-cellular components. Cellular components consist of tumour cells and stromal cells such as blood vessels, immune cells, fibroblasts and myofibroblasts (Neesse et al., 2011). Non-cellular components consist of ECM containing proteins such as collagens, laminin, proteoglycans, glycoproteins, polysaccharides and fibronectin and several signalling molecules. Desmoplastic reaction is a characteristic feature of PDAC (Hidalgo, 2010). Pancreatic tumour microenvironment consists of abundant stromal cells, especially pancreatic stellate cells (PSC) (Haqq et al., 2014). High stromal activity as well as high-level expression of ECM have been shown to be associated with poor prognosis in PDAC, suggesting that stroma has a significant impact on the progression of PDAC (Erkan et al., 2007, 2008; Mantoni et al., 2008). This reflects the bipolar nature of the mechanisms by which the tumour cells escape proliferation regulation; besides enhancing proliferation itself, they also inhibit the proliferative suppression control of the surrounding environment (Caon et al., 2020). The number and activity of TILs also have an impact on PDAC patients' survival (Ino et al., 2013). Stroma, immune cells and cancer cells have multiple and complicated interactions with each other.

## 2.8.3    Pancreatic stellate cells and cancer-associated fibroblasts

PSCs are quiescent in normal pancreas. They can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm (Bachem et al., 1998). PSCs undergo  morphological and functional changes during the carcinogenesis process, and are assumed to play a pivotal role in the development of pancreatic fibrosis in PDAC (Masamune & Shimosegawa, 2013). Among several other factors, such as cytokines and growth factors, ethanol and its metabolites have been shown to induce these changes (Apte et al., 2000). During this activation, quiescent PSCs become myofibroblast-like cells and start expressing α-smooth muscle actin (α-SMA), losing lipid droplets, actively proliferating and migrating (Apte et al., 1998; Bachem et al., 1998; Erkan et al., 2012; Masamune et al., 2009; Masamune & Shimosegawa, 2009; Omary et al., 2007; Vonlaufen et al., 2008). These cells are also called cancer-associated fibroblasts (CAFs) and they produce large numbers of ECM components, a phenomenon which, in turn, is associated with a poor prognosis (Erkan et al., 2008). CAFs have the ability not only to produce, but also to degrade ECM, thus regulating ECM turnover (Phillips et al., 2003).

CAFs have moreover been shown to express toll-like receptors (TLRs) and a wide range of pro- and anti-inflammatory cytokines, growth factors and adipocytokines, which therefore also implies a role in inflammation regulation (Masamune et al., 2008, 2010; Masamune & Shimosegawa, 2013; Vonlaufen et al., 2007). They are also able to perform endocytosis and phagocytosis of foreign bodies, thus taking part in the local immune functions in the pancreas. Moreover, observations have been reported suggesting that PSCs have some role in glucose metabolism by expressing glucose transporters (Masamune & Shimosegawa, 2013).

Two distinct CAF subtypes have recently been identified: myofibroblastic or inflammatory phenotypes (Bernard et al., 2019; Öhlund et al., 2017). Myofibroblastic CAFs (myCAFs) express markers of myofibroblasts, while inflammatory CAFs (iCAFs) express inflammatory markers like interleukin 6 (IL-6) and leukaemia inhibitory factor (LIF). MyCAFs seem to be located adjacent to tumour cells and have been proposed to inhibit tumour progression (Biffi et al., 2019; Özdemir et al., 2014; Rhim et al., 2014), contrary to iCAFs located farther away within the dense stroma believed to promote tumour progression, chemoresistance and also immune suppression (Biffi et al., 2019; Flint et al., 2016; Hwang et al., 2008). The two subtypes of CAFs described above are suggested to be interconvertible cell states rather than endpoints of differentiation.

Tumour-growth factor-β (TGF-β) and interleukin-1 (IL-1), both secreted by tumour cells, have been demonstrated to be the key signalling molecules responsible for CAF polarization, the former inducing myCAF and the latter iCAF formation (Biffi et al., 2019). TGF-β has been shown to antagonize the IL-1-mediated JAK-STAT-pathway (Zhao et al., 2008) which, in turn, is the key pathway inducing iCAF formation, as mentioned above.

## 2.8.4    Hyaluronan

### 2.8.4.1    The nature of hyaluronan

Hyaluronan (HA), also known as hyaluronic acid, hyaluronate, is a glycosaminoglycan, which, like other glycosaminoglycans, consists of unbranched single-chain polymers of disaccharide units containing hexose (D-glucuronic acid ) and N-acetylhexosamine (N-acetyl-D-glucosamine) linked by a β bond. There are, however, several respects in which HA differs from other glycosaminoglycans: unlike other glycosaminoglycans, HA contains no peptide in its primary structure. It

is also distinct from other glycosaminoglycans regarding the site of synthesis; instead of in the Golgi, it is synthesized in the plasma membrane. Moreover, its molecular weight usually reaches the millions compared to the molecular weight of other glycosaminoglycans which lie around $5-50 \times 10^3$. (Fraser et al., 1997; Liang et al., 2016)

**Figure 4.**  Structural formula of disaccharide unit of hyaluronan.
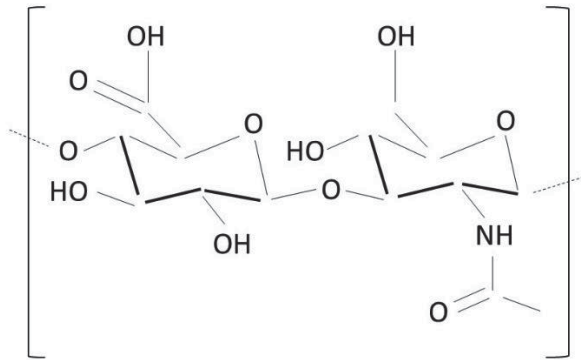


Although more than half of total HA is believed to be present in skin, it is present in all tissues, the highest concentrations being in the connective tissues like the umbilical cord, synovial fluid, skin and the vitrious body (Fraser et al., 1997).

## 2.8.4.2   Metabolism of hyaluronan

The metabolism of HA is extremely dynamic, such that as much as one third of the body's HA has been estimated to undergo daily turnover (Laurent et al., 1996). The regulation of HA metabolism is complex, occurring at multiple levels including enzyme expression, post-transcriptional control by micro-RNAs (mi-RNA) and antisense HAS expression as well as posttranslational modifications (Garantziotis & Savani, 2019).The activity of the HA metabolic enzymes has also been described to generate a self-regulating feedback loop (Caon et al., 2020).

The synthesis of HA occurs mainly in mesenchymal cells even though it can be produced by many cell types (Liang et al., 2016). There are three hyaluronan synthases (HAS) named HAS1-3, of which HAS2 is believed to be the most important isoform (Caon et al., 2020).

The catabolism of HA is carried out by hyaluronidases. Several hyaluronidases have been identified, of which Hyal1, Hyal2 and PH20 are the best-known proteins. Various hyaluronidases are known to degrade HA-chains of different sizes. Hyal2 hydrolyses high-molecular-weight HA into intermediate length HA, while Hyal1 carries out further degradation. In addition, HA fragmentation can also occur by reactive oxygen and nitrogen species. (Garantziotis & Savani, 2019).

### 2.8.4.3    Functions of hyaluronan

Several HA receptors have been described and characterized, among which CD44 is the best characterized. It mediates several functions including cell motility, inflammation, lymphocyte homing and cell growth, but also participates in HA clearance and injury resolution. The other known receptors include hyaluronic acid receptor for endocytosis (HARE), the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), the receptor for HA-mediated motility (RHAMM), the toll like receptor 4  (TLR4) and layilin. (Caon et al., 2020; Garantziotis & Savani, 2019).

The signalling pathways that HA activates via its receptors are multiple: CD44 activation in turn activates RhoGTPase signalling, leading through different effectors (PI3K, Akt, mTOR) to cytoskeletal organization, chemoresistance, cell growth and proliferation (Caon et al., 2020; Dibble & Cantley, 2015; Manning & Toker, 2017). The interaction between HA and CD44 also activates Cdc42 signalling which, in turn, results in activation of ERK1/2 with significant consequences for cell-growth, cytoskeletal re-arrangement and actin remodelling (Vigetti et al., 2008). CD44 activation by HA has also been reported to interact with oncoprotein Vav2 leading to an increase in ovarian tumour cell growth and migration, as well as causing antiapoptosis and chemoresistance of breast tumour cells through a mechanism involving protein kinase C and the production of miR-21 (L. Y. Bourguignon et al., 2001; L. Y. W. Bourguignon et al., 2009). There is some evidence demonstrating that HA may affect pathways involving p53 and mitochondrial apoptosis (Kumar et al., 2018; Y.-J. Lee et al., 2016)via CD44. The binding of HA to RHAMM, another receptor of HA, has effects on adhesion and cell motility (Vigetti et al., 2014). HARE, instead, has the ability to trigger the ERK1/2 pathway with the consequences mentioned before and also the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Pandey et al., 2016). Finally, HA-LYVE-1 binding has been shown to promote proliferation of lymphatic endothelial cells (Wu et al., 2014).

The biophysical functions of HA depend significantly on its molecular size (Tavianatou et al., 2019). High molecular weight HA (HMW-HA) has been shown to have more anti-angiogenic, anti-proliferative and immunosuppressive effects and, in turn, low molecular weight HA (LMW-HA) seems to induce more inflammation, angiogenesis and proliferation (Tavianatou et al., 2019). Short HA fragments are able disrupt the HA-CD44 interaction by binding to CD44 and therefore attenuating the biological effects of HA (C. Yang et al., 2012). There are publications emphasizing the role of LMW-HA in the progression of cancer in terms of invasion and metastasis, as well as cancer cell motility stimulation (Sugahara et al., 2006; Wu et al., 2015).

### 2.8.4.4    Hyaluronan and cancer

HA has been shown to accumulate in the stroma of most malignant tumours, including breast and colorectal cancer and prostate, ovarian, bladder, endometrial, gastric and thyroid carcinomas as well as lung and pancreatic adenocarcinomas (Afify et al., 2005; Anttila et al., 2000; Auvinen et al., 2000; Böhm et al., 2002; Cheng et al., 2013; Lipponen et al., 2001; Lokeshwar et al., 2001; Pirinen et al., 2001; Tiainen et al., 2015). HA accumulation can change the tissue dramatically, which is the case in grade 3 ovarian cancer, for example, with a 49-fold increase in median HA concentration compared to normal circumstances (Hiltunen et al., 2002).

Strong evidence shows hyaluronan accumulation in the tumour cells and/or peritumoral stroma to be related to tumour progression and poor survival in several cancer types, including PDAC (Anttila et al., 2000; Auvinen et al., 2000; Böhm et al., 2002; Cheng et al., 2013; Köbel et al., 2004; Lipponen et al., 2001; Ropponen et al., 1998; Setälä et al., 1999; Tiainen et al., 2015). This is reasonable, given the broad scale of effects of HA on cell motility, proliferation, apoptosis and tissue remodelling. Besides these, HA has also been shown to have a role in neo-angiogenesis in tumour microenvironment via the expression of angiogenic factors and matrix metalloproteinases, adhesion and penetration of primary tumour cells to metastasize to target and in the reprogramming of energy metabolism (Caon et al., 2020; Fieber et al., 2004; Ghose et al., 2018; Karousou et al., 2017).

Even though HA has the well-known ability to modulate immune responses (Jiang et al., 2011), the data on its role in immune escape in malignant conditions are limited. However, several mechanisms have been proposed. First of all, the HA-rich tumour microenvironment has been demonstrated to act as a barrier insulating tumour cells from immune cells (Evanko et al., 2012; Singha et al., 2015). It has been

suggested that HA could be important for T cell migration and extravasation in light of observations of increasing T cell-HA binding during T cell activation (Lee-Sayer et al., 2015). HA has also been shown to be associated with macrophage polarization into the anti-inflammatory and pro-tumoural M2 type (Kobayashi et al., 2010; Kuang et al., 2007; Tiainen et al., 2015). Regarding the literature, no studies examining the association between HA accumulation and immune responses in PDAC have been presented.

## 2.8.5    Immune response at the tumour site

Tumour-associated inflammation is a well-known characteristic of cancer. As in several other organs, chronic inflammatory conditions in the pancreas are risk factors for cancer development (Duell et al., 2012). On the other hand, an efficient host immune system is widely considered to be an essential factor preventing tumour development and progression, and the immune cell infiltration has been shown to be an independent prognostic factor in PDAC (Ino et al., 2013).

It has been hypothesized that an irregularity in tissue structure at the site of the developing tumour is handled by the host immune system like invading pathogens or dying cells, if not disturbed by factors like immune escape mechanisms of tumours. In normal circumstances, the initial inflammatory response to such inflammation triggering stimulus includes degranulation of neutrophils and platelets followed by the activation of tissue macrophages. Chemokines, cytokines, lipid mediators such as prostaglandins and chemotactic peptides are released during this inflammatory response resulting in the recruitment, differentiation and activation of circulating lymphocytes and monocytes which congregate at the site of the source of the alarm. Dendritic cells (DCs), the most common antigen-presenting cells (APC), become activated, after which they have the ability to take up antigens and present them to naive T cells (Shalapour & Karin, 2019). Monocytes convert to macrophages, key players in tumour immunity with the ability to kill tumour cells direct by releasing cytolytic enzymes, secreting cytotoxic factors such as tumour necrosis factor (TNF) and presenting tumour antigens to activate specific T cell response (S. Yang et al., 2020). The activated T cells include both cytotoxic CD8+ and CD4+ helper T cells (Th). The response includes both the clonal proliferation of activated T cells and the release of a wide array of cytokines and chemokines. Some of these cytokines, such as interferon γ (IFN-γ), kill tumour cells directly. Activated DCs and macrophages can express several co-stimulatory molecules and

immunomodulatory cytokines such as IL-12 thus potentiating T cell activation (Shalapour & Karin, 2019; Teng et al., 2015).

In the physiological inflammation process, the chain of events described above represents the initiation and amplification phases of the inflammation process. Once the target of the process has been eliminated, it is essential to limit the damage which the inflammation process causes. This phase is called the resolution phase.

DCs and macrophages, in addition to pro-inflammation molecules, also express immunoregulatory cytokines, such as IL-10 and IL-23, that suppress the following immune response (Merad et al., 2013; Shalapour & Karin, 2019). As a result, the generation of Tregs and regulatory B cells is enhanced, which in turn leads to the suppression of the immune reaction.

The antitumour immune response at the tumour site changes significantly during the progression of PDAC (Hiraoka et al., 2006; Liu et al., 2016). The prevalence of Tregs and myeloid derived suppressor cells (MDSCs), which are strongly immunosuppressive cells, increases, resulting in a decrease in the number of effector T cells (Teff) such as CD8+ T cells (Bayne et al., 2012; Hiraoka et al., n.d.; Ino et al., 2013). The effect of Tregs has long been attributed to FoxP3+ Tregs, but observations have recently been published suggesting an even more significant role of FoxP3-Tregs in Treg-dependent immune suppression, at least in PDAC. Tregs produce IL-10 and transforming growth factor β (TGFβ) thus attenuating the function of both APCs and effector T cells. The suppressive function of FoxP3-Tregs seems also to be associated to their ability to kill both CD8 T cells and myeloid cells through the perforin-granzyme B pathway (Barilla et al., 2019; Seo & Pillarisetty, 2017).

CD4+ Th cells, which can differentiate into subtypes with distinctive phenotypes, differentiate predominantly into tumour-promoting and immunosuppressing Th2 cells instead of Th1 polarization (Tassi et al., n.d.). Th1 -cells express transcription factor T bet and Th2 cells GATA-3, which in research settings can be used as a means to distinguish them from each other. This deviation of Th cell polarization seems to be due to the chain of events where activated CAFs secrete thymic stromal lymphopoietin (TSLP), which in turn induces activation of tumour antigen-loaded DCs. These DCs are believed to have an essential role in Th polarization by activating Th2 -cells, probably in the draining lymph nodes. Intratumoural Th2 infiltrate has been shown to predict poorer survival of patients with PDAC (De Monte et al., 2011).

Similarly, the cancer-associated polarization of tumour-associated macrophages (TAMs) into protumour M2 macrophages is associated with poorer survival (S. Yang

et al., 2020). TAMs are known to produce immunosuppressive cytokines, the inhibitory B7 family molecules such as programmed cell death ligand 1 (PD-L1) and B7-H4 as well as a tryptophan hydrolyzing enzyme indoleamine 2,3-dioxygenase (IDO), which is known to attenuate effector T cell functions (DeNardo & Ruffell, 2019; Kuang et al., 2009; S. Yang et al., 2020).

The factors leading to immune suppression are multiple and not yet fully understood. Possibly the most essential factor behind the immune suppression is the variety of signals that the tumour cells themselves produce thus attenuating the attack against it. This phenomenon, immune escape of the tumour, is considered in the next section. There are, however, other factors modulating the immune microenvironment to an immunosuppressive deviation such as chronic tumour-associated inflammation, tumour-associated hypoxia and endoplasmic reticulum (ER) stress.

Chronic tumour-associated inflammation leads to the sustained production of TGFβ, which supports differentiation of immunosuppressive cells like Tregs and plasmocytes that can express immunoglobulin A (Massagué, 2008). Tumour growth leads to increased need for oxygen due to the high glycolytic rate of rapidly proliferating tumour cells, which in turn causes hypoxia when the angiogenesis cannot meet the need. Hypoxia induces the activation of hypoxia-inducible factor 1 (HIF-1α) and further the secretion of TGFβ (Ammirante et al., 2014) and a number of chemokines making the microenvironment more immunosuppressive (Cruz et al., 2017). ER stress is another reported phenomenon in tumour cells that amplifies tumour-associated inflammation with the immunosuppressive consequences described above (Oh et al., 2013; M. Wang & Kaufman, 2014). ER stress also suppresses anti-tumour immunity by disrupting DC homeostasis (Cubillos-Ruiz et al., 2015).

## 2.8.6 Immune escape of the tumour

Despite the wide variety of foreign proteins in tumour cells, PDAC becomes less immunogenic over time. The immune suppressing mechanisms that tumour cells develop have been proposed to be behind this phenomenon, known as immune escape. These mechanisms are partly linked with each other and with the other microenvironment-modulating factors discussed above, since many of the signal molecules are common. Thus, it is not always clear what is the cause and what is the

consequence. The mechanisms also vary between PDAC tumours depending on the mutation profile of the tumour (Karamitopoulou, 2019).

Several immune escape mechanisms have been described in the literature. First, tumour cells support the induction and recruitment of various inhibitory immune cells such as Tregs, M2-macrophages, Th2 cells and MDSCs (Ghalamfarsa et al., 2019; Karamitopoulou, 2019). The accumulation and action of these cells then have the immunosuppressive consequences described above. One example of this mechanism, already described by DeMonte et al. in 2011 is the secretion of proinflammatory cytokines (TNF and IL-1β), which cause CAFs to release TSLP, ultimately leading to Th2 polarization (De Monte et al., 2011).

Second, cancer cells can avoid recognition by effector T cells by downregulating antigen presentation pathways such as major histocompatibility complex (MHC) I protein or transporter associated with antigen processing (TAP), or even losing their tumour antigens during constant cell division, a phenomenon called immunoediting (Martinez-Bosch et al., 2018).

Third, secretion of immunomodulating cell-surface proteins such as the immune checkpoint molecules PD-1/PD-L1 or cytotoxic T-lymphocyte-associated protein (CTLA) 4 lead to inhibition of T cell response by binding to their ligands/receptors on the surface of antigen-presenting cells or T cells. (Zhou et al., 2019b) In normal circumstances, these immune checkpoint molecules are expressed by T cells and when binding to its receptor PD-1 on the surface of the immunosuppressive cells, like Tregs, activate them thereby limiting the immune response. Overexpression of these molecules by tumour cells is one method to attenuate immune response.

Fourth, PDAC tumours secrete a wide variety of immunosuppressive chemokines like stromal cell-derived factor 1 and cytokines such as IL-1, IL-6, IL-10, TGF-β, TNF-α or vascular endothelial growth factor (VEGF) (Martinez-Bosch et al., 2018).

Fifth, tumour cells increase their resistance to apoptosis by overexpression of STAT3 or BCL-2, thus annihilating the immune response (Martinez-Bosch et al., 2018).

**Figure 5.**  Examples of the immune escape mechanisms of the PDAC tumour cells.



Figure content labels:
- Th2
- M2
- T-reg
- Decrease in the number and function of effector T-cells  −
- T-eff, T-eff, T-eff, T-eff, T-eff
- Activation
- Induction and recruitment of inhibitory immune cells  +
- Suppression  −
- Secretion of immunosuppressive chemokines and cytokines
- Overexpression of PD-L1
- Overexpression of STAT3 -> apoptosis ↓
- Tumour cell
- CD73
- AMP
- Immunosuppressive effects of adenosine  −
- Overexpression of CD73  -> Adenosine ↑↑

## 2.9    Prognosis

Despite intense attempts to detect PDAC in its early stage, improvements in surgical treatment and the extensive development of new drugs, the prognosis in PDAC has remained poor. The worldwide five-year survival rate for pancreatic cancer patients is around 6%, with a relatively wide range from 2% to 10% in the literature (McGuigan et al., 2018; Mizrahi et al., 2020). A systematic review study assessing the burden of PDAC in Europe based on 91 studies showed a poor median survival of 4.6 months from diagnosis (Carrato et al., 2015). According to the same study, the median survival of patients treated with curative-oriented surgery ranged from 11 to 25.7 months. Only approximately 20% of patients with localised, resectable tumours are alive five years after surgery (Mizrahi et al., 2020).

The main reason for the poor survival of patients with PDAC is the late onset of symptoms, leading to late diagnosis; four out of five patients have either unresectable or metastatic disease at the time of diagnosis, which eliminates the option for surgery (D. Li et al., 2004; Mizrahi et al., 2020). However, as mentioned above, prognosis is currently only slightly better in patients who undergo curatively aimed surgery.

The best means to improve prognosis are debated. Some improvement would surely be possible to achieve by detecting early stage PDAC and optimizing the

outcome of surgery performed (Ahola et al., 2017). As survival rates remain poor even after surgery with curative intent, more expectations are focused on novel drugs such as immunotherapy, which has shown remarkable effects in several malignancies.

# 3   AIMS OF THE STUDY

The purpose of the study was to determine the prognostic role of immune cell infiltration in PDAC, to optimize the method of measuring it, and examine the mechanisms of immune escape of the tumour.

The specific aims for each part of the study were:

I   To examine the prognostic role of T lymphocyte CD3+/CD8+ ICS and its relation to MLH1 expression in consecutive series of PDAC patients.

II   To test the prognostic significance of ICS in another consecutive series of PDAC patients from Northern Finland. The secondary aim of this part was to compare TMA-like hotspot and whole tissue section techniques in the analysis of ICS.

III   To ascertain whether cell-specific CD73 acts as a prognostic factor in PDAC and to evaluate its relationship to other factors in the microenvironment, such as PD-L1 and ICS.

IV   To examine the prognostic role of stromal HA accumulation and its relation to immune cell infiltration and the immune-suppressing molecules CD73 and PD-L1 in PDAC.

# 4   PATIENTS AND METHODS

## 4.1   Patients

The study population consisted of 129 consecutive patients who underwent surgery aiming at a cure for PDAC at Central Finland Central Hospital (CFCH) in Jyväskylä between 2000 and 2016 (I, III and IV). After excluding TNM stage 3 and 4 patients and the patients with insufficient resection margins (R2), a total of 108 patients were included in the Study (I). A total of 110 patients were included in the third part of the study, of whom five were excluded from the survival analysis because of the TNM stage of 3-4 leading to a total of 103 patients (III). In Part IV of the study, a further two patients were excluded due to unavailability of eligible archive samples, leading to a total of 103 patients (IV).

For the second part of the study, the study population consisted of 95 consecutive patients who underwent curative-aiming surgery for PDAC at Oulu University Hospital in the period 1993-2015. The exclusion criteria were the same as for the other parts of the study: TNM stage 3 and 4 (seventh edition) and R2 surgical margin.

Detailed information on patients and tumour characteristics, surgical treatment and complications, oncological treatment and follow-up were retrieved from the prospectively maintained and continuously updated database (I, II and IV). The data were then confirmed by patient chart review. For the second part of Study (II), the data were obtained from patient records and patient survival data. Cause of death was ascertained from the Cause of Death Registry maintained by Statistics Finland.

None of the patients received neoadjuvant therapy (I-IV). The mean age of patients was 66.9 years [standard deviation (SD) 8.2] (Parts I, III and IV) and 64 years (SD 9.3) (Part II). The median follow-up time was 44 months (I, III and IV) for those alive at the end of follow up.

All studies were conducted in accordance with the principles of the Declaration of Helsinki and the guidelines for good clinical practice. The studies were approved by the authorities of both CFCH and Oulu University Hospital: Oulu University Hospital Ethics Committee and the Finnish Authority for Medicolegal Affairs (Valvira).

**Table 4.** Clinical characteristics of study patients. The data are presented separately for Jyväskylä and Oulu cohorts.

| Characteristic | Distribution of patiens | |
|---|---|---|
| | Jyväskylä cohort | Oulu cohort |
| Mean age at diagnosis | 66.9 | 64.0 |
| Sex | Male 57 (53%), Female 51 (47%) | Male 41(52%), Female 38(48%) |
| TNM stage | 7th edition | 8th edition |
| Ia | 5 (4.6%) | 8 (10.1%) |
| Ib | 7 (6.5%) | 17 (21.5%) |
| IIa | 23 (21.3%) | 11 (13.9%) |
| IIb | 74 (67.6%) | 29 (36.7%) |
| III | 0 (0.0%) | 14 (17.7%) |
| Grade | | |
| I | 31 (30.0%) | 15 (19.0%) |
| II | 64 (62.1%) | 29 (36.7%) |
| III | 8 (7.7%) | 17 (21.5%) |
| Perineural invasion | | |
| Positive | 68 (63.0%) | 37 (46.8%) |
| Negative | 36 (33.3%) | 42 (53.2%) |
| Resection margin | R0 51 (47.2%), R1 57 (52.8%) | R0 50 (63.6%), R1 29 (36.7%) |

## 4.2 Histopathological examination

The histopathological reviews of tumour specimens were performed by an experienced gastrointestinal pathologist. Tumour staging was done according the seventh edition of UICC/AJCC TNM categories at the time of patient selection (I, III and IV). For the second part of the study, re-staging was done according to the eighth edition during the study. The grading was performed according to the WHO classification of tumours 2010 (Bosman FT, Carneiro F, Hruban RH, 2010). The completeness of tumour resection was defined using the guidelines described by Verbeke et al. (Verbeke et al., 2006), according to which R0 resection is defined as a microscopically complete resection of PDACs with margin >1mm, whereas R1 resection indicates resection margin ≤1mm and R2 resection macroscopic residual disease respectively.

## 4.3 Tumour sampling, immunohistochemistry and immunofluorescence

Tissue microarray blocks were constructed from formalin-fixed paraffin-embedded tumour samples. Two tissue cores 0.6mm in diameter were taken both from the core of the tumour and the invasive margin from representative tumour blocks using Manual Tissue Microarrayer MTA-1 (Beecher Instruments, Inc). The most representative areas in relation to immune cells were chosen from the HE -stained slides by a histopathologist.

Sections of 2 μm thickness were used for immunohistochemistry (IHC) analyses (I, III and IV). For the second part of the study, the thickness of the sections was 3 μm.

Staining for CD3 and CD8 was conducted with anti-CD3 (LN 10, 1:200; Novocastra) and anti-CD8 (SP16, 1:400; Thermo Scientific) antibodies, using a Lab Vision Autostainer 480 (Immunovision Technologies Inc.) (I, III and IV). For the second part of the study, the antibodies used were Novocastra, NCL-L-CD3-565, clone LN10, 1:50 for CD3 and Novocastra, NCL-L-CD8, Clone 4B11, 1:200 for CD8. Samples were incubated with antibody dilutions for 30 minutes at room temperature, and antigen retrieval was performed for 20 minutes. (II)

Novocastra, NCL-L-MLH1, clone ES05, 1:50 antibody was used for staining for MLH1. Staining for PD-L1 was conducted with anti-PD-L1 (E1L3N, 1:100; Cell Signalling Technology) antibody, using a BOND-III stainer (Leica Biosystems). PD-L1 staining was performed using whole tissue sections.

HA staining was performed as described in the original publication: a complex containing the G1 domain of cartilage aggrecan and link protein was labelled with biotin (bHABC), diluted to 3 μg/ml of 1% bovine serum albumin in phosphate buffer, and incubated overnight at 4°C on sections pre-treated with H2O2 and 1% bovine serum albumin to block endogenous peroxidases and unspecific binding respectively. After one hour's incubation in avidin-biotin-peroxidase (Vector Laboratories, Irvine, CA; 1:200 dilution) the sections were washed with PBS and incubated in 0.05% 3.3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.03% H2O2 in the phosphate buffer, followed by nuclear counterstaining with Mayers haematoxylin. (IV)

**Figure 6.** Photomicrograph image of hyaluronan staining of a PDAC sample.
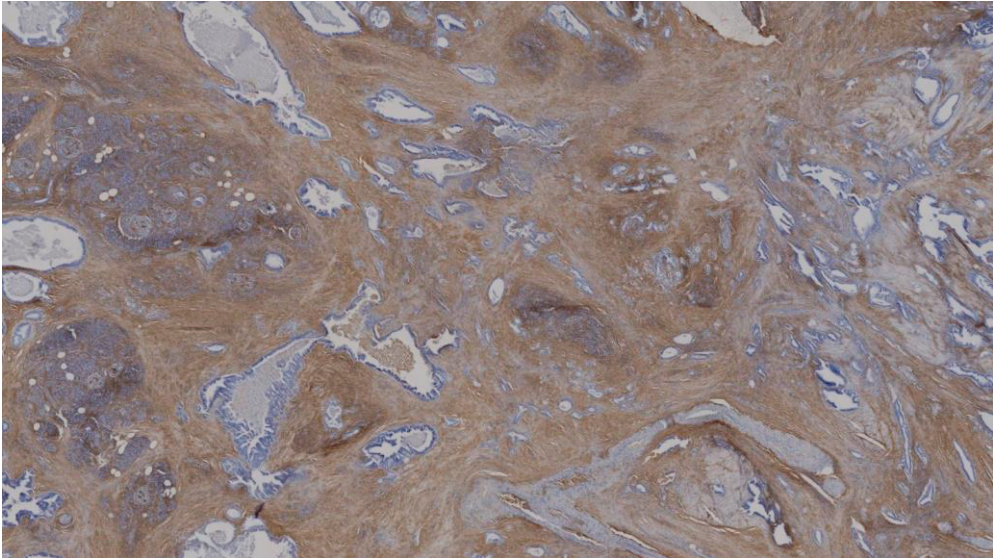


Signal visualization for all IHC was done by diaminobenzidine and sections were counterstained with haematoxylin.

For immunofluorescence staining of FFPE samples, Alexa Fluor 488-conjugated anti-pan-cytokeratin (eBioscience #53-9003-80) and Cy3-conjugated anti-α-smooth muscle actin (Sigma #C6198 both mouse monoclonal antibodies) were used together with rabbit anti-human CD73 antibody (D7F9A), which was visualized using Alexa647-conjugated goat anti-rabbit IgG (Invitrogen #A32733) as a second-stage reagent. Pannoramic Midi FL slide scanner (3DHISTECH) was used for imaging and Case viewer 1.4. program to analyze the stained sections.

## 4.4    Immune cell score determination

CD3+ and CD8+ cells were assessed by digital image analysis without knowledge of the clinical data. Aperio digital slide scanner (Leica Biosystems) was used for scanning the stained TMA and whole tissue sections, followed by analysis using an ImageJ-based program to count the staining positive cells. The analysis was based on separating haematoxylin and diaminobenzidine colour layers and then applying a brightness threshold, as described in detail by Vayrynen et al (Vayrynen et al., 2012).

The automatically analysed samples were reviewed individually by the researcher and, in case of significant clustering bias, the counting was manually corrected. For the second part of the study, in addition to the whole tissue section analysis, immune cell hotspot areas (0.28 mm2) were defined digitally according to ICS protocol both in the tumour area and on the invasive margin, thus simulating the original TMA technique.

Samples with the higher lymphocyte count both from the tumour core and from the invasion margin were selected. Samples were divided into "low" and "high" groups based on the calculated values for positively stained lymphocytes (cells/mm$^2$). Receiver operating characteristic (ROC) curves drawn for each group in relation to disease-specific 3-year mortality was used to determine cut-off values. Examples of samples with low and high densities of CD8+ lymphocytes are shown in Fig 6.

**Figure 7.** Photomicrograph images of tissue micro array samples with low (A) and high (B) densities of CD8+ cells.
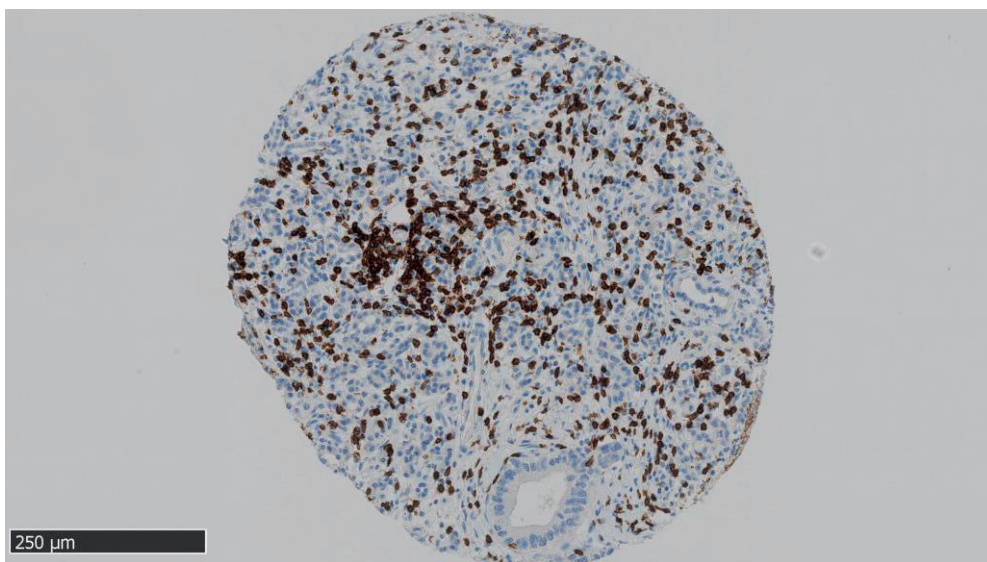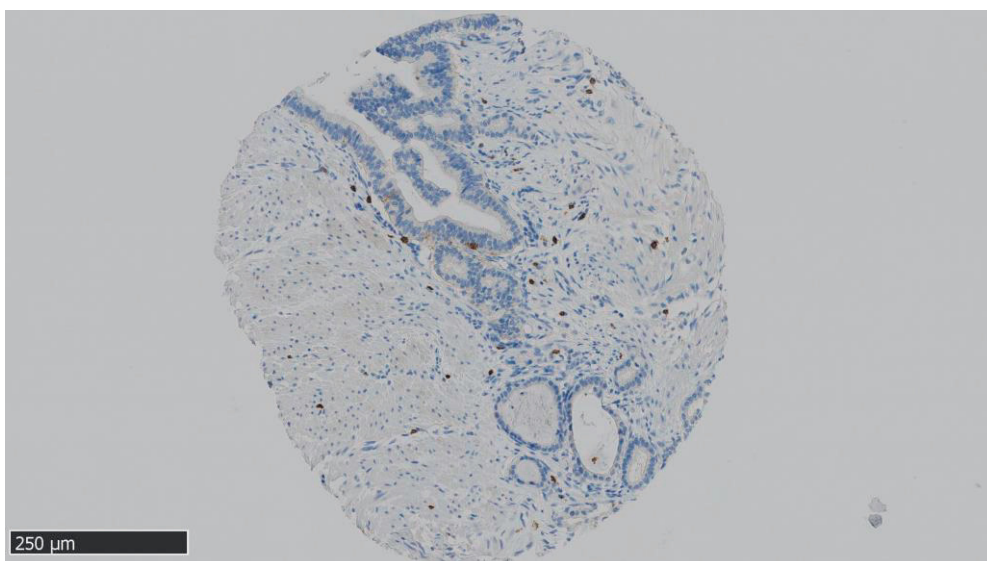




According to the original Immunoscore® method described by Galon et al. (J Galon et al., 2014), the "high" and "low" groups were used to form the ICS from ICS 0 (low densities of both cell types in both regions) to ICS 4 (high densities of both cell

types in both regions). This led to five ICS groups (ICS 0, ICS 1, ICS 2, ICS 3 and ICS 4). ICS 0 and ICS 1 groups formed the low ICS group. ICS 2 formed the moderate ICS group and ICS 3-4 the high ICS group.

**Table 5.**      Immune cell score determination.

| CD3+ density in tumour core | CD3+ density in invasion margin | CD8+ density in tumour core | CD8+ density in invasion margin | Immune cell score | Immune cell score group |
|---|---|---|---|---|---|
| HIGH | HIGH | HIGH | HIGH | 4 | HIGH |
| HIGH | HIGH | HIGH | LOW | 3 | HIGH |
| HIGH | HIGH | LOW | LOW | 2 | MODERATE |
| HIGH | LOW | LOW | LOW | 1 | LOW |
| LOW | LOW | LOW | LOW | 0 | LOW |

## 4.5    Assessment of MLH-1

MLH-1 staining was assessed from four TMA punches per tumour by two independent researchers. Tumours were determined to be MLH-1 positive when there was staining positivity in any of the cancer cells. Normal pancreatic cells or/and inflammatory cells were used as positive controls.

## 4.6    Assessment of CD73

The intensity and proportion of staining on the cell surfaces were assessed independently by four researchers. This was done blind to the clinical data. In case of disagreement consensus was reached by three researchers.

In the case of tumour cell staining assessment, intensity was graded from 1 to 3 and the final score (0-300) was calculated by multiplying the proportion of stained tumour cells (0-100%) by the staining intensity. Patients were then divided into two groups according to the final score, using receiver operating characteristic (ROC) curves drawn in relation to disease-specific 3-year mortality to find a cut-off value of 90.

The proportion of CD73 positive TILs, tumour stroma and vascular structures was also assessed. A positive staining proportion of >3% of TILs in the tumour sample was considered positive. In the case of tumour stroma, CD73 staining positivity was graded weak, moderate or strong when respectively < 5%, 5-16% or

> 17% of the stromal area was stained. When assessing the vascular structures, 95% was set as a cut-off value for CD73 positivity due to the strong staining intensity.

## 4.7    Assessment of PD-L1

PD-L1 expression was assessed independently by three researchers by estimating the proportion of PD-L1 positivity on the tumour cell surface and in tumour stroma (II). A tumour sample was considered positive when more than 1% of the tumour cells expressed PD-L1, or, in case of stromal PD-L1 expression, more than 5% of the stromal cells expressed PD-L1.

## 4.8    Assessment of hyaluronan accumulation

Expression of HA was assessed by digital image analysis using QuPath v.0.1.2. The average intensity of the cores from the tumour centre and the invasive margin was used in the analyses. The samples were divided into two groups: low and high stromal HA expression.  ROC curve drawn in relation to disease-specific 3-year mortality was used to determine the cut-off value.

## 4.9    Statistical analysis

Statistical analyses were performed with IBM SPSS statistics 23 for Windows (I) and IBM SPSS statistics 24 for Windows (IBM Corporation, Armonk, NY, USA) (II-IV). Chi-square test was used to compare categorical variables. The Kaplan-Meier method with log-rank test was used to calculate disease-specific survival (DSS) and OS. Univariate and multivariable Cox proportional hazards regression model was used to calculate hazard ratios for OS and DSS. A p-value less than 0.05 was considered significant. All statistical tests were two-sided.

# 5   RESULTS

## 5.1   Immune cell score predicts survival in pancreatic cancer

In Study I, infiltration of CD3+ and CD8+ lymphocytes was successfully analysed in relation to survival in 108 patients with PDAC. In the assessment, 36 (33.3%) patients were found to have low ICS(0-1), moderate ICS (2) as well as high ICS (3-4), making the ICS groups identical in size. TNM stage distribution in the 108 patients was as follows: 1A 5 (4.6%), 1B 7 (6.5%), 2A 23 (21.3%) and 2B 73 (67.6%). The respective 1-year, 3-year and 5-year DSS rates in the study population were 75.2 %, 32.4 % and 18.8 %; the respective OS rates were 70.2 %, 29.1 % and 16.9 %. Median OS of the study population was 22 [95% CI (17.9-26.1)] months. ICS was not associated with any of the clinicopathological parameters studied.

Low ICS was strongly associated with diminished DSS [univariate hazard risk (HR) 2.5 (95% CI 1.38-4.52)] and OS [HR 2.5 (1.41-4.34)] in the study cohort (Fig. 7.). Multivariable analysis confirmed ICS as an independent prognostic factor with adjusted HR of 4.44 (2.21-8.91) (DSS) and 4.2 (2.18-8.17) (OS) for low ICS. The 5-year DSS rates for ICS groups were as follows: low ICS 5.0%, moderate ICS 15.2% and high ICS 33.4%. The other independent prognostic factors in the study population were histological tumour grade and perineural invasion. High ICS was significantly associated with prolonged survival, also within the TNM stage 2B.

The impact on survival of each ICS component was also assessed. High CD3+ T cell infiltration in the tumour core showed the strongest association with DSS, but all the components were significantly associated with DSS.

Five (4.6%) MLH1-negative tumours were found, and there was no association between MLH1 -status and ICS or survival.

**Figure 8.** Kaplan-Meier graph showing the differences in overall survival between low, moderate and high ICS groups in the Jyväskylä cohort.
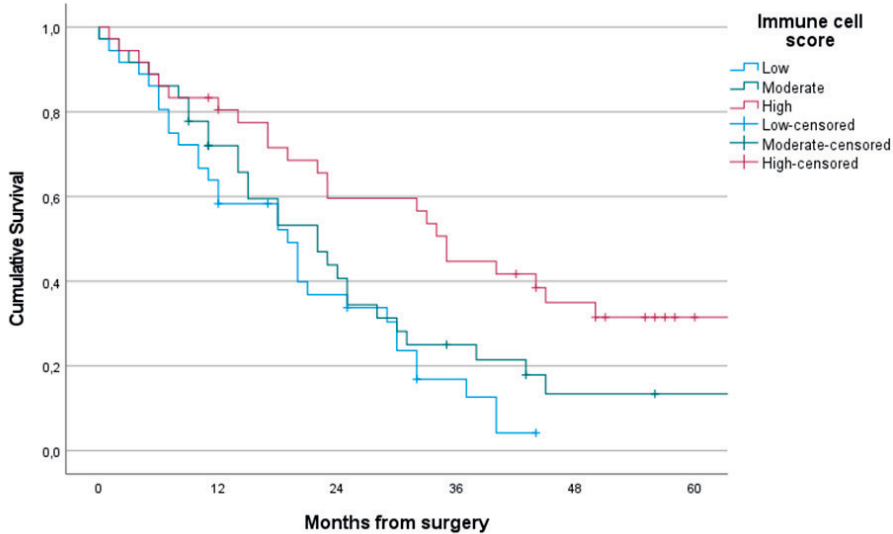


## 5.2 Whole-section technique performs better than tissue micro array-like hotspot technique when assessing immune cell score

In Study II, the ICS for patients in the study population was determined using two different techniques: TMA-like hotspot technique and whole-section technique. The ICS distribution in the 79 patients was as follows: low 39 (49.4%), moderate 22 (27.8%) and high 18 (22.8%). The respective 1-year, 3-year and 5-year DSS rates in the study population were 72.0 %, 27.5 % and 19.5 %; the respective OS rates 70.0 %, 25.3 % and 17.9 %. Median OS of the study population was 20 months. Again, no significant associations were found between ICS and any of the clinicopathological parameters.

When the whole-section technique was used, ICS was confirmed to be an independent prognostic factor with an HR of 0.22 (0.08-0.60) (DSS) and 0.27 (0.11-0.67) (OS) for the high ICS-group compared to the low ICS-group. The 5-year DSS rates for the ICS groups were 5.4% (low), 26.4% (moderate) and 55.6% (high).

**Table 6.** 5-year disease-specific survival (DSS) and overall survival (OS) in Jyväskylä and Oulu cohorts stratified by ICS groups.

|  | Jyväskylä cohort | | | | Oulu cohort* | | | |
|---|---|---|---|---|---|---|---|---|
|  | ICS 0–1 | ICS 2 | ICS 3 | Total | ICS 0–1 | ICS 2 | ICS 3 | Total |
| 5-year DSS (%) | 5.0 | 15.2 | 33.4 | 18.8 | 5.4 | 26.4 | 55.6 | 19.5 |
| 5-year OS (%) | 4.2 | 13.4 | 31.5 | 16.9 | 5.3 | 26.4 | 43.8 | 17.9 |

*Whole tissue sections were used to determine ICS

Instead, when using the hot-spot technique, statistical significance for the association between ICS and survival was not reached, although a similar trend was observed. There were significant differences between the immune cell counts depending on the technique used. First, the median number of lymphocytes per square millimetre was significantly higher in hotspots than in whole sections. Second, both the range and the interquartile range of immune cells counted using whole section technique were significantly smaller than the ranges of the average immune cell densities observed in hot spot technique.

**Table 7.** Adjusted hazard ratios (HRs) for the Oulu cohort with 95% confidence intervals (CI) of disease-specific and overall mortality of PDAC patients with low (0-1), moderate (2) and high (3-4) ICS, presented separately for whole tissue sections and hotspots.

|  | Number of patients | Immune cell score 0–1 HR (95% CI) | Immune cell score 2 HR (95% CI) | Immune cell score 3-4 HR (95% CI) |
|---|---|---|---|---|
| Whole sections |  |  |  |  |
| Disease-specific mortality | 79 | 1.00 Reference | 0.45 (0.21–0.95) | 0.22 (0.08–0.60) |
| Overall mortality | 79 | 1.00 Reference | 0.42 (0.20–0.88) | 0.27 (0.11–0.67) |
| Hotspots |  |  |  |  |
| Disease-specific mortality | 79 | 1.00 Reference | 1.10 (0.52–2.34) | 0.64 (0.26–1.58) |
| Overall mortality | 79 | 1.00 Reference | 1.41 (0.69–2.89) | 0.70 (0.30–1.66) |

## 5.3 High expression of CD73 in pancreatic cancer tumour cells predicts poor survival independently of the number of tumour infiltrating lymphocytes

In Study III, expression of CD73 was successfully assessed separately in tumour cells, vascular structures, TILS and tumour stroma. TMA was used for staining after the correspondence of TMA and whole section samples was found to be 100% in the 16 corresponding TMA and whole section samples.

The associations between clinical and histopathological variables and cell-specific CD73 positivity were assessed. High CD73 expression in tumour cells was found to be associated with PD-L1 expression, perineural invasion and histopathological grade. Moreover, high expression of CD73 in TILs was found to be associated with lymph node metastasis. In addition, there was a statistically significant association between high expression of CD73 in TILs, vascular structures and stroma. CD73 positivity in any cells assessed was not associated with ICS in the study cohort.

When expression of PD-L1 was assessed, only five (4.5%) patients were found to be PD-L1 positive. PD-L1 positivity in tumour cells was associated with high CD73 expression in tumour stroma, high histopathological grade and with low T class of the primary tumour.

The impact of high CD73 expression on the survival of patients with PDAC was evaluated. High expression of CD73 in tumour cells was found to be significantly associated with poor DSS (p=0.021) and OS (p=0.016) (Fig. 8.). In multivariable analysis, CD73 positivity in tumour cells was found to be an independent negative prognostic factor. Instead, expression of CD73 in TILs, tumour stroma or vascular structures was not associated with survival.

**Figure 9.** Overall survival of patients with PDAC, stratified by CD73 expression in tumour cells.
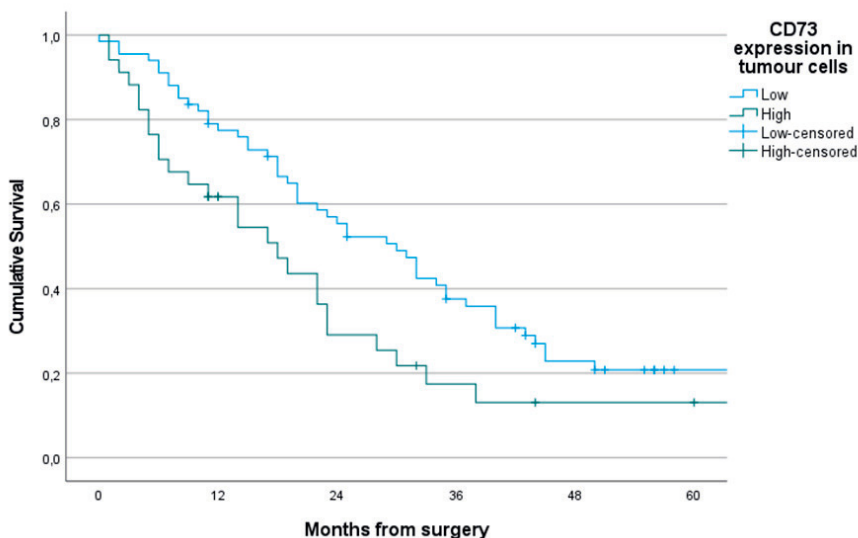
## 5.4 Stromal hyaluronan accumulation is associated with low immune cell score and poor survival in pancreatic cancer

In Study IV, expression of HA in tumour stroma of 101 PDAC patients was assessed using an HA-specific probe and digital image analysis. HA staining was clearly seen in all specimens with little visible variation between the cases. The expression of PD-L1 in tumour stroma was also assessed.

When the associations between stromal HA expression and other histopathological parameters were assessed, a significant association between high stromal expression and low ICS was found. Stromal expression was not associated with any other parameter.

High stromal expression of HA was found to be significantly associated with poor DSS (p=0.037) and OS (p=0.013) (Fig. 9). When the multivariable analysis was performed, stromal HA accumulation was found to be an independent negative prognostic factor together with TNM stage, high CD73 expression in tumour cells and low ICS.

**Figure 10.** Overall survival of patients with PDAC, stratified by HA expression.
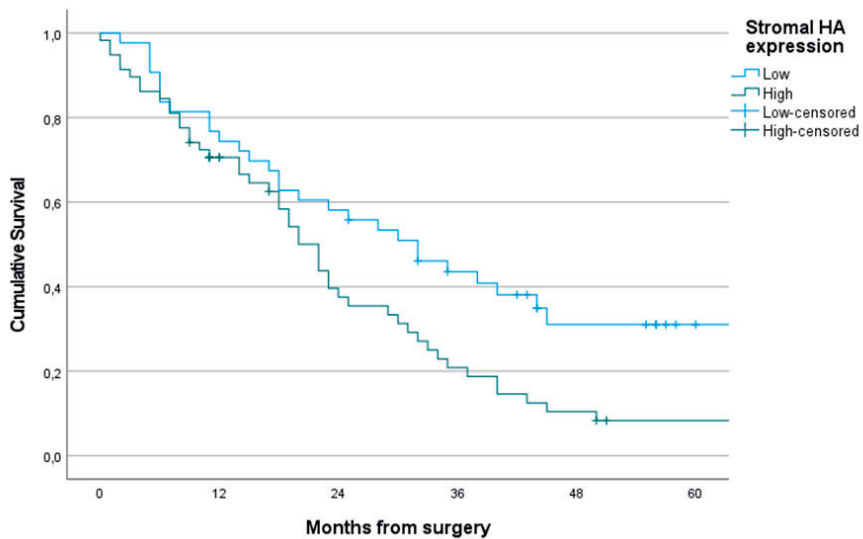
# 6   DISCUSSION

This thesis summarizes the findings regarding the impact of host immune response, the measurement of this response and the immune escape mechanisms in PDAC reported in four original articles.

According to the results, the host immune response significantly impacts the progression of PDAC. ICS, reflecting the level of this immune response, can be used for the prediction of the survival of PDAC patients. Using whole section samples has become easy and seems to yield more accurate results when estimating the number of TILs. Finally, various factors in the tumour microenvironment, such as CD73 and changes in the metabolism of HA, have an important and even a prognostic role in the tumour environment and complicated regulation of host immune response in PDAC.

## 6.1   Patient material and survival

The study population consisted of consecutive patients who had undergone a curative-aimed surgery at a single hospital. This fact, combined with the prospective data collection, significantly improves the reliability of the results. Excluding patients with TNM stage 3 and 4 made the study population more homogenous in relation to disease progression, thus making it easier to recognize the impact of tumour microenvironment on survival.

The 5-year OS rates for the study population were 16.9% (I, III, IV) and 17.9% (II). Survival rates of 30-55% in PDAC patients treated with curative intent have been published, but the reports of actual 5-year survival in PDAC after curative aimed surgery vary between 0-20% (Ahola et al., 2017; Carpelan-Holmström et al., 2005). Taking into account the double-checking of histological diagnoses during our study, the survival rates in study populations appear to be somewhat higher than expected. The exclusion of patients with TNM stages 3-4 and with R2 resection margin from the study provide one explanation for this.

## 6.2     Surgery and adjuvant therapy

All the patients in this study had undergone surgery with curative intent. During the study period the surgical technique did not significantly change; the pylorus-preserving pancreaticoduodenal resection was the main surgical procedure in CFCH while standard Whipple operation was mainly performed in Oulu University Hospital. There are no data showing that surgical technique has an impact on the immune response and in turn on the main results of this study.

None of the patients in the Jyväskylä cohort nor in the Oulu cohort received neoadjuvant therapy, which is rapidly becoming more common in the treatment of PDAC as described above. By contrast, 87 % of patients in the Jyväskylä cohort and 69% in the Oulu cohort with information available received adjuvant chemotherapy consisting mainly of gemcitabine or 5-fluorouracil-leucovorin. The effect of adjuvant therapy on patient survival in this study cohort cannot be reliably estimated. However, the adjuvant therapy was given according to the current guidelines.

## 6.3     Immune cell score in pancreatic cancer

In the first part of the study, we showed that the immune response -reflecting immune cell score is a prognostic factor independent of TNM stage or histopathological grade.

The impact of host immune response has been the object of intensive research throughout the last decade. The majority of studies concerning the impact of TILs in PDAC have demonstrated the effect by dividing the study population into two categories based on the TIL counts. A recent meta-analysis summarized the evidence of the prognostic value of TILs in PDAC. The authors included 39 studies in their meta-analysis, among them the first and the second parts of this study (Orhan et al., 2020). The main results of this meta-analysis are clear, and in line with our results: The abundant infiltration of effector T cells in the tumour area predicts prolonged survival, reflecting the impact of host immune response suppressing the progression of the disease. Although several studies have been conducted on the impact of TILs in PDAC, our study was the first to apply the structured scoring system, taking account of the type, number and location of immune cells in PDAC patients.

There are several immune cell populations infiltrating the PDAC tumours, each of them affecting the big picture of tumour microenvironment. In our study, the impact of two cell populations, CD3+ and CD8+ T cells, was assessed in two

different locations. All these components of ICS were significantly associated with the DSS of the patients, but the best prognostic value was achieved when combining them to form the ICS. According to the meta-analysis by Orhan et al, T cell infiltration located at the tumour centre has the greatest impact on survival (Orhan et al., 2020). In our series, the location of TILs had only a minimal effect on the prognostic value. This, however, is in line with the subgroup analysis of the same meta-analysis showing no significant differences between the locations.

TNM has been the cornerstone for years when estimating the prognoses of patients with PDAC, also guiding the treatment decisions. The prognostic value of ICS appeared to be greater than the value of TNM stage in our study population. The survival of patients varied significantly within a single TNM stage depending on the ICS. This becomes understandable given that TNM stage describes the progression of the disease more than the biological features of the tumour cells or microenvironment, while both of these factors as well as the grade of differentiation have a significant impact on survival. In this era of rapidly developing immune-modulating drugs, the information provided by TNM alone is not sufficient. Rather, a combination of these classifications is urgently needed.

Because of the incomplete data on the significance of microsatellite instability (MSI) in PDAC, we also performed MLH1 staining to determine if MSI is associated with ICS as it is in colorectal cancer. There were only five (4-6%) MLH1-positive tumours in our series, and we found no association between MLH1-status and ICS. Indeed, according to the literature, MSI is a rare event in PDAC with the incidence varying 0-1.3% in all PDAC (Eso et al., 2020; Grant et al., 2020; Z. I. Hu et al., 2018), although observations of greater incidence have also been made in small cohorts (Nakata et al., 2002).

In the second part of the study, we confirmed the prognostic value of ICS with another cohort of PDAC patients. In addition to this, we successfully demonstrated the differences between the use of whole tissue sections and TMA. TMA has been a useful tool when assessing large materials in research settings, but the rapid evolution of digital pathology image analysis has partly decreased the relevance of TMA. TMA has some well-known weaknesses, such as sampling error and tears in the TMA tissue sections. It is possible that these limitations played some role in our results of the second part of the study. Nevertheless, the use of whole tissue sections appeared to yield more reliable results taking account of the small variance between cases and the better performance in predicting survival.

To become reproducible, such a classification system as ICS should be validated with larger populations to determine the optimal cut-off values. In our study, the

cut-off values between the first and the second parts of the study varied significantly due to some differences between the research methods used, such as thickness of the tissue slice and the methods of determining the hot spot.

## 6.4    Other factors in tumour microenvironment

In the first part of the study, MLH-1 staining was carried out to evaluate the possible role of MSI in the immune cell infiltration of tumours within the study cohort. The method was chosen given that MLH-1 is supposed to identify most MSI tumours. MLH-1 positivity was not associated with survival in the study cohort. Somewhat surprisingly, there was also no association between MLH-1 positivity and ICS in study population. This may be due to the relatively small size of the study population.

 In the third part of the study, we assessed the impact of CD73 and PD-L1 on the survival of PDAC patients and their relationship to immune cell infiltration in the tumour area. In line with other studies published on CD73 in PDAC (Q. Chen et al., 2020; Zhou et al., 2019a), we found that high CD73 expression in tumour cells predicts poor survival in PDAC patients.

 Paying attention to the fact that CD73 is known to suppress immune response via its G-protein-coupled receptors -activating function, it may be surprising that expression of CD73 showed no association with ICS in our study population. However, high CD73 expression has been associated with exhausted phenotype of T cells in a mouse experiment (Deng et al., 2018). In light of this, it is possible that the immune suppressing impact of CD73 on T cells is more to deactivate than to decrease their number. On the other hand, CD73 is also known to have non-enzymatic functions promoting the proliferation and migration of cancer cells (Z.-W. Gao et al., 2017). These functions may also have a role in the observed survival-diminishing impact of CD73.

 By means of cell-specific assessment of CD73 expression, we were able to demonstrate, for the first time, that high expression of CD73 in TILs is associated with metastatic spread in lymph nodes. This increased expression of adenosine A2A receptor has previously been linked to lymph node metastasis in head and neck carcinoma (Ma et al., 2017). This makes our finding the more convincing and confirms the role of adenosine pathway in cancer progression.

 On the contrary, PD-L1 did not show significant prognostic value in our study population. The literature gives a partly inconsistent picture of the impact of PD-L1 on survival in PDAC, although the meta-analyses summarizing the older studies

indicate that it may have prognostic significance (H.-L. Gao et al., 2018; Y. Hu et al., 2019; Zhuan-Sun et al., 2017). The PD-L1 positive rate has also been greater on average than in our study population. This may be due to the differences in the estimation methodology of PD-L1 since there is no consensus on how PD-L1 expression should be reported in PDAC. Interestingly, we found an association between high expression of PD-L1 and CD73. This is in line with the earlier observation of an association between these two immunosuppressive molecules by Deng et al. (Deng et al., 2018).

In the fourth part of the study, we performed a computer-assisted evaluation of expression of HA in PDAC tumour stroma. High expression of HA in tumour stroma was demonstrated to be an independent prognostic factor in PDAC. This concurs with earlier results published by Cheng et al., carried out with a relatively small study population already in 2013 (Cheng et al., 2013), and more recent results published by Franklin et al (Franklin et al., 2019).

In addition to the prognostic value of stromal HA expression, we demonstrated an association between the stromal HA accumulation and low-level immune response as judged by the low ICS. This corroborates earlier findings of the role of HA accumulation in the differentiation of macrophages into immunosuppressive M2 -phenotype (Kuang et al., 2007; Tiainen et al., 2015), which can be deduced to lead to lower infiltrations of T cells in the tumour area, as shown in our study.

We found no association between expression of CD73 and HA in our study population. This can be explained given that CD73 seems to suppress the immune response more by impacting the function of the T cells rather than their number, while HA accumulation is associated with TIL counts.

Although the association between the accumulation of HA and the progression of cancer is well evidenced in various malignancies, and now also in PDAC, the total amount of HA is likely not the decisive element itself but rather the result of the enormous change in HA metabolism during cancer progression. The different influence of HA particles depending on the length of the HA chains is well documented (Jiang et al., 2011). There are data, for instance, showing that short HA chains can induce angiogenesis while the high-molecular mass HA is rather anti-angiogenic (Deed et al., 1997). As an indicator of the significant role of the changes in the length of the HA chains, there are data according to which the cell surface hyaluronidase TMEM2 is an independent negative prognostic factor in PDAC. From this point of view it is not so surprising that the clinical trials combining new HA degrading drugs with cytostatic drugs have been disappointing (Ramanathan et al., 2019; Van Cutsem et al., 2020).

## 6.5   Strengths and limitations of the studies

The strengths of this study include the consecutive patient series, making selection bias minimal. Data collection was mainly carried out prospectively and the review of the patient records was done carefully. The causes of death were obtained from hospital records and the National Cause of Death Registry, making the DSS rates reliable.

In addition, the study patients, although treated in two hospitals, received quite similar treatment as regards surgical procedures and oncological treatment. Although the study period was quite long (2000-2016), no significant changes in treatment occurred during those years. We did not include palliatively treated patients or patients who were operated on with curative intention but who turned out to have disease at TNM stage 3 or 4.

The selective use of digital image analysis definitely offered some advantages. Compared to manual counting of immune cell numbers, digital image analysis probably yields more exact results with no miscalculations or exhaustion even when analysing large numbers of samples. This requires, however, the careful calibration of the softa to distinguish the targeted cells, and that is what we did. Regarding the estimation of HA staining intensity, the preliminary estimation of HA staining intensity showed that the differences between the cases were sometimes minimal and the scoring could be done more reliably with digital image analysis.

There are also some weaknesses in this study. First, the number of patients was relatively small, making it more difficult to find survival differences, for instance, between the different TNM stages. Moreover, the use of TMA may cause sampling error. To reduce this bias, two tissue cores were taken from each location (I) and the whole section technique was used in the second part of the study.

Furthermore, we were not able to validate the cut-off values determined in the Jyväskylä cohort in the Oulu cohort. This was due to the differences between the manual TMA and the hot-spot techniques and probably also because of the differences between the laboratories regarding the thickness of the tissue slice.

## 6.6   Future prospects

As the strong evidence establishes the impact of the host immune system on the survival of the patients with PDAC, international consensus on the optimal methodology should be achieved in the near future to describe the immune response

in a way that can be replicated. ICS, as we have demonstrated, has potential to act as such a scoring system. Whatever the immune scoring system chosen is called, international validation will be needed to confirm optimal cut-off values and to ensure that the chosen classification is reproducible in different milieus.

We have shown the great variation in survival of patients with PDAC within a single TNM stage, indicating the shortcomings of the TNM classification system; alone, it lacks the ability to predict survival as accurately as is needed. In future, a combination of the TNM staging system and an immune cell infiltration-based scoring system should be taken into clinical use to improve the prediction of survival of patients with PDAC.

An apparent shortcoming of ICS and similar methodologies, compared to the TNM staging system, is the lack of an option to estimate the score preoperatively even tentatively when the surgical specimen is not yet available. Non-invasive methods to estimate the levels of immune cell infiltration have already been investigated (Levi et al., 2019), and the need for research in this field is obvious, also in PDAC. The opportunity to obtain the combined prognostic value of the TNM staging system and the immune cell infiltration-based scoring system for use preoperatively would have a notable effect on the treatment of patients with PDAC by means of improved patient selection and management.

As new drugs against PDAC are developed, the majority of which target the immune escape methods of the tumour or aim to modulate the host immune response, the need for predictive markers will not decrease, quite the reverse. It is not on the immediate horizon to find a drug with great impact suitable for all PDAC patients, rather we are rapidly moving toward personalized medicine. This will increase the need for predictive markers, such as PD-L1, CD73, or HA to identify those patients who will benefit from the increasing number of the drugs available. The possibility to obtain information on such biomarkers noninvasively via imaging would naturally open up vast new possibilities, for example, in the field of personalized neoadjuvant treatment.

# 7 CONCLUSIONS

This study contributes information on the impact of the host immune response on the progression of PDAC and provides a scoring system enabling classification of patients based on immune cell infiltration. This study also shows the prognostic value of CD73 and HA accumulation in PDAC and increases the understanding of the mechanisms beyond the battle between tumour and host immune system.

The detailed conclusions are as follows:

1. ICS seems to be a suitable methodology to measure immune response in PDAC for clinical purposes, having a prognostic value superior to that of TNM classification.

2. ICS was confirmed to predict survival in another cohort of consecutive PDAC patients. ICS determined in whole tissue sections provided higher prognostic value than the ICS determined in hotspots.

3. High CD73 expression in tumour cells is an independent prognostic factor for poor survival in association with perineural invasion and PD-L1 expression in PDAC. In addition, high expression of CD73 in TILs was associated with positive lymph node status.

4. Stromal HA accumulation represents an independent negative prognostic factor in PDAC. In addition, it was associated with low ICS, suggesting that it has a role in immune regulation in tumour microenvironment.

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# ORIGINAL COMMUNICATIONS

# PUBLICATION
# I

**High immune cell score predicts improved survival in pancreatic cancer**

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# High Immune Cell Score Predicts Improved Survival in Pancreatic Cancer

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**Abbreviations**: IS: immune cell score; PDAC: pancreatic ductal adenocarcinoma; DSS: disease-spesific survival; OS: overall survival; TMA: tissue microarray; MSI: microsatellite instability

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**Abstract**

Increasing evidence suggests that cancer progression is strongly influenced by host immune response, which is represented by immune cell infiltrates. T -lymphocyte -based immunoscore has proved to be a prognostic factor in colon cancer, but its significance in pancreatic cancer is poorly known.

Total of 108 patients operated (R0/R1) for pancreatic ductal adenocarcinoma (PDAC) (TNM stage I-II) were included in the study. Immune cell score (IS) was determined by scoring the samples from grade 0 to 4 according to the number of immune cells ($CD3^+$ and $CD8^+$) in tumor core and invasion margin using tissue microarrays, immunohistochemistry and digital analysis. Tumors with microsatellite instability were identified by MLH1 immunostaining.

High IS and low histological grade were significantly associated with better disease-spesific survival (DSS) and overall survival (OS). The 5-year DSS rate for low, moderate and high IS groups were 5.0 %, 15.2 % and 33.4 %, respectively (p=0.007). The 5-year OS rate for the low, moderate and high IS groups were 4.2 %, 13.4 % and 31.5 %, respectively (p=0.004). In addition, IS and prognosis varied within a single TNM stage. There was no association between IS and any of the clinicopathological variables. IS was shown to be an independent prognostic factor for better DSS and OS in multivariate analysis, together with the histological grade of the tumor and perineural invasion. Five MLH1 negative tumors (4.6%) were found showing no correlation with IS.

IS could be a useful prognostic marker in patients with PDAC treated by primary surgery.

**Keywords:** Immunoscore, pancreatic cancer, microsatellite instability, microenvironment, immune cell score

**Introduction**


Pancreaticoduodenal resection remains the only potentially curative treatment for pancreatic ductal adenocarcinoma (PDAC). Patients with resectable PDAC have a 5-year overall survival (OS) of 15% to 25% after radical resection and adjuvant chemotherapy [1, 2]. Approximately 80 % of patients have unresectable tumors at the time of diagnosis due to advanced locoregional or metastatic disease [3].

The American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) Tumor-Node-Metastasis (TNM) classification has provided the basic platform for prognostication and treatment of pancreatic cancer [4]. It is well known, however, that TNM classification describes tumor burden and associates with survival, but does not provide information of the biological behavior of PDAC.

The accumulation of successive genetic mutations seems to be the preliminary step for the development of PDAC [5]. Subtypes defined by different mutational background are associated with distinct histopathological characteristics and survival [5, 6]. PDAC is usually stroma-rich composed of almost 90% extracellular matrix with a complex assembly of fibroblasts, immune and neural cells, endothelial cells and a vast collection of growth factors, adhesion molecules, and structural compounds such as collagen, fibronectin and hyaluronic acid [7]. Extracellular matrix creates a dynamic compartment that plays a pivotal role in the process of tumor formation, progression, invasion, and metastasis.

The role of the immune system in cancer development, progression and survival has been well demonstrated [8]. Particularly, previous studies have provided a strong evidence of the role of cytotoxic immune response for patient survival in various tumors [8]. Systematic analysis of tumor infiltrating immune cells in colorectal cancer, and their impact on survival has led to introduction of immunoscore, which is based on calculation of cytotoxic lymphocytes in tumor and invasion margin, and has been recently patented. There is some evidence showing that in colorectal cancer immunoscore gives even better prognostic accuracy than TNM classification system [9, 10].

Microsatellite instability (MSI) has been associated with better survival in gastric and colorectal cancers [11, 12]. Enhanced immune response is believed to be the main factor behind the prolonged survival of MSI patients [13]. There is controversial data of the epidemiology of MSI and its potential prognostic role in

PDAC [14-16]. Only one study has shown association between prolonged survival of patients with MSI tumors and better immune response in PDAC [15].

PDAC has been traditionally regarded as a cancer that evades host immune system. Based on previous studies, it seems that immune cell infiltration may provide a tool to estimate the prognosis of the patients also in pancreatic cancer [17-20]. The aim of this study was to examine prognostic role of T -lymphocyte - based immune cell score (IS), following similar principles as the original immunoscore, and its relation to MLH1 expression in PDAC.

## Material and methods

### Patients

From 2000 to 2016, 241 consecutive patients underwent pancreaticoduodenal resection at our institution. Of these, 129 patients (53.5 %) had PDAC. Thoracoabdominal computed tomography, magnetic resonance cholangiopancreatography and endoscopic retrograde cholangiopancreatography were used to diagnose and stage pancreatic tumors. Total of 108 patients with stage 1A-2B disease were included in the current study. Patients having locally inoperable disease, peritoneal carcinosis or distant metastases were excluded. Data were retrieved from our prospectively maintained and continuously updated database established in 2000, with detailed information on patient and tumor characteristics, surgical treatment and complications, oncological treatments and follow-up. Data was confirmed by patient chart review. None of the included patients received neoadjuvant chemotherapy before surgery. Operations were initially done according to standard Kausch-Whipple technique [21]. Since 2004 pylorus-preserving pancreaticoduodenal resection became the main surgical procedure performed [22]. Extended pancreaticoduodenectomies, including total pancreatectomy, radical lymphadenectomy or portal vein resection were performed very selectively. Ninety-four out of the 108 patients (87%) received adjuvant chemotherapy consisting mainly of gemcitabine or 5-fluorouracil-leucovorin.

### Histopathological examination

Histopathological examination of tumor specimens was done by histopathologists according to TNM categories, and all histopathological slides were reviewed by experienced gastrointestinal pathologist (JB). Tumor staging was done according to the 7th edition of the UICC/AJCC TNM categories [23]. R0-resection was defined as a microscopically complete resection of PDACs with margin >1mm, whereas R1 resection indicated resection margin ≤1mm according to Verbeke et al [24]. R2-resection indicated macroscopic residual disease, and these were excluded from this study.

**Tumor sampling**

Tissue microarrays (TMAs) were constructed using method described earlier [25]. Two tissue cores with diameter of 0.6mm were taken both from the core of the tumor and the invasive margin. The invasive margin was defined as a region of 0.5mm on each side of the border between tumor cells and normal pancreatic tissue. The most representative areas with immune cells were chosen from the HE stained slides by histopathologist (JB).

**Immunohistochemistry**

Two-µm-thick TMA block sections were immunohistochemically stained using CD3 (Novocastra, NCL-L-CD3, clone PS1, 1:100), CD8 (Thermo Scientific, RM-9116, clone SP16, 1:100) and MLH1 (Novocastra, NCL-L-MLH1, clone ES05, 1:50) antibodies and standard procedures using the BOND-III staining instrument (Leica Biosystems) and Bond Polymer Refine Detection Kit (Leica Biosystems). Samples were incubated with antibody dilutions for 30 minutes at room temperature, and antigen retrieval was performed for 20 minutes (CD3 and CD8) or 30 minutes (MLH1) using Bond Epitope Retrieval Solution 2 (Leica Biosystems).

**Immune cell score and microsatellite instability status determination**

Immunohistochemical stainings were assessed without knowledge of the clinical data. CD3^{+} and CD8^{+} cells were assessed by digital image analysis. Stained TMA sections were scanned using an Aperio digital slide scanner (Leica Biosystems), followed by analysis using an ImageJ-based program to count the positive cells. Cells were identified by method described earlier by Väyrynen et al [26], based on separating hematoxylin and diaminobenzidine color layers and then applying a brightness threshold using open source image analysis software ImageJ. The numbers of CD3^{+} and CD8^{+} cells were count from representative areas of the tumor center and invasion margin. Both intraepithelial and intrastromal immune cell infiltrates were included in cell count. All of the automatically analyzed samples were individually reviewed and in case of significant clustering bias the counting was corrected manually. In the case of tumor not filling the whole area, the cell counts were corrected according to percentage of tumor in the punch. A sample with the

higher lymphocyte count from the tumor center and from the invasion margin was selected. Samples were divided into two groups based on the calculated values for positively stained lymphocytes (cells/mm$^2$): "high" and "low" cell densities (**Fig. 1**). To determine cut-off values for IS with optimal sensitivity and specificity, we used receiver operating characteristic (ROC) curves drawn for each group in relation to disease-specific 3-year mortality as previously described by Wirta et al [13]. The cut off -values were as follows (cells/mm$^2$): 527 for CD3$^+$ in tumor core, 674 for CD3$^+$ in invasive margin, 297 for CD8$^+$ in tumor core and 320 for CD8$^+$ in tumor margin (**Table 1**).

**Table 1. Immune cell score cut-off values**

| | Cut-off values (cells/mm2) | AUC (95% CI) | Sensitivity | Spesificity | Likelihood ratio |
|---|---|---|---|---|---|
| CD3$^+$ Tumor core | 527 | 0.61 (0.50-0.72) | 0.578 | 0.591 | 1.45 |
| CD3$^+$ Invasive margin | 674 | 0.52 (0.41-0.63) | 0.625 | 0.432 | 1.10 |
| CD8$^+$ Tumor core | 297 | 0.60 (0.50-0.71) | 0.500 | 0.682 | 1.57 |
| CD8$^+$ Invasive margin | 320 | 0.54 (0.43-0.65) | 0.453 | 0.636 | 1.24 |

**Figure 1. Examples of tissue microarray block sections of low (A) and high CD3+ (B), and CD8+ (C,D) T cell densities.**
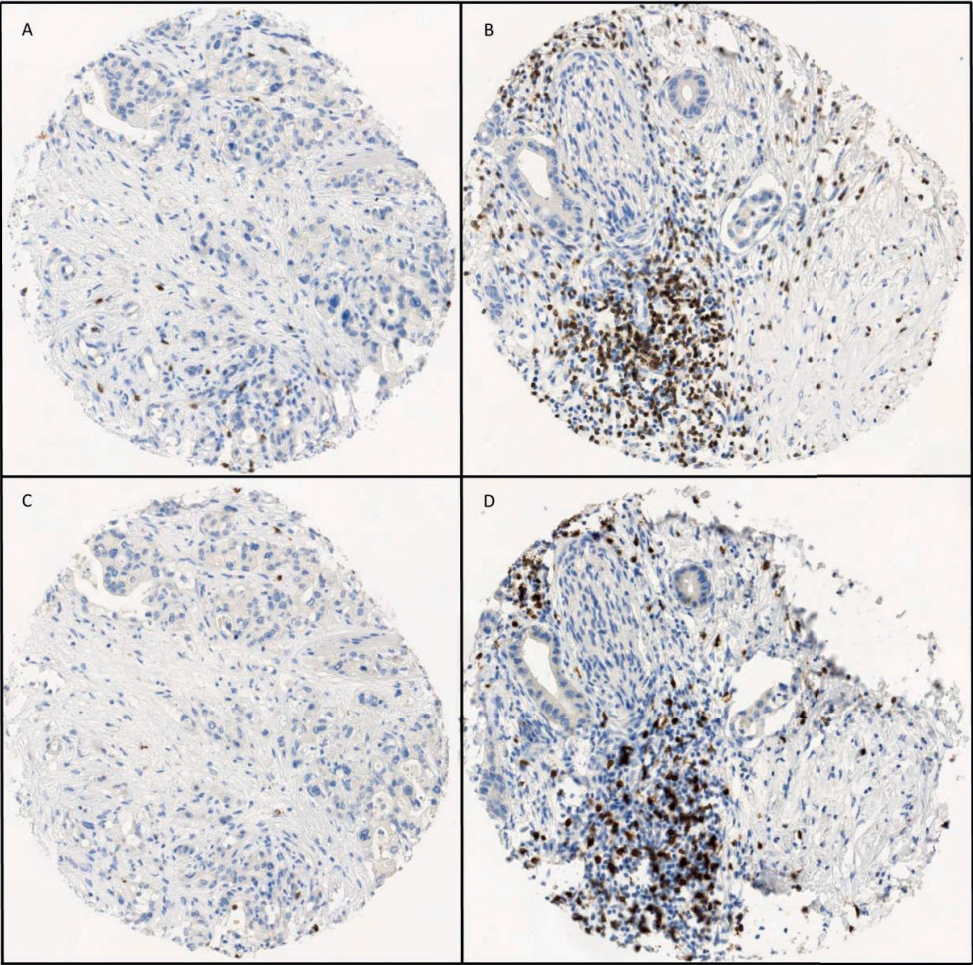
The "high" and "low" groups were used to form the IS from IS0 (low densities of both cell types in both regions) to IS 4 (high densities of both cell types in both regions) as described previously [13]. This led to five IS groups (IS0, IS1, IS2, IS3 and IS4). Due to limited number of patients we included IS 0 and IS 1 groups to one group (low IS group), IS 2 formed moderate IS group and IS 3-4 the high IS group. MLH1 staining was evaluated from four TMA punches per every tumor by two independent researchers. Tumors were determined MLH1 negative when there was no staining positivity in any cancer cell. Either normal pancreatic cells or inflammatory cells were used as positive controls.

**Statistical analysis**

Chi-square test was used to calculate differences between IS groups and clinicopathological variables. The Kaplan-Meier method was used to calculate DSS and OS, and the differences between the study groups were compared with the log-rank test. Due to a small number of stage 1A and stage 1B patients , these stages were combined as stage 1 for survival analysis.  Survival times were calculated from the date of surgery until the time of death or the end of follow-up (December, 31, 2016). The causes of death were obtained from hospital records and the National Cause of Death Registry. Univariate and multivariate Cox proportional hazards regression model was used to calculate hazard ratios for OS and DSS. Only variables with p<0.20 at univariate analysis were entered in the multivariate analysis. All statistical tests were two-sided. A *p*-value less than 0.05 was considered significant. The statistical analysis was performed with IBM SPSS statistics 23 for Windows (IBM Corporation, Armonk, NY, USA).

**Results**

**Clinicopathological characteristics, MLH1 staining and their association with immune cell score**

No association with studied clinicopathological variables and IS were observed (**Table 2**).The mean age of the study population was 66.9 years (SD 8.2). Median body mass index was 25.3 kg/m² (IQR 14.5-40.0). TNM stage distribution in this study population was as follows: Stage 1A+1B 11.2%, stage 2A 21.3%, stage 2B 67.6%. The IS distribution was as follows: IS0-1 33.3%, IS2 33.3%, IS3-4 33.3% (**Table 2**). IS

distribution by stage is shown in **Table 2** and **Fig. 2.** We found five (4.6%) MLH1 negative tumors. MSI status was not associated with IS or survival **(Table 2 and 4).**

**Table 2. Clinicopathological variables and their association with immune cell score (IS).**

| | IS 0-1 n (% of row total) | IS 2 n (% of row total) | IS 3-4 n (% of row total) | Total, n(%) | p |
|---|---|---|---|---|---|
| Total, n (%) | 36 (33.3%) | 36 (33.3%) | 36 (33.3%) | 108 (100%) | |
| **Age groups, n (%) years** | | | | | |
| <65 | 14 (32.6%) | 13 (30.2%) | 16 (37.2%) | 43 (39.8 %) | |
| 65 to 75 | 14 (29.8%) | 16 (34.0%) | 17 (36.2%) | 47 (43.5 %) | 0.565 |
| >75 | 8 (44.4%) | 7 (38.9%) | 3 (16.7%) | 18 (16.7 %) | |
| **Gender, n (%)** | | | | | |
| Male | 21 (36.8%) | 20 (35.1%) | 16 (28.1%) | 57 (52.8 %) | 0.458 |
| Female | 15 (29.4%) | 16 (31.4%) | 20 (39.2%) | 51 (47.2 %) | |
| **BMI groups, no (%)** | | | | | |
| <20(kg/m2) | 0 (0.0%) | 4 (50%) | 4 (50%) | 8 (7.4 %) | |
| 20-25(kg/m2) | 12 (30%) | 15 (37.5%) | 13 (32.5%) | 40 (37.0%) | 0.410 |
| 25-30(kg/m2) | 16 (38.1%) | 12 (28.6%) | 14 (33.3%) | 42 (40.0 %) | |
| >30(kg/m2 | 7 (46.7%) | 4 (26.7%) | 4 (26.7%) | 15 (14.3 %) | |
| **T-stage, n (%)** | | | | | |
| pT1 | 1 (25.0%) | 3 (75.0%) | 0 (0.0%) | 4 (3.7 %) | |
| pT2 | 6 (26.1%) | 5 (21.7%) | 12 (52.2%) | 23 (21.3 %) | 0.101 |
| pT3 | 29 (35.8%) | 28 (34.6%) | 24 (29.6%) | 81 (75.0%) | |
| **N-stage, n (%)** | | | | | |
| pN0 | 10 (28.6%) | 15 (42.9%) | 10 (28.6%) | 35 (32.4 %) | 0.301 |
| pN1 | 26 (35.6%) | 21 (28.8%) | 26 (35.6%) | 73 (67.6 %) | |
| **Stage, n (%)** | | | | | |
| IA | 1 (20%) | 3 (60%) | 1 (20%) | 5 (4.6 %) | |
| IB | 1 (14.3%) | 2 (28.6%) | 4 (57.1%) | 7 (6.5 %) | |
| IIA | 8 (34.8%) | 10 (43.5%) | 5 (21.7%) | 23 (21.3 %) | 0.430 |
| IIB | 26 (35.6%) | 21 (28.8%) | 26 (35.6%) | 73 (67.6 %) | |
| **Histological grade, n (%)** | | | | | |
| 1 | 11 (35.5%) | 11 (35.5%) | 9 (29.0%) | 31 (30.0 %) | |
| 2 | 21 (32.8%) | 19 (29.7%) | 24 (37.5%) | 64 (62.1%) | 0.895 |
| 3 | 2 (25.0%) | 4 (50.0%) | 2 (25.0%) | 8 (7.7 %) | |
| **Perineural invasion, n (%)** | | | | | |
| Positive | 20 (29.9%) | 22 (32.8%) | 25 (37.3%) | 67 (65.0 %) | 0.630 |
| Negative | 14 (38.9%) | 11 (30.6%) | 11 (30.6%) | 36 (35.0 %) | |
| **Resection margin** | | | | | |
| R0 | 15 (29.4%) | 19 (37.3%) | 17 (33.3%) | 51 (47.2 %) | 0.640 |
| R1 | 21 (36.8%) | 17 (29.8%) | 19 (33.3%) | 57 (52.8 %) | |
| **Microsatellite status** | | | | | |
| Unstable | 2 (40.0%) | 1 (20.0%) | 2 (40.0%) | 5 (4.6%) | 0.811 |
| Stable | 34 (33.0%) | 35 (34.0%) | 34 (33.0%) | 103 (95.4%) | |

**Figure 2. Distribution of immune cell score classes between different TNM stages.**
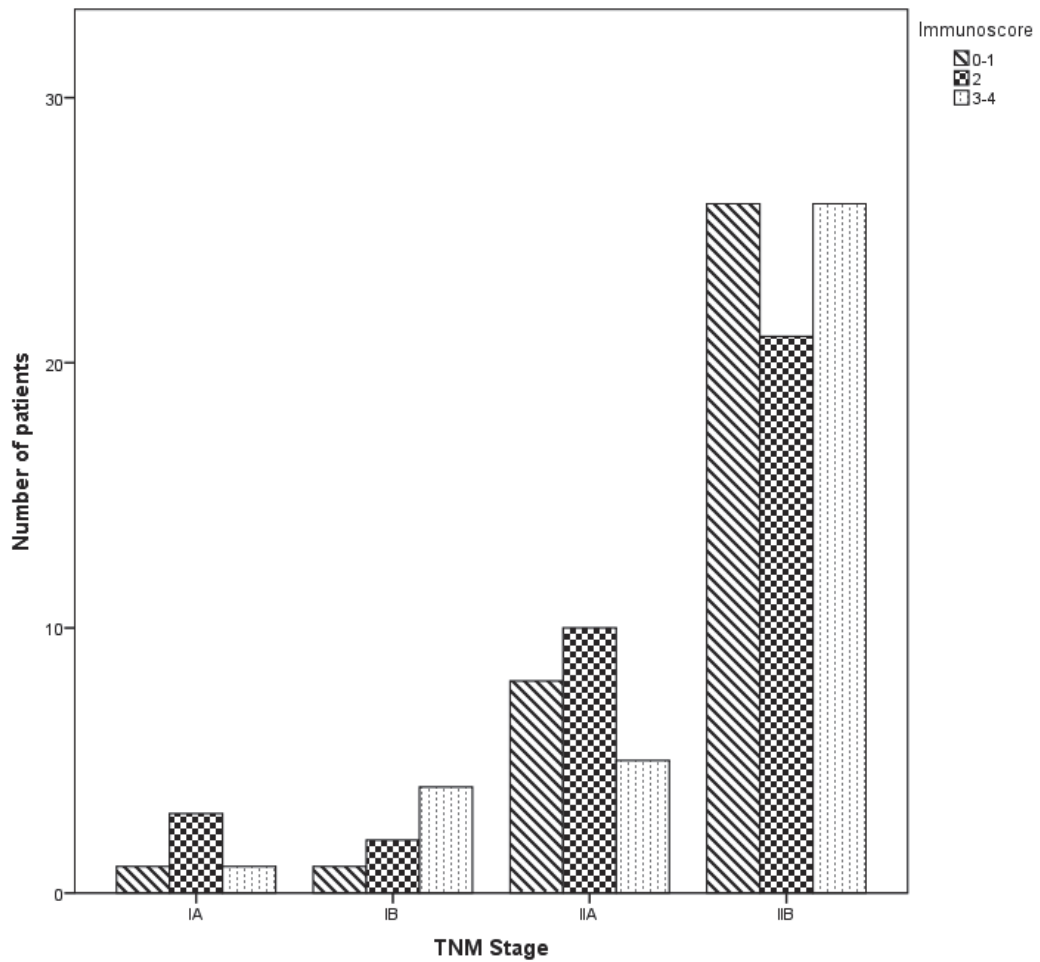


**Prognostic impact of immune cell score on survival**

Median follow–up time was 44 (IQR 15.8 to 57.3) months for those alive at the end of follow-up. Median OS of the 108 patients was 22 [95% CI: (17.9-26.1)] months. The 1-year, 3-year and 5-year DSS rates were 75.2 %, 32.4 % and 18.8 % and OS rates 70.2 %, 29.1 % and 16.9 %, respectively. Median OS was 30 months (95% CI 8.1-51.9) for stage 1A+B, 25 months (95% CI 7.9-42.1) for stage 2A and 21 months (95% CI 17.0-25.0) for stage 2B (p=0.249).

Median DSS for low, moderate and high IS groups were 20 (95% CI 16.2-23.8), 22 (95% CI 14.9-29.1) and 35 months (95% CI 25.4-44.6), respectively. The 5-year DSS rate for low, moderate and high IS groups

were 5.0 %, 15.2 % and 33.4 %, p=0.007 (**Fig. 3A**). Median OS for low (0-1), moderate (2) and high (3-4) IS was 19 (95% CI 11.8-26.2), 22 (95% CI 13.3-30.7) and 35 (95% CI 30.8-39.2) months, respectively. The 5-year OS rate for the low, moderate and high IS groups were 4.2 %, 13.4 % and 31.5 %, respectively, p=0.004 (**Fig. 3B**). High IS was significantly associated with improved survival within stage 2B (**Fig. 4**).

**Figure 3. Prognostic impact of immune cell score on DSS and OS.**
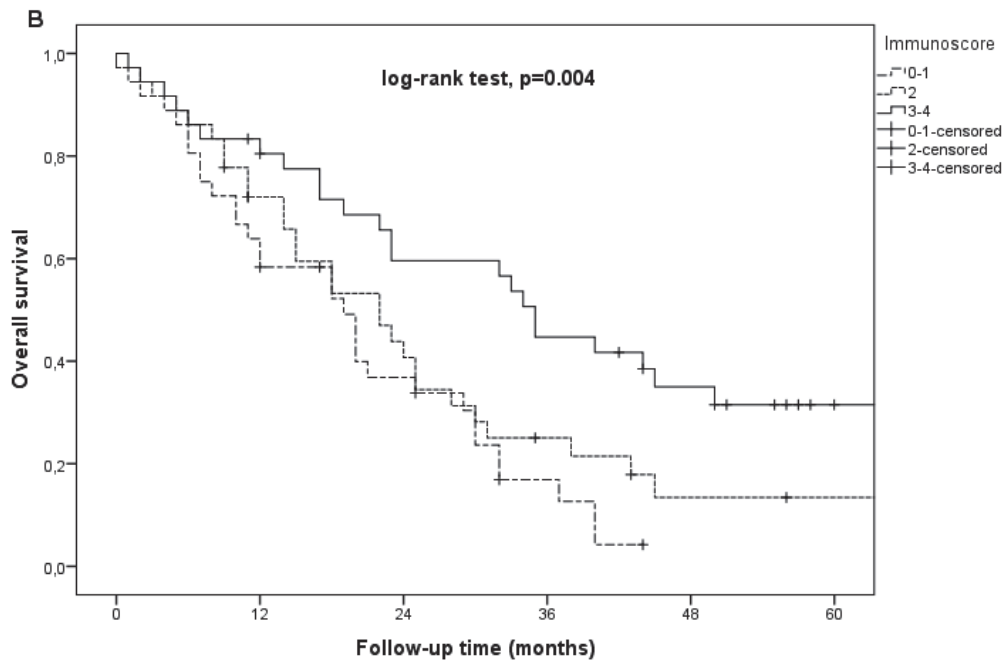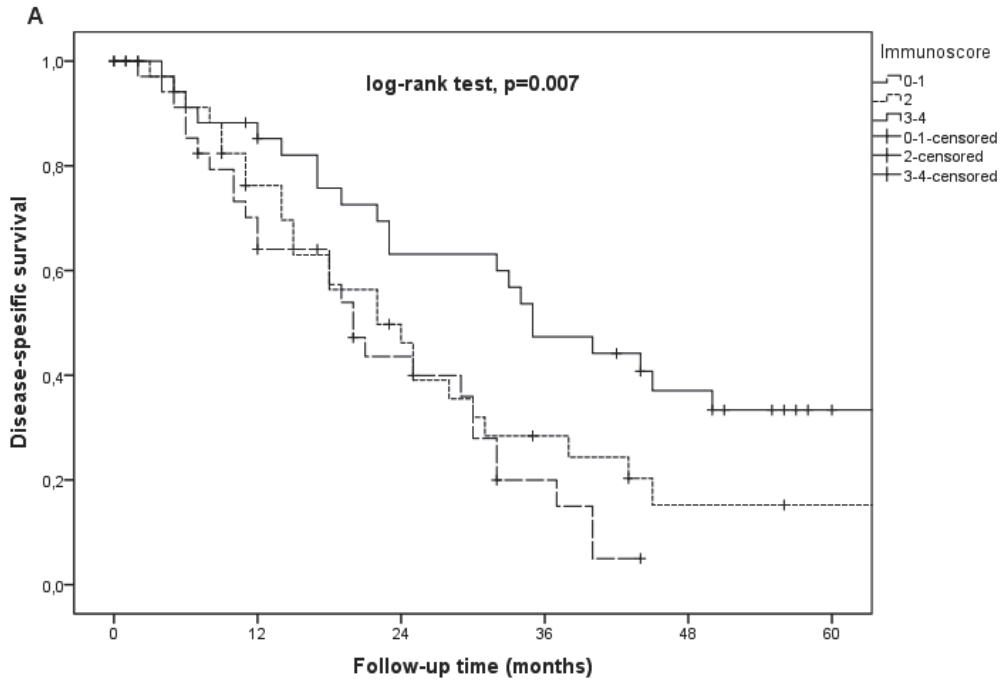
**Figure 4. Prognostic impact of immune cell score on DSS and OS within TNM stage 2B.**
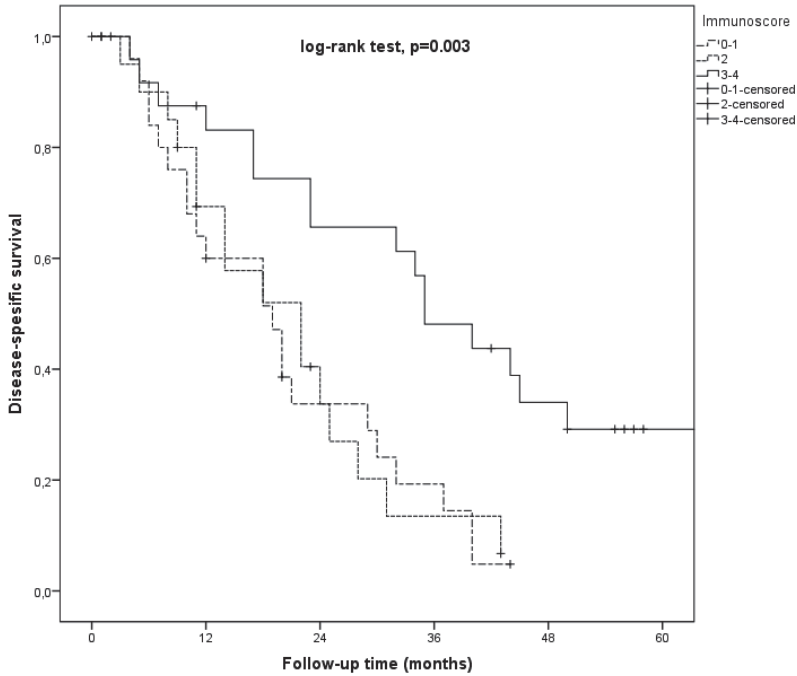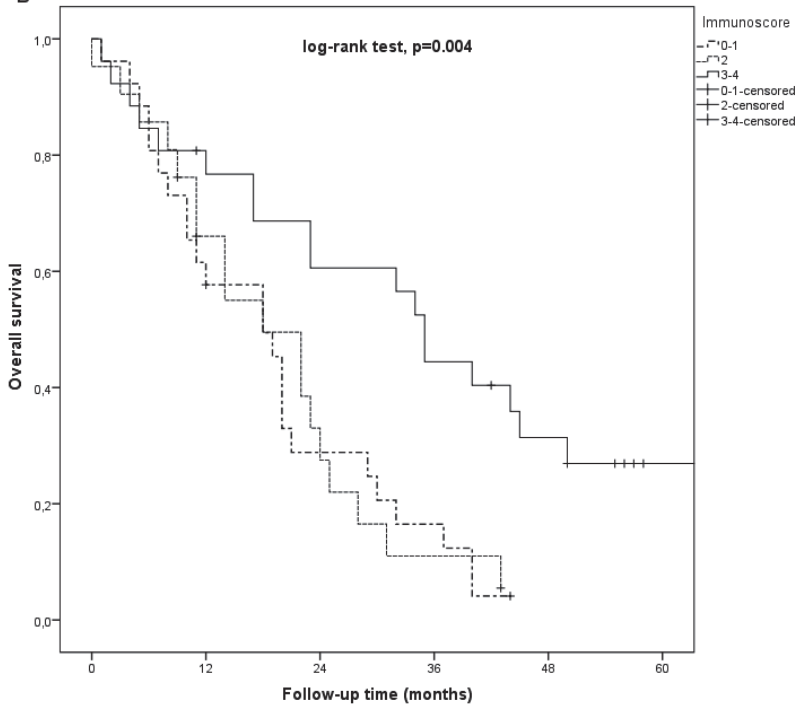
When assessing the impact of each IS component on 5-year DSS and OS we found a significant association between better DSS and OS, and high number of CD3+ cells in both tumor core (OS) and invasive margin. CD8+ cells in tumor core were significantly associated to better DSS and there was a trend for improved OS. Higher number of CD8+ cells in invasive margin showed a significant association with prolonged OS as well (**Table 3**).

**Table 3. Survival according to CD3+ and CD8+ lymphocytes in tumor center and invasion margin**

| | | Disease-spesific survival (n=108) | | | Overall survival (n=108) | |
|---|---|---|---|---|---|---|
| | Total (n) | 5-year survival | p | Total (n) | 5-year-survival | p |
| CD3+ tumor core | | | | | | |
| High | 47 | 28.7% | **0.012** | 47 | 26.9% | **0.009** |
| Low | 51 | 6.0% | | 51 | 5.2% | |
| CD3+ invasive margin | | | | | | |
| High | 43 | 28.6% | **0.023** | 43 | 26.1% | **0.020** |
| Low | 65 | 10.7% | | 65 | 9.5% | |
| CD8+ tumor core | | | | | | |
| High | 62 | 22.6% | **0.048** | 62 | 20.5% | 0.054 |
| Low | 46 | 14.3% | | 46 | 12.6% | |
| CD8+ invasive margin | | | | | | |
| High | 63 | 25.0% | **0.043** | | 23.% | **0.026** |
| Low | 45 | 7.7% | | | 6.6% | |

**Prognostic factors for survival**

A univariate Cox proportional hazards regression model was used to analyze the relationship between survival, clinicopathological variables and IS (**Table 4**). In multivariate analysis high IS, low histological grade of the tumor and negative perineural invasion were independent prognostic factors of improved 5-year DSS and OS (**Table 5**). In our series, TNM stage was not correlated with survival **(Fig. 5)**.

**Table 4. Univariate analysis with Cox proportional hazard model.**

| | | Disease-free survival | | | | Overall survival | | |
|---|---|---|---|---|---|---|---|---|
| | Total (n) | 5-year survival | HR(95%CI) | p | Total (n) | 5-year-survival | HR (95%CI) | p |
| All | 108 | 18.8% | | | 108 | 16,9% | | |
| **Age (years):** | | | | | | | | |
| <65 | 43 | 12.1% | 1 | 0.976 | 43 | 11.0% | 1 | 0.916 |
| 65 to 75 | 47 | 25.1% | 1.02(0.62-1.68) | | 47 | 22.3% | 1.05(0.65-1.68) | |
| >75 | 18 | 26.9% | 1.08(0.54-2.15) | | 18 | 23.9% | 1.15(0.61-2.17) | |
| **Gender:** | | | | | | | | |
| Female | 57 | 17.4% | 1 | 0.261 | 57 | 14.8% | 1 | 0.110 |
| Male | 51 | 20.7% | 1.30(0.82-2.07) | | 51 | 19.5% | 1.43(0.92-2.21) | |
| **BMI (kg/m2)** | | | | | | | | |
| <20 | 8 | 12.5% | 1 | 0.590 | 8 | 12.5% | 1 | 0.769 |
| 20-25 | 40 | 6.5% | 1.15(0.50-2.63) | | 40 | 5.8% | 1.24(0.55-2.83) | |
| 25-30 | 42 | 25.6% | 0.81(0.35-1.86) | | 42 | 22.2% | 0.96(0.42-2.18) | |
| >30 | 15 | 25.4% | 1.14(0.43-3.01) | | 15 | 25.4% | 1.12(0.43-2.94) | |
| **T-stage** | | | | | | | | |
| pT1 | 4 | 0.0% | 1 | 0.889 | 4 | 0.0% | 1 | 0.579 |
| pT2 | 23 | 15.2% | 0.74(0.17-3.20) | | 23 | 15.2% | 0.52(0.15-1.78) | |
| pT3 | 81 | 20.7% | 0.71(0.17-2.93) | | 81 | 18.2% | 0.57(0.18-1.82) | |
| **N-stage** | | | | | | | | |
| pN0 | 34 | 28.6% | 1 | 0.199 | 34 | 25.9% | 1 | 0.236 |
| pN1 | 74 | 15.0% | 1.41(0.84-2.38) | | 74 | 13.3% | 1.34(0.82-2.19) | |
| **Stage** | | | | | | | | |
| IA+IB | 9 | 28.1% | 1 | 0.168 | 4 | 25.6% | 1 | 0.209 |
| IIA | 23 | 33.8% | 0.84(0.32-2.25) | | 23 | 30.5% | 0.85(0.34-2.10) | |
| IIB | 74 | 13.3% | 1.47(0.63-3.42) | | 74 | 11.8% | 1.39(0.63-3.03) | |
| **Histological grade** | | | | | | | | |
| 1 | 31 | 29.8% | 1 | **0.016** | 31 | 28.8% | 1 | **0.008** |
| 2 | 64 | 12.8% | 1.89(1.11-3.20) | | 64 | 11.8% | 1.92(1.15-3.20) | |
| 3 | 8 | 14.3% | 3.00(1.20-7.50) | | 8 | 12.5% | 3.12(1.32-7.38) | |
| **Perineural invasion** | | | | | | | | |
| Negative | 36 | 20.8% | 1 | 0.168 | 36 | 19.8% | 1 | 0.079 |
| Positive | 67 | 15.8% | 1.41(0.87-2.30) | | 67 | 13.9% | 1.52(0.95-2.45) | |
| **Resection margin** | | | | | | | | |
| R0 | 51 | 20.5% | 1 | 0.948 | 51 | 18.4% | 1 | 0.899 |
| R1 | 57 | 16.8% | 1.02(0.64-1.61) | | 57 | 15.0% | 1.03(0.67-1.59) | |
| **Microsatellite status** | | | | | | | | |
| Stable | 103 | | 1 | 0.177 | 103 | | 1 | 0.298 |
| Unstable | 5 | | 1.86(0.75-4.63) | | 5 | | 1.61(0.65-4.00) | |
| **Immune cell score** | | | | | | | | |
| 3-4 | 36 | 33.4% | 1 | **0.008** | 36 | 31.5% | 1 | **0.005** |
| 2 | 36 | 15.2% | 1.86(1.03-3.34) | | 36 | 13.4% | 1.84(1.05-3.21) | |
| 0-1 | 36 | 5.0% | 2.50(1.38-4.52) | | 36 | 4.2% | 2.48(1.41-4.34) | |

Table 5. Multivariate analysis with Cox proportional hazard model.

| | Univariate analysis p | Disease-spesific survival HR (95% CI) | p | Overall survival HR(95% CI) | p |
|---|---|---|---|---|---|
| **Gender:** | | | | | |
| Female | 0.260 | 1 | | 1 | |
| Male | | 1.222(0.76-1.97) | 0.410 | 1.405(0.89-2.22) | 0.146 |
| **Age:** | | | | | |
| <65 years | 0.049 | 1 | | 1 | |
| 65 to 75 years | | 1.271(0.74-2.17) | 0.381 | 1.303(0.78-2.18) | 0.314 |
| >75 years | | 0.568(0.27-1.20) | 0.138 | 0.626(0.31-1.26) | 0.188 |
| **Immune cell score** | | | | | |
| IS 3-4 | **0.008** | 1 | | 1 | |
| IS 2 | | 2.989(1.56-5.72) | **<0.001** | 2.537(1.36-4.73) | **<0.001** |
| IS 0-1 | | 4.437(2.21-8.91) | **0.001** | 4.216(2.18-8.17) | **0.003** |
| **Histological grade** | | | | | |
| 1 | **0.016** | 1 | | 1 | |
| 2 | | 2.246(1.29-3.93) | **0.005** | 2.327(1.35-4.02) | **0.002** |
| 3 | | 4.314(1.58-11.75) | **0.004** | 3.140(1.17-8.40) | **0.023** |
| **Stage** | | | | | |
| IA+IB | 0.168 | 1 | | 1 | |
| IIA | | 1.076(0.37-3.10) | 0.892 | 1.102(0.38-2.69) | 0.977 |
| IIB | | 1.909(0.72-5.08) | 0.195 | 1.484(0.60-3.68) | 0.394 |
| **Perineural invasion** | | | | | |
| Negative | 0.168 | 1 | | 1 | |
| Positive | | 1.711(1.00-2.93) | 0.050 | 1.888(1.12-3.18) | **0.017** |

**Figure 5. Prognostic impact of TNM stage on DSS and OS.**
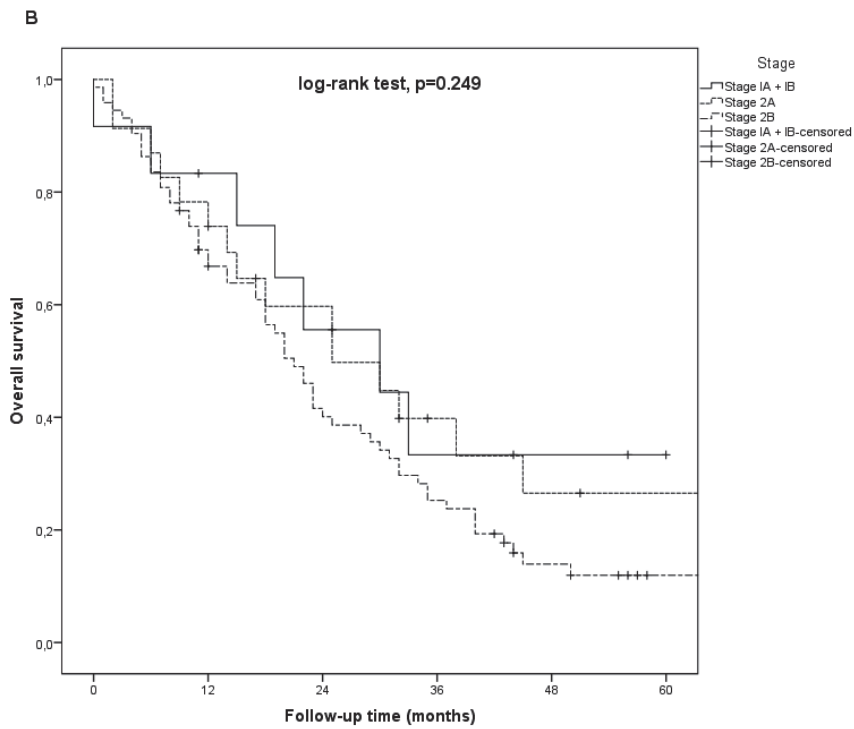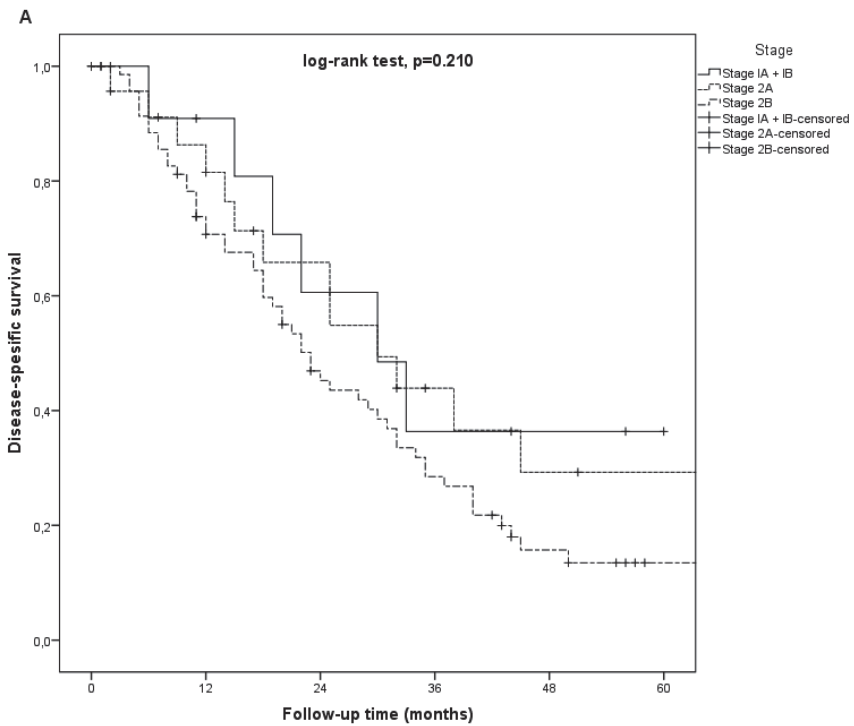
**Discussion**

The importance of adaptive immune response and immune environment has been recognized in multiple cancer types, such as colon, rectal, hepatocellular and also pancreatic cancer [18, 19, 27-30]. To our knowledge, this study utilizes for the first time the IS scoring system in PDAC and shows that it is an independent prognostic factor regardless of TNM stage. Moreover, the prognostic value of IS was more significant than the value of TNM stage in our series. In addition, IS and prognosis varied within a single tumor stage possibly reflecting some features of tumor cell biology and underlining the significance of cytotoxic inflammation.

Different combinations of tumoral and peritumoral immune cell infiltrations have been proposed to be used as an indicator of immune microenvironment [18, 19, 27-29]. Previously, high number of tumoral and juxtatumoral cytotoxic lymphocytes (CD8^{+}) have been reported to be a good prognostic factor in pancreatic cancer [18, 29, 31]. The association between high number of CD4^{+} lymphocytes in tumor core and improved survival has been documented as well [27, 29]. Ino et al. demonstrated that PDAC tumors are infiltrated by a combination of T cells. They showed that tumors with abundant CD4^{+}/CD8^{+} T cells but low number of regulatory T cells had better prognosis [29]. Recently, Carstens et al reported similar results of the impact of CD3^{+} and CD8^{+} cell infiltration for survival, but not that of regulatory T cells [32]. In our study, high CD3^{+} and CD8^{+} densities in tumor core or invasive margin alone correlated to the more favourable prognosis, but their combination into IS showed the best prognostic value.

To find out if microsatellite instability has any effect to IS we carried out immunohistochemical staining for MLH1, which is supposed to recognize most of the MSI tumors. Probably because of small number of MSI negative cases, we could not find any clinical significance for survival. We could not find association between MSI status and IS either. The literature shows controversial data of significance of the MSI in PDAC. The prevalence of MSI varies depending on the definition of MSI and the technique used, and the impact on survival is not clear [14-16].

To date TNM staging is the best prognostic indicator in PDAC [33]. However, while it reflects tumor burden at the time of diagnosis, it does not provide information about the biological features of PDAC. In addition, over 90% of curatively operated cases which are included in this kind of studies represent stage 2

A/B. Therefore significance of TNM as prognostic factor remains limited in PDAC as seen also in our study. TNM stage was not significantly associated with survival and survival inside a single stage was highly variable.

Grade of differentiation holds information about mitotic rate and nuclear morphology, however it is not well known how this relates to genetic background of PDAC and production of immunoactivating neoantigens. In our study the grade of differentiation was significantly associated with DSS and OS as reported earlier [34]. IS was not associated with grade of differentiation, suggesting that these factors reflect different features of biology in PDAC.

At present, there is no consensus of immune response measurement in pancreatic carcinoma as is the case in colon carcinoma [10]. As compared to colon carcinoma, the microenvironment of PDAC is characterized by a dense and abundant stromal tissue enabling possibility for regional variation of lymphocytic density [7]. It is well known that the density of inflammatory cells varies in different regions of the tumor [17, 35]. In this study we formed the IS by counting the number of $CD3^+$ and $CD8^+$ lymphocytes in the most representative areas of the tumor core and invasion margin thus taking into account regional variation in the density of cytotoxic lymphocytes within tumor and invasive front. According to immunoscore principle, we used only $CD3^+$ and $CD8^+$ cell populations acknowledging that several other cells affect immune response. However, these specific populations have the best documented impact on survival while simultaneously being simple enough for potential utilization in clinical setting.

Selection of cut-off values is critical for reproducibility in this kind of quantitative classification. Previously IS cut-off values have been selected with the minimum P-value method [36]. In this study, we determined cut-off values with optimal sensitivity and specificity based on ROC curves related to disease-specific 3-year mortality, which needs validation in other studies. Moreover, the use of tissue microarray blocks may cause sampling error. To reduce this possibility two tissue cores were taken from tumor center and two from invasion margin. For IS determination the one with higher count of cells was used. Our patients come from a single geographical area and were treated by similar guidelines. However, the number of patients is relatively small to detect survival differences. There were very few Stage IA and IB patients in our study and stage III and IV patients were excluded due to dismal prognosis.

In conclusion, IS seems to be a suitable way to measure immune response for clinical purposes. It could also provide important data to select those patients who will benefit of immune modulating therapy. The use of IS together with TNM staging and histological grade could provide better characterization of a single tumor.

## Compliance with ethical standards

The use of patient samples and the data inquiry were approved by the Oulu University Hospital Ethics Committee. The need to obtain a written or oral consent from the patients for using the samples in research was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA, Dnro 10832/06.01.03.01/2014).

## Conflict of interest

The authors state no potential conflicts of interest.

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# PUBLICATION
## II

Immune cell score in pancreatic cancer – comparison of hotspot and whole section techniques

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**ORIGINAL ARTICLE**

# Immune cell score in pancreatic cancer—comparison of hotspot and whole-section techniques

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## Abstract

An immune cell score (ICS) was introduced for predicting survival in pancreatic ductal adenocarcinoma (PDAC). Few studies have compared different methods of evaluating immune infiltrate. This study compared ICSs determined in whole sections or tissue microarray-like hotspots for predicting survival after PDAC surgery. We included in 79 consecutive patients from a single geographical area that underwent surgery for PDAC (R0/R1, stages I–III). We performed digital image analyses to evaluate CD3 and CD8 staining. ICSs were classified as low, moderate, or high, based on the numbers of immune cells in the tumour core and invasive margin. We compared ICS groups determined with the hotspot and whole-section techniques. Associations between ICS and survival were analysed with Cox regression models, adjusted for sex, age, tumour stage, differentiation grade, perineural invasion, and resection radicality. In hotspot ICS analysis, 5-year overall survival rates for low, moderate, and high groups were 12.1%, 26.3%, and 26.8%, respectively ($p = 0.193$). In whole-section analyses, overall survival rates were 5.3%, 26.4%, and 43.8%, respectively ($p = 0.030$). In the adjusted Cox model, whole-section ICS groups were inversely associated with the overall mortality hazard ratio (HR): low, moderate, and high ICS groups had HRs of 1.00, 0.42 (95% CI 0.20–0.88), and 0.27 (95% CI 0.11–0.67), respectively. The number of immune cells per square millimetre in the tumour core and the invasive margin were significantly higher and had a wider range in hotspots than in whole-tissue sections. Accordingly, ICS could predict survival in patients with PDAC after surgery. Whole tissue section ICSs exhibited better prognostic value than hotspot ICSs.

**Keywords** Immune cell score · Pancreatic cancer · Whole section · Microenvironment · CD3 · CD8

## Abbreviations

| | |
|---|---|
| ICS | Immune cell score |
| PDAC | Pancreatic adenocarcinoma |
| DSS | Disease-specific survival |
| OS | Overall survival |
| TMA | Tissue microarray |

## Introduction

Cancer progression is known to be strongly influenced by the host immune response, which is represented by immune cell infiltrates [1, 2]. Multiple scoring systems have been developed to evaluate the association between the host immune response and survival of patients with

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cancer [3–6]. Traditional tumour node metastasis (TNM) staging is the gold standard for evaluating prognosis in most solid tumours, and TNM staging was recently updated by the American Joint Committee on Cancer (AJCC) in the 8th edition of the staging manual [7]. However, TNM staging ignores the impact of the host immune response. Nevertheless, it has been shown that tumour-infiltrating lymphocytes were correlated with survival in rectal, oesophageal, and gastric cancers [8–10]. Indeed, in colorectal cancer, a computer-assisted evaluation of the quantity of $CD3^+$ and $CD8^+$ lymphocytes, known as the Immunoscore®, was found to be a reproducible, independent prognostic parameter [11]. Thus, it was suggested that integrating the TNM and Immunoscore® might provide more accurate staging.

Pancreatic ductal adenocarcinoma (PDAC) is the seventh deadliest cancer worldwide [12]. It is typically diagnosed in the late stages, which rules out curative surgery [13]. PDAC is characterised by a vast stromal reaction, inflammatory response, and neovascularization, which all contribute to resistance against anti-cancer drugs [14]. Even with surgery, survival rates remain low [15]. Inflammatory response and tumour microenvironment have been researched in recent years, and their significance in pancreatic cancer is becoming evident [16]. Various biomarkers have been investigated to enhance determinations of prognosis and to find the most suitable therapeutic approaches [17]. Although various combinations of intra-tumoural and peri-tumoural immune cells have been proposed as prognostic factors in pancreatic cancer [5], complete knowledge is lacking, due to the complex interplay between chronic inflammation and the immune response.

We recently introduced a T lymphocyte-based immune cell score (ICS), which we applied to pancreatic ductal adenocarcinoma (PDAC). This ICS showed good correlation with survival [18]. That study was based on tissue microarrays (TMAs), which are widely used in research to facilitate investigations of specific characteristics in a large number of tissue samples. Nevertheless, the TMA technique has a well-known risk of sampling error. Investigators have attempted to avoid this problem by acquiring multiple tissue cores from several hotspots. However, very few studies have compared TMA and whole-tissue techniques for evaluating immune cell infiltrates.

The primary aim of the present study was to test the prognostic significance of ICS in a separate consecutive series of patients with PDAC in Northern Finland. The secondary aim was to compare the TMA-like hotspot technique and whole-tissue section technique for efficacy in an ICS analysis. This study was designed and performed according to reporting recommendations for tumour marker prognostic studies [19].

## Materials and methods

### Patients

This retrospective cohort study included paraffin-embedded archival specimens of 95 consecutive patients with PDAC that received surgical treatment in 1993–2015 at Oulu University Hospital. Patients were excluded when they had advanced disease or distant metastases (stages 3–4, according to TNM, 7th edition, which was in use at the time of patient selection) or R2 resection margins. The final series consisted of 79 patients with stages 1-2B (TNM 7th edition) and R0/R1 tumours. During the present study, we re-staged the specimens, according to the TNM, 8th edition, which resulted in the distribution of stages described in Table 1. The histological diagnoses were confirmed by an expert gastrointestinal pathologist. All patients underwent either a pancreaticoduodenectomy, according to Whipple ($n = 72$), or a total pancreatectomy ($n = 7$). No patient received neoadjuvant therapy. The mean patient age at diagnosis was 64 years (SD 9.3). Patient characteristics are described in Table 1.

The clinical data were obtained from patient records and patient survival data. Cause of death was obtained from the Cause of Death Registry from Statistics Finland. Our use of the samples and patient data were approved by the Oulu University Hospital Ethics Committee and by the National Authority for Medicolegal Affairs (VALVIRA).

### Histopathological examination

Histopathological reviews of tumour specimens were performed by two experienced gastrointestinal pathologists (TK, JB). Tumour stage was determined according to the 7th edition of the UICC/AJCC TNM categories, which were current at the time of patient selection, and restaging was performed according to the 8th edition, later during the study.

### Immunohistochemistry

Tissue sections (3-μm thick) from a representative tumour tissue block were immunohistochemically stained with anti-CD3 antibodies (Novocastra, NCL-L-CD3-565, clone LN10, 1:50) and anti-CD8 antibodies (Novocastra, NCL-L-CD8, Clone 4B11, 1:200). Antigen retrieval was performed with tris-EDTA buffer at pH 9 in a microwave at 98 °C for 15 min. Samples were incubated with diluted antibodies at room temperature for 30 min. Bound antibodies were detected with the Dako Envision Kit (DAKO,

**Table 1** Clinical characteristics and immune cell score based on whole sections

| Variables | Total $N = 79$ | Immune cell score 0–1 $N = 39$ | Immune cell score 2 $n = 22$ | Immune cell score 3–4 $n = 18$ | $p$ value |
|---|---|---|---|---|---|
| **Sex** | | | | | |
| Male | $n = 41$ | $n = 18$ | $n = 10$ | $n = 13$ | 0.145 |
| Female | $n = 38$ | $n = 21$ | $n = 12$ | $n = 5$ | |
| **Age** | | | | | |
| < 65 | $n = 36$ | $n = 18$ | $n = 10$ | $n = 8$ | 1.000 |
| 65–75 | $n = 35$ | $n = 17$ | $n = 10$ | $n = 8$ | |
| > 75 | $n = 8$ | $n = 4$ | $n = 2$ | $n = 2$ | |
| **Tumour** | | | | | |
| T1a | $n = 0$ | $n = 0$ | $n = 0$ | $n = 0$ | 0.096 |
| T1b | $n = 5$ | $n = 2$ | $n = 1$ | $n = 2$ | |
| T1c | $n = 14$ | $n = 4$ | $n = 8$ | $n = 2$ | |
| T2 | $n = 43$ | $n = 21$ | $n = 10$ | $n = 12$ | |
| T3 | $n = 17$ | $n = 12$ | $n = 3$ | $n = 2$ | |
| **Node** | | | | | |
| N0 | $n = 36$ | $n = 20$ | $n = 11$ | $n = 5$ | 0.361 |
| N1 | $n = 29$ | $n = 12$ | $n = 9$ | $n = 8$ | |
| N2 | $n = 14$ | $n = 7$ | $n = 2$ | $n = 5$ | |
| **Stage** | | | | | |
| IA | $n = 8$ | $n = 2$ | $n = 4$ | $n = 2$ | |
| IB | $n = 17$ | $n = 10$ | $n = 5$ | $n = 2$ | |
| IIA | $n = 11$ | $n = 8$ | $n = 2$ | $n = 1$ | 0.356 |
| IIB | $n = 29$ | $n = 12$ | $n = 9$ | $n = 8$ | |
| III | $n = 14$ | $n = 7$ | $n = 2$ | $n = 5$ | |
| **Grade** | | | | | |
| I | $n = 15$ | $n = 6$ | $n = 7$ | $n = 2$ | 0.473 |
| II | $n = 29$ | $n = 13$ | $n = 9$ | $n = 7$ | |
| III | $n = 17$ | $n = 10$ | $n = 2$ | $n = 5$ | |
| **Perineural invasion** | | | | | |
| Negative | $n = 42$ | $n = 22$ | $n = 10$ | $n = 10$ | 0.694 |
| Positive | $n = 37$ | $n = 17$ | $n = 12$ | $n = 8$ | |
| **Resection** | | | | | |
| R0 | $n = 50$ | $n = 22$ | $n = 15$ | $n = 13$ | 0.441 |
| R1 | $n = 29$ | $n = 17$ | $n = 7$ | $n = 5$ | |
| **Disease-specific survival (%)** | | | | | |
| 1 year | 72.0 | 61.0 | 90.9 | 69.5 | **0.020** |
| 3 years | 27.5 | 10.9 | 39.6 | 55.6 | |
| 5 years | 19.5 | 5.4 | 26.4 | 55.6 | |
| **Overall survival (%)** | | | | | |
| 1 year | 70.0 | 59.1 | 90.9 | 65.7 | |
| 3 years | 25.3 | 10.5 | 39.6 | 43.8 | **0.030** |
| 5 years | 17.9 | 5.3 | 26.4 | 43.8 | |

*P*-values showing significant association (< 0.05) are marked in bold

Copenhagen, Denmark). DAB was used as a chromogen, and haematoxylin was used as a counterstain.

## Immune cell score determination

Immunohistochemical stains were assessed without knowledge of the clinical data. $CD3^+$ and $CD8^+$ cells were assessed with digital image analysis. Stained whole-tissue sections were scanned with an Aperio digital slide scanner AT2 Console (Leica Biosystems Imaging Inc., Nussloch, Germany), then analysed with the ImageJ program and a previously validated cell counting method [20]. Immune cell hotspot areas (0.28 mm$^2$) were defined digitally according to an ICS protocol, in both the tumour area and the invasive margin, which simulated the original TMA technique [18]. As previously described, the selected hotspot areas were both representative of the tumour and rich in immune cells. Tertiary lymphoid structures were not included in the hotspots. The invasive margin was defined as a 0.5-mm-wide region on each side of the tumour, which included cells at the border between the tumour cells and normal pancreatic tissue. $CD3^+$ and $CD8^+$ cells were counted separately in hotspot areas, in the whole tumour area, and in the invasive margin area (Fig. 1). Samples were divided into two groups of "high" and "low" cell densities, based on the calculated numbers of positively stained lymphocytes (cells/mm$^2$).

To determine the cut-off values for an ICS with optimal sensitivity and specificity, we used receiver operating characteristic (ROC) curves for each group, based on disease-specific 3-year mortality. The cut-off values for the hotspot counts were, as follows (cells/mm$^2$): 1116 for $CD3^+$ in the tumour core, 1314 for $CD3^+$ in the invasive margin, 1185 for $CD8^+$ in the tumour core, and 998 for $CD8^+$ in the invasive margin. The cut-off values for the whole-section counts were 396, 370, 120, and 157, respectively.

The high and low groups were used to construct ICS groups, which ranged from ICS 0 (low $CD3^+$ and $CD8^+$ densities in both regions) to ICS 4 (high $CD3^+$ and $CD8^+$ densities in both regions), as described previously [18]. According to the ICS protocol, three groups were formed (high, moderate, and low).

## Statistical analysis

We used the chi-square test to calculate differences in clinicopathological variables between groups. We used the Kaplan–Meier method and log-rank test to evaluate disease-specific survival (DSS) and overall survival (OS). Survival times were calculated from the date of surgery to the time of death or the end of follow-up (December 31, 2015). We performed univariate and multivariate Cox proportional hazards regression models to calculate hazard ratios for DSS and OS. These models were adjusted with the following a-priori determined confounders: age, sex, tumour stage (according to the 8th
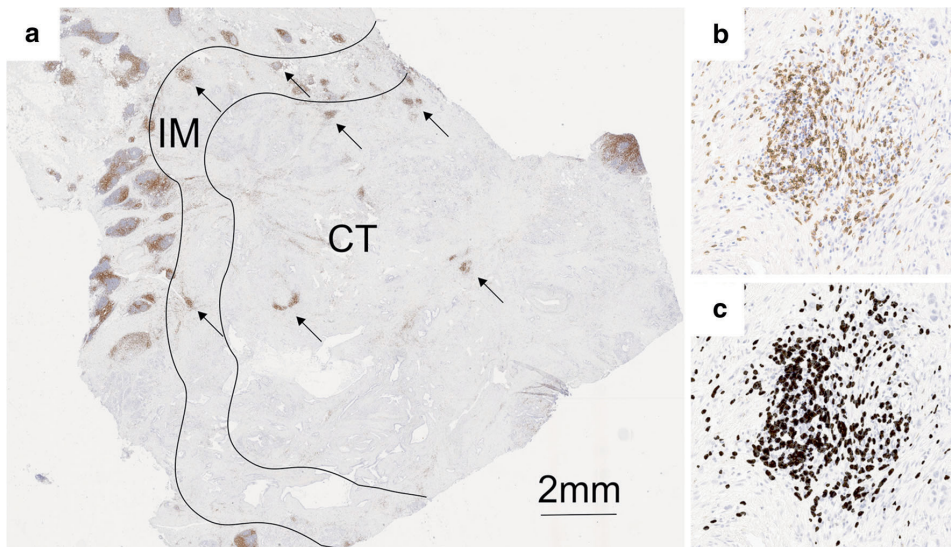
**Fig. 1** T cell infiltration in different regions of pancreatic ductal adenocarcinoma. **a** Image of a whole section of anti-CD3-stained tissue displaying the centre of the tumour (CT) and the invasive margin (IM). The highest densities of CD3$^+$ T cells were found in lymphoid aggregates, localised in the IM or the CT (arrows). **b** A hotspot in the CT that contains a lymphoid aggregate shows numerous positive T cells and fewer scattered tumour cells. The hotspot T cell density was frequently much higher than the average whole-section T cell density. **c** The corresponding image analysis shows the counted cells (dark grey shading)

edition of the UICC/AJCC TNM categories), differentiation grade, and resection radicality (R0/R1). *P* values less than 0.05 were considered significant. The statistical analysis was performed with IBM SPSS statistics 24 for Windows (IBM Corporation, Armonk, NY, USA).

## Results

### Clinicopathological characteristics and their association with the immune cell score

The clinicopathological parameters and their relationships to the ICSs are shown in Table 1. We found no significant association between the ICS and any of the clinicopathological parameters.

### Prognostic impact of ICS on survival

Regarding the whole study group, the median follow-up time was 14 months. The estimated median OS was 20 months (95% CI 16.3–24.1). The 1-year, 3-year, and 5-year DSS rates were 72.0%, 27.5%, and 19.5%, respectively; the respective OS rates were 70.0%, 25.3%, and 17.9%.

For the hotspot ICS analysis, the median DSS times for the low (ICS 0–1), moderate (ICS 2), and high (ICS 3–4) groups were 21 (95% CI 13.7–28.5), 15 (95% CI 5.4–23.7), and 37 months (95% CI 14.5–59.4), respectively. The 5-year DSS rates for the low, moderate, and high ICS groups were 12.1%, 29.4%, and 32.2%, respectively (*p* = 0.150; Fig. 2). The median OS times for the low, moderate, and high ICS groups were 21 months (95% CI 13.7–28.5), 12 months (95% CI 7.9–16.7), and 37 months (95% CI 14.6–59.3), respectively. The 5-year OS rates for the low, moderate, and high ICS groups were 12.1%, 26.3%, and 26.8% (*p* = 0.193).

The whole-section ICS analysis showed that the median DSS times for the low and moderate ICS groups were 14 (95% CI 7.1–21.9) and 28 (95% CI 13.3–42.2), respectively. A median DSS time was not reached in the high ICS group. The 5-year DSS rates for the low, moderate, and high ICS groups were 5.4%, 26.4%, and 55.6%, respectively (*p* = 0.020; Fig. 3). The median OS times for the low, moderate, and high ICS groups were 14 months (95% CI 10.4–18.5), 28 months (95% CI 13.3–42.2), and 26 months (95% CI 8.9–42.9), respectively. The 5-year OS rate for the low, moderate, and high ICS groups were 5.3%, 26.4%, and 43.8%, respectively (*p* = 0.030).

### Comparison of the measurement results (hotspots vs. whole sections)

We found that, when whole tissue sections were used to determine the number of immune cells, the ICS could predict patient survival (Fig. 3; Table 2). In contrast, the hotspot technique showed a similar trend but failed to show a significant association between the ICS and survival (Fig. 2; Table 2).

**Fig. 2** Disease-specific survival of patients with pancreatic ductal adenocarcinoma, stratified by low, moderate, and high immune cell scores, determined in hotspots
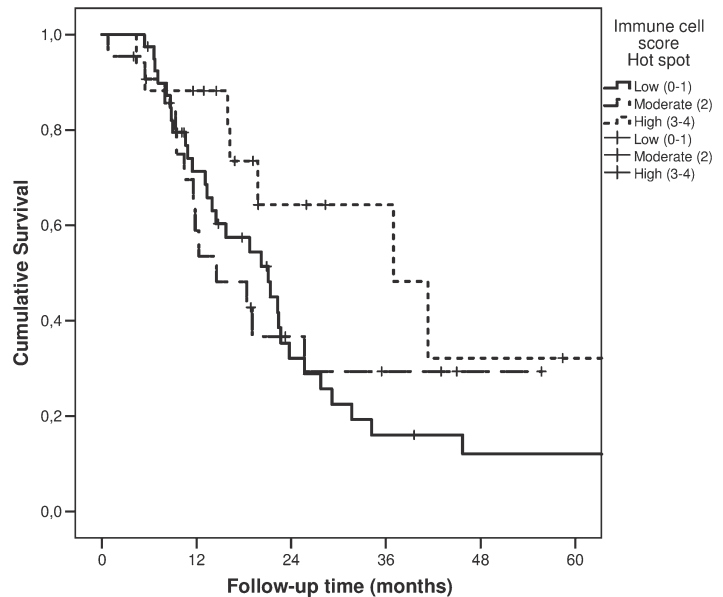


Table 2 shows the Cox regression results from the unadjusted (crude) and adjusted models. The DSS and OS were analysed with ICSs determined with either the whole-section or hotspot technique. Results were similar when the models used the 7th edition of the UICC/AJCC TNM categories.

Table 3 shows the unadjusted (crude) impact of each ICS component. The association between T cell densities and survival was stronger with the whole-section technique than with the hotspot technique. When we compared the numbers of immune cells between whole sections and hotspots, we found that the median number of immune cells per square millimetre was significantly higher in hotspots than in whole tissue sections. In particular, both the range and the interquartile range of immune cell counts determined with the hotspot technique were significantly larger than the ranges of the average immune cell densities observed in whole tissue sections (Fig. 4).

## Discussion

This study confirmed the significance of ICS as a prognostic factor in pancreatic cancer. We also found visible differences between the TMA-like hotspot and the whole-section techniques. Our analyses suggested that the whole-section technique was superior to the TMA-like hotspot technique. The whole-section technique indicated that the immune response had a significant impact on survival. With the hotspot technique, a similar trend was observed, but without statistical significance.

The association between the immune microenvironment of the tumour and the survival of patients with cancer had been well documented for PDAC [5, 21–23]. This association was also clearly shown in the present study. ICS was developed based on this association, combined with the notion of bringing together the prognostic value of several immune cell populations in different locations to obtain better prognostic value [1, 11, 18]. This study showed that the ICS determined with the whole-section technique predicted patient survival better than the ICS determined with the hotspot technique. However, the density of immune cells determined with the hotspot technique turned out to be significantly higher in this study compared to the previous study, despite the fact that we used hotspot sizes identical to the punch size used for TMA in the original ICS study [18]. This difference might be explained by the changes in immune cell densities from one level of section to the next. Moreover, in the TMA technique, small punches from hotspot areas are used to obtain a picture of the characteristics of the whole tumour. The inaccuracy of manual tissue punching makes it impossible to achieve consistency in selecting the most representative part of a tumour for TMA. In contrast, greater consistency can be achieved when the target area is defined from scans of the whole tissue section. Consequently, the TMA technique is more likely to show lower immune cell densities than the hotspot technique. In addition, tears in the TMA tissue sections can cause problems in estimating the sizes of analysed areas.

**Fig. 3** Disease-specific survival of patients with pancreatic ductal adenocarcinoma, stratified by low, moderate, and high immune cell scores, determined on whole tissue sections
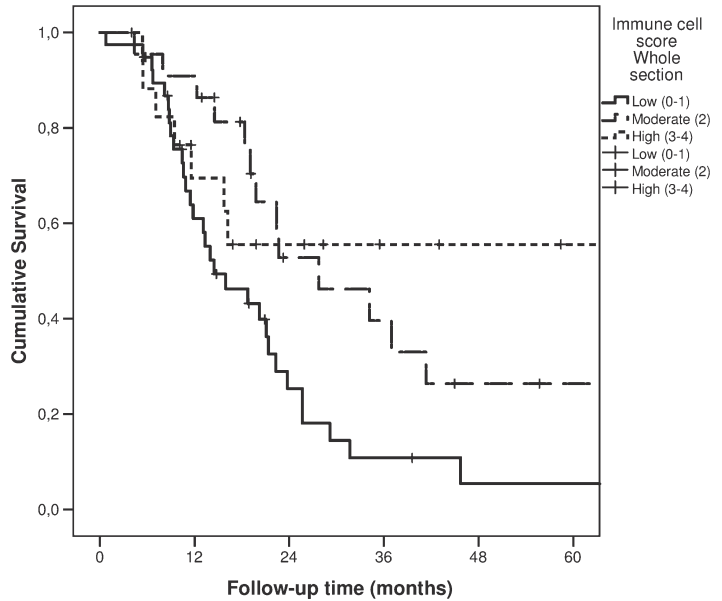


Tissue punches represent a small part of the whole tumour area. Alternatively, the development of image analysis has made it possible to count different cell populations rapidly and reliably in the whole tumour area [24]. In this study, the reliability of the whole tissue section technique over the hotspot technique was evidenced by the smaller variability between cases and the better performance in predicting survival. In future, the TMA technique will continue to play a role in research, when investigating specific characteristics in a large number of tissue samples. However, because the

**Table 2** Hazard ratios (HRs) with 95% confidence intervals (CI) of disease-specific and overall mortality of pancreatic ductal adenocarcinoma patients with low (0–1), moderate (2), and high (3–4) immune reaction based on immune cell score. Results based on whole sections and hotspots are presented separately

|  | Number of patients | Immune cell score 0–1 HR (95% CI) | Immune cell score 2 HR (95% CI) | Immune cell score 3–4 HR (95% CI) |
|---|---|---|---|---|
| Whole sections |  |  |  |  |
| Disease-specific mortality |  |  |  |  |
| All patients (crude) | 79 | 1.00 (Reference) | 0.48 (0.25–0.92) | 0.41 (0.18–0.94) |
| All patients (adjusted)* | 79 | 1.00 (Reference) | 0.45 (0.21–0.95) | 0.22 (0.08–0.60) |
| Overall mortality |  |  |  |  |
| All patients (crude) | 79 | 1.00 (Reference) | 0.47 (0.25–0.89) | 0.51 (0.24–1.07) |
| All patients (adjusted)* | 79 | 1.00 (Reference) | 0.42 (0.20–0.88) | 0.27 (0.11–0.67) |
| Hotspots |  |  |  |  |
| Disease-specific mortality |  |  |  |  |
| All patients (crude) | 79 | 1.00 (Reference) | 0.92 (0.48–1.77) | 0.45 (0.20–1.03) |
| All patients (adjusted)* | 79 | 1.00 (Reference) | 1.10 (0.52–2.34) | 0.64 (0.26–1.58) |
| Overall mortality |  |  |  |  |
| All patients (crude) | 79 | 1.00 (Reference) | 1.06 (0.57–1.98) | 0.52 (0.24–1.13) |
| All patients (adjusted)* | 79 | 1.00 (Reference) | 1.41 (0.69–2.89) | 0.70 (0.30–1.66) |

*Adjusted for sex, age, tumour stage (TNM 8th edition), grade of differentiation, perineural invasion, radicality of resection (R0/R1)

**Table 3** Non-adjusted hazard ratios (HRs) with 95% confidence intervals (CI) of disease-specific and overall mortality of pancreatic ductal adenocarcinoma patients based on number of T cells (low and high). Results of whole sections and hotspots are presented separately

| | Number of patients | Low HR (95% CI) | High HR (95% CI) |
|---|---|---|---|
| **Whole sections** | | | |
| Disease-specific mortality | | | |
| CD3 core (crude) | 79 | 1.00 (Reference) | 0.35 (0.13–0.98) |
| CD3 front (crude) | 79 | 1.00 (Reference) | 0.18 (0.39–1.20) |
| CD8 core (crude) | 79 | 1.00 (Reference) | 0.83 (0.47–1.46) |
| CD8 front (crude) | 79 | 1.00 (Reference) | 0.46 (0.26–0.81) |
| Overall mortality | | | |
| CD3 core (crude) | 79 | 1.00 (Reference) | 0.42 (0.17–1.06) |
| CD3 front (crude) | 79 | 1.00 (Reference) | 0.72 (0.42–1.23) |
| CD8 core (crude) | 79 | 1.00 (Reference) | 0.88 (0.51–1.52) |
| CD8 front (crude) | 79 | 1.00 (Reference) | 0.52 (0.30–0.90) |
| **Hotspots** | | | |
| Disease-specific mortality | | | |
| CD3 core (crude) | 79 | 1.00 (Reference) | 0.67 (0.36–1.23) |
| CD3 front (crude) | 79 | 1.00 (Reference) | 0.56 (0.29–1.08) |
| CD8 core (crude) | 79 | 1.00 (Reference) | 0.89 (0.47–1.70) |
| CD8 front (crude) | 79 | 1.00 (Reference) | 0.80 (0.46–1.42) |
| Overall mortality | | | |
| CD3 core (crude) | 79 | 1.00 (Reference) | 0.74 (0.41–1.32) |
| CD3 front (crude) | 79 | 1.00 (Reference) | 0.65 (0.35–1.19) |
| CD8 core (crude) | 70 | 1.00 (Reference) | 0.91 (0.49–1.71) |
| CD8 front (crude) | 70 | 1.00 (Reference) | 0.92 (0.53–1.59) |

whole-section analysis is rapidly becoming easier, it may become the gold standard for estimating immune status in future, as evidenced by, e.g., the Immunoscore® method applied in colorectal cancer [11].

The mechanism that underlies the effect of immune cell infiltration on patient survival has not been fully established. Jamieson et al. showed that a large amount of immune cell infiltrate was associated with factors related to less malignancy, smaller tumour size, no lymph node metastases or intravenous invasion, and a lower stage of pancreatic cancer [23]. It has been suggested that the co-expression of $CD4^+$ and $CD8^+$ cells could serve as a prognostic factor in pancreatic cancer [5, 18, 22]. Immune cells might function, in the early stages of carcinogenesis, by preventing the spread of tumour cells to distant sites. Accordingly, pancreatic cancers with low amounts of immune cell infiltrate, as observed in our material, might have more invasive and metastatic potential, due to a greater ability to evade the immune system. Carstens et al. showed that effective cytotoxic T cell function appeared to require a location close to the cancer cells [25]. It was previously suggested that strong desmoplasia must play an important role in pancreatic cancer immune evasion. However, a recent study challenged this conviction by showing that desmoplasia did not impair T cell infiltration into pancreatic cancer tissue [25].

Given the fact that the impact of novel immunomodulating therapies appears to depend on the state of the local host immune system, it is important to continue developing tools for measuring that state. Our results indicated that the ICS could provide important additional information to traditional methods of TNM-staging in PDAC. However, before the ICS can be used in routine clinical applications, future studies are needed to provide a methodological validation of optimal cut-off values, based on several study populations [24]. Moreover, the value of this method must be validated in a prospective, multi-institutional setting.

This study had some limitations. The number of patients was relatively small, which limited the size of ICS subgroups, and resulted in low statistical power. Moreover, the follow-up time was relatively short, a common problem in PDAC, due to high mortality. We could not validate the previously determined cut-off values in this population because the immune cell densities varied significantly, due to differences between the manual TMA and hotspot techniques and due to technical differences
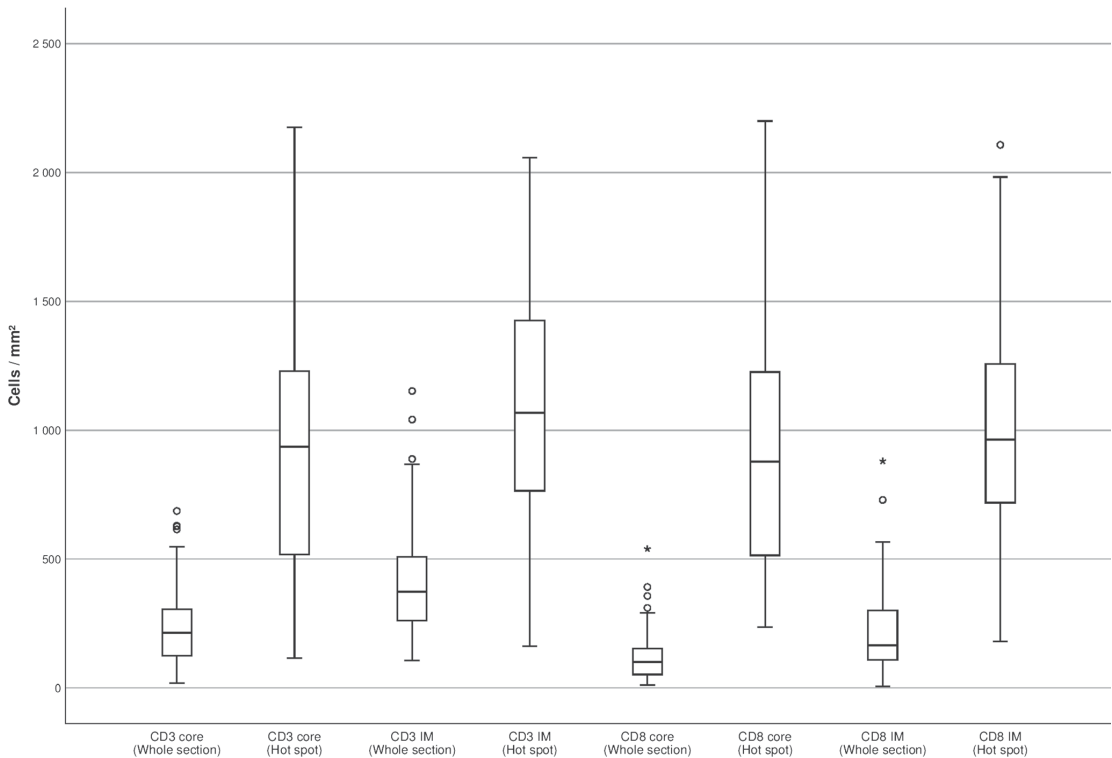
**Fig. 4** CD3$^+$ and CD8$^+$ T cell densities in the tumour core and invasive margin, presented separately for hotspots and whole tissue sections

between laboratories [18]. The main strength of the present study was the use of a consecutive patient series from a single geographical area of Northern Finland; thus, we could avoid a selection bias.

In conclusion, we confirmed that the ICS could predict post-surgical survival in patients with PDAC. In addition, our results suggested that the ICS determined in whole tissue sections provided higher prognostic value than the ICS determined in hotspots.

## Compliance with ethical standards

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# PUBLICATION
## III

**Prognostic impact of CD73 expression and its relationship to PD-L1 in patients with radically treated pancreatic cancer**

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**ORIGINAL ARTICLE**

# Prognostic impact of CD73 expression and its relationship to PD-L1 in patients with radically treated pancreatic cancer

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## Abstract

Immune suppressing molecule CD73 is overexpressed in various cancers and associated with poor survival. Little is so far known about the predictive value of CD73 in pancreatic ductal adenocarcinoma (PDAC). The purpose of this study was to investigate the prognostic significance of CD73 in PDAC. The study material consisted of 110 radically treated patients for PDAC. Tissue microarray blocks were constructed and stained immunohistochemically using CD73 antibody. Staining intensity and numbers of stained tumour cells, inflammatory cells, stroma, and blood vessels were assessed. High-level CD73 expression in tumour cells was positively associated with PD-L1 expression, perineural invasion, and histopathological grade. CD73 positivity in tumour-infiltrating lymphocytes was significantly associated with lymph node metastasis. Lymphocytic CD73 positivity was also associated with staining positivity in both stroma and vascular structures. In addition, CD73 positivity in vascular structures and stroma were associated with each other. There were no significant associations between CD73 positive tumour cells and CD73 positivity in any other cell types. PD-L1 expression was associated with CD73 staining positivity in stroma ($p = 0.007$) and also with histopathological grade ($p = 0.033$) and T class ($p = 0.016$) of the primary tumour. CD73 positivity in tumour cells was significantly associated with poor disease-specific ($p = 0.021$) and overall survival ($p = 0.016$). In multivariate analysis, CD73 positivity in tumour cells was an independent negative prognostic factor together with histopathological grade, TNM stage, and low immune cell score. In conclusion, high CD73 expression in tumour cells is associated with poor survival in PDAC independently of the number of tumour-infiltrating lymphocytes or TNM stage.

**Keywords** Pancreatic cancer · Microenvironment · CD73 · PD-L1 · Prognosis

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This article is part of the Topical Collection on *Quality in Pathology*

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**Abbreviations**

| | |
|---|---|
| PDAC | Pancreatic ductal adenocarcinoma |
| TIL | Tumour infiltrating lymphocyte |
| DSS | Disease-specific survival |
| OS | Overall survival |
| ICS | Immune cell score |

## Introduction

Tumour microenvironment has been shown to impact on cancer progression [1–4]. Malignant tumours like pancreatic ductal adenocarcinoma (PDAC) are known to develop several mechanisms in order to suppress the host immune system [5, 6]. In line with others, our previous results have shown an association between the number of tumour-infiltrating lymphocytes (TILs) and survival in various cancer types ([7–9]).

PDAC is the seventh deadliest cancer worldwide [10]. Approximately 80% of patients have an unresectable tumour at the time of diagnosis due to advanced disease [11] and survival rates remain low even after attempted curative surgery [12]. Despite the promising results of immune-modulating agents in many other cancers, the results in PDAC have been disappointing.

CD73, also called ecto-5′-nucleotidase (NT5E), is one of the major nucleotide metabolizing enzymes having an essential role in sustaining immune homeostasis. It dephosphorylates adenosine monophosphate (AMP) to adenosine, which in turn activates specific G protein-coupled receptors (GPCR) and suppresses immune reaction. The apical distribution of CD73 in normal pancreatic duct epithelial cells has been shown to shift to a more diffuse distribution in PDAC [13]. This probably promotes cancer cell aggressiveness, angiogenesis and metastasis [14–16]. CD73 also has non-enzymatic functions in cells, and there is evidence suggesting that CD73 also promotes the proliferation and migration of cancer cells independently of its enzymatic activity [17].

There are reports of CD73 overexpression in various cancers [18–24] showing an association with poor survival [16, 25]. However, opposite associations have also been reported [26]. One reason may be that CD73 is expressed in a variety of cell types such as certain lymphocyte populations, lymphatic and blood endothelial cells, subsets of epithelial cells, fibroblasts and cancer cells (Fig. 1). Often cell-specific expression has not been taken into account in these prognostic analyses. The prognostic value of CD73 in PDAC is still limited [27].

PD-L1 (also called B7-H1 or CD274) is an immunosuppressive molecule. According to earlier studies, high-level PD-L1 expression seems to be associated with poor differentiation, neural invasion and poor survival in PDAC [28].

Little is known so far about the prognostic impact of CD73 in PDAC, and there are no studies concerning co-expression and a possible interrelationship between CD73 and PD-L1.

Targeting CD73 could be a novel cancer treatment strategy; it is currently under intensive research and several clinical trials are ongoing (www.clinicaltrials.gov).

The aim of this study was to ascertain whether cell-specific CD73 acts as a prognostic factor in PDAC and to evaluate its relationship to other factors in microenvironment, such as PD-L1 and immune cell score (ICS). This study was designed and performed according to the reporting recommendations for prognostic studies on tumour markers [29].

## Material and methods

From 2000 to 2016, a total of 110 patients with stage I-IV PDAC were operated on in the Central Hospital of Central Finland, Jyväskylä, Finland. The surgical procedures included 20 classic pancreaticoduodenectomies, 82 pylorus-preserving pancreatoduodenectomies, 4 total pancreatectomies, and 4 distal pancreatic resections. Data were retrieved from our prospectively maintained and continuously updated population-based database established in 2000, including detailed information on patient and tumour characteristics, surgical treatment and complications, oncological treatments and follow-up. Patients with tumour-node-metastasis (TNM) stage III-IV ($n = 7$) were excluded from the survival analysis. Neoadjuvant chemotherapy was not given to any of the patients, whereas 95% of patients received adjuvant chemotherapy.

### Histopathological examination

All histopathological tumour specimens were reviewed by an experienced gastrointestinal histopathologist (JB). Tumour staging was done according to the 7th edition of the UICC/AJCC TNM categories [30]. The grading was performed according to the WHO classification of tumours 2010 [31].
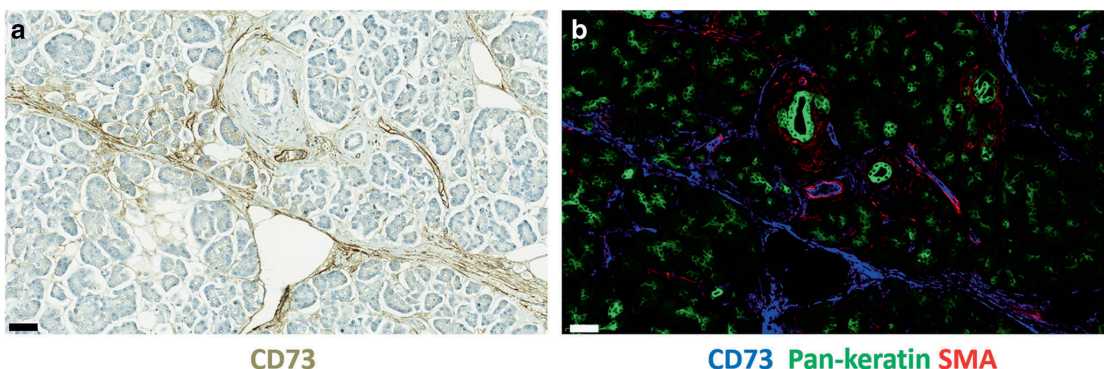


CD73   CD73 Pan-keratin SMA

Fig. 1 CD73 expression in normal pancreas. a Immunoperoxidase staining of normal pancreas for CD73 (brown). b Multicolour immunofluorescence staining of a consecutive section of normal pancreas for CD73 (blue), pan-cytokeratin (green) and alpha-smooth muscle actin (red). Bars, 50 μm

## Tumour sampling and immunohistochemistry

Tissue microarray blocks were constructed from formalin-fixed paraffin-embedded primary PDAC patient tumour samples. Two tissue cores 0.6 mm in diameter were taken both from the core of the tumour and the invasive margin from representative tumour blocks. Sections of 2 μm thickness were used for immunohistochemical (IHC) analyses. Staining for CD73 was conducted with rabbit monoclonal anti-CD73 antibody (D7F9A, Cell Signalling) and ultraView Universal DAB detection kit (Roche) for Ventana. Staining for CD3 and CD8 was conducted with anti-CD3 (LN 10, 1:200; Novocastra) and anti-CD8 (SP16, 1:400; Thermo Scientific) antibodies, using a Lab Vision Autostainer 480 (ImmunoVision Technologies Inc.). Staining for PD-L1 was conducted with anti-PD-L1 (E1L3N, 1:100; Cell Signalling Technology) antibody, using a BOND-III stainer (Leica Biosystems). PD-L1 staining was carried out using whole tissue sections.

Signal visualization for all IHC was done by diaminobenzidine and sections were counterstained with haematoxylin. In order to validate our TMA method for CD73, we analyzed the expression of CD73 in tumour cells (TC) using whole-section samples from 16 corresponding cases. The correspondence between whole sections and TMA punches was 100%; when assessing tumour cells separately in both groups, the same 4 samples out of 16 were considered CD73 positive in both groups.

ICS was determined using TMA technique as described earlier [7]. Briefly, ICS describes the immune response represented by CD3 and CD8 immune cells in the tumour centre and at the invasive margin.

For immunofluorescence stainings of FFPE samples, Alexa Fluor 488-conjugated anti-pan-cytokeratin (eBioscience #53-9003-80) and Cy3-conjugated anti-α-smooth muscle actin (Sigma #C6198 both mouse monoclonal antibodies) were used together with the rabbit anti-human CD73 antibody (D7F9A), which was visualized using Alexa647-conjugated goat anti-rabbit IgG (Invitrogen #A32733) as a second-stage reagent. The stained sections were imaged using Pannoramic Midi FL slide scanner (3DHISTECH) and analyzed using Case Viewer 1.4 program.

## Quantitative evaluation of CD73 and PD-L1 expression

IHC-stained TMA slides were assessed independently by four researchers (IK, JL, KT, JB) blinded to the clinical data. In case of disagreement, consensus was reached by three observers. In the case of CD73 staining, the intensity (1–3) and the proportion of staining on the cell surfaces (0–100%) were assessed. The final score (0–300) was calculated by multiplying the proportion of stained tumour cells by the staining intensity. Patients were divided into two groups using a cut-off value of 90, which was selected by using receiver operating characteristic (ROC) curves drawn in relation to disease-specific 3-year mortality (Fig. 2).

In addition, the percentage of CD73 positive TILs, tumour stroma and vascular structures were assessed. In the case of TILs, the sample was considered positive if > 3% of lymphocytes were positive for CD73. Tumour stroma positivity was considered weak, moderate or strong when < 5%, 5–16% or > 17% of the stromal area was stained respectively. Due to the strong staining intensity of vascular structures, 95% was set as a cut-off value for CD73 positivity of vascular structures.

PD-L1 expression was evaluated by estimating the proportion of PD-L1 positivity on the tumour cell surface. If over 1% of the tumour cells expressed PD-L1, the tumour was considered positive. There is no consensus on how PD-L1 expression should be reported in PDAC, and therefore none of the schemes like tumour proportion scale (TPS) or combined positive score (CPS) was used.

## Statistical analyses

The associations between clinical and histopathological variables, cell-specific CD73 positivity and PD-L1 positivity in tumour cells were analyzed using chi-square test. Univariate and multivariate Cox proportional hazards regression model was used to calculate hazard ratios for OS and DSS. Only variables with $p < 0.05$ in univariate analysis were entered into the multivariate analysis despite the a priori determined confounder, tumour stage ($p = 0.158$). All statistical tests were two-sided. A $p$ value less than 0.05 was considered significant. The statistical analyses were performed with IBM SPSS statistics 24 for Windows (IBM Corporation, Armonk, NY, USA).

# Results

## Patient demographics

A total of 110 of PDAC patients were included in this study. The distribution of samples regarding different variables is shown in Table 1.

## Associations between CD73 expression and other histopathological variables

We analyzed the associations between clinical and histopathological variables, cell-specific CD73 positivity and PD-L1 positivity in tumour cells (Tables 2 and 3).

High-level CD73 expression in tumour cells (CD73^{+}TC) was positively associated with PD-L1 expression, perineural invasion and histopathological grade (Table 2). CD73
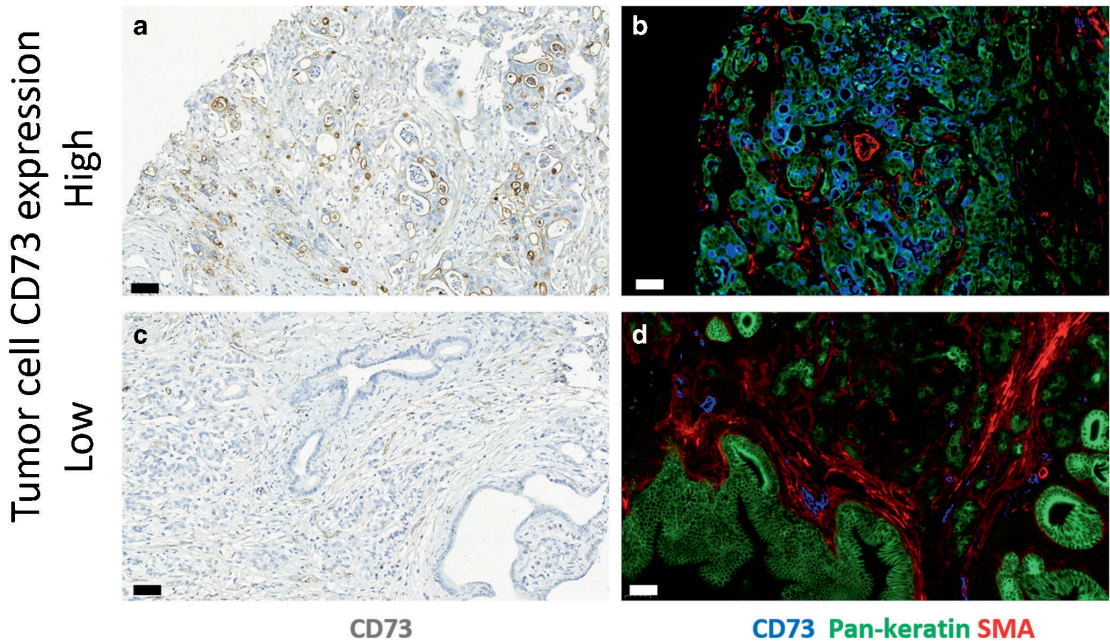
**Fig. 2** CD73 expression in adenocarcinoma of pancreas. Representative immunoperoxidase stainings (a and c) for CD73 (brown) and multicolour immunofluorescence stainings (b, d) for CD73 (blue), pan-cytokeratin (green) and alpha-smooth muscle actin (red). Bars, 50 μm

positivity in TILs was significantly associated with lymph node metastasis. Lymphocytic CD73 positivity was also associated with staining positivity in both stroma and vascular structures (Table 3). In addition, CD73 positivity in vascular structures and stroma was associated with each other. There were no associations between CD73$^+$TC and CD73 positivity in any other cell types in the tumour area.

PD-L1 positivity in tumour cells was also associated with CD73 staining positivity in stroma and also with high histopathological grade and low T class of the primary tumour.

## CD73 expression and survival

Regarding the whole study group, the median follow-up time was 44 (IQR 12.0 to 57.0) months for those alive at the end of follow-up. The estimated median overall survival (OS) for all patients was 23 [95% CI: (18.6–27.4)] months. CD73$^+$TC was significantly associated with poor disease-specific survival (DSS) ($p = 0.021$) and OS ($p = 0.016$) (Fig. 3). In the multivariate analysis, CD73$^+$TC was an independent negative prognostic factor together with histopathological grade, TNM stage and low ICS (Table 4).

High-level CD73 expression in tumour stroma, TILs or vascular structures did not show any significant correlation with survival (Table 3).

## Discussion

Our results show that a high CD73 expression in tumour cells is associated with poor survival in PDAC independently of ICS or TNM stage. We moreover found an association between high expression of CD73+ in tumour cells and perineural invasion. PD-L1 expression and high CD73 expression in both tumour cells and in stroma were significantly associated with each other. Moreover, we demonstrated that patients with high CD73 expression in TILs were more likely to have lymph node metastasis.

Earlier studies have reported similar results concerning the impact of CD73 on PDAC survival [28]. In a mouse experiment published by Stagg et al., CD73 deficiency led to increased number of CD8+ T cells in tumours. This was thought to be one factor behind the protective effect of CD73 deficiency [32]. According to another mouse model, high CD73 expression in T-lymphocytes was associated with an "exhausted" phenotype of T cells [33]. According to the present study, it is possible that, in PDAC, CD73 suppresses immune response by impacting on TILs activity rather than their number.

To the best of our knowledge, these results show for the first time the association between high CD73 expression in tumour cells and perineural invasion indicating that CD73 overexpression may be implicated in this process.

**Table 1** Clinicopathological characteristics

n = 110

|  | n/median | %/min-max |
|---|---|---|
| Age, years | 67.0 | 45–82 |
| Gender | | |
| Male | 58 | 52.7 |
| Female | 52 | 47.3 |
| T-stage | | |
| pT1 | 3 | 2.7 |
| pT2 | 22 | 20.0 |
| pT3 | 81 | 73.6 |
| pT4 | 4 | 3.6 |
| N-stage | | |
| pN0 | 32 | 29.1 |
| pN1 | 78 | 70.9 |
| Stage | | |
| IA | 3 | 2.7 |
| IB | 7 | 6.4 |
| IIA | 20 | 18.2 |
| IIB | 73 | 66.4 |
| III | 3 | 2.7 |
| IV | 4 | 3.6 |
| Histological grade | | |
| 1 | 30 | 27.3 |
| 2 | 65 | 59.1 |
| 3 | 7 | 6.4 |
| Perineural invasion | | |
| Positive | 36 | 34.6 |
| Negative | 68 | 65.4 |
| PD-L1 in tumour cells[a] | | |
| Positive | 5 | 4.5 |
| Negative | 105 | 95.5 |
| CD73+ tumour cell score[b] | | |
| High | 37 | 33.6 |
| Low | 73 | 66.4 |
| CD73+ TILs[c] | | |
| Positive | 55 | 53.9 |
| Negative | 47 | 46.1 |
| CD73+ stroma[d] | | |
| Strong | 38 | 34.5 |
| Moderate | 37 | 33.6 |
| Weak | 35 | 31.8 |
| CD73+ vessels[e] | | |
| High | 68 | 61.8 |
| Low | 42 | 38.2 |

[a] Tumour samples were considered PD-L1 positive when > 1% of the tumour cells were positive for PD-L1

[b] The score (0–300) was formed by multiplying the proportion of stained tumour cells by the staining intensity (0–3). 90 was set as a cut off value

[c] Tumour samples were considered positive when >3% of lymphocytes were positive for CD73

[d] Tumour stroma positivity was considered weak, moderate or strong when < 5%, 5–16% or > 17% of the stromal area was stained, respectively

[e] Due to the strong staining intensity of vascular structures, 95% was set as a cut off value for CD73 positivity of vascular structures

According to the literature, perineural invasion can be found in some form in almost all surgically removed PDACs when searched with thin slice thickness and also taking account of perineural invasion with low severity.

**Table 2** Clinicopathological variables and their association with CD73 expression in tumour cells (CD73 + TC)

|  | CD73 + TC high n = 37 | | CD73 + TC low n = 73 | | p value |
|---|---|---|---|---|---|
|  | n | % | n | % |  |
| T-class | | | | | 0.985 |
| 1 | 1 | 2.8 | 2 | 2.7 | |
| 2 | 7 | 19.4 | 15 | 20.5 | |
| 3 | 27 | 75.0 | 5 | 72.6 | |
| 4 | 1 | 2.8 | 3 | 4.1 | |
| N-class | | | | | 0.916 |
| 0 | 11 | 29.7 | 21 | 28.8 | |
| 1 | 26 | 70.3 | 52 | 71.2 | |
| Grade | | | | | *0.013* |
| 1 | 5 | 14.7 | 25 | 36.8 | |
| 2 | 24 | 70.6 | 41 | 60.3 | |
| 3 | 5 | 14.7 | 2 | 2.9 | |
| Perineural invasion | | | | | *0.041* |
| Negative | 7 | 20.6 | 29 | 40.8 | |
| Positive | 27 | 79.4 | 42 | 59.2 | |
| CD73+ vessels | | | | | 0.377 |
| Negative | 12 | 32.4 | 30 | 41.1 | |
| Positive | 25 | 67.6 | 43 | 58.9 | |
| CD73+ TILs | | | | | 0.930 |
| Negative | 15 | 45.5 | 32 | 46.4 | |
| Positive | 18 | 54.5 | 37 | 53.6 | |
| CD73+ Stroma | | | | | 0.328 |
| Weak | 9 | 24.3 | 26 | 35.6 | |
| Moderate | 12 | 32.4 | 25 | 34.2 | |
| Strong | 16 | 43.2 | 22 | 30.1 | |
| PD-L1 TC | | | | | *0.025* |
| 0 | 33 | 89.2 | 72 | 98.6 | |
| 1 | 4 | 10.8 | 1 | 1.4 | |
| Immune cell score | | | | | 0.947 |
| Low | 11 | 29.7 | 23 | 32.4 | |
| Moderate | 13 | 35.1 | 23 | 32.4 | |
| High | 13 | 35.1 | 25 | 35.2 | |

However, according to a meta-analysis of 3538 patients, the incidence of perineural invasion was 71.7%, which is in line with that found in our study population [34]. This discrepancy between the incidences found in routine histopathological analysis and in a meticulous search with thin slice thickness is thought to reflect the variable severity of perineural invasion. In other words, perineural invasion with low severity is sometimes not found in histopathological analysis when using routine slice thickness. The same meta-analysis, however, shows that perineural invasion found in routine histopathological analysis seems to be an independent prognostic factor for poor survival.

**Table 3**　Interrelationship between clinicopathological variables and their association to 5-year DSS

| Variables | T class | N class | Perineural invasion | Vessels CD73+ | LC CD73+[a] | Stroma CD73+ | TC CD73+[b] | PD-L1 TC + | ICS[c] | 5-year DSS[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| Grade | NS | NS | NS | 0.073↑ | NS | 0.060↑ | 0.013↑ | 0.010↑ | NS | 0.008↓ |
| T class | | 0.057↑ | NS | NS | NS | NS | NS | 0.016↓ | 0.032↓ | NS |
| N class | | | NS | NS | 0.001↑ | NS | NS | NS | NS | NS |
| Perineural invasion | | | | NS | NS | NS | 0.041↑ | NS | NS | NS |
| Vessels CD73+ | | | | | 0.004↑ | >0.001↑ | NS | 0.072↑ | 0.056↑ | NS |
| LC CD73+[a] | | | | | | 0.023↑ | NS | 0.059↑ | 0.038↑ | NS |
| Stroma CD73+ | | | | | | | NS | 0.007↑ | 0.030↑ | NS |
| TC CD73+[b] | | | | | | | | 0.025↑ | NS | 0.021↓ |
| PD-L1 TC + | | | | | | | | | NS | NS |
| ICS[c] | | | | | | | | | | 0.014↑ |

Nonsignificant (NS), $p > 0.1$; bold value, $p < 0.05$. Chi-square test. Arrows show the direction of association

[a] CD73+ Lymphocytes (low/high)

[b] CD73+ Tumour cells (low/high)

[c] Immune cell score

[d] Log rank test used

In our study cohort, high CD73 expression in both tumour cells and in stroma was significantly associated with PD-L1 expression in tumour cells. Similar findings have been reported in gastrointestinal neuroendocrine neoplasms [35]. Deng et al. demonstrated a close connection between these two immunosuppressive molecules in their recent mouse experiment concerning head and neck cancer [33]. They showed that blockade of CD73 reversed the exhausted T cell phenotype through downregulation of PD-1 and CTLA-4 on T cells. Mice studies have also proven that blocking adenosine receptor A2 (A2AR) enhances the efficacy of anti-PD-1 antibodies through enhanced antitumour T cell responses [36, 37].

Although the evidence of the pro-tumoural effect of high CD73 expression is increasing, the impact of CD73 expression in TILs is far from clear. Immunosuppressive regulatory T cells ($T_{reg}$) as well as T helper 17 cells and myeloid-derived suppressor cells are known to express CD73 [38–40].We showed that PDAC patients with CD73 + TILs were more likely than the controls to develop lymph node metastases. We think this association may reflect the impact of immunosuppressive cells mentioned above. However, double staining of immune cells is needed in the future to confirm this hypothesis. Correspondingly, Ma et al. [41] showed that the increased expression of A2AR correlated with positive lymph
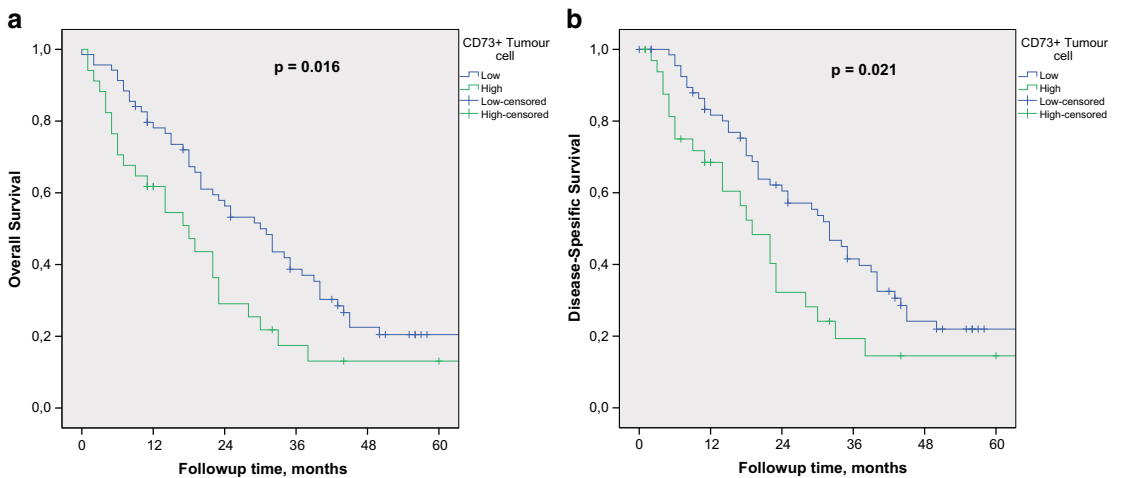


**Fig. 3** Prognostic impact of CD73+TC on DSS (a) and OS (b)

**Table 4** Uni- and multivariate analysis with Cox proportional hazard model

|  | Univariate analysis (OS)[a] HR (95% CI) | p | Univariate analysis (DSS)[b] HR (95% CI) | p | Multivariate analysis (OS) HR (95%CI) | p | Multivariate analysis (DSS) HR (95% CI) | p |
|---|---|---|---|---|---|---|---|---|
| CD73 (TC) |  |  |  |  |  |  |  |  |
| Negative | 1 | 0.019 | 1 | 0.025 | 1 | 0.035 | 1 | 0.043 |
| Positive | 1.78 (1.10–2.88) |  | 1.79 (1.08–2.97) |  | 1.81 (1.04–3.15) |  | 1.83 (1.02–3.29) |  |
| Tumour grade |  |  |  |  |  |  |  |  |
| 1 | 1 | 0.006 | 1 | 0.012 | 1 | 0.006 | 1 | 0.011 |
| 2 | 1.96 (1.15–3.34) |  | 1.96 (1.13–3.39) |  | 1.96 (1.13–3.40) |  | 2.00 (1.13–3.54) |  |
| 3 | 3.90 (1.55–9.85) |  | 3.70 (1.37–10.04) |  | 5.25 (1.69–16.30) |  | 5.10 (1.48–17.61) |  |
| TNM Stage |  |  |  |  |  |  |  |  |
| IA | 1.13 (0.27–4.64) | 0.234 | 1.29 (0.31–5.32) | 0.181 | 1.74 (0.40–7.66) | 0.041 | 1.83 (0.41–8.14) | 0.027 |
| IB | 0.54 (0.19–1.48) |  | 0.57 (0.21–1.58) |  | 0.33 (0.10–1.10) |  | 0.36 (0.11–1.22) |  |
| IIA | 0.56 (0.29–1.07) |  | 0.49 (0.24–1.00) |  | 0.48 (0.25–.094) |  | 0.40 (0.19–0.84) |  |
| IIB | 1 |  | 1 |  | 1 |  | 1 |  |
| ICS[a] |  |  |  |  |  |  |  |  |
| High | 1 | 0.019 | 1 | 0.018 | 1 | 0.004 | 1 | 0.003 |
| Moderate | 1.82 (1.03–3.19) |  | 1.82 (1.01–3.30) |  | 2.19 (1.17–4.13) |  | 2.47 (1.27–4.82) |  |
| Low | 2.25 (1.26–4.05) |  | 2.38 (1.29–4.40) |  | 2.82 (1.51–5.23) |  | 3.07 (1.59–5.95) |  |

[a] Immune cell score

node status in head and neck squamous cell carcinoma. This refers to the significance of the immunosuppressive adenosine pathway in cancer progression.

Our study has some limitations. Sampling error is a well-known risk related to the use of TMA. To minimize this risk, we analyzed whole sections of 16 cases to validate our method, and the correspondence between TMA and whole sections was excellent. The use of consecutive patient series from a single geographical area to avoid a selection bias strengthens our study. In addition, double assessing of IHC staining by two independent researchers increases the reliability of the results.

The development of the combined treatments of anti-CD73 with other immune-modulating agents such as anti-PD1 will potentially bring new hope for patients with PDAC. In the future, personalized cancer therapy will lead to an increasing need for applicable biomarkers. There also remains a need for basic research on our fine-tuned immune system.

In conclusion, our study shows that high expression of CD73 is an independent prognostic factor in PDAC also associated with perineural invasion. We furthermore demonstrate an association between CD73 and PD-L1 expression in pancreatic tumour cells. In addition, our study shows for the first time that patients with high CD73 expression in TILs are more likely to have lymph node metastasis.

**Contributions** All authors met the criteria listed in the ICMJE recommendations for the qualification of authorship.

**Authors' contributions** Drs. Tahkola and Böhm had full access to all the data and take responsibility for the integrity of the data and the accuracy of the analysis.

Concept and design: Tahkola, Mecklin, Jalkanen and Böhm.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Tahkola, Salmi and Böhm.
Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: Tahkola and Böhm.
Administrative, technical, or material support: Ahtiainen, Kellokumpu, Mecklin, Laukkarinen, Laakkonen, Kenessey, Jalkanen, Salmi and Böhm.
Supervision: Mecklin, Laukkarinen, Kellokumpu and Böhm.

## Compliance with ethical standards

The use of patient samples and the data inquiry were approved by the Oulu University Hospital Ethics Committee. The need to obtain written or oral consent from patients to use their samples in research was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA, Dnro 10832/06.01.03.01/2014).

**Conflict of interest** The authors declare that they have no conflict of interest.

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# PUBLICATION
## IV

**Stromal hyaluronan accumulation is associated with low immune response and poor prognosis in pancreatic cancer**

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# scientific reports

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# Stromal hyaluronan accumulation is associated with low immune response and poor prognosis in pancreatic cancer

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Hyaluronan (HA) accumulation has been associated with poor survival in various cancers, but the mechanisms for this phenomenon are still unclear. The aim of this study was to investigate the prognostic significance of stromal HA accumulation and its association with host immune response in pancreatic ductal adenocarcinoma (PDAC). The study material consisted of 101 radically treated patients for PDAC from a single geographical area. HA staining was evaluated using a HA-specific probe, and the patterns of CD3, CD8, CD73 and PD-L1 expression were evaluated using immunohistochemistry. HA staining intensity of tumour stromal areas was assessed digitally using QuPath. CD3- and CD8-based immune cell score (ICS) was determined. High-level stromal HA expression was significantly associated with poor disease-specific survival ($p = 0.037$) and overall survival ($p = 0.013$) In multivariate analysis, high-level stromal HA expression was an independent negative prognostic factor together with histopathological grade, TNM stage, CD73 positivity in tumour cells and low ICS. Moreover, high-level stromal HA expression was associated with low ICS ($p = 0.017$). In conclusion, stromal HA accumulation is associated with poor survival and low immune response in PDAC.

**Abbreviations**
HA      Hyaluronan
ICS     Immune cell score
PDAC    Pancreatic ductal adenocarcinoma
DSS     Disease-specific survival
OS      Overall survival
TMA     Tissue microarray

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid malignancies with 5-year survival rates of 2–9%[1,2]. This is partly related to advanced disease stage at the time of diagnosis ruling out curative surgery. Tumour cells are in constant interaction with non-neoplastic cells, and the tumour microenvironment influences cancer progression. PDAC has been shown to develop mechanisms that suppress the host immune response against the tumour[3]. The first signs of this immune suppression are seen already in the premalignant lesions[4]. PDAC is characterized by an abundant desmoplastic stroma, which has been suggested to facilitate the escape from the immune surveillance[5,6].

Hyaluronan (HA) is one of the main components of the extracellular matrix. In normal physiological conditions, it is strongly expressed during wound healing and at sites of inflammation, including cancer[7]. It also

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influences immune responses[8]. A complex regulation system controls HA metabolism, mainly dependent on HA-producing synthases and degrading hyaluronidases. The activation of the cell surface HA receptors such as CD44[9] and RHAMM[10] modulate cell proliferation, aggregation, migration and angiogenesis[11] and may also be involved in the HA-induced epithelial–mesenchymal transition[12] and stem cell functions[13]. HA has been shown to be overexpressed in most human malignancies[14–23]. Several studies have indicated that hyaluronan accumulation in the tumour cells and/or peritumoral stroma is related to tumour progression and poor survival in many cancer types[23–31]. However, the mechanisms underlying the association between accumulation of HA, host immune response and poor survival remain unclear, especially in PDAC.

The association between a strong immune response and better survival is well established in various cancers[32–36]. We have previously introduced a T-lymphocyte-based immune cell score (ICS) as a strong favourable prognostic factor in PDAC[37]. Extensive alterations occur in the complex PDAC microenvironment during the tumorigenesis. Multiple mechanisms, such as overexpression of the immunosuppressive molecules CD73 and PD-L1, may lead to immune suppression[38–41]. There is some evidence showing that HA plays a role in immune response regulation[42,43]. According to our hypothesis this might be one of the key factors explaining the association between HA accumulation and low survival among cancer patients[25].

The aim of the present study was to examine the prognostic role of stromal HA accumulation and its relation to immune cell infiltration and the immune-suppressing molecules CD73 and PD-L1 in PDAC.

## Methods

### Patients.
From 2000 to 2016, a total of 129 patients with PDAC were operated on with curative intent in the Central Hospital of Central Finland, Jyväskylä, Finland. Patients with locally inoperable tumour, peritoneal carcinosis or distant metastases were excluded, resulting in the 101 patients with stage IA-IIB disease. Detailed information on patient and tumour characteristics, surgical treatment and complications, oncological treatment and follow-up were collected prospectively, updated and confirmed by a review of patient records review. None of the included patients received neoadjuvant chemotherapy before surgery.

### Histopathological examination.
All histopathological tumour specimens were reviewed by an experienced gastrointestinal histopathologist (JB). Tumour staging was done according to the 7th edition of the UICC/AJCC TNM categories[44]. The grading was performed according to the WHO classification of tumours 2010[45].

### Tumour sampling, HA assay, and immunohistochemistry.
Tissue microarray (TMA) blocks were constructed as described previously, from formalin-fixed paraffin-embedded primary PDAC patient tumour samples. Two tissue cores 0.6 mm in diameter were taken both from the core of the tumour and the invasive margin from representative tumour blocks. Sections of 2 μm thickness were used for immunohistochemical (IHC) analyses[37].

Hyaluronan was stained as described previously[26]. Briefly, a complex containing the G1 domain of cartilage aggrecan and link protein was labeled with biotin (bHABC), diluted to 3 μg/ml of 1% bovine serum albumin in phosphate buffer, and incubated overnight at 4 °C on sections pretreated with $H_2O_2$ and 1% bovine serum albumin to block endogenous peroxidases and unspecific binding, respectively. After one hour incubation in avidin–biotin–peroxidase (Vector Laboratories, Irvine, CA; 1:200 dilution) the sections were washed with PBS, and incubated in 0.05% 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.03% $H_2O_2$ in the phosphate buffer, followed by nuclear counterstaining with Mayers hematoxylin (Fig. 1). Staining for CD73 was conducted as described previously, with rabbit monoclonal anti-CD73 antibody (D7F9A, Cell Signalling) and ultraView Universal DAB detection kit (Roche) for Ventana[39] (Fig. 2). Staining for CD3 and CD8 was conducted with anti-CD3 (LN 10, 1:200; Novocastra) and anti-CD8 (SP16, 1:400; Thermo Scientific) antibodies, using a Lab Vision Autostainer 480 (Immunovision Technologies Inc.) (Fig. 3). Staining for PD-L1 was conducted as described previously, with anti-PD-L1 (E1L3N, 1:100; Cell Signalling Technology) antibody, using a BOND-III stainer (Leica Biosystems). PD-L1 staining was carried out using whole tissue sections [37] (Fig. 4).

Signal visualization for all IHC was done by diaminobenzidine and sections were counterstained with haematoxylin.

In general, HA staining was clearly seen in all specimen both in stroma and in tumour epithelium. Stained TMA sections were scanned using an Aperio digital slide scanner (Leica Biosystems), followed by analysis using QuPath v 0.1.2 as described below.

ICS was determined using the TMA technique as described earlier[37]. Briefly, ICS describes the immune response represented by the density of CD3 and CD8 positive immune cells in the tumour centre and at the invasive margin. PD-L1 expression was evaluated by estimating the proportion of PD-L1 positivity on the tumour cell surface as described earlier[39]. In addition, we analyzed also the proportion of PD-L1 expression in stromal cells using the 5% staining proportion as a cutoff.

### Quantitative evaluation of HA staining.
HA was evaluated using QuPath v 0.1.2[46]. First, the stain vectors and background values were estimated using the *Estimate stain vectors* command to facilitate stain separation with the color deconvolution method. *Simple tissue detection* command was used to delineate the tissue area from the white background. This area was manually edited with the brush tool to exclude tumour epithelial regions. *SLIC superpixel segmentation* was used to divide the area into superpixels (neighboring groups of pixels sharing similar characteristics). DAB intensity was calculated for each superpixel, and the data were exported at individual superpixel level. R statistical programming language version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) was used to summarize the mean intensity for each case. The distribution of DAB intensities was similar in different TMAs suggesting that the assay had performed uniformly (Fig. S.1). The cores
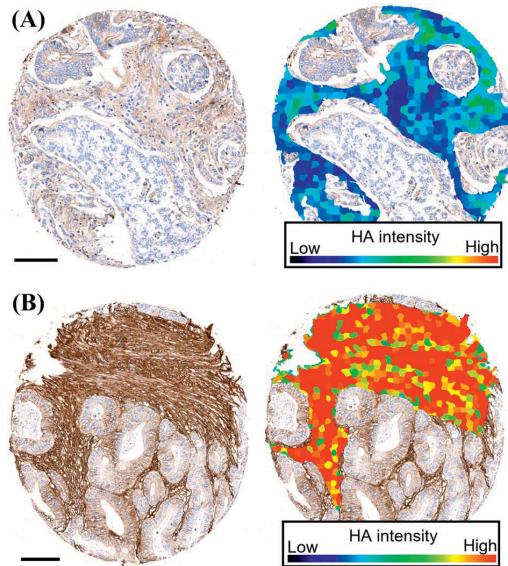
**Figure 1.** Representative examples of tissue microarray cores with low (**A**) and high (**B**) stromal hyaluronan (HA) intensity. The images show the exclusion of the tumour parenchyma and the delineation of the superpixels formed by the program used for analysis. Scale bars indicate 100 μm.
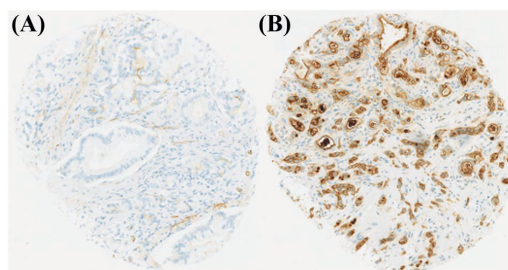


**Figure 2.** Representative examples of tissue microarray cores with low (**A**) and high (**B**) expression of CD73.

from tumour centre and invasive margin had a strong positive correlation (Spearman's rho = 0.69), and average HA intensity of all available tumour regions was therefore used in the main analyses.

Samples were divided into two groups based on the mean intensity value: high and low stromal HA expression. To determine cut-off values for HA expression with optimal sensitivity and specificity, we used receiver operating characteristic (ROC) curve drawn in relation to disease-specific 3-year mortality.

**Statistical analyses.** The chi-square test was used when analysing the associations between HA and clinical and histopathological variables, CD73 positivity and PD-L1 positivity in tumour cells. The estimates for hazard ratios for overall survival (OS) and disease specific survival (DSS) were calculated using univariate and multivariate Cox proportional hazards regression model. Only variables with $p < 0.05$ in univariate analysis were entered into the multivariate analysis despite the a-priori determined confounder, tumour stage ($p = 0.117$). All statistical tests were two-sided. A $p$ value less than 0.05 was considered significant. The statistical analyses were performed and Fig. 5 created with IBM SPSS statistics 24 for Windows (IBM Corporation, Armonk, NY, USA, https://www.ibm.com/analytics/spss-statistics-software).

**Compliance with ethical standards.** The use of patient samples and the data inquiry were approved by the Oulu University Hospital Ethics Committee. The need to obtain written or oral consent from patients to use their samples in research was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA,
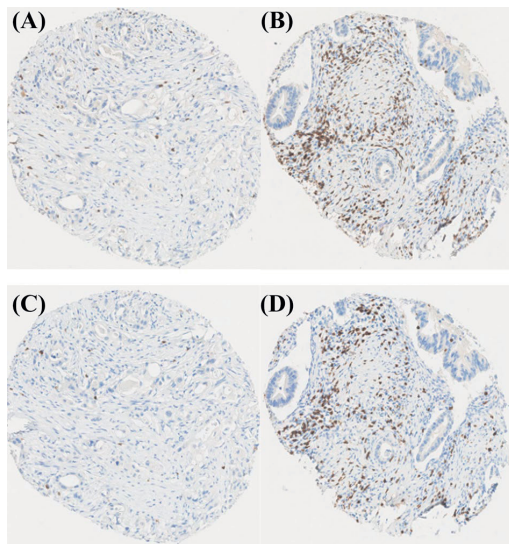
**Figure 3.** Representative examples of tissue microarray cores with low (**A**) and high CD3+ (**B**), and CD8+ (**C,D**) T-cell densities.
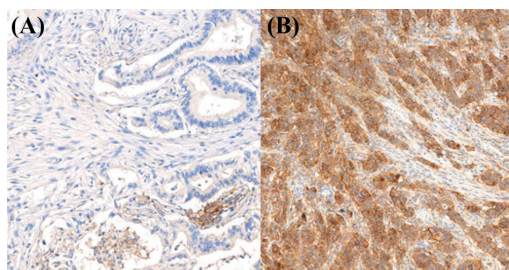


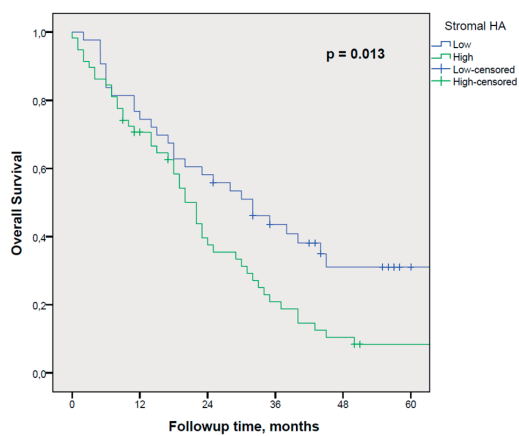**Figure 4.** Representative examples of PD-L1 negative (**A**) and positive (**B**) tumour samples.



**Figure 5.** Prognostic impact of stromal HA content on survival on OS.

| Total, n | 101 |
|---|---|
| **Age, years** | |
| Median | 67 |
| Range | 45–82 |
| **Gender, n (%)** | |
| Male | 53 (52.5) |
| Female | 48 (47.5) |
| **T-stage, n (%)** | |
| pT1 | 3 (3.0) |
| pT2 | 22 (21.8) |
| pT3 | 76 (75.2) |
| pT4 | 0 (0) |
| **N-stage, n (%)** | |
| pN0 | 30 (29.7) |
| pN1 | 71 (70.3) |
| **Stage, n (%)** | |
| IA | 3 (3.0) |
| IB | 7 (6.9) |
| IIA | 20 (19.8) |
| IIB | 71 (70.3) |
| **Histological grade, n (%)** | |
| 1 | 29 (28.7) |
| 2 | 60 (59.4) |
| 3 | 7 (6.9) |
| Unknown | 5 (5.0) |
| **Perineural invasion, n (%)** | |
| Positive | 33 (32.7) |
| Negative | 64 (63.4) |
| Unknown | 4 (4.0) |
| **PD-L1 in tumour cells, n (%)** | |
| Positive | 3 (3.0) |
| Negative | 98 (97.0) |
| **PD-L1 in tumour stroma, n (%)** | |
| Positive | 12 (11.9) |
| Negative | 89 (88.1) |
| **Immune cell score, n (%)** | |
| Low | 65 (64.4) |
| High | 34 (35.6) |
| **Stromal hyaluronan expression, n (%)** | |
| Low | 43 (42.6) |
| High | 58 (57.4) |
| **CD73 expression in tumour cells, n (%)** | |
| Low | 67 (66.3) |
| High | 34 (33.7) |

**Table 1.** Clinicopathological characteristics.

## Results

**Patient demographics.** A total of 101 of PDAC patients were included in this study. The distribution of key clinicopathological variables among these patients is shown in Table 1.

**Associations between stromal HA expression and other histopathological variables.** Stromal HA accumulation appeared to associate with low ICS ($p = 0.017$). The associations between stromal HA expression and other clinical and histopathological variables, cell-specific CD73 positivity and PD-L1 positivity in tumour cells were also assessed and are shown in Table 2. Stromal HA accumulation was not associated with

| | HA high, n (%) | HA low, n (%) | *p* Value |
|---|---|---|---|
| **Gender** | | | |
| Male | 33 (56.9) | 20 (46.5) | 0.301 |
| Female | 25 (43.1) | 23 (53.5) | |
| **T-stage** | | | |
| pT1 | 2 (3.4) | 1 (2.3) | 0.742 |
| pT2 | 14 (24.1) | 8 (18.6) | |
| pT3 | 42 (72.4) | 34 (79.1) | |
| pT4 | 0 | 0 | |
| **N-stage** | | | |
| pN0 | 19 (32.8) | 11 (25.6) | 0.435 |
| pN1 | 39 (67.2) | 32 (74.4) | |
| **Stage** | | | |
| IA | 2 (3.4) | 1 (2.3) | 0.979 |
| IB | 4 (6.9) | 3 (7.0) | |
| IIA | 12 (20.7) | 8 (18.6) | |
| IIB | 40 (69.0) | 21 (72.1) | |
| **Histological grade** | | | |
| 1 | 16 (30.2) | 13 (30.2) | 0.994 |
| 2 | 33 (62.3) | 27 (62.8) | |
| 3 | 4 (7.5) | 3 (7.0) | |
| **Perineural invasion** | | | |
| Positive | 35 (64.8) | 29 (67.4) | 0.786 |
| Negative | 19 (35.2) | 14 (32.6) | |
| **PD-L1 in tumour cells** | | | |
| Positive | 1 (1.7) | 2 (4.7) | 0.392 |
| Negative | 57 (98.3) | 41 (95.3) | |
| **PD-L1 in tumour stroma** | | | |
| Positive | 6 (10.3) | 6 (14.0) | 0.579 |
| Negative | 52 (89.7) | 37 (86.0) | |
| **Immune cell score** | | | |
| Low | 43 (74.1) | 22 (51.2) | *0.017* |
| High | 15 (25.9) | 21 (48.8) | |
| **CD73 in tumour cells** | | | |
| Low | 39 (67.2) | 28 (65.1) | 0.823 |
| High | 19 (32.8) | 15 (34.9) | |

**Table 2.** Clinicopathological variables and their association with stromal hyaluronan (HA).

other clinicopathological parameters, including CD73 positivity in tumour cells and PD-L1 positivity in tumour cells and stromal cells.

**Stromal HA accumulation and survival.** Regarding the whole study group, the median follow-up time was 44 (IQR 15.0 to 57.0) months for those alive at the end of follow-up. The estimated median OS for all patients was 25 months [95% CI: (17.7–32.3)]. Stromal HA accumulation was significantly associated with poor DSS ($p = 0.037$) and OS ($p = 0.013$) (Fig. 5).

In the multivariate analysis, stromal HA accumulation was an independent negative prognostic factor together with histopathological grade, TNM stage, CD73 positivity in tumour cells and low ICS (Table 3).

## Discussion

In the present study, using a larger, consecutive patient series from a single geographical area of Northern Finland without apparent selection bias, we showed the role of stromal HA accumulation as an independent prognostic factor for poor survival in pancreatic cancer. We also found an association between the HA accumulation and low immune response as judged by the tumour-infiltrating T-cell densities.

While the number of patients in the present work was higher than in previous studies on HA in PDAC[6,24], an even larger material would probably have allowed a connection between T-cell score and hyaluronan stronger than that now established. The small tissue cores turned out to be quite acceptable for the analysis since in preliminary tests the HA intensities were strongly correlated between different cores of the same tumour even between the tumour centre and invasive margin. The computer-assisted evaluation of HA staining adopted for

| | Univariate analysis (OS)[a] HR (95% CI) | p | Univariate analysis (DSS)[b] HR (95% CI) | p | Multivariate analysis (OS) HR (95%CI) | p | Multivariate analysis (DSS) HR (95% CI) | p |
|---|---|---|---|---|---|---|---|---|
| **Stromal HA** | | | | | | | | |
| Low | 1 | 0.015 | 1 | 0.042 | 1 | 0.019 | 1 | 0.048 |
| High | 1.80 (1.22–2.88) | | 1.67 (1.02–2.72) | | 1.85 (1.11–3.10) | | 1.71 (1.00–2.92) | |
| **CD73 (TC)[c]** | | | | | | | | |
| Negative | 1 | 0.022 | 1 | 0.029 | 1 | 0.015 | 1 | 0.023 |
| Positive | 1.76 (1.09–2.84) | | 1.76 (1.06–2.93) | | 2.01 (1.14–3.53) | | 2.00 (1.10–3.62) | |
| **Tumor grade** | | | | | | | | |
| 1 | 1 | 0.006 | 1 | 0.013 | 1 | 0.012 | 1 | 0.019 |
| 2 | 1.95 (1.14–3.34) | | 1.95 (1.12–3.39) | | 1.99 (1.15–3.44) | | 2.01 (1.14–3.56) | |
| 3 | 3.88 (1.54–9.79) | | 3.69 (1.36–10.01) | | 3.96 (1.36–11.52) | | 3.80 (1.18–12.01) | |
| **TNM stage** | | | | | | | | |
| IA + IB | 0.64 (0.28–1.48) | 0.234 | 0.69 (0.30–1.61) | 0.117 | 0.53 (0.21–1.36) | 0.055 | 0.58 (0.22–1.52) | 0.036 |
| IIA | 0.55 (0.29–1.06) | | 0.49 (0.24–0.99) | | 0.49 (0.25–0.95) | | 0.41 (0.20–0.85) | |
| IIB | 1 | | 1 | | 1 | | 1 | |
| **ICS[d]** | | | | | | | | |
| High | 1 | 0.007 | 1 | 0.008 | 1 | 0.006 | 1 | 0.002 |
| Low | 1.99 (1.21–3.30) | | 2.05 (1.21–3.47) | | 2.23 (1.26–3.95) | | 2.54 (1.40–4.63) | |

**Table 3.** Multivariate analysis with Cox proportional hazard model. [a]Overall survival. [b]Disease specific survival. [c]Tumour cell. [d]Immune cell score.

the present work was felt easier than manual scoring of sometimes minor differences in intensity. It can also be recommended for future studies due to its independence of personal variation between evaluators.

Stromal HA accumulation in malignancies originating from non-stratified epithelium is associated with a poor survival in a number of solid tumours[7]. Indeed, given the large desmoplastic stroma in PDAC, a major role of HA was expected in the progression of this cancer. The idea was further supported for example by a fact that a drug specifically reducing HA synthesis inhibits human PDAC cell growth in vitro and in mice in vivo[49]. However, clinical trials combining enzymatic removal of HA and cytostatic drugs have been disappointing[50,51] suggesting that it is not just the content of HA that enhances malignant growth. Rather, activated synthesis and concurrent degradation of HA probably provide an environment supporting cancer spreading[7]. This becomes understandable by considering the two opposite influences of HA on cell migration. By its swelling pressure HA gel creates free space for cells to move in, while at the same time blocks attachment to adjacent cells and matrix proteins.

Indeed, the cell surface hyaluronidase TMEM2 is an independent negative prognostic factor in PDAC[52], demonstrating the importance of HA degradation in PDAC progression. TMEM2 associates to integrins and clears HA to facilitate cancer cell adhesion and migration[53]. Besides facilitating migration in HA-rich matrix the fragments created by hyaluronidase act as a signal that amplify inflammation.

Increasing numbers of studies have shown the impact of HA on the host immune response: It is suggested to protect tumour cells against immune attack by forming peri-cellular coats[54]. Moreover, HA accumulation seems to facilitate tumour-associated macrophage infiltration and their differentiation into the pro-tumoral M2 phenotype[25,42] with an immunosuppressive effect preventing antitumour immunity by T-cells. Interestingly, in the present study we demonstrate an inverse correlation between T-cell-based ICS and stromal HA accumulation in PDAC. This supports the idea that an HA-rich extracellular matrix not only acts as a shield between T-cells and tumour cells, but also prevents T-cell infiltration in the whole tumour microenvironment. We have previously published a paper showing that CD73 positivity in PDAC cells is a prognostic factor in PDAC independently of ICS and hypothesized that CD73 suppresses immune response by impacting on the activity of the tumour infiltrating lymphocytes rather than their number[39]. This might also explain why stromal HA expression associates with ICS but not with CD73 or PD-L1.

Recently, different phenotypes of ECM-producing cancer-associated fibroblasts (CAFs) have been described, including inflammatory CAFs and myofibroblastic CAFs[55]. Inflammatory CAFs are supposed to be tumour-promoting via immune suppression[56]. In future, it would be reasonable to find out if the HA accumulation associates with the polarization of CAFs, since this would further give some insight into the potential mechanism behind the association between HA and immune status. One possible link between CAF polarisation and HA synthesis is the STAT3-signaling pathway, since the inhibition of STAT3—pathway has been shown to downregulate HA—synthesis[57] and, on the other hand, to promote differentiation of CAFs into myCAFs[56].

As far as we know, the association of HA on T-cell immune response has not been studied earlier in PDAC but the present finding clearly warrants further expansion of the studies to obtain a more detailed view of the interactions between HA and lymphocytes in this disease with such a bleak prognosis. In future studies, information of physical properties (for example molecular mass) of HA molecules is also needed, since there are data indicating that molecular weight affects the biological functions of HA molecules[58].

In conclusion, our study indicates that stromal HA accumulation may be associated with low T cell densities in the PDAC microenvironment, but still represents an adverse prognostic parameter independent of T

cell densities, tumour stage, tumour grade, and CD73 expression. The results warrant further definition of the interactions between T-cell immunity and hyaluronan in the tumour microenvironment.

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## Acknowledgements

## Author contributions

All authors met the criteria listed in the ICMJE recommendations for the qualification of authorship. K.T. and J.B. had full access to all the data and take responsibility for the integrity of the data and the accuracy of the analysis. Concept and design: K.T., J.M. and J.B. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: K.T., J.V. and J.B. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: K.T. and J.B. Administrative, technical, or material support: All authors. Supervision: J.M., J.L., I.K. and J.B.

## Funding

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at https://doi.org/ 10.1038/s41598-021-91796-x.

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