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PROGNOSTIC BIOMARKERS IN PANCREATIC DUCTAL ADENOCARCINOMA

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ACADEMIC DISSERTATION

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To my dear family

CONTENTS

1 ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers worldwide. Radical surgical resection combined with chemotherapy, the only potentially curative treatment, is possible in only a small proportion of patients. Although overall survival is poor, marked variation exists between patients of the same tumor stage. New biomarkers could be helpful in predicting prognosis. Despite considerable research on potential biomarkers, since the discovery of CA19-9, none has gained a role in clinical practice. Identification of new biomarkers to predict PDAC patient outcome more accurately and to enhance our knowledge of the molecular mechanisms behind the disease is crucial. Differential diagnosis between PDAC and chronic pancreatitis (CP) can be challenging. A pancreatic mass can prove to be benign or malignant. A clear preoperative diagnosis would be valuable for patients to avoid unnecessary and extensive surgery. The standard serum-based marker for diagnosis of PDAC, CA19-9, has diagnostic limitations because it can be normal in patients with localized disease or high in patients with benign pancreatic disease, including CP.

The aim of this thesis was to explore, tissue expression of tumor biomarkers in PDAC. The prognostic significance of these biomarkers in patient survival was evaluated. In each study, we used different biomarkers: podocalyxin (PODXL), PROX1, β-catenin, UCHL5, and REG4. In the last study, we also evaluated the diagnostic significance of serum REG4 levels in patients with PDAC and in those with CP. Immunohistochemical expression of tumor markers was evaluated in 154 surgical specimens and serum REG4 level in 130 samples from PDAC patients treated between 2000 and 2011. The CP control group comprised 34 patients who underwent resection because of suspicion of malignancy.

PODXL, PROX1, β-catenin, and UCHL5 were independent prognostic markers. High tissue expression of PODXL prognosticated poor survival among PDAC patients compared with low tissue expression, whereas high tissue expression of both PROX1 and β-catenin was associated with increased survival. Positive nuclear UCHL5 expression was an independent factor for favorable prognosis. REG4 failed to be an independent marker of prognosis in PDAC, but serum REG4 levels were higher in PDAC than in CP suggesting its utility in differential diagnosis.

These studies provide novel knowledge of potential prognostic tumor markers in PDAC. Moreover, we identified a serum biomarker, REG4, that may be useful in differential diagnosis between PDAC and CP.

2 FINNISH SUMMARY

Haiman duktaalinen adenokarsinooma on yksi huonoennusteisimmista syöpätaudeista. Suomessa haimasyöpä aiheuttaa kolmanneksi eniten syöpäkuolemia. Radikaali kirurgia yhdistettynä sytostaattihoitoon on ainoa mahdollinen parantava hoitomuoto, mutta vain pieni osa haimasyöpäpotilaista soveltuu tällaiseen hoitoon. Vaikka taudin yleinen ennuste on huono, saman levinneisyysasteen potilailla ennuste saattaa vaihdella paljonkin. Uudet biomarkkerit auttaisivat potilaiden ennusteen arvioinnissa. Vaikka lukuisia potentiaalisia markkereita on tutkittu vuosikausien ajan, yhtään markkeria ei ole otettu kliiniseen käyttöön CA 19-9:n löytämisen jälkeen. On välttämätöntä löytää uusia biomarkkereita, jotta haimasyöpäpotilaiden ennustetta voidaan arvioida tarkemmin ja täsmällisemmin. Tämä parantaisi myös tietämystä haimasyövän kehittymisen taustasta molekyylitasolla. Erotusdiagnostiikka haimasyövän ja kroonisen haimatulehduksen välillä voi ajoittain olla hankalaa. Haiman kuvantamistutkimuksissa nähdyt muutokset voivat olla pahan- tai hyvänlaatuisia. Tarkka diganoosi ennen mahdollista leikkausta hyödyttäisi potilaita, joita ei välttämättä tällöin tarvitsisi altistaa raskaalle kirurgiselle hoidolle. CA19-9, jota käytetään laajasti leikkausta edeltävässä diagnosoinnissa, on osin ongelmallinen, koska se voi olla normaalitasolla syövästä tai koholla hyvänlaatuisesta muutoksesta huolimatta.

Tutkimuksemme tavoitteena oli tutkia kudosmarkkereiden ennusteellista arvoa haimasyövässä. Jokaisessa osatyössä tutkimme eri biomarkkereita. Tutkitut markkerit olivat podokalyksiini, PROX1, β-catenin, UCHL5 ja REG4. Viimeisessä osatyössä tutkimme myös REG4:n diagnostista arvoa haimasyövän ja kroonisen pankreatiitin välillä. Markkereiden immunohistokemiallinen ilmentyminen arviotiin 154 leikatusta haimasyöpäpotilaasta vuosina 2000-2011. Kroonisen pankreatiitin kontrolliryhmään kuului 34 potilasta, jotka oli leikattu haiman pahanlaatuisen kasvainepäilyn takia.

PODXL, PROX1, β-catenin ja UCHL5 olivat itsenäisiä ennustemarkkereita. Korkea PODXL:n kudosilmentyminen ennusti huonompaa selviytymistä syövästä verrattuna matalaan ilmentymiseen. Korkea PROX1:n ja β-cateninin kudosilmentyminen sen sijaan ennusti parempaa selviytymistä. Positiivinen UCHL5:n ilmentyminen liittyi myös parempaan ennusteeseen verrattuna negatiiviseen ilmentymiseen. REG4:n osalta taudin ennusteessa ei ollut merkittäviä eroja kudosilmentymisen mukaan, mutta REG4:n pitoisuudet olivat merkittävästi korkeammat haimasyövässä kuin kroonisessa haimatulehduksessa. Tutkimuksemme toi uutta tietoa potentiaalisista ennusteellisista biomarkkereista. Löysimme myös uuden mahdollisen biomarkkerin, REG4:n, jota voidaan mahdollisesti käyttää tulevaisuudessa apuna diagnostiikassa.

3 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-IV):

I Podocalyxin is a marker of poor prognosis in pancreatic ductal adenocarcinoma. Saukkonen K, Hagström J, Mustonen H, Juuti A, Nordling S, Fermér C, Nilsson O, Seppänen H[1], Haglund C.[1] PLoS ONE 2015; 10(6): e0129012.

II PROX1 and β-catenin are prognostic markers in pancreatic ductal adenocarcinoma. Saukkonen K, Hagström J, Mustonen H, Juuti A, Nordling S, Kallio P, Alitalo K, Seppänen H[1], Haglund C.[1] BMC Cancer 2016; 16:472.

III Nuclear ubiquitin C-terminal hydrolase L5 expression associates with increased patient survival in pancreatic ductal adenocarcinoma. Arpalahti L[2], Saukkonen K[2], Hagström J, Mustonen H, Seppänen H[1], Haglund C[1], Holmberg CI[1]. Tumour Biology 2017; 39(6):1010428317710411.

This publication was also included in the thesis of the other first author Leena Arpalahti entitled "The Proteasome-Associated Deubiquitinase UCHL5/UBH-4 in Proteasome Modulation and as a Prognostic Marker in Gastrointestinal Cancers" (ISBN: 978-951-4017-3).

IV Prognostic and diagnostic value of REG4 serum and tissue expression in pancreatic ductal adenocarcinoma. Saukkonen K, Hagström J, Mustonen H, Lehtinen L, Carpen O, Andersson LC, Seppänen H[1], Haglund C[1]. Tumour Biology 2018; 40(3):1010428318761494

[1] = equal last authorship
[2] = equal contribution

4 ABBREVIATIONS

5-FU	5-fluorouracil
AJCC	American Joint Committee on Cancer
APC	adenomatous polyposis coli
AUC	area under the curve
BMI	body mass index
CA	celiac axis
CA 19-9	carbohydrate antigen 19-9
CC	Creative Commons
CEA	carcinoembryonic antigen
CHA	common hepatic artery
CI	confidence interval
CP	chronic pancreatitis
CRC	colorectal cancer
CRP	C-reactive protein
CSS	cancer-specific survival
CT	computed tomography
CTNNB1	protein β-catenin encoding gene
DM	diabetes mellitus
DNA	deoxyribonucleic acid
DUB	deubiquitinating enzyme
ECM	extracellular matrix
ERCP	endoscopic retrograde cholangiopancreatography
EUS	endoscopic ultrasound
FNA	fine-needle aspiration
GDA	gastroduodenal artery
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCC	hepatocellular carcinoma
HES9	monoclonal antibody against human embryonic stem cells
HR	hazard ratio
IBD	inflammatory bowel disease
IHC	immunohistochemistry
IQR	interquartile range
IPMN	intraductal papillary mucinous neoplasm
ISGPS	International Study Group of Pancreatic Surgery
IVC	inferior vena cava
LNR	lymph node ratio
mAb	monoclonal antibody
MCN	mucinous cystic neoplasm
MRCP	magnetic resonance cholangiopancreatography
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid

NCCN	National Comprehensive Cancer Network
pAb	polyclonal antibody
PanIN	pancreatic intraepithelial neoplasia
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PET	positron emission tomography
PODXL	podocalyxin-like 1 protein
PROX1	prospero homeobox protein 1
PSC	pancreatic stellate cell
PTC	papillary thyroid cancer
PV	portal vein
r	correlation coefficient
REG4	regenerating islet-derived protein 4
RNA	ribonucleic acid
ROC	receiver operating characteristic
SE	standard error
SMA	superior mesenteric artery
SMV	superior mesenteric vein
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGF-β	transforming growth factor-beta
TMA	tissue microarray
TNM	tumor-node-metastasis
UCHL5	ubiquitin c-terminal hydrolase L5
UPS	ubiquitin-proteasome system
US	ultrasonography

5 INTRODUCTION

Pancreatic malignancy has a notoriously dim prognosis, with an overall 5-year survival of less than 8% [1]. In the United States, it is the fourth leading cause of cancer-related deaths, and in Finland it is the third (Finnish Cancer Registry). Pancreatic ductal adenocarcinoma (PDAC) accounts for over 90% of pancreatic cancers, and it metastasizes rapidly in its early stages. There are also other tumors of the pancreas with different prognoses and treatment methods, but this study focuses entirely on PDAC.

The underlying causes of pancreatic cancer are still to be resolved. Accumulating gene mutations leading to dysplasia and malignant tumor development are regarded as the most common cause [2,3]. Smoking and chronic pancreatitis are some of the most important risk factors. Sadly, no suitable population-based screening tests exist. At the early stage of PDAC, patients' symptoms are often vague and may be completely missing. Nevertheless, early detection would be critical for patient outcome. Curative treatment is based on radical surgical resection followed by chemotherapy. However, only a minor proportion of patients, roughly 10-20%, are eligible for surgery because of concomitant advanced disease or metastases [2].

The prognosis of PDAC is based on stage of the disease, tumor-free resection margins, histological type and differentiation of the tumor, lymph node metastases, and tumor size. Although overall survival is poor, marked variation exists between patients of the same tumor stage. New diagnostic, prognostic, and predictive biomarkers are urgently needed. Since the discovery of biomarker CA19-9, no biomarker has gained a role in clinical practice despite comprehensive research.

The aim of this study was to examine the prognostic significance of five tissue biomarkers in surgically treated patients. We also evaluated the differential diagnostic usefulness of REG4 between PDAC and chronic pancreatitis.

6 REVIEW OF THE LITERATURE

6.1 EPIDEMIOLOGY

Pancreatic cancer is the world's 12th most common cancer type with nearly 340 000 new cases annually. However, it is the seventh leading cause of cancer deaths globally, with incidence and mortality rates being almost identical [4]. In developed countries, pancreatic cancer causes almost 190 000 deaths every year, with incidence of 8.6 in men and 5.9 in women per 100 000. In less developed areas, the recorded incidence rate is slightly lower, from 2.4 to 3.3 per 100.000, and an almost identical mortality rate [5]. In the United States, pancreatic cancer is the fourth leading cause of cancer deaths, with incidence of 14.1 in men and 10.9 in women and overall mortality rates of 12.5 and 9.5, respectively [1,6].

According to the Finnish Cancer Registry, pancreatic cancer age-adjusted incidence rate in Finland in 2015 was 9.0 for men and 7.1 for women per 100.000 inhabitants and more than 1000 new cases (Finnish Cancer Registry, available at www.cancerregistry.fi). With almost the same mortality rate, 8.8 in men and 6.9 in women, pancreatic cancer is the third leading cause of cancer deaths (Figure 1). At Helsinki University Hospital, the 5-year survival rate was 22% for those patients resected for PDAC between 2000 and 2013 [7]. However, the overall survival for pancreatic cancer, including all subtypes of pancreatic malignancies, remains at about 5% in Finland according to the Finnish Cancer Registry. With distant metastases, PDAC is fatal, as 5-year survival rate drops to near zero [1].

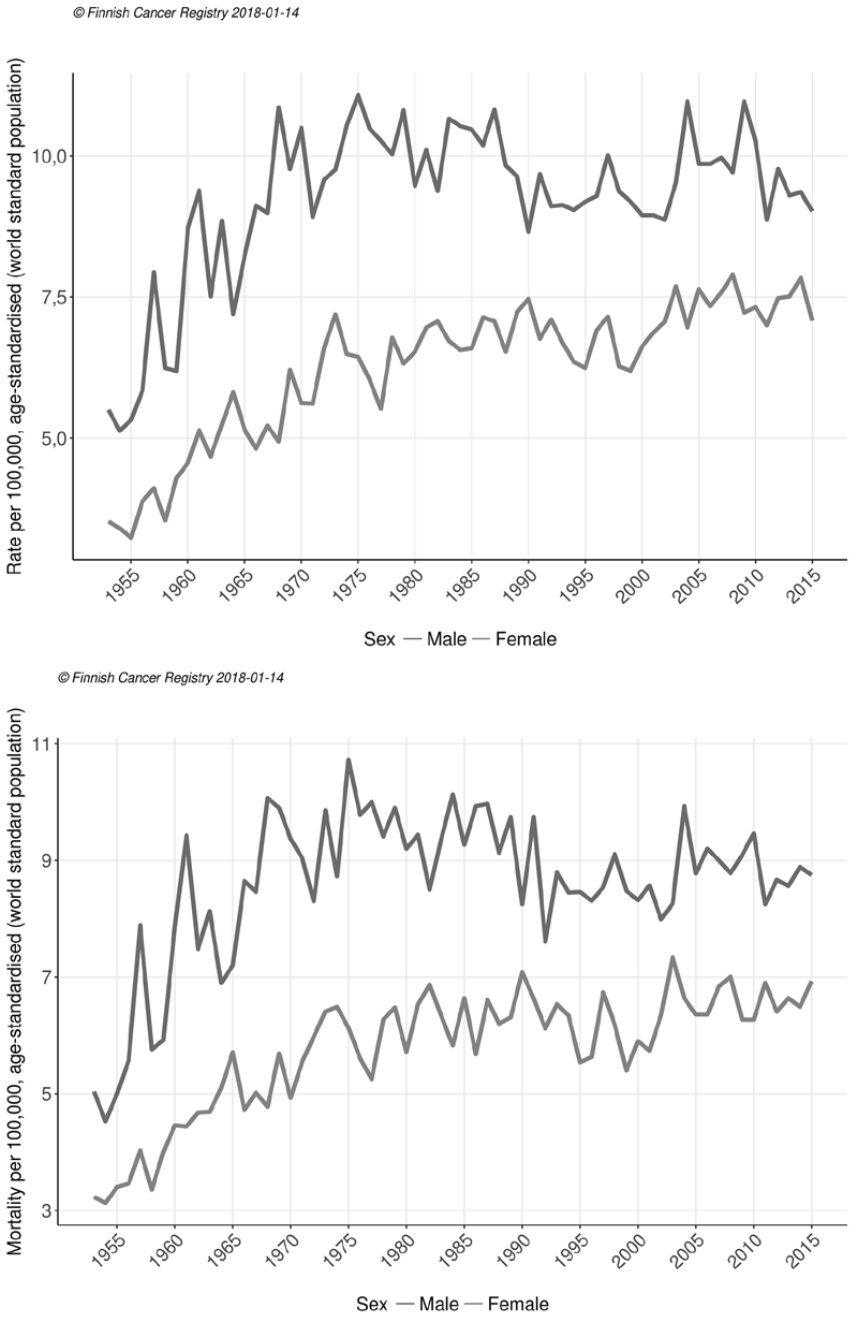

Figure 1. Annual incidence and mortality of pancreatic cancer by time period. Source: Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi, updated 13 March 2018.

6.2 ETIOLOGY

6.2.1 RISK FACTORS

Pancreatic cancer is a multifactorial and complex disease. Lifetime risk for pancreatic cancer is less than 1%, being more frequent in developed countries than in less developed areas [5]. A family history of pancreatic cancer, smoking, chronic pancreatitis, and advanced age are regarded as constant risk factors for pancreatic cancer. Other risk factors include diabetes mellitus, excessive alcohol consumption, obesity, Western dietary habits, and non-O blood group [3,8,9].

6.2.1.1 Genetic predisposition/family history

About 10% of pancreatic cancer patients have a familial basis [10-12]. Risk for pancreatic cancer is roughly doubled with one first-degree relative diagnosed with the disease, rising to even 57-fold in families with four or more affected members [11,13]. Pancreatic cancer can develop in many genetic syndromes, which, however, only partly cover the cases of familial pancreatic cancers. The most common syndromes with mutated genes and risk ratios are described in Table 1.

Table 1. Hereditary cancer syndromes with increased risk for pancreatic cancer and affected genes. The table is modified from earlier studies [3,10,14].

Genetic syndrome	Affected gene(s)	Risk ratio
Familial breast and ovarian cancer	BRCA2, BRCA1	3.5-10
Familial atypical multiple mole melanoma syndrome	CDKN2A (p16)	9-47
Peutz-Jeghers syndrome	STK11 (LKB1)	132
Hereditary pancreatitis	PRSS1, SPINK1	50-80
Hereditary non-polyposis colorectal cancer (Lynch syndrome)	Multiple	9
Familial adenomatous polyposis (FAP)	APC	4.5
Familial pancreatic cancer	PALB2	6
Familial pancreatic cancer (monoallelic); ataxia-telangiectasia (biallelic)	ATM	Unknown

6.2.1.2 Smoking

Smoking is a recognized risk factor for pancreatic cancer. Current cigarette smoking leads to a 1.8-3.4-fold risk relative to non-smokers [15-17]. The risk ratio increases with the number of cigarettes smoked. Former smokers have approximately a 1.2-fold risk relative to non-smokers. The risk for former smokers remains elevated for 10-20 years after cessation. The starting age of cigarette smoking has no effect on the risk.

6.2.1.3 Chronic pancreatitis

Pancreatitis, an inflammation of the pancreas, destroys normal pancreatic tissue and can lead to irreversible changes in pancreatic cells. Accumulating evidence shows that longstanding existing chronic pancreatitis is a strong risk factor for pancreatic cancer [18-20]. The latent time between diagnosis of chronic pancreatitis and pancreatic cancer can be as long as 10-20 years. The risk can increase to 5-fold with unspecified pancreatitis, to 13- to 16-fold with chronic pancreatitis, and to even 69-fold with hereditary pancreatitis [18,20]. The risk seems to be highest in newly diagnosed (<2 years) chronic pancreatitis patients, with the risk diminishing over time [20]. Even though the link between chronic pancreatitis and pancreatic cancer is strong, only about 5% of patients with chronic pancreatitis develop pancreatic cancer over a 20-year period.

6.2.1.4 Diabetes mellitus

Diabetes mellitus (DM) leads to elevated blood glucose levels and can be divided into insulin deficiency (type 1 DM) and insulin resistance (type 2 DM) types. Type 2 DM is associated with obesity and unhealthy nutritional habits, and its incidence is rapidly rising. DM is associated with a twofold increased risk for pancreatic cancer compared with healthy subjects [21,22]. The risk is highest for a recently diagnosed DM and decreases over time. An elevated risk remains even 20 years after DM diagnosis. Up to 40% of the patients with pancreatic cancer report new-onset DM within three years prior to the cancer diagnosis [23,24]. However, the absolute risk is low, since less than 1% of newly diagnosed patients with DM develop pancreatic cancer during a five-year follow-up [23,25].

6.2.1.5 Other (lifestyle-related) risk factors

Risk for pancreatic cancer increases with age, being very uncommon in patients under 40 years. The median age of onset is 65-75 years. Over 80% of patients diagnosed are aged between 60 and 80 years.

Obesity is associated with an increased risk for pancreatic cancer, even without DM [26]. It increases the risk 25-50% compared with normal-weight individuals, regardless of diabetes or smoking status [27]. The risk ratio appears to depend on body mass index (BMI): the higher the BMI, the greater the risk for pancreatic cancer. With a stepwise rise of 5 kg/m^2 in BMI, the overall risk increases 2-12% [27]. In addition to obesity, low physical activity and such dietary habits as high intake of red meat and low intake of fruits and vegetables and polyunsaturated fatty acids may have an effect on the increased risk [28].

Excessive alcohol consumption as an independent risk factor for pancreatic cancer has been under debate. Current evidence indicates that heavy alcohol consumption (>50 g alcohol per day) can increase the risk of pancreatic cancer [29]. The most recent meta-analysis suggests that even alcohol consumption of ≥24 g per day may increase the risk [30].

6.2.2 SCREENING

Screening for pancreatic cancer is problematic. The malignant nature of invasive pancreatic cancer and the perception that most patients present with advanced stage disease lead to efforts to identify early invasive pancreatic cancers and pre-invasive lesions. However, identifying pre-invasive lesions may result in overdiagnosis and treatment of patients. In addition, pancreatic cancer has a rather low incidence, and there is no simple and accurate screening method. For that reason, possible screening ought to be focused on high-risk individuals with genetic predisposition syndrome associated with pancreatic cancer, family history of pancreatic cancer, or patients more than 50 years old with several known risk factors such as new-onset diabetes and smoking history [31].

However, screening for pancreatic cancer in high-risk individuals is controversial in assessing the survival of patients [32-34]. There is disagreement about the age to initiate screening and about the best imaging method for screening, but endoscopic ultrasound (EUS) and magnetic resonance-based imaging (MRI/MRCP) appear to be the most suitable methods [35].

6.3 PATHOGENESIS

6.3.1 PRECURSOR LESIONS

Pancreatic ductal adenocarcinoma is the most common pancreatic malignancy. PDAC develops from non-invasive precursor lesions, most commonly from epithelial proliferations within the pancreatic ducts, referred to as pancreatic intraepithelial neoplasias (PanINs) [36]. PanINs are non-invasive and microscopic (<5 mm) lesions classified by their epithelial atypia. PanIN lesions are traditionally divided into three grades: PanIN-1A/B, PanIN-2, and PanIN-3 [37,38]. The higher the PanIN grade, the closer the lesion is to progressing to invasive carcinoma (Figure 2). However, in 2015, consensus recommendations suggested using a two-class grading (low vs. high grade) [39]. Low-grade PanINs (PanIN-1) are common with increasing age, and high-grade PanINs (PanIN-3) are usually present in the pancreas with invasive cancer.

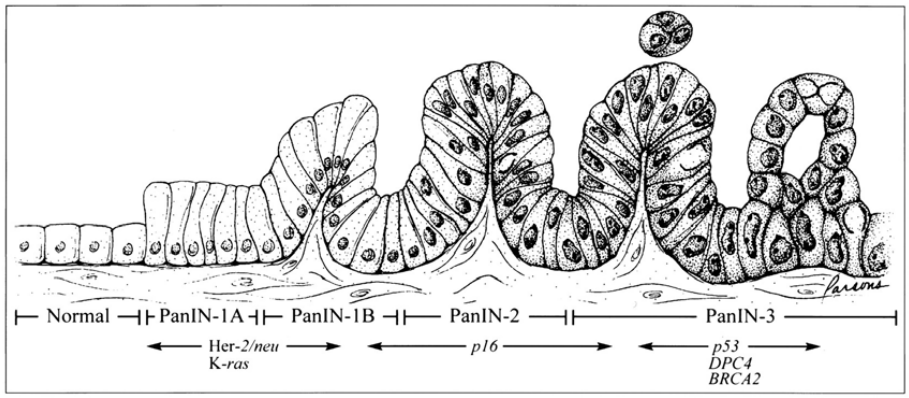

Figure 2. PanIN progression model showing genetic alterations. PanIN = pancreatic intraepithelial neoplasia. Reprinted [36] with permission of the American Association for Cancer Research.

Some adenocarcinomas can develop from macroscopic cystic tumors or precursors: from intraductal papillary mucinous neoplasms (IPMNs) or from mucinous cystic neoplasms (MCNs). IPMNs are mucinous cysts affecting the pancreatic duct system and are more than 5-10 mm in size [40]. They are categorized into low-grade (adenoma), intermediate/moderate (borderline), or high-grade (carcinoma *in situ*) based on the degree of dysplasia in the epithelium. In addition, multiple histological subtypes are identified [41].

MCNs are less common than IPMNs and occur mainly in women. They are usually located in the body or tail of the pancreas and do not communicate with the pancreatic ducts [42,43]. MCNs also are categorized as IPMNs, and their key feature is ovarian-type stroma underlying the epithelium, which differentiates MCNs from other mucin-producing neoplasms. International guidelines have been established to standardize and direct the follow-up and treatment of these cystic neoplasms [44-46] .

6.3.2 GENE MUTATIONS

The genetic basis of pancreatic cancer is complex and heterogeneous, creating a challenge for treatment. However, four major genetic alterations are responsible for PDAC. KRAS is the most frequently mutated oncogene. Mutations in KRAS occur in over 90% of the tumors, which leads to increased proliferation, cell survival, and suppressed apoptosis [47,48]. CDKN2A, encoding a protein with a critical role in cell-cycle regulation, is the most frequently altered tumor suppressor gene in more than 90% of PDACs [49,50]. KRAS mutations are common also in PanINs, suggesting they may be one of the first alterations in pancreatic tumorigenesis, whereas inactivation of CDKN2A does not typically occur in the low-grade precursor lesions [51]. Somatic mutations in the TP53 tumor suppressor gene occur in about 75% of pancreatic malignancies. The protein encoded by TP53 has a crucial role in apoptotic signaling and control of DNA damage during the cell cycle [52]. As the fourth most commonly mutated gene, tumor suppressor SMAD4 is mutated in about 55% of cases, regulating the transforming growth factor-beta (TGF-β) signaling pathway [53,54].

In recent years, an explosion of knowledge about the genetic alterations underlying the pathogenesis of pancreatic cancer has occurred. Studies have identified, through genome sequencing, mutations in hundreds of genes [50,55]. The difficulty is in identifying which genes drive or enhance the tumorigenesis of pancreatic neoplasms.

6.3.3 TUMOR MICROENVIRONMENT

The microenvironment of pancreatic adenocarcinoma is complex, affecting both tumor growth and therapeutic response. It is characteristically stroma-rich, consisting of proliferating myofibroblasts (pancreatic stellate cells (PSCs)), extracellular matrix (ECM) such as type I collagen and hyaluronic acid, and many types of inflammatory cells including macrophages, mast cells, lymphocytes, and plasma cells [56]. The stroma functions not only as a mechanical barrier, but also forms a dynamic environment involved in tumor formation, progression, invasion, and metastasis [57,58]. Pancreatic cancer is characterized by low microvascular density with limited perfusion, leading to intratumoral hypoxia. The fibrous stroma may contribute to reduced blood

flow, and constant production of ECM increases the interstitial pressure, compressing the capillary vessels and impairing drug delivery [59,60].

An additional relevant feature of the microenvironment of pancreatic adenocarcinoma is restraining of immune surveillance and creating an inflammatory response that supports tumorigenesis through cross-talk between tumor cells and immune cells [61]. Immunosuppressive regulatory T-lymphocytes and myeloid cells are recruited to the tumor stroma from the early stages of tumor formation, which leads to a block in T-cell-mediated antitumor immunity. This recruitment is induced by oncogenic activation of KRAS in pancreatic cells, resulting in production of granulocyte-macrophage colony-stimulating factor (GM-CSF) [62,63].

The central role of the tumor microenvironment makes it an important focus for novel therapy targets. Therapies aimed at depleting or modifying cellular and acellular components of the stroma are intriguing and they may provide a new approach for immune-based or other targeted therapies [63,64].

6.4 DIAGNOSIS

6.4.1 CLINICAL PRESENTATION

Most pancreatic cancers do not elicit symptoms in the early stage. The possible symptoms depend on the location of the tumor within the pancreas. Most symptoms are vague and non-specific such as nausea, vomiting, weight loss, or abdominal discomfort. Tumors in the head of the pancreas (60-70% of cases) can cause obstructive cholestasis and jaundice, which may lead to earlier detection of the disease. Abdominal pain occurs more commonly in later stages, and it typically feels dull and deep in the epigastrium. As the cancer infiltrates the retroperitoneum, back pain may appear. New-onset DM and degenerating glucose balance with diagnosed DM can be a manifestation of the failure of the endocrine pancreas. Weight loss can arise from anorexia, maldigestion from pancreatic ductal obstruction, or cachexia. Infrequently, obstruction of the pancreatic duct can provoke acute pancreatitis. Deep or superficial venous thrombosis may occur as a sign of malign disease. Other rare symptoms may be panniculitis, liver function abnormalities, gastric outlet obstruction, increased abdominal girth, or depression [3,9,65].

Physical examination may reveal upper abdominal resistance, jaundice (first detectable in the sclera), lymphadenopathy, hepatomegaly, painlessly enlarged gallbladder (Courvoisier's sign), and ascites. Abnormalities in routine blood tests are non-specific and include hyperglycemia, anemia, and abnormalities in liver function tests [8].

6.4.2 IMAGING

Transabdominal ultrasonography (US) can be used as a standard or first-line diagnostic imaging modality in a patient with upper abdominal pain or to evaluate dilatation of the bile and pancreatic ducts. US is safe, readily available, and cost-effective, but it lacks the sensitivity and specificity to detect pancreatic tumors.

When suspecting a pancreatic tumor, multiphase-multidetector computed tomography (CT) with pancreatic protocol and with intravenous administration of constrast material is the imaging technique of choice for the initial evaluation. Overall, CT detects solid pancreatic masses with a sensitivity of 90% and a specificity of 99% [66,67]. However, in small pancreatic lesions (<2 cm), the sensitivity of CT imaging drops to 70% or below [68]. CT enables visualization of the primary tumor in relation to blood vessels, namely superior mesenteric artery (SMA), celiac axis, superior mesenteric vein (SMV), and portal vein (PV), but also to adjacent and distant organs. By CT imaging, the initial staging and management plan can

generally be confirmed. In diagnosis of vascular invasion by pancreatic cancer, a sensitivity of 71% and a specificity of 92% are reported [69].

Magnetic resonance imaging (MRI) combined with magnetic resonance cholangiopancreatography (MRCP) can be used instead of CT if the patient cannot tolerate intravenous contrast of CT. MRI seems to be as sensitive and specificas CT in assessing the vascular invasion [69]. No significant difference in evaluating the resectability of pancreatic cancer is present between MRI and CT [70]. A disadvantage of MRI is its unsuitability for tissue sampling.

Endoscopic retrograde chloangiopancreatography (ERCP) demonstrates the anatomy of pancreatic and bile ducts, and it can be used as a treatment tool in patients with obstructive jaundice, in whom an endoscopic stent is needed to relieve obstruction [71]. ERCP also allows brushing and lavage of the duct system, providing cells for diagnosis.

Some patients require additional diagnostic imaging. Endoscopic ultrasonography (EUS) can be used in patients with suspected pancreatic cancer, but with no visible mass in CT scan. EUS may be superior to CT in tumor detection and staging, but is comparable in nodal staging and evaluating resectability [72,73]. It is useful in detecting small tumors (<2 cm) that are undetected by other imaging modalities [72]. For EUS, a meta-analysis showed sensitivity of 72% and specificity of 90% for T1-2 stages and sensitivity of 90% and specificity of 72% for T3-4 tumors [74]. When available, it is the preferred method of acquiring tissue for cytology or histological diagnosis. If cytology is needed, CT-guided fine-needle aspiration (FNA) can be carried out. EUS combined with FNA reaches a sensitivity of up to 90% for detecting pancreatic cancer [75].

Postiron emission tomography (PET) is not routinely used in diagnosis or staging since it has no obvious advantage compared with current diagnostic methods [76]. However, it may provide additional information after CT if distant metastases are suspected, but the lesions remain indefinable [77,78]. For defining nodal stage (N status), PET is equal to CT in sensitivity [78]. PET scanning seems not to show any additional advantage in differential diagnostics between chronic pancreatitis and pancreatic cancer relative to other imaging techniques [76]. If none of the imaging techniques clarify the clinical stage of the pancreatic cancer, a diagnostic laparoscopy or laparotomy can be performed to determine metastatic spread.

6.4.3 DIAGNOSTIC TUMOR MARKERS

Research on potential diagnostic tumor markers in PDAC has been intense over the years. Carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic

antigen (CEA) are the only routinely used markers in diagnosis and follow-up. However, they both have limited sensitivity and specificity and they cannot be used in early diagnosis. Numerous potential tumor markers have been investigated, but none has achieved a role in clinical practice [79-83]. In order to describe the extent of studies on potential tumor markers, more than 2500 genes are showed to be involved in pancreatic cancer in more than 2500 studies [84].

CA 19-9, also known as sialylated Lewis A antigen, is an antibody binding to the tumor surface marker sialyl Lewis a [85]. In Caucasian populations, about 5-10% are negative for Lewis a and express no CA 19-9. Thus, negative values do not rule out pancreatic cancer. Sensitivity of CA 19-9 for pancreatic cancer is about 70-90% and specificity 68-91% [86]. In non-jaundiced patients, CA 19-9 may complement other diagnostic methods. However, it must be borne in mind that several benign diseases, such as chronic and acute pancreatitis, liver cirrhosis, cholangitis, and obstructive jaundice, may elevate CA 19-9 levels. Also, in other gastrointestinal cancers, such as bile duct, gastric, colorectal, esophageal, and hepatocellular carcinomas, levels of CA 19-9 can be increased [87]. In addition, CA 19-9 lacks sensitivity in small-diameter (<3 cm), and dedifferentiated PDACs. Even though CA 19-9 fails to meet the criteria for a diagnostic marker, it is a beneficial tool in PDAC assessement. Increased pretreatment CA 19-9 levels >100 kU/l may predict unresectable or metastasized disease, and a decrease in post-treatment CA 19-9 level may be associated with prolonged survival and responsiveness to chemotherapy [88,89].

CEA is usually used in colorectal cancer as a diagnostic and prognostic marker, but it can also be elevated in gastric and pancreatic cancers. In pancreatic cancer, a sensitivity of 40-45 % and a specificity of 81-89% are reported [90,91].

In addition to the conventional biomarkers described above, a great array of novel biomarkers has been introduced, including micro-RNAs and other non-coding RNAs, multimarker panels, proteomics, cytokines, and genetic and epigenetic markers [80-83]. While some progress has been made, the evidence for their efficacy still remains insufficient.

6.4.4 DIFFERENTIAL DIAGNOSIS BETWEEN PANCREATIC CANCER AND CHRONIC PANCREATITIS

Differential diagnosis between pancreatic cancer and chronic pancreatitis (CP) can be challenging, especially in patients with pancreatic mass, which can prove to be either benign or malignant [92]. Mass-forming chronic pancreatitis should preoperatively be differentiated from pancreatic cancer in

order to possibly avoid unnecessary and extensive surgery. Unfortunately, no blood test exists for chronic pancreatitis, and the disease is mainly diagnosed by imaging and symptoms.

Some cases of mass-forming pancreatitis are autoimmune-based. Autoimmune pancreatitis can be divided into two subtypes depending on the relation to IgG4. Type 1 autoimmune pancreatitis is characterized by lymphoplasmacytic sclerosing histological feature, and it is an IgG4-related disease. In contrast, type 2 features neutrophilic infiltration of the epithelium of the pancreatic duct in histology and is not related to IgG4 [93]. Autoimmune pancreatitis and pancreatic cancer share many clinical features such as age over 60 years, obstructive jaundice, new-onset diabetes, and elevated levels of serum tumor markers [94]. The differential diagnosis between autoimmune pancreatitis and pancreatic cancer is based on clinical, serological, imaging, and pathological findings. Imaging techniques include CT/MRI, ERCP, and EUS-guided FNA [95].

In non-autoimmune chronic pancreatitis, serum marker CA 19-9 has its diagnostic limitations and is not sufficient alone to differentiate pancreatic cancer from CP [86]. With CT and MRI imaging, CP is characterized by calcifications of the pancreas, pancreatic duct dilatation, and enlargement or atrophy of the pancreas [75]. In the early stages of CP, these findings may be absent, however. Typically, pancreatic adenocarcinoma is an ill-defined hypodense mass on CT. Smaller cancers (<2 cm) can be isodense, which makes it difficult to detect these cancers. Furthermore, conventional CT may have difficulties in differentiating between inflammatory and neoplastic masses. When using EUS for diagnosis, abnormalities found at the earliest stages of developing pancreatic cancer can be identical to CP [96].

Despite developments in established imaging modalities (MRI, CT, EUS, and PET), differential diagnosis between inflammatory and neoplastic pancreatic masses remains a challenge. New imaging technologies, such as contrast-enhanced EUS, EUS elastography, and molecular imaging, are on their way, but they lack standardized protocols and are in a too early stage of development for clinical practice [75,92].

6.5 TREATMENT

6.5.1 STAGING AND OVERVIEW OF TREATMENT

To evaluate resectability, PDAC is staged based on imaging according to tumor-node-metastasis (TNM) classification. The American Joint Committee on Cancer (AJCC) has recently published a new version (8th edition) of the staging system for pancreatic cancer (Table 2) [97]. A few major modifications were made from the older 7th edition staging system, which was introduced in 2010 [98]. First, instead of designating extrapancreatic invasion, which can be difficult to predict accurately before surgery, T3 tumors are now defined as larger than 4 cm. Second, nodal involvement has been revised from a binary system to a three-class categorization based on extent of nodal involvement: N0, N1 (1-3 positive regional lymph nodes), and N2 (≤4 positive regional lymph nodes) [99,100].

Surgical resection with oncological treatment is regarded as the only curative treatment and can result in significantly longer survival than the other treatment options. In general, stages I and II are considered resectable, and stage IV unresectable. Stage III can be considered both. Pancreatic cancer without distant metastasis can be divided into three more clinically relevant categories: resectable, borderline resectable, and locally advanced. Certain parameters, based on CT imaging, determine PDAC resectability [68,101,102].

A resectable tumor shows no evidence of extrapancreatic disease and there is a patent SMV or PV. Also, a normal tissue plane is identified between the tumor and the celiac trunk, common hepatic artery (CHA), or SMA. With a borderline resectable tumor, there is involvement of SMV or PV with tumor contact ≥180° or bilateral narrowing or occlusion of the vein not exceeding the inferior border of the duodenum [89]. There can also be evidence of tumor abutment <180° without showing deformity or stenosis against celiac trunk, CHA, or SMA. In a locally advanced tumor, which is considered unresectable, SMV or PV is bilaterally narrowed or occluded exceeding the inferior border of the duodenum [89]. Furthermore, arterial involvement of the celiac trunk, CHA, or SMA is advanced with tumor contact or invasion >180°. If there is evidence of metastatic spread (typically to the liver, peritoneum or lung), the tumor is considered unresectable.

Table 2. Staging system for pancreatic cancer according to the American Joint Committee on Cancer (AJCC) 7th and 8th edition. Used with permission of the American College of Surgeons, Chicago, Illinois, USA. The original and primary source for this information is the AJCC Cancer Staging Manual, 7th (2010) and 8th (2017) edition published by Springer International Publishing *[97,98]*.

Stage	Primary tumor (T)	Regional lymph nodes (N)	Distant metastases (M)	Characteristics
AJCC 7th edition				
IA	T1	N0	M0	Tumor limited to pancreas ≤2 cm
IB	T2	N0	M0	Tumor limited to pancreas <2 cm
IIA	T3	N0	M0	Tumor extends beyond pancreas without involvement of CA or SMA
IIB	T1-T3	N1	M0	Regional lymph node metastasis
III	T4	Any N	M0	Tumor involves CA or SMA (unresectable primary tumor)
IV	Any T	Any N	M1	Distant metastasis
AJCC 8th edition				
IA	T1	N0	M0	Maximum tumor diameter ≤2 cm
IB	T2	N0	M0	Maximum tumor diameter between 2-4 cm
IIA	T3	N0	M0	Tumor diameter >4 cm
IIB	T1-T3	N1	M0	Metastasis in 1-3 regional lymph nodes
III	Any T	N2	M0	Metastasis in ≥4 regional lymph nodes
	T4	Any N	M0	Tumor involves CA or SMA (unresectable primary tumor)
IV	Any T	Any N	M1	Distant metastasis

Abbreviations: CA = celiac axis; SMA = superior mesenteric artery

The International Study Group of Pancreatic Surgery (ISGPS) has defined borderline resectable pancreatic cancer in their consensus statement according to the National Comprehensive Cancer Network's (NCCN) previous recommendation [102]. Patients with borderline resectable pancreatic cancer are a challenging subgroup, because they are in the stage between a straightforward resectable disease and a technically unresectable disease. In the presence of venous involvement (SMV or PV), exploration, resection, and possible reconstruction of the vein is recommended, if achievable. On the other hand, arterial resections are associated with increased morbidity and mortality and should not be performed routinely. They are acceptable in selected cases, but in general, patients with arterial infiltration should be first treated with neoadjuvant therapy and then re-evaluated for surgery after the treatment depending on patients' performance status [102]. NCCN has just recently updated the criteria for resectability status [103]. The criteria are described in detail in Table 3.

Patients with borderline resectable pancreatic cancer may benefit from an attempt to downsize the tumor with neoadjuvant therapy [104]. This has the potential to elicit a positive tumor response and to downstage patients eligible for surgery [105]. Neoadjuvant therapy can detect patients with rapidly progressing disease who would not benefit from surgical resection. In contrast, tumor response rates to current neoadjuvant treatments are not high, and delaying surgical resection can also lead to progression of the disease. Because of this, patients undergoing neoadjuvant therapy should have restaging by imaging before the planned surgery [9].

Table 3. Criteria defining resectability status according to National Comprehensive Cancer Network (NCCN) guidelines, version 2.2017. Table modified *[103]* and reproduced with permission by NCCN.

Resectability status	Arterial	Venous
Resectable	No arterial tumor contact (CA, SMA, or CHA)	No tumor contact with SMV or PV; or ≤180° contact without vein contour irregularity
Borderline resectable	Pancreatic head: Solid tumor contact with • CHA allowing resection and reconstruction • SMA of ≤180° • Variant arterial anatomy (presence and degree of tumor contact should be noted) Pancreatic body/tail: Solid tumor contact with • CA of ≤180° • CA of >180° without involvement of aorta, and with intact GDA	Solid tumor contact with • SMV or PV of >180° • SMV or PV ≤180° with contour irregularity, or thrombosis, but suitable for complete resection and reconstruction • IVC
Unresectable	Distant metastasis Pancreatic head: Solid tumor contact with • SMA >180° • CA >180° • First jejunal SMA branch Pancreatic body/tail: Solid tumor contact with • SMA or CA >180° • CA and aortic involvement	• Unreconstructible SMV/PV due to tumor involvement or occlusion • Contact with most proximal draining jejunal branch into SMV

Abbreviations: CA = Celiac axis; SMA = Superior mesenteric artery; CHA = Common hepatic artery; SMV = Superior mesenteric vein; PV = Portal vein; GDA = Gastroduodenal artery; IVC = Inferior vena cava

A significant proportion of pancreatic cancer patients develop jaundice due to biliary obstruction, which can be relieved by endoscopic stenting via ERCP. Preoperative biliary drainage should only be carried out in patients with active cholangitis, or if the resection cannot be scheduled within 2 weeks of diagnosis [105]. Metallic stents may have fewer obstruction- and stent-related complications than plastic stents [106,107]. If the life expectancy of the patient is less than 4 months, a plastic stent can be inserted. In patients who are candidates for neoadjuvant therapy, plastic or metallic stents may be used, with a preference for metallic stents [108]. The disadvantage of metallic stents is that they are expensive and their exchange is more challenging than with plastic stents. Staging laparoscopy is recommended to avoid unnecessary laparotomies, although a pancreatic tumor is staged as resectable by imaging, especially in large tumors and in cases with high CA 19-9 level, because distant metastases are found in 10-20% during the operation [109-111].

6.5.2 PANCREATIC SURGERY

Radical surgery combined with oncological treatment is the only potentially curative treatment. However, while 20% of the patients are candidates for surgery at the time of diagnosis, many of these patients are found to have microscopically positive margins at the time of operation [1,112]. Surgery and possible adjuvant therapy can extend the median survival time from about 5 months (all stages at diagnosis) to 25 months [7,113,114]. The 5-year survival of surgically treated patients can reach about 20% [7,100]. Perioperative mortality from pancreatic resection, ranging from 1% to 4%, is rather low at most centers, especially in high-volume centers, despite the invasive and demanding surgery [7,115,116]. Several studies show that centralization of these operations into high-volume hospitals leads to better patient outcome, fewer complications, and lower mortality [115,117,118]. With pancreatic resection, the goal is to achieve total extirpation of the tumor with clear resection margins ≥1 mm (R0 resection). There is great variation in achieving the R0 resection, which a range from 26% to 74% [119].

The location and size of the tumor determine the type of surgery. Since the majority of operable pancreatic tumors localize in the head of pancreas, the standard surgical procedure is pancreaticoduodenectomy (Whipple procedure) [120]. The procedure consists of removing the head of the pancreas, duodenum, proximal part of jejunum, gallbladder and its cystic duct, common bile duct (ductus choledochus), distal part of stomach (antrum), and regional lymph nodes. During the operation three anastomoses are performed: pancreaticojejunostomy, choledochojejunostomy, and gastrojejunostomy with possible entero-enteral anastomosis. The alternative method is to perform

pancreaticoduodenectomy by a pylorus-preserving method. Current evidence shows no relevant differences in mortality, morbidity, and survival between these two operations [121]. The dissection can be extended along the right hemi-circumference of the SMA to the coeliac trunk to possibly improve clearance and the rate of R0 resection [105].

For patients with tumors in the body or tail of the pancreas, distal pancreatectomy is usually performed. This procedure includes the resection of the body and the tail of the pancreas and usually the spleen. At times, total pancreatectomy is required. A laparoscopic approach in surgery for pancreatic cancer is used in specialized, high-volume centers. The exact role of the laparoscopic approach in pancreatic cancer surgery remains undefined, since there is lack of multi-center randomized trials and standardization of the procedure [122-124]. One recently published randomized trial showed no significant differences in postoperative overall complications between laparoscopic and open pancreatoduodenectomy [125]. By far, open surgery remains the gold standard of care.

Standard lymphadenectomy during pancreaticoduodenectomy is recommended. It comprises the supra- and infrapyloric lymph nodes, the lymph nodes in the anteriosuperior group along the CHA, along the bile and cystic duct, on the posterior aspect of the superior portion of the head of pancreas, on the inferior aspect of the head of pancreas, on the right lateral side of SMA, and on the anterior surface of the superior and inferior portion of the head of pancreas [126]. For tumors of the body and tail of the pancreas, removal of the following lymph nodes is recommended: lymph nodes at the splenic hilum, along the splenic artery, and on the inferior margin of the pancreas [126]. Further, standard lymphadenectomy should include ≥12 lymph nodes to allow adequate pathologic staging. The total number of lymph nodes examined, and lymph node ratio (LNR) (number of involved lymph nodes/number of lymph nodes examined) should be reported. Along with the new 8th AJCC staging edition, LNR has been replaced by N1/N2 status. Attempts to improve survival by extended lymphadenectomy have failed to show survival benefit [127], and the patients who underwent extended lymphadenectomy have considerably increased perioperative complications [128,129]. Hence, extended lymphadenectomy is not recommended [126].

6.5.3 ONCOLOGICAL TREATMENT

6.5.3.1 Neoadjuvant therapy

The goal of neoadjuvant therapy (given prior to operation) is to downsize the tumor from unresectable disease to resectable disease, and, ultimately, to

improve survival of patients. Whether neoadjuvant therapy is given to resectable pancreatic cancer patients or not is considered on a case-to-case basis. Resection rates of up to 70% after neoadjuvant therapy with initially radiographically resectable PDAC are reported [104,130]. Pancreatic resection after neoadjuvant therapy seems to be safe [131]. Neoadjuvant therapy with gemcitabine or FOLFIRINOX (a chemotherapy combination of folinic acid, 5-fluorouracil (5-FU), irinotecan, and oxaliplatin), followed by chemoradiation (combination of capecitabine and radiotherapy), if suitable, is considered if the tumor is local but primarily borderline resectable or unresectable [105].

6.5.3.2 Adjuvant therapy

Postoperative adjuvant therapy reduces the risk for recurrence and improves patient outcome. Chemotherapy with either 5-FU or gemcitabine after pancreatic resection is considered standard adjuvant therapy after surgery, and it improves disease-free and overall survival of PDAC patients [132-134]. 5-FU and gemcitabine seem to be equally effective, with median overall survival of 23.0 and 23.6 months, respectively [113]. The aim of adjuvant therapy after surgery for PDAC is to introduce 6 months (six cycles) of postoperative adjuvant chemotherapy, which can be initiated up to 12 weeks after surgery [135]. Gemcitabine combined with erlotinib seems not to improve disease-free or overall survival of PDAC patients after radical surgery relative to gemcitabine treatment alone [136]. Adjuvant therapy with gemcitabine plus capecitabine compared with gemcitabine alone may have a favorable effect on overall survival (median overall survival 28.0 vs. 25.5 months) [137]. Recently, S-1 treatment (combination of tegafur (prodrug of fluorouracil), gimeracil, and oteracil potassium) was showen to be superior to gemcitabine treatment in a Japanese study (median overall survival 46.5 vs. 25.5 months) [138]. In periampullary cancer, adjuvant therapy after surgery with either 5-FU or with gemcitabine has provided a survival benefit compared with observation only, with median survival of 43.1 and 35.2 months, respectively [139].

Adjuvant radiotherapy is mainly used in the United States and rarely in Europe. The benefit of radiotherapy is controversial [3,68,105,132]. In brief, adjuvant radiotherapy is not generally recommended.

6.5.3.3 Palliative therapy

The goal of palliative therapy is to prolong life expectancy and to reduce symptoms in metastatic or unresectable disease. When the tumor is unresectable and locally advanced, regardless of the treatment method, the average overall survival of these patients remains modest (approximately one

year). The standard care has been 6 months of gemcitabine. By adding radiotherapy or chemoradiation to chemotherapy alone, the median overall survival seems not to be improved [140-142].

In advanced or metastasized disease, much effort has been directed to finding a gemcitabine chemotherapy combination to improve survival. The results have been unsatisfactory. According to a meta-analysis in 2013, combination therapy compared with gemcitabine alone significantly improved overall survival, but the advantage was only marginal [143]. Furthermore, combination therapy induced more treatment-related toxicity. Promising results are reported with gemcitabine treatment combined with nab-paclitaxel, which prolongs the overall survival by about two months (8.5 vs. 6.7 months) [144]. Also, gemcitabine combined with capecitabine improved median overall survival by three months (10.3 vs. 7.5 months) relative to gemcitabine alone [145].

However, major improvement has been achieved with FOLFIRINOX therapy, which is superior to gemcitabine alone in terms of efficacy [146]. Median overall survival in the FOLFIRINOX group was 11.1 months, whereas in the group treated with gemcitabine it was 6.8 months. This multimodal chemotherapy has more toxicity, unfortunately, which limits its usage. FOLFIRINOX is an option for the treatment of patients with metastatic pancreatic cancer and good performance status.

6.6 PROGNOSIS

6.6.1 PATIENT-RELATED PROGNOSTIC FACTORS

The incidence of pancreatic cancer increases with age. However, it is most frequently diagnosed among people aged 65-74 years, with a median age of 70 years at diagnosis. With increasing age, also comorbidities become more common. Patients older than 80 years may have an increased incidence of postoperative mortality, morbidity, and cardiac complications and longer hospital stays than younger patients [147,148]. In general, pancreatic surgery can be recommended only in selected patients older than 80 years. Pancreatic cancer is slightly more common in men than in women, and mortality is also slightly higher in men [1,114]. Diabetes, common among pancreatic cancer patients, is associated with worse survival [149,150].

6.6.2 TUMOR-RELATED PROGNOSTIC FACTORS

Tumor stage and resectability are the main determinants of prognosis in pancreatic cancer [100]. From the resected tumor specimen, many prognostic factors can be determined. Radicality of the resection is one of the key elements. The goal is to achieve radical resection (R0), meaning a ≥1 mm resection margin of tissue without macroscopic or microscopic tumor infiltration. Other prognostic factors of the tumor specimen include tumor size (T stage), lymph-node metastasis (N stage), LNR, differentiation grade, and neural and vascular invasion [116,151-153].

6.6.3 PROGNOSTIC SERUM AND TISSUE TUMOR MARKERS

Tissue biomarkers have been widely investigated over the years with the aim of enhancing prognostication and prediction of clinical outcome after surgery. Despite numerous studies evaluating the potential prognostic value of biomarkers, no biomarker is routinely used in clinical practice. There are a few meta-analyses and reviews recapitulating the current knowledge of these potential biomarkers [154,155] (Table 4). However, the tissue biomarkers studied in this thesis have not been investigated before in PDAC.

Table 4. Independent prognostic and predictive markers in patients with pancreatic ductal adenocarcinoma. This table is reproduced with modifications [154] with permission from the Britsh Journal of Surgery. Studies with <70 patients were excluded from the original table.

Biomarker	Reference	Year	*n*	Hazard ratio (95% CI)	*P*
Self-sufficiency in growth signals					
Cyclin E	Skalicky	2006	118	1·71 (1·12-2·63)	0·013
Ki-67	Karamitopo	2010	77	3·63 (1·9-6·9)	< 0·001
HER2	Komoto	2009	129	1·81 (1·07-3·04)	0·026
IGF2BP3	Schaeffer	2010	127	3·34 (1·92-8·96)	0·051
Midkine	Maeda	2007	75	2·14 (1·29-3·71)	0·003
Insensitivity to growth-inhibitory signals					
MUC2	Takikita	2009	120	1·6 (1·1-2·4)	< 0·05
	Juuti	2003	76	0·6 (0·3-1·0)	< 0·05
SMAD4	Tascilar	2001	249	0·74 (0·55-0·99)	0·042
SMAD7	Wang	2009	71	0·39 (0·18-0·83)	0·014
TGF-ß1	Nio	2005	91	0·49 (0·30-0·79)	0·003
Evasion of apoptosis					
IEX-1	Sasada	2008	78	0·66 (0·50-0·86)	0·002
XAF1	Huang	2010	89	0·48 (0·28-0·82)	0·007
Sustained angiogenesis					
CD34	Fujioka	2001	104	1·94 (1·12-3·36)	0·019
COX-2	Juuti	2006	128	1·6 (1·1-2·4)	0·018
	Matsubayashi	2007	299	1·41 (1·08-1·84)	0·01
Dkk3	Fong	2009	114	0·61 (0·40-0·94)	0·024
PEDF	Uehara	2004	80	0·39 (0·22-0·70)	0·002
Tissue factor	Nitori	2005	113	2·01 (1·21-3·37)	0·008
VEGFR1 (FLT-1)	Chung	2006	76	0·10 (0·02-0·49)	0·004
Tissue invasion and metastasis					
ALCAM/CD166	Kahlert	2009	97	2·87 (1·69-4·87)	< 0·001
Caveolin-1	Suzuoki	2002	79	1·88 (1·04-3·39)	0·036
CCR7	Nakata	2008	89	1·95 (1·04-3·64)	0·036
Cytokeratin 20	Matros	2006	103	2·41 (1·09-2·25)	0·016
CXCR4	Maréchal	2009	71	2·54 (1·27-5·10)	< 0·001
Dysadherin	Shimamura	2003	125	2·17 (1·14-4·14)	0·019
E-cadherin	Shimamura	2003	125	0·55 (0·35-0·85)	0·008
Ezrin	Yeh	2005	73	2·73 (1·65-5·37)	0·03
Galectin-3	Shimamura	2002	104	0·49 (0·29-0·81)	0·006
GDNF	Ben	2010	94	2·10 (1·23-3·58)	0·007
HMGA1	Liau	2008	89	12·47 (2·71-57·52)	0·001
L1-CAM	Ben	2010	94	2·05 (1·20-3·49)	0·009
LI-cadherin	Takamura	2003	102	0·49 (0·28-0·86)	0·01
MMP-7	Yamamoto	2001	70	4·85 (1·22-10·8)	0·022
MUC4	Saitou	2005	135	1·96 (1·13-3·38)	0·017

S100A4	Oida	2006	72	1·81 (1·01-3·27)	0·048
Escape from immune surveillance					
RCAS1	Hiraoka	2002	80	3·09 (1·33-7·21)	0·009
Epigenetic modifications					
Histone H3K4me2	Manuyakorn	2010	140	0·42 (0·27-0·65)	< 0·001
Histone H3K9me2	Manuyakorn	2010	140	0·62 (0·40-0·96)	0·032
Histone H3K18ac	Manuyakorn	2010	140	0·60 (0·40-0·92)	0·018
Histone H3K27me3	Wei	2008	165	0·49 (0·32-0·75)	0·001
Resistance to chemotherapy					
hENT1	Farrell	2009	91	0·40 (0·22-0·75)	0·03
Other markers					
CD133	Maeda	2008	80	2·15 (1·21-3·87)	0·009
HOXB2	Segara	2005	74	2·69 (1·39-5·20)	0·003
LMO2	Nakata	2009	164	0·43 (0·28-0·67)	< 0·001
p97	Yamamoto	2004	83	2·42 (1·11-2·26)	< 0·01
Thymidylate synthase	Hu	2003	132	1·66 (1·05-2·63)	0·029
TROP2	Fong	2008	197	1·8 (1·1-3·1)	0·01

6.6.3.1 CA19-9

Besides being used as a diagnostic marker, CA 19-9 has been studied also as a prognostic marker. Most clinics use the value of 37 kU/l as the standard cut-off point. At this value, CA 19-9 seems not to be an independent prognostic factor. For patients with localized disease undergoing resection, the preoperative CA 19-9 is generally a poor prognostic biomarker because of possible falsely elevated levels in the case of biliary obstruction. Preoperative CA 19-9 levels are most informative when there is no biliary obstruction or when it has been decompressed and the serum bilirubin has normalized. With postoperative values over 180, CA 19-9 can function as an independent prognostic factor [156,157]. Also, the decrease in CA 19-9 level from the baseline value during chemotherapy predicts prognosis [158,159]. Lack of decrease in CA 19-9 levels during chemotherapy seems to have the strongest negative impact on survival. However, patients with very high preoperative CA 19-9 levels may live as long as patients with normal levels, assuming that the CA 19-9 level falls into the normal range after resection [160].

6.6.3.2 Podocalyxin

Podocalyxin-like 1 protein (PODXL) regulates cell-to-cell adhesions through charge-repulsive effects, but also contributes to cell morphology. It is a transmembrane glycoprotein closely related to the hematopoietic stem cell marker CD34 and to endoglycan [161]. PODXL is normally expressed in hematopoietic progenitor cells [162], vascular endothelial cells [163], and renal podocytes [164]. It was first identified in the kidney in helping to maintain filtration pathways [165]. When normal PODXL expression is lost, it is associated with glomerulopathies – mainly with nephrotic syndrome [166].

PODXL overexpression has been described in many cancer types such as leukemia and breast, colorectal, urothelial bladder, hepatocellular, prostate, gastric, esophageal, and lung cancers [167-176]. It is an independent factor for poor outcome in renal cell carcinoma and, breast, colorectal, gastric, and esophageal cancers [168-170,174,175,177,178]. PDAC cells are generally positive for PODXL, but other adenocarcinomas of the biliary and gastrointestinal tract are mainly negative [179]. Membranous PODXL expression correlates with poor prognosis in CRC and urothelial bladder cancer [169-171,178]. Polyclonal antibody is commercially available for PODXL. High cytoplasmic expression of PODXL by a novel monoclonal antibody has been shown to also be a marker of poor prognosis in CRC [180]. At the time of our study of PODXL, no reports existed about the prognostic significance of PODXL in PDAC. Since then, two studies have been published, in which PODXL has been shown to be associated with unfavorable prognosis in pancreatic cancer [181,182].

6.6.3.3 PROX1 and β-catenin

The Wnt/β-catenin signaling pathway takes part in regulating cellular processes, such as organ development and differentiation, and tissue homeostasis in adults [183]. Aberrant signaling can lead to cancer development [184]. A key molecule in this pathway is β-catenin, which is an intracellular protein localizing in cell membrane, cytoplasm, and nucleus. Binding of Wnt ligand to its receptors inhibits β-catenin phosphorylation, which allows β-catenin to escape from degradation. β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it activates a target gene expression via interaction mainly with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors [185,186]. In CRC, most tumors have mutations in key regulatory factors of the Wnt/β-catenin pathway such as adenomatous polyposis coli (APC) or protein β-catenin encoding gene (CTNNB1) [185].

In PDAC, the role of the Wnt/β-catenin signaling pathway is controversial because of varying and sometimes paradoxical effects in the pancreas. Although genetic alterations of the Wnt signaling pathway are involved in PDAC [50], mutations of APC or CTNNB1 are rare [187]. Immunohistochemical membranous expression of β-catenin is correlated with loss of tumor differentiation in PDAC [188]. β-catenin may be upregulated in PDAC by both immunohistochemistry and polymerase chain reaction (PCR) [187,189]. Prognostic significance of β-catenin expression in PDAC has been investigated in a few studies with rather short follow-up times [190-193].

Transcription factor PROX1 has been established to be a downstream target of the Wnt/β-catenin pathway in colorectal tumor neoplastic transformation and progression [194]. PROX1 is a transcriptional regulator and a part of the homeobox transcription factor family [195] with a key role in the development of the central nervous system [196], lens [197], liver [198], pancreas [198], lymphatic system [199], and heart [200]. In addition, it has oncogenic properties through alterations in its expression. Depending on the tissue, it can act either as a tumor suppressor or as an oncogene [201]. PROX1 is less expressed in pancreatic cancer cells than in the normal exocrine pancreas [202]. Gene expression level of PROX1 has been shown to be lower in patients with survival of less than 6 months than in patients with longer survival [202]. Recently, high PROX1 expression was demonstrated to predict better prognosis in gastric cancer [203].

6.6.3.4 UCHL5

The ubiquitin-proteasome system (UPS) is an essential cellular protein degradation system. Protein substrates are targeted for degradation by polyubiquitination [204]. Ubiquitins are small molecules that adhere to protein polypeptide chains and target them for degradation by the proteasome. Before degradation, the attached polyubiquitin chains are removed by deubiquitinating enzymes (DUBs). The human genome contains around 80 known DUBs, from which, some 40 of which are associated with various types of cancer [205,206]. UCHL5/Uch37, a cysteine protease from the family of ubiquitin C-terminal hydrolases (UCHs), is one of the three proteasome-associated DUBs. It interacts with the 26S-proteasome subunit, inducing its DUB activity [207]. Disturbances in this function are associated with malignant processes.

UCHL5 expression levels and intracellular location vary in both cancerous and normal tissues (The Human Protein Atlas Project, available at: www.proteinatlas.org/ENSG00000116750-401 UCHL5/tissue, accessed 23rd of December 2017). In esophageal squamous cell carcinoma, hepatocellular carcinoma, and epithelial ovarian cancer, high UCHL5 expression is reported

to be associated with poor survival and increased cancer recurrence [208-210]. In contrast, strong, but also negative, UCHL5 expression is correlated with better patient survival in lymph node-positive rectal cancer [211]. Before our study, UCHL5 expression has not been reported in PDAC.

6.6.3.5 REG4

The regenerating islet-derived (REG) proteins are a group of small secretory proteins involved in regulation of cell regeneration and proliferation [212,213]. Among the four REG families (1 to 4), REG4 is the most recently discovered. It was identified and isolated in 2001 from a cDNA library of ulcerative colitis tissue [214]. REG4 is physiologically expressed in the colon and small intestine with high expression in enteroendocrine cells [215,216], but not in pancreatic islets. Upregulation of REG4 expression occurs in inflammatory bowel diseases (IBDs) [214,215], but its expression is also increased in many gastrointestinal cancers. In CRC, high tissue expression of REG4 is associated with poor prognosis [217-219], but positive tissue REG4 expression has been shown to be associated with better prognosis in non-mucinous CRC [220]. In gastric cancer, high tissue REG4 expression predicts poor survival [221] and possibly promotes peritoneal metastasis [222]. In gallbladder cancer, positive tissue REG4 expression favors better prognosis [223]. Overexpression of REG4 is also expected to play a role in gastric [224] and colorectal [225] carcinogenesis.

Several reports show REG4 expression in pancreatic cancer cells to be increased relative to normal pancreatic cells, and REG4 to promote invasiveness and proliferation of cancer cells [226-228]. In addition, REG4-expressing pancreatic tumors tend to grow larger, while knockdown of REG4 expression leads to tumor shrinking or impaired growth of cancer cells *in vivo* and *in vitro* [226,229]. More intense resistance to radiation and chemotherapy (mainly gemcitabine) occurs in pancreatic cancer cell models *in vivo* and *in vitro* along with REG4 expression [229,230]. The prognostic value of REG4 expression in PDAC is unknown. Tissue REG1A/B expression is associated with prognosis of PDAC [231].

Elevated REG4 serum levels and positive immunohistochemical staining are present in PDAC patients, suggesting that REG4 may serve as a diagnostic marker [226,231,232].

7 AIMS OF THE STUDY

- To evaluate the relationship of PODXL with clinicopathological parameters and its role as a prognostic marker in PDAC by two different antibodies

- To examine the association of PROX1 and β-catenin with clinicopathological parameters and to determine their role as prognostic markers in PDAC

- To explore UCHL5 tumor tissue expression in PDAC and to assess UCHL5 expression as a prognostic marker in PDAC

- To evaluate tumor tissue and serum REG4 expression in PDAC and to investigate the value of serum REG4 level in differential diagnosis between patients with PDAC and those with CP.

8 PATIENTS AND METHODS

8.1 PATIENTS

Between 2000 and 2011, altogether 188 PDAC patients underwent surgery with curative intent at the Department of Surgery, Helsinki University Hospital, Finland. Of these, 34 patients were excluded from this study: 22 who received neoadjuvant chemotherapy, 8 who were eventually diagnosed with stage IV disease, and 4 in whom stage was not reliably documented. Other types of pancreatic cancer (e.g. cystic or mucinous malignancies) were not included in the study. Patients' median age at surgery was 64 (range 39-83) years. The median follow-up was 2.0 (range 0.2-13.1) years. The TNM staging was based on the 7th edition of the AJCC staging system for pancreatic cancer. For Study IV, the control group for serum REG4 analysis consisted of 34 patients with histopathologically verified CP who underwent pancreatic surgery because of suspicion of malignancy between 2000 and 2008. The median age for this patient group was 54 (range 35-74) years. Clinical data were derived from patient records. The Finnish Population Registry provided survival data, and Statistics Finland provided cause of death for those deceased. Clinicopathological characteristics of the study population are described in Table 5.

All studies followed the tenets of the Declaration of Helsinki and were approved by the Surgical Ethics Committee of Helsinki University Hospital, and the National Supervisory Authority of Welfare and Health (Valvira).

Table 5. Clinicopathological characteristics of the study population for immunohistochemistry.

	PDAC study population
n(%)	154
Age, years	
<65	77 (50.0)
≥65	77 (50.0)
Gender	
Male	84 (54.5)
Female	70 (45.5)
T	
1	11 (7.1)
2	39 (25.3)
3	101 (65.6)
4	3 (1.9)
N	
0	48 (31.2)
1	106 (68.8)
Stage (AJCC 7th edition)	
IA	9 (5.8)
IB	18 (11.7)
IIA	20 (13.0)
IIB	104 (67.5)
III	3 (1.9)
Lymph node ratio	
<20 %	118 (77.6)
≥20 %	34 (22.4)
Missing	2
Histological grade	
1	18 (11.8)
2	110 (71.9)
3	25 (16.3)
Missing	1
Perineural invasion	
Yes	101 (77.1)
No	30 (22.9)
Missing	23
Perivascular invasion	
Yes	43 (34.1)
No	83 (65.9)
Missing	28

8.2 TUMOR TISSUE SPECIMENS (STUDIES I-IV)

Formalin-fixed and paraffin-embedded tumor samples were obtained from the archives of the Department of Pathology, Helsinki University Hospital, Finland. Experienced pathologists re-evaluated all samples for confirmation of the histopathological diagnosis of PDAC. Representative areas of tumor specimens were defined and marked on hematoxylin- and eosin-stained slides for preparation of tissue microarray blocks (TMA). In order to evaluate TMA representativeness compared with whole tissue blocks, six 1.0-mm cores were taken from each tumor block from both the invasive front and the central part of the tumor with a semiautomatic tissue microarrayer (Tissue Arrayer 1, Beecher Instruments Inc., Silver Spring, MD, USA). In Study II, we also chose 13 whole tumor tissue blocks and the corresponding lymph node metastases from the patient cohort to compare PROX1 expression in the tumor and in its lymph node metastases.

8.3 IMMUNOHISTOCHEMISTRY AND ANTIBODIES

Tumor-tissue microarray blocks were freshly cut into 4-μm sections. After deparaffinization in xylene and rehydration through a gradually decreasing ratio of ethanol to distilled water, slides were treated in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) in antibody-specific buffer for 20 minutes at 98°C to retrieve antigen. Staining of sections was performed in an Autostainer 480 (Lab Vision Corp.) by the Dako REAL EnVision Detection system, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark) or by ImmPRESS HRP Polymer Detection Kit, Peroxidase, Anti-Goat IgG (Vector Laboratories, Burlingname, CA, USA). Tissues were incubated with the chosen antibody for one hour or overnight at room temperature. Antibodies and variations in pre-treatment, dilution, and positive control are described in Table 6.

The novel monoclonal in-house antibody (HES9) used in the study I recognizes amino acid residues 189-192 of PODXL. The polyclonal antibody (HPA2110, Atlas Antibodies, Stockholm, Sweden) recognizes amino acid recidues 278-415. The specificity of the polyclonal antibody has been validated by Western blotting and protein arrays, and PODXL protein expression has been mapped by immunohistochemistry in normal tissues and common cancers [233,234].

Table 6. Antibodies for immunohistochemistry.

Antibody	Clone	Source	Pre-treatment	Dilution	Positive control
PODXL HES9	mAb	In-house	Tris-HCl (pH 8.5)	1:800	colon
PODXL	pAb, HPA 2110	Atlas Antibodies, Sweden	Tris-HCl (pH 8.5)	1:250	colon
PROX1	pAb, Anti-human PROX1 Antibody	R&D Systems, USA	Tris-HCl (pH 8.5)/Tris-EDTA (pH 9.0)	1:1500	Colon, lymph node
β-catenin	pAb, Beta-Catenin Antibody	Invitrogen, Thermo Fisher Scientific, USA	Tris-HCl (pH 8.5)/Tris-EDTA (pH 9.0)	1:500	Colon, lymph node
UCHL5	pAb, HPA005908	Sigma Aldrich, USA	Tris-HCl (pH 8.5)	1:800	Colon
REG4	mAb	In-house	Tris-HCl (pH 8.5)	1:50	Colon, lymph node

Abbreviations: mAb = monoclonal antibody; pAb = polyclonal antibody

The monoclonal antibody has previously been described in detail [180]. In brief, mice were immunized with the undifferentiated human embryonic (hEs) stem cell line SA167 (Cellartis, Gothenburg, Sweden, www.cellartis.com). By conventional hybridoma technology, hybridoma cell lines were established that produced monoclonal antibodies against hES cells. The target antigen was identified as PODXL. Both epitopes occur in the extracellular part of the PODXL molecule. The epitope sequence of the HPA2110 matches 100% three protein coding PODXL splice variants (PODXL 001, 005, and 201, The Human Protein Atlas). The fourth splice variant provides an 87% match (PODXL 202). The epitope sequences of the HES9 matches 100% all splice variants.

8.4 STAINING PATTERN AND SCORING OF SAMPLES

Immunostaining was in each project independently scored by two investigators (Studies I, II, and IV: Kapo Saukkonen and Jaana Hagström; Study III: Leena Arpalahti and Jaana Hagström), and they were blinded to clinical data and outcome. Differences in scoring were discussed until consensus was reached. The highest score of each patient was regarded as representative for analysis in Studies I, II, and IV. In Study III, the median

score of each patient served in further analysis because of variation of expression in pancreatic tumor tissue.

In Study I, PODXL expression by monoclonal antibody (mAb) HES9 was evenly distributed in the cytoplasm and was often granular in appearance. By polyclonal antibody (pAb), PODXL expression was also cytoplasmic with no nuclear expression. A distinct membranous positivity emerged in many cases, regardless of cytoplasmic staining intensity. Cytoplasmic staining was scored as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3) according to staining intensity. For cases with membranous staining by the pAb, the score was recorded as 3, regardless of cytoplasmic staining intensity.

In Study II, cytoplasmic staining of PROX1 and β-catenin was scored as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3) according to staining intensity. With β-catenin, also membranous staining was evaluated. In the samples with no membranous staining, there was no cytoplasmic staining either.

In Study III, cytoplasmic and membranous staining of UCHL5 was scored separately. According to staining intensity, cytoplasmic staining was scored as negative (0), low positive (1), moderate positive (2), or high positive (3). Nuclear staining was scored according to the proportion of positive nuclei in the tumor tissue: 0% to 10% positive nuclei scored as 0, 11% to 40% as 1, 40% to 75% as 2, and 76% to 100% as 3.

In Study IV, cytoplasmic staining of REG4 was scored as either negative or positive when any degree of staining was present.

8.5 ELISA (STUDY IV)

The REG4 sandwich ELISA assays were performed using the Human REG4 ELISA Pair Set (SEK11186, Sino Biological Inc., Beijing, China) in accordance with the manufacturer's instructions. The primary anti-REG4 antibody (2 μg/ml in CBS buffer containing 0.05M Na_2CO_3, 0.05M $NaHCO_3$, pH 9.6) was immobilized to a 96-well plate overnight at 4°C, after which the wells were blocked with 1% BSA on 0.05% TBST for one hour at RT. Serum samples of the 130 patients with PDAC and the 34 CP controls were diluted 1:10 in sample buffer (0.1% BSA in 0.05% TBST) and incubated in duplicate wells (100 μl per well) for 2 hours at RT. The HRP-conjugated secondary anti-REG4 antibody (0.5 μg/ml in 0.5% BSA in 0.05% TBST) was allowed to bind for one hour at RT, after which TMB substrate solution was added and allowed to react for 20 minutes at RT. The color reaction was stopped with 1

N H_2SO_4 and the absorbance (450 nm) was measured with a Victor 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA).

8.6 STATISTICAL ANALYSES

For statistical purposes, dichotomization of tumor marker immunohistochemical expression was performed (Studies I-III): PODXL mAb expression into low (scores 0-2) and high (3), PODXL pAb expression into non-membranous (0-2) and membranous (3), PROX1 and β-catenin expression into low (0-1) and high (2-3), UCHL5 cytoplasmic expression into low (0-1) and high (2-3), and UCHL5 nuclear expression into negative (<10% nuclear positivity, score 0) and positive (>10% nuclear positivity, scores 1-3). These divisions were done similarly as in previous studies by immunohistochemistry for more reliable evaluation of the results [178,180,193,203,235].

To evaluate different PODXL antibodies, and β-catenin and PROX1 together, a categorization with three classes was created (Studies I and II): low (PODXL mAb low, and PODXL pAb non-membranous; and PROX1 and β-catenin low), moderate (either PODXL mAb high or PODXL pAb membranous; and either PROX1 or β-catenin high), and high (both PODXL mAb high and PODXL pAb membranous; and PROX1 and β-catenin high).

The association between tumor marker expression and relevant clinicopathological parameters was evaluated by Fisher's exact test or by the linear-by-linear test. The Spearman correlation coefficient was calculated to explore correlations between tumor marker expression and specific laboratory parameters.

To determine the difference between serum REG4 levels of PDAC and CP, the Mann-Whitney U test was used. Higher quarter of interquartile range (IQR) of serum REG4 level of CP patients served as the cut-off point for assessing survival of PDAC patients. PDAC patients were dichotomized into a low (<4.10 ng/ml) and a high group (≥4.10 ng/ml) according to serum REG4 level (Study IV). Receiver operating characteristic (ROC) curves were established, and the area under the curve (AUC) values calculated to evaluate tumor markers. By maximizing Yonden's index, optimal cut-off values were determined. Multivariate logistic regression analysis was used to discover independent risk factors for PDAC (Study IV).

Survival analyses were performed by the Kaplan-Meier method and compared by log-rank test or by Breslow test. The Bonferroni correction was applied for multiple comparisons by dividing the probability level with the number of comparisons. The Cox proportional hazard model was performed

in uni- and multivariate analyses adjusted for age, gender, stage, lymph node status, perivascular invasion, and postoperative adjuvant therapy. To simplify the model, stage and LNR were combined into a single variable since they are internally correlated. Interaction terms were considered. Testing of the Cox model assumption of constant hazard ratios over time involved the inclusion of a time-dependent covariate separately for each testable variable. All tests were two-sided. A p-value of 0.05 or less was considered significant. Statistical analyses were with SPSS (IBM SPSS Statistics, for Mac/Windows; SPSS, Inc., Chicago, IL, USA).

9 RESULTS

9.1 IMMUNOHISTOCHEMISTRY

9.1.1 PODXL (STUDY I)

PODXL expression by the pAb was cytoplasmic in tumor cells, but in some specimens, a distinct membranous expression pattern was apparent, which did not correlate with intensity of the cytoplasmic expression. Such a staining pattern was not visible for the mAb; instead the staining was cytoplasmic and evenly distributed.

PODXL staining by the pAb could be evaluated in 166 specimens (98.8%): 13 (7.8%) showing negative, 71 (42.8%) weak, 55 (33.1%) moderate, and 27 (16.3%) strong staining. By the pAb, non-membranous staining was evaluated in 93 specimens (56.0%), and membranous staining in 73 specimens (44.0%). PODXL staining by the mAb was evaluated in 165 specimens (98.2%): 21 (12.7%) showing negative, 69 (41.8%) weak, 39 (23.6%) moderate, and 36 (21.8%) strong staining. Expression of each tumor marker is presented in Table 7, and representative staining patterns are illustrated in Figure 3.

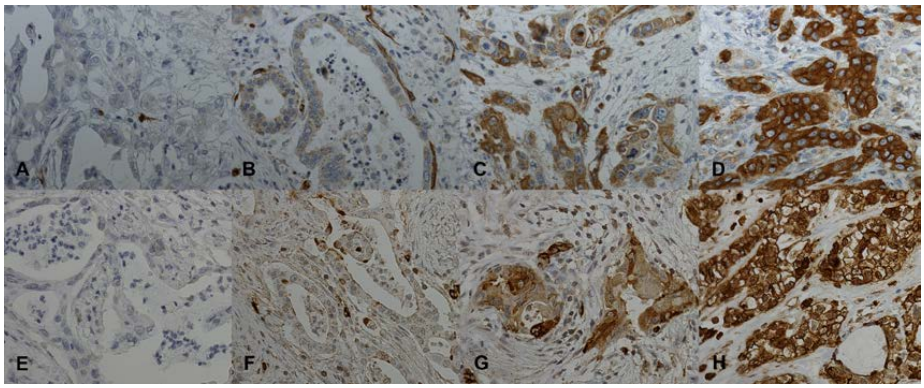

Figure 3. Immunohistochemical staining pattern of PODXL by polyclonal antibody HPA2110 and by monoclonal antibody HES9 in pancreatic ductal adenocarcinoma. Representative images of PODXL expression in PDAC by pAb HPA2110 from A to D (negative-weak-moderate-strong). Representative images of PODXL expression in PDAC by mAb HES9 from E to H (negative-weak-moderate-strong). Modified from Study I with permission by Creative Commons (CC) BY.

9.1.2 PROX1 AND β-CATENIN (STUDY II)

PROX1 expression was evenly distributed in the cytoplasm without distinctive membranous staining. In normal pancreatic tissue at the edge of the tumor, clear nuclear staining was present, even though all of the nuclei were not stained. We detected staining of the nuclei in two cancer tissue samples, and the cytoplasmic staining scores in these samples were 1 and 3. Otherwise, cancer specimens were negative for nuclear staining. In the whole tumor specimens, no nuclear staining was present in metastases, as only negative or weak cytoplasmic staining was present (Figure 4).

β-catenin expression was present both in the cell membrane and within the cytoplasm. In few exceptions, the staining was not uniform in the tumor cell. Cytoplasmic staining was stronger with more intense membranous staining. Cytoplasmic expression pattern showed two different staining types: homogeneous and granular. Nuclear staining was not seen and only three specimens lacked membranous staining (Figure 4). Scoring membranous and cytoplasmic staining separately was not possible. Therefore, cytoplasmic expression was used in statistical analyses.

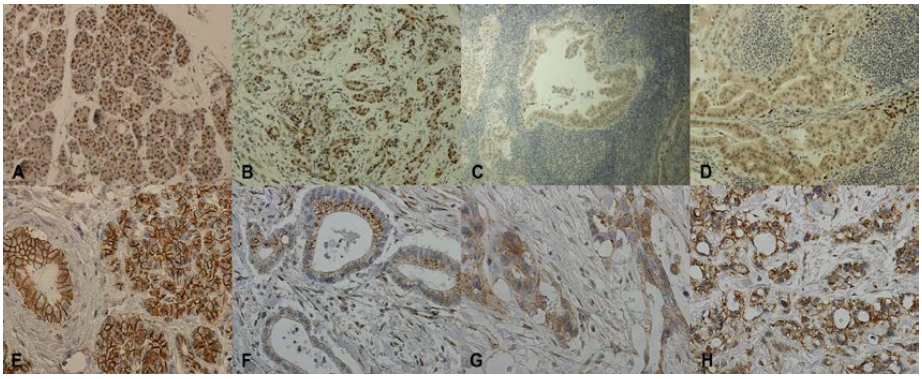

Figure 4. Immunohistochemical staining pattern of PROX1 in normal pancreatic tissue (A), in the transitional zone of normal pancreatic tissue and cancerous tissue (B), and in metastasized lymph node (C-D). Immunohistochemical staining pattern of β-catenin in normal pancreatic tissue (E). Weak cytoplasmic β-catenin expression positivity in PDAC with no membranous expression (F), and with some membranous positivity (G). Moderate cytoplasmic β-catenin expression in PDAC (H). Modified from Study II with permission by CC BY.

PROX1 staining was evaluable in the tumor tissue of 154 specimens (98.7%): 20 (13.0%) showing negative, 60 (39.0%) weak, 66 (42.9%) moderate, and 8 (5.2%) strong staining (Table 7). β-catenin cytoplasmic staining was

evaluable in 153 specimens (98.1%): 1 (0.7%) showing negative, 52 (34.0%) weak, 63 (41.2%) moderate, and 37 (24.2%) strong staining (Table 7). Combined PROX1 and β-catenin expression was evaluated in 152 tumors (97.4%): 38 (25.0%) showing low, 56 (36.8%) moderate, and 58 (38.2%) high staining pattern.

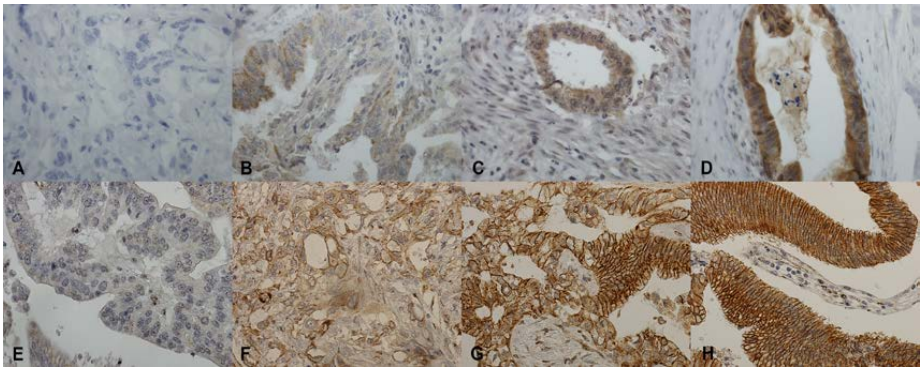

Figure 5. Immunohistochemical staining pattern of PROX1 and β-catenin expression in PDAC. Negative (A), weak (B), moderate (C), and strong (D) cytoplasmic PROX1 expression. Negative (E), weak cytoplasmic and membranous (F), moderate cytoplasmic (G), and strong cytoplasmic and membranous positivity (H) of β-catenin expression. Modifed from Study II with permission by CC BY.

9.1.3 UCHL5 (STUDY III)

When cytoplasmic UCHL5 expression was observed, the staining was uniform and ubiquitous throughout the tumor tissue (Figure 6). Cytoplasmic and nuclear UCHL5 expression could be evaluated in the tumor tissue of 153 specimens (99.4%). The cytoplasmic immunoexpression was negative in 94 (61.4%), low in 51 (33.3%), moderate in 7 (4.6%), and strongly positive in 1 (0.7%) specimen. Nuclear expression was evaluated according to the proportion of positive nuclei in the tumor tissue as 0 in 74 (48.4%), as 1 in 50 (32.7%), as 2 in 20 (13.1%), and as 3 in 9 (5.9%) tumor specimens (Table 7).

The one patient with strong cytoplasmic UCHL5 expression scored 3 for nuclear expression, while the seven patients with moderate cytoplasmic expression scored mainly 0 or 1. Normal-appearing cells next to the tumor tissue showed principally low or negative cytoplasmic staining and a low proportion of nuclear positive staining.

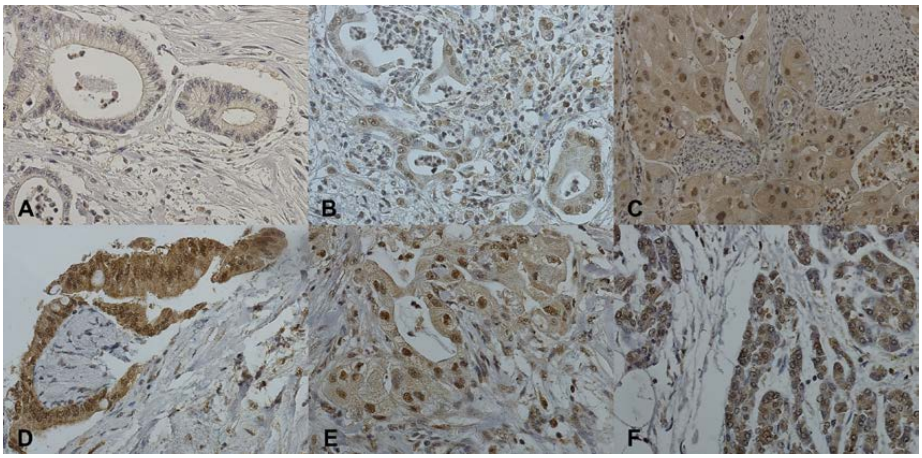

Figure 6. Immunohistochemical staining pattern of UCHL5 expression in PDAC and in normal pancreatic tissue. Negative-low-moderate-strong cytoplasmic UCHL5 expression (A-D). Positive nuclear UCHL5 expression (E). UCHL5 staining in normal-appearing pancreatic tissue next to the tumor tissue (F). Modified from Study III with permission by CC BY NC.

9.1.4 REG4 (STUDY IV)

Positive REG4 immunoexpression in the tumor cells was cytoplasmic with a granular distribution when present. Expression was predominantly located on the apical cell surface with no distinct nuclear expression (Figure 7).

Immunostaining was evaluable in 153 cases (99.4%): 110 (71.9%) were negative and 43 (28.1%) positive (Table 7).

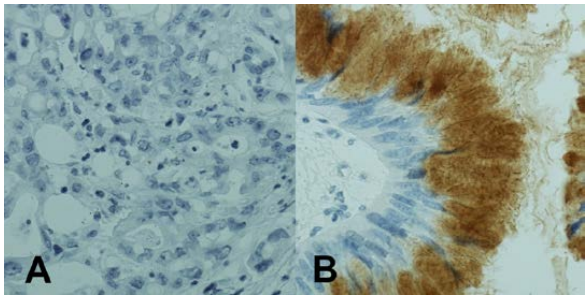

Figure 7. Immunohistochemical staning pattern of REG4 expression. Negative (A) and positive (B) tissue REG4 expression in PDAC. Modified from Study IV with permission by CC BY NC.

Table 7. Immunohistochemical expression of the studied tumor markers in pancreatic ductal adenocarcinoma.

Marker expression	**Staining pattern**				
	Patients	N (%)			
PODXL pAb		**Non-membranous**			**Membranous**
	166	93 (56.0)			73 (44.0)
PODXL mAb		**Negative**	**Weak**	**Moderate**	**Strong**
	165	21 (12.7)	69 (41.8)	39 (23.6)	36 (21.8)
PROX1		**Negative**	**Weak**	**Moderate**	**Strong**
	154	20 (13.0)	60 (39.0)	66 (42.9)	8 (5.2)
β-catenin		**Negative**	**Weak**	**Moderate**	**Strong**
	153	1 (0.7)	52 (34.0)	63 (41.2)	37 (24.2)
UCHL5 cytoplasmic		**Negative**	**Weak**	**Moderate**	**Strong**
	153	94 (61.4)	51 (33.3)	7 (4.6)	1 (0.7)
UCHL5 nuclear		**0**	**1**	**2**	**3**
	153	74 (48.4)	50 (32.7)	20 (13.1)	9 (5.9)
REG4		**Negative**			**Positive**
	153	110 (71.9)			43 (28.1)

Abbreviations: pAb = polyclonal antibody, mAb = monoclonal antibody

9.2 ASSOCIATION OF BIOMARKER EXPRESSION WITH CLINICOPATHOLOGICAL PARAMETERS (STUDIES I-IV)

Membranous PODXL expression by the pAb was associated significantly with advanced T stage of the tumor (p=0.045) and with positive perineural invasion (p=0.005) (Table 8). High PODXL expression by the mAb was associated with poor differentiation of the tumor (p=0.033). Combined PODXL expression with both the pAb and the mAb was associated with poor differentiation of the tumor (p=0.014) and with positive perineural invasion (p=0.007).

For PROX1 expression, a significant association was detected with age; patients with low PROX1 expression were younger than patients with high PROX1 expression (p=0.038) (Table 9). Low β-catenin expression was associated with poor differentiation of the tumor (p=0.025). PROX1 and β-catenin expression correlated with each other (Spearman correlation coefficient=0.371; 95% CI 0.24-0.50; p<0.001).

Patients with positive nuclear UCHL5 expression had smaller tumors than patients with negative nuclear UCHL5 expression (p=0.018), and these patients were also significantly older (p=0.004) (Table 10). Cytoplasmic and nuclear UCHL5 expression showed no significant correlation with each other (p=0.117; Spearman correlation).

Tissue REG4 expression was associated with tumor histological grade; patients with positive tissue REG4 expression had more differentiated tumors than patients with negative tissue REG4 expression (p=0.025) (Table 11). Serum REG4 level showed no significant associations with ordered parameters.

Table 8. Association of clinicopathological parameters with PODXL expression by pAb HPA2110, mAb HES9, and their combination in PDAC.

	PODXL expression by pAb			PODXL expression by mAb			Combined PODXL expression			
	Non-membranous	Membranous		Low	High		Low	Moderate	High	
n(%)	**93 (56.0)**	**73 (44.0)**	**p**	**129 (78.2)**	**36 (21.8)**	**p**	**87 (53.0)**	**45 (27.4)**	**32 (19.6)**	**p**
Age, years										
<65	46 (49.5)	36 (49.3)	1.000	63 (48.8)	18 (50.0)	1.000	43 (49.4)	22 (48.9)	16 (50.0)	1.000
≥65	47 (50.5)	37 (50.7)		66 (51.2)	18 (50.0)		44 (50.6)	23 (51.1)	16 (50.0)	
Gender										
Male	52 (55.9)	40 (54.8)	1.000	73 (56.6)	18 (50.0)	0.570	50 (57.5)	24 (53.3)	17 (53.1)	0.689
Female	41 (44.1)	33 (45.2)		56 (43.4)	18 (50.0)		37 (42.5)	21 (46.7)	15 (46.9)	
T										
1	10 (11.0)	2 (2.7)	**0.045**	10 (7.9)	2 (5.7)	0.776	10 (11.7)	0 (0.0)	2 (6.3)	0.227
2	25 (27.5)	18 (24.7)		31 (24.4)	12 (34.3)		22 (25.9)	12 (26.7)	9 (28.1)	
3	54 (59.3)	50 (68.5)		83 (65.4)	21 (60.0)		52 (61.2)	31 (68.9)	20 (62.5)	
4	2 (2.2)	3 (4.1)		3 (2.3)	1 (2.9)		1 (1.2)	2 (4.4)	1 (3.1)	
Missing	2			2	1		2			
N										
0	26 (28.6)	23 (31.5)	0.733	38 (29.9)	11 (30.6)	1.000	25 (29.4)	14 (31.1)	10 (31.3)	0.829
1	65 (71.4)	50 (68.5)		89 (70.1)	25 (69.4)		60 (70.6)	31 (68.9)	22 (68.7)	
Missing	2			2			2			
Stage (AJCC 7th edition)										
IA	7 (7.9)	2 (2.8)	0.606	7 (5.6)	2 (5.6)	0.531	7 (8.4)	0 (0.0)	2 (6.3)	0.419
IB	9 (10.1)	9 (12.3)		15 (12.0)	3 (8.3)		8 (9.7)	8 (17.8)	2 (6.3)	
IIA	10 (11.2)	10 (13.7)		16 (12.8)	4 (11.1)		10 (12.0)	6 (13.3)	4 (12.5)	
IIB	58 (65.2)	46 (63.0)		80 (64.0)	24 (66.6)		54 (65.1)	28 (62.2)	21 (65.6)	
III	0 (0.0)	3 (4.1)		2 (1.6)	1 (2.8)		0 (0.0)	2 (4.5)	1 (3.1)	
IV	5 (5.6)	3 (4.1)		5 (4.0)	2 (5.6)		4 (4.8)	1 (2.2)	2 (6.3)	
Missing	4			4			4			
Lymph node ratio										
<20%	68 (75.6)	56 (77.8)	0.852	96 (76.8)	27 (75.0)	0.826	63 (75.0)	35 (79.5)	24 (75.0)	0.907
≥20%	22 (24.4)	16 (22.2)		29 (23.2)	9 (25.0)		21 (25.0)	9 (20.5)	8 (25.0)	
Missing	3	1		4			3	1		
Grade										
1	17 (21.2)	7 (11.1)	0.103	20 (18.2)	4 (12.5)	**0.033**	16 (21.6)	5 (12.8)	3 (10.7)	**0.014**
2	53 (66.3)	44 (69.8)		78 (70.9)	18 (56.3)		51 (68.9)	28 (71.8)	17 (60.7)	
3	10 (12.5)	12 (19.1)		12 (10.9)	10 (31.2)		7 (9.5)	6 (15.4)	8 (28.6)	
Missing	13	10		19	4		13	6	4	
Perineural invasion										
Yes	51 (67.1)	57 (87.7)	**0.005**	80 (73.4)	27 (87.1)	0.151	48 (67.6)	32 (75.0)	26 (92.9)	**0.007**
No	25 (22.9)	8 (12.3)		29 (26.6)	4 (12.9)		23 (32.4)	8 (25.0)	2 (7.1)	
Missing	17	8		20	5		16	5	4	
Perivascular invasion										
Yes	23 (30.7)	26 (43.3)	0.151	32 (30.8)	15 (50.0)	0.081	19 (27.1)	15 (41.7)	13 (48.1)	0.039
No	52 (69.3)	34 (56.7)		72 (69.2)	15 (50.0)		51 (72.9)	21 (58.3)	14 (51.9)	
Missing	18	13		25	6		17	9	5	

Fisher's exact test was used for 2x2 tables, and the linear-by-linear association test for tables with more than two rows. Missing data were excluded from the analyses. Modified from Study I with permission by CC BY.

Table 9. Association of clinicopathological parameters with PROX1 expression, β-catenin expression, and their combined expression.

	PROX1 expression			β-catenin expression			Combined PROX1 and β-catenin expression			
	Low	High		Low	High		Low	Moderate	High	
n(%)	**80 (51.9)**	**74 (48.1)**	**p**	**53 (34.6)**	**100 (65.4)**	**p**	**38 (25.0)**	**56 (36.8)**	**58 (38.2)**	**p**
Age, years										
<65	46 (57.5)	30 (40.5)	**0.038**	28 (52.8)	48 (48.0)	0.613	21 (55.3)	32 (57.1)	23 (39.7)	0.121
≥65	34 (42.5)	44 (59.5)		25 (47.2)	52 (52.0)		17 (44.7)	24 (42.9)	35 (60.3)	
Gender										
Male	45 (56.3)	40 (54.1)	0.871	32 (60.4)	52 (52.0)	0.394	23 (60.5)	31 (55.4)	30 (51.7)	0.409
Female	35 (43.7)	34 (45.9)		21 (39.6)	48 (48.0)		15 (39.5)	25 (44.6)	28 (48.3)	
T										
1	5 (6.3)	7 (9.5)	0.274	3 (5.7)	8 (8.0	0.602	2 (5.3)	4 (7.1)	5 (8.6)	0.343
2	18 (22.5)	22 (29.7)		14 (26.4)	26 (26.0)		10 (26.3)	12 (21.4)	18 (31.0)	
3	56 (70.0)	43 (58.1)		34 (64.2)	65 (65.0)		25 (65.8)	39 (69.6)	34 (58.6)	
4	1 (1.3)	2 (2.7)		2 (3.8)	1 (1.0)		1 (2.6)	1 (1.8)	1 (1.7)	
N										
0	23 (28.8)	25 (33.8)	0.602	15 (28.3)	32 (32.0)	0.714	10 (26.3)	17 (30.4)	20 (34.5)	0.436
1	57 (71.2)	49 (66.2)		38 (71.7)	68 (68.0)		28 (73.7)	39 (69.6)	38 (65.5)	
Stage (AJCC 7th edition)										
IA	4 (5.0)	5 (6.8)	0.550	3 (5.7)	6 (6.0)	0.590	2 (5.3)	3 (5.4)	4 (6.9)	0.412
IB	8 (10.0)	10 (13.5)		6 (11.3)	12 (12.0)		4 (10.5)	6 (10.7)	8 (13.8)	
IIA	11 (13.8)	9 (12.2)		5 (9.4)	14 (14.0)		4 (10.5)	7 (12.5)	8 (13.8)	
IIB	56 (70.0)	48 (64.9)		37 (69.8)	67 (67.0)		27 (71.1)	39 (69.6)	37 (63.8)	
III	1 (1.3)	2 (2.7)		2 (3.8)	1 (1.0)		1 (2.6)	1 (1.8)	1 (1.7)	
Lymph node ratio										
<20%	57 (71.3)	60 (83.3)	0.086	40 (75.5)	77 (78.6)	0.687	29 (76.3)	38 (67.9)	49 (87.5)	0.138
≥20%	23 (28.7)	12 (16.7)		13 (24.5)	21 (21.4)		9 (23.7)	18 (32.1)	7 (12.5)	
Missing		2			2				2	
Grade										
1	10 (14.7)	12 (19.0)	0.543	5 (10.9)	17 (19.8)	**0.025**	4 (12.5)	7 (14.0)	11 (22.4)	0.059
2	47 (69.1)	42 (66.7)		29 (63.0)	60 (69.8)		20 (62.5)	36 (72.0)	33 (67.3)	
3	11 (16.2)	9 (14.3)		12 (26.1)	9 (10.5)		8 (25.0)	7 (14.0)	5 (10.2)	
Missing	12	11		7	14		6	6	9	
Perineural invasion										
Yes	49 (73.1)	51 (82.3)	0.291	34 (77.3)	66 (77.6)	1.000	25 (73.5)	32 (76.2)	42 (80.8)	0.438
No	18 (26.9)	11 (17.7)		10 (22.7)	19 (22.4)		9 (26.5)	10 (23.8)	10 (19.2)	
Missing	13	12		9	15		4	14	6	
Perivascular invasion										
Yes	23 (36.9)	19 (32.2)	0.706	19 (43.2)	24 (30.0)	0.169	13 (38.2)	17 (42.5)	13 (26.5)	0.247
No	41 (63.1)	40 (67.8)		25 (56.8)	56 (70.0)		21 (61.8)	23 (57.5)	36 (73.5)	
Missing	15	15		9	20		4	16	9	

Fisher's exact test was used for 2x2 tables, and the linear-by-linear association test for tables with more than two rows. Missing data were excluded from the analyses. Modified from Study II with permission by CC BY.

Table 10. Association of UCHL5 cytoplamic and nuclear expression with clinicopathological parameters.

	UCHL5 cytoplasmic expression			UCHL5 nuclear expression		
	Low	High	p	Negative	Positive	p
n (%)	**145 (94.7)**	**8 (5.3)**		**74 (48.4)**	**79 (51.6)**	
Age (years)						
<65	43 (29.7)	0 (0.0)	0.106	29 (39.2)	14 (17.7)	**0.004**
≥65	102 (70.3)	8 (100.0)		45 (60.8)	65 (82.3)	
Gender						
Male	78 (53.8)	6 (75.0)	0.295	40 (54.1)	44 (55.7)	0.872
Female	67 (46.2)	2 (25.0)		34 (45.9)	35 (44.3)	
T						
1	10 (6.9)	1 (12.5)	1.000	2 (2.7)	9 (11.4)	**0.018**
2	39 (26.9)	1 (12.5)		17 (23.0)	23 (29.1)	
3	93 (64.1)	6 (75.0)		53 (71.6)	46 (58.2)	
4	3 (2.1)	0 (0.0)		2 (2.7)	1 (1.3)	
N						
0	44 (30.3)	4 (50.0)	0.260	21 (28.4)	27 (34.2)	0.488
1	101 (69.7)	4 (50.0)		53 (71.6)	52 (65.8)	
Stage (AJCC 7th edition)						
IA	9 (6.2)	1 (12.5)	0.341	2 (2.7)	8 (10.1)	0.237
IB	16 (11.0)	1 (12.5)		10 (13.5)	7 (8.9)	
IIA	18 (12.4)	2 (25.0)		8 (10.8)	12 (15.2)	
IIB	99 (68.3)	4 (50.0)		52 (70.3)	51 (64.6)	
III	3 (2.1)	0 (0.0)		2 (2.7)	1 (1.3)	
Lymph node ratio						
<20%	111 (77.1)	7 (87.5)	0.685	53 (72.6)	65 (82.3)	0.175
≥20%	33 (22.9)	1 (12.5)		20 (27.4)	14 (17.7)	
Missing	1			1		
Grade						
1	17 (13.5)	1 (16.7)	1.000	6 (9.1)	12 (18.2)	0.258
2	90 (71.4)	4 (66.7)		49 (74.2)	45 (68.2)	
3	19 (15.1)	1 (16.7)		11 (16.7)	9 (13.6)	
Missing	19	2		8	13	
Perineural invasion						
Yes	97 (78.9)	4 (57.1)	0.185	49 (76.6)	52 (78.8)	0.834
No	26 (21.1)	3 (42.9)		15 (23.4)	14 (21.2)	
Missing	22	1		10	13	
Perivascular invasion						
Yes	41 (34.5)	2 (33.3)	1.000	20 (33.3)	23 (35.4)	0.852
No	78 (65.5)	4 (66.7)		40 (66.7)	42 (64.6)	
Missing	26	2		14	14	

Fisher's exact test served for 2x2 tables and the linear-by-linear association test for tables with more than two rows. Missing data were excluded from the analyses. Modified from Study III with permission by CC BY NC.

Table 11. Association of tissue REG4 expression and serum REG4 levels with clinicopathological parameters.

	Tissue REG4 expression			Serum REG4 level		
	Negative	**Positive**	**p**	**Low**	**High**	**p**
n (%)	**110 (71.9)**	**43 (28.1)**		**60 (46.2)**	**70 (53.8)**	
Age (years)						
<65	34 (30.9)	9 (20.9)	0.238	16 (26.7)	23 (32.9)	0.565
≥65	76 (69.1)	34 (79.1)		44 (73.3)	47 (67.1)	
Gender						
Male	63 (57.3)	21 (48.8)	0.371	32 (53.3)	44 (62.9)	0.289
Female	47 (42.7)	22 (51.2)		28 (46.7)	26 (37.1)	
T						
1	7 (6.4)	4 (9.3)	0.892	4 (6.7)	3 (4.3)	0.203
2	30 (27.3)	10 (23.3)		19 (31.7)	16 (22.9)	
3	72 (65.5)	27 (62.8)		36 (60.0)	49 (70.0)	
4	1 (0.9)	2 (4.7)		1 (1.7)	2 (2.9)	
N						
0	35 (31.8)	13 (30.2)	1.000	18 (30.0)	19 (27.1)	0.846
1	75 (68.2)	20 (69.8)		42 (70.0)	51 (62.9)	
Stage (AJCC 7th edition)						
IA	7 (6.4)	3 (7.0)	0.640	3 (5.0)	3 (4.3)	0.844
IB	13 (11.8)	4 (9.3)		7 (11.7)	7 (10.0)	
IIA	15 (13.6)	5 (11.6)		7 (11.7)	10 (14.3)	
IIB	74 (67.3)	29 (67.4)		42 (70.0)	48 (68.6)	
III	1 (0.9)	2 (4.7)		1 (1.7)	2 (2.9)	
Lymph node ratio						
<20%	85 (77.3)	33 (78.6)	1.000	48 (80.0)	51 (73.9)	0.531
≥20%	25 (22.7)	9 (21.4)		12 (20.0)	18 (26.1)	
Missing		1			1	
Grade						
1	7 (6.4)	11 (26.2)	**0.025**	9 (15.3)	6 (8.6)	0.132
2	84 (76.4)	25 (59.5)		43 (72.9)	50 (71.4)	
3	19 (17.3)	6 (14.3)		7 (11.9)	14 (20.0)	
Missing		1		1		
Perineural invasion						
Yes	75 (81.5)	26 (68.4)	0.111	38 (73.1)	46 (79.3)	0.504
No	17 (18.5)	12 (31.6)		14 (26.9)	12 (20.7)	
Missing	18	5		8	12	
Perivascular invasion						
Yes	33 (37.1)	10 (27.8)	0.407	19 (38.0)	18 (32.1)	0.547
No	56 (62.9)	26 (72.2)		31 (62.0)	38 (67.9)	
Missing	21	7		10	14	

Fisher's exact test served for 2x2 tables and the linear-by-linear association test for tables with more than two rows. Missing data were excluded from analyses. Modified from Study IV with permission by CC BY NC.

9.3 SURVIVAL ANALYSES

9.3.1 PODXL (STUDY I)

For patients with membranous PODXL expression by the pAb, CSS was significantly poorer than for patients with non-membranous PODXL expression (p=0.006, Figure 8A). Five-year CSS in the membranous PODXL expression group was 14.0% (95% CI 5.2-22.8%) and in the non-membranous PODXL expression group 24.8% (95% CI 15.0-34.6%).

PDAC patients with high PODXL expression by the mAb had significantly poorer cancer-specific survival (CSS) than patients with low PODXL expression (p=0.001) (Figure 8B). Five-year CSS for PDAC patients with high PODXL expression was 4.4% (95% CI -3.6-12.4%) and for those with low PODXL expression 24.8% (95% CI 16.4-33.3%).

The combination of the mAb and the pAb PODXL expression showed a significantly poorer CSS for patients with high combined expression than for those with low combined expression (p=0.001, Figure 8C). Such a survival difference was not detected between patients with moderate and high combined expression (p=0.37), or between patients with moderate and low combined expression (p=0.020, Bonferroni correction). Five-year CSS for patients with combined high expression was 5.0% (95% CI -4.2-14.2%), for patients with moderate expression 18.7% (95% CI 6.5-30.9%), and for patients with low expression 26.6% (95% CI 16.2-37.0%).

Cox regression uni- and multivariate analyses confirmed these results. In multivariate analyses, high PODXL expression by the mAb and membranous PODXL expression by the pAb were independent markers of poor prognosis in PDAC (HR=2.36, 95% CI 1.47-3.80, p<0.001; and HR=2.03, 95% CI 1.32-3.13, p=0.001, respectively). Their combination was also an independent marker of poor prognosis in PDAC (moderate vs. low HR=2.07, 95% CI 1.25-3.44, p=0.005; and high vs. low HR=2.67, 95% CI 1.55-4.59, p<0.001). The multivariate analysis is presented in Table 12.

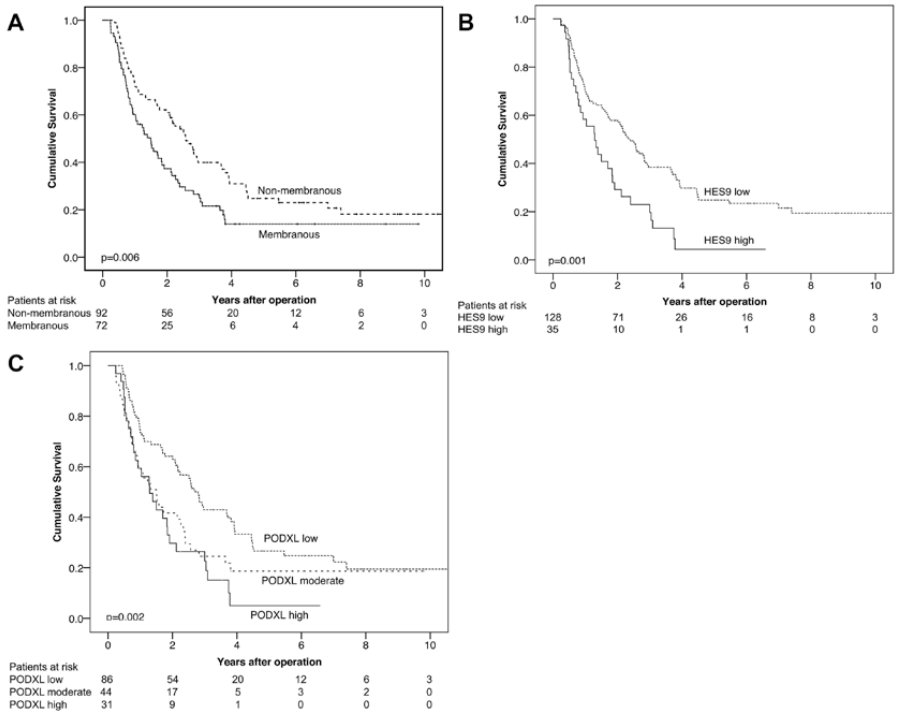

Figure 8. Cancer-specific survival according to the Kaplan-Meier method for PODXL expression by pAb HPA2110 (A), by mAb HES9 (B), and by combined pAb and mAb (C). Modified from Study I with permission by CC BY.

Table 12. Multivariate analysis of relative risk of death from pancreatic ductal adenocarcinoma by PODXL expression.

Polyclonal antibody HPA2110			Monoclonal antibody HES9			Combined		
	HR (95% CI)	**p-value**		**HR (95% CI)**	**p-value**		**HR (95% CI)**	**p-value**
PODXL expression								
Non-membranous	1.00		Low	1.00		Low	1.00	
Membranous	2.03 (1.32-3.13)	**0.001**	High	2.36 (1.47-3.80)	**<0.001**	Moderate	2.07 (1.25-3.44)	**0.005**
						High	2.67 (1.39-3.54)	**<0.001**
Age								
<65	1.00			1.00			1.00	
≥65	1.13 (0.87-1.46)	0.370		1.05 (0.81-1.37)	0.715		1.12 (0.86-1.46)	0.370
Gender								
Female	1.00			1.00			1.00	
Male	1.15 (0.76-1.75)	0.501		1.34 (0.81-1.87)	0.323		1.15 (0.76-1.75)	0.501
Stage and LNR								
IA-IIA	1.00			1.00			1.00	
IIB-III and LNR<20%	1.24 (0.75-2.08)	0.404		1.21 (0.73-2.01)	0.462		1.26 (0.75-2.08)	0.373
IIB-III and LNR ≥20%	3.01 (1.70-5.35)	**<0.001**		2.64 (1.49-4.67)	**0.001**		3.00 (1.67-5.36)	**<0.001**
Perivascular invasion								
No	1.00			1.00			1.00	
Yes	1.88 (1.21-2.91)	**0.005**		1.92 (1.23-3.01)	**0.004**		1.82 (1.17-2.85)	**0.008**

Abbreviations: HR = hazard ratio, CI = confidence interval, LNR = lymph node ratio.

9.3.2 PROX1 AND β-CATENIN (STUDY II)

Five-year CSS did not differ significantly between PDAC patients with low PROX1 expression and those with high PROX1 expression (p=0.174, Figure 9A). Five-year CSS for PDAC patients with low PROX1 expression was 15.5% (95% CI 6.7-24.3%) and for patients with high PROX1 expression, 20.0% (95% CI 9.2-30.8%).

In PDAC patients with low β-catenin expression, five-year CSS was significantly poorer than in patients with high β-catenin expression (p=0.007, Figure 9B). CSS for PDAC patients with low β-catenin expression was 11.3% (95% CI 2.1-20.5%) and 22.4% (95% CI 13.0-31.8%) for those with high β-catenin expression.

Combined low expression of PROX1 and β-catenin showed significantly poorer survival for PDAC patients than combined high expression (p=0.013, Figure 9C). Between patients with combined moderate and low expression (p=0.092), and patients with combined moderate and high expression (p=0.435), no significant difference in CSS was seen. Five-year CSS for patients with combined low expression was 10.3% (95% CI -0.7-21.3%), for patients with combined moderate expression 18.7% (95% CI 9.9-29.5%), and for patients with combined high expression 21.3% (95% CI 8.1-34.5%).

Multivariate analysis showed that both high PROX1 expression (HR=0.63, 95% CI 0.42-0.95, p=0.026) and high β-catenin expression (HR=0.54, 95% CI 0.35-0.82; p=0.004) were independent markers of better prognosis in PDAC (Table 13). The combined high expression of PROX1 and β-catenin also independently showed better prognosis in PDAC (HR=0.46, 95% CI 0.28-0.76, p=0.002).

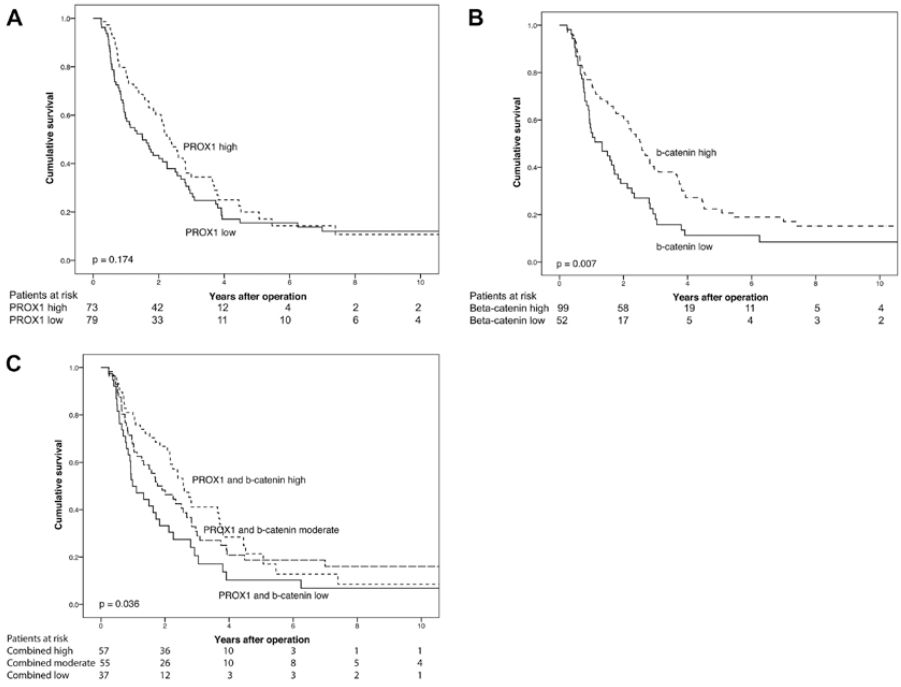

Figure 9. Cancer-specific survival analysis according to the Kaplan-Meier method in PDAC for PROX1 expression (A), for β-catenin expression (B), and for their combined expression (C). Modified from Study II with permission by CC BY.

Table 13. Cox multivariate analysis of relative risk of death from PDAC by PROX1, β-catenin, and their combined expression.

	PROX1 expression		β-catenin expression		Combined PROX1 and β-catenin expression	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Low	1.00		1.00		1.00	
Moderate					0.61 (0.36-1.03)	0.063
High	0.63 (0.42-0.95)	**0.026**	0.54 (0.35-0.82)	**0.004**	0.46 (0.28-0.76)	**0.002**
Age						
<65	1.00		1.00		1.00	
≥65	1.12 (0.75-1.67)	0.589	0.99 (0.66-1.49)	0.977	1.04 (0.69-1.56)	0.847
Gender						
Female	1.00		1.00		1.00	
Male	1.20 (0.80-1.79)	0.379	1.19 (0.79-1.77)	0.403	1.19 (0.80-1.78)	0.398
Stage and LNR						
IA-IIA	1.00		1.00		1.00	
IIB-III and LNR<20%	1.63 (0.98-2.72)	0.061	1.45 (0.87-2.42)	0.152	1.48 (0.88-2.48)	0.141
IIB-III and LNR ≥20%	3.46 (1.91-6.28)	**<0.001**	3.41 (1.89-6.16)	**<0.001**	3.51 (1.94-6.36)	**<0.001**
Perivascular invasion						
No	1.00		1.00		1.00	
Yes	1.96 (1.28-3.01)	**0.002**	2.06 (1.34-3.16)	**0.001**	2.08 (1.35-3.21)	**0.001**
Postoperative adjuvant therapy						
No	1.00		1.00		1.00	
Yes	0.50 (0.33-0.76)	**0.001**	0.45 (0.29-0.68)	**<0.001**	0.47 (0.31-0.71)	**<0.001**

Abbreviations: HR = hazard ratio, CI = confidence interval, LNR = lymph node ratio

9.3.3 UCHL5 (STUDY III)

PDAC patients with either high cytoplasmic UCHL5 expression (p=0.034) or positive nuclear UCHL5 expression (p=0.005) had significantly better five-year CSS. Five-year CSS for patients with high cytoplasmic expression was 57.1% (95% CI 17.2-83.7%) and for patients with low cytoplasmic expression 18.1% (95% CI 12.2-24.9%) (Figure 10A). For patients with positive nuclear UCHL5 expression five-year CSS was 22.1% (95% CI 13.5-32.1%) and for those with negative expression 17.4% (95% CI 9.7-27.0%) (Figure 10B).

Tumor stage categorization demonstrated a more explicit beneficial survival trend with nuclear UCHL5 expression. Differences in survival were not significant in patients with stage IA-IIA disease. However, in stage IIB-III patients with positive nuclear expression five-year CSS was 19.9% (95% CI 10.2-31.9%), whereas in patients with negative expression CSS was 10.4% (95% CI 3.8-20.8%, p=0.007) (Figure 10C). For patients over 65 years and with positive nuclear UCHL5 expression five-year CSS was 19.1% (95% CI 10.3-29.8%) relative to 13.1% (95% CI 5.2-25.1%) for those with negative expression (p<0.001) (Figure 10D). Furthermore, in the subgroup of patients over 65 years, five-year CSS was 15.4% (95% CI 6.3-28.4%) for those with stage IIB-III disease and positive nuclear expression, relative to 8.1% (95% CI 1.7-21.3%, p=0.002) for those with negative expression (Figure 10E). Also, in the subgroup of lymph node-positive patients, a significant survival difference was detected. Patients in this subgroup with positive nuclear UCHL5 expression had five-year CSS of 19.9% (95% CI 10.2-31.9%), while patients with negative expression had CSS of 10.6% (95% CI 3.9-21.2%, p=0.006) (Figure 10F).

Cytoplasmic UCHL5 expression was not an independent prognostic factor in multivariate analysis (HR=0.47, 95% CI 0.17-1.29, p=0.144). However, positive nuclear UCHL5 expression independently predicted better survival (HR=0.63, 95% CI 0.44-0.90, p=0.012) (Table 14).

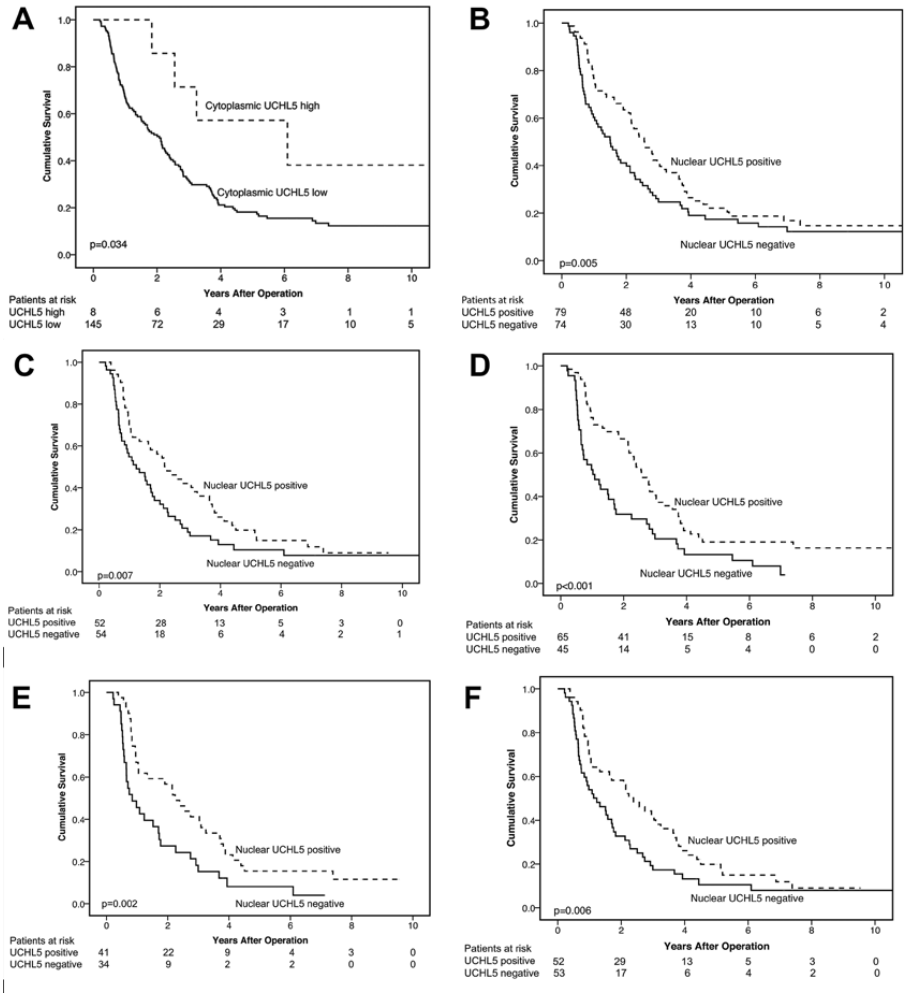

Figure 10. Cancer-specific survival according to the Kaplan-Meier method in PDAC for cytoplasmic UCHL5 expression (A) and for nuclear UCHL5 expression (B). Cancer-specific survival in subgroups of patients with stage IIB-III disease (C), patients over 65 years (D), patients over 65 years and with stage IIB-III disease (E), and patients with lymph node-positive disease (F). Modified from Study III with permission by CC BY NC.

Table 14. Cox multivariate analysis of relative risk of death from PDAC by UCHL5 expression.

	Nuclear UCHL5 expression		Cytoplasmic UCHL5 expression	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
UCHL5 expression				
Negative/Low	1.00		1.00	
Positive/High	0.63 (0.44-0.90)	**0.012**	0.47 (0.17-1.29)	0.144
Age				
<65	1.00		1.00	
≥65	1.18 (0.93-1.49)	0.166	1.14 (0.91-144)	0.254
Gender				
Male	1.00		1.00	
Female	0.91 (0.63-1.32)	0.615	0.97 (0.67-1.40)	0.868
Stage and LNR				
IA-IIA	1.00		1.00	
IIB-III and LNR < 20%	1.59 (1.01-2.49)	**0.046**	1.55 (0.98-2.44)	0.060
IIB-III and LNR ≥ 20%	3.36 (2.02-5.60)	**<0.001**	3.04 (1.83-5.05)	**<0.001**
Postoperative adjuvant therapy				
No	1.00		1.00	
Yes	0.65 (0.45-0.94)	**0.021**	0.71 (0.49-1.03)	0.068

Abbreviations: HR = hazard ratio, CI = confidence interval, LNR = lymph node ratio. Modified from Study III with permission by CC BY NC.

9.3.4 REG4 (STUDY IV)

Neither negative nor positive REG4 tissue expression predicted survival differences in PDAC patients (p=0.496, Figure 11A). Five-year CSS for patients with negative REG4 tissue expression was 19.7% (95% CI 12.1-27.3%) and for patients with positive expression 20.1% (95% CI 7.8-32.4%).

Likewise, CSS did not differ between patients with high and low serum REG4 level (p=0.146) (Figure 11B). Five-year CSS for patients with high serum REG4 level was 16.2% (95% CI 7.4-25.0%) relative to 25.5% (95% CI 14.1-36.9%) for those with low serum REG4 level.

Tumor stage division created a survival benefit in the subgroup of patients with non-metastasized stage IA-IIA disease by serum REG4 level (p=0.046) (Figure 11C). Five-year CSS for patients with high serum REG4 level was 21.3% (95% CI 2.9-39.7%) relative to 52.9% (95% CI 29.2-76.6%) for those with low serum REG4 level.

In patients with histological grade I disease, positive tissue REG4 expression favoured better prognosis when relative to negative tissue expression (five-year CSS 36.4% vs. 0.0%, p=0.006, Figure 11D). No such difference was detected in patients with histological grade II-III.

In multivariate analysis, neither tissue REG4 expression nor serum REG4 level was an independent prognostic factor (HR=0.87, 95% CI 0.55-1.35, p=0.528; and HR=1.18, 95% CI 0.78-1.78, p=0.417, respectively) (Table 15). However, a significant interaction occurred between grade and tissue REG4 expression. The interaction model suggested that positive tissue REG4 expression was a protective factor for survival in patients with grade I disease (HR=0.14, 95% CI 0.03-0.68, p=0.015).

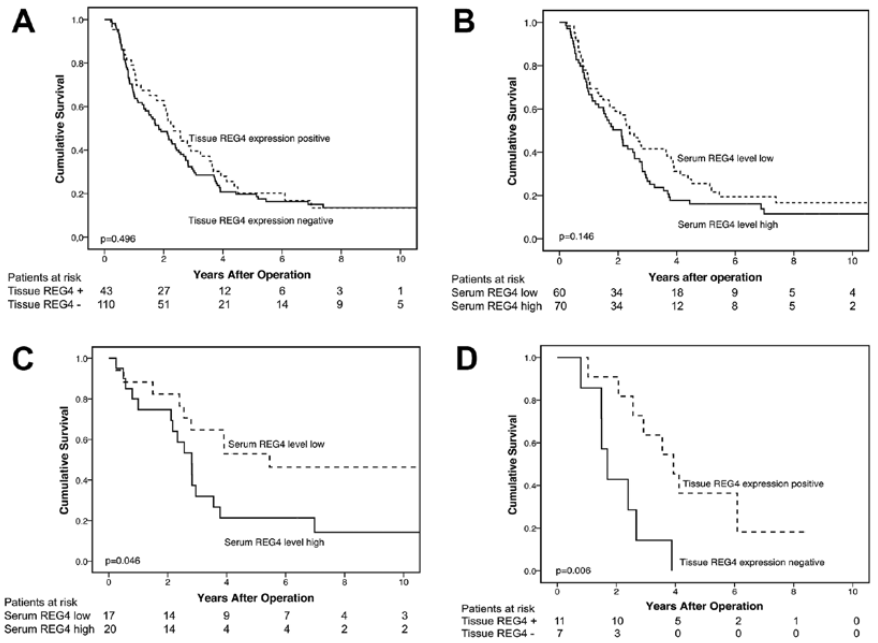

Figure 11. Cancer-specific survival in PDAC according to the Kaplan-Meier method for tissue REG4 expression (A), serum REG4 level (B), serum REG4 level in patients with stage IA-IIA disease (C), and for tissue REG4 expression in patients with grade I disease (D). Modified from Study IV with permission by CC BY NC.

Table 15. Cox multivariate analysis of relative risk of death from PDAC by REG4 expression.

	Tissue REG4 expression		Serum REG4 level	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
REG4 expression				
Negative/Low	1.00		1.00	
Positive/High	0.87 (0.55-1.35)	0.528	1.18 (0.79-1.78)	0.417
Age				
<65	1.00		1.00	0.249
≥65	1.20 (0.79-1.84)	0.393	1.30 (0.83-2.05)	
Gender				
Male	1.00		1.00	
Female	1.06 (0.73-1.53)	0.771	1.29 (0.85-1.96)	0.227
Grade				
1	1.00	**<0.001**	1.00	**0.001**
2	2.14 (1.42-3.24)		2.22 (1.38-3.59)	
3	4.59 (2.01-10.47)		4.93 (1.89-12.87)	
Stage and LNR				
IA-IIA	1.00		1.00	
IIB-III and LNR < 20%	1.55 (0.99-2.43)	0.055	1.95 (1.16-3.28)	**0.012**
IIB-III and LNR ≥ 20%	3.47 (2.09-5.78)	**<0.001**	4.18 (2.34-7.47)	**<0.001**
Postoperative adjuvant therapy				
No	1.00		1.00	
Yes	0.72 (0.49-1.05)	0.088	0.83 (0.55-1.27)	0.387

Abbreviations: HR = hazard ratio, CI = confidence interval, LNR = lymph node ratio

9.4 SERUM REG4 LEVELS AND DIAGNOSTIC ACCURACY (STUDY IV)

9.4.1 SERUM REG4 LEVELS IN PANCREATIC DUCTAL ADENOCARCINOMA AND CHRONIC PANCREATITIS

We assessed serum REG4 levels in 130 PDAC patients. The median serum REG4 level in PDAC was 4.90 (range 1.0-59.1) ng/ml. Serum REG4 levels were significantly higher in PDAC patients than in CP patients (median 4.90 vs. 3.05 ng/ml; p=0.002, Figure 12 and Table 16). Table 16 summarizes serum REG4 levels in different stages of PDAC. Significant differences between tumor stages in serum REG4 levels were not found.

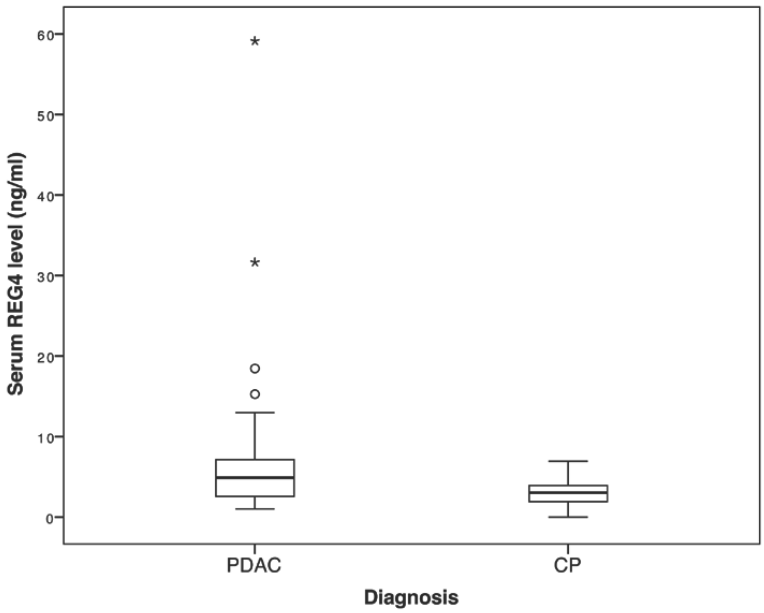

Figure 12. Serum REG4 levels are significantly higher in pancreatic ductal adenocarcinoma (PDAC) than in chronic pancreatitis (CP) (median 4.90 vs. 3.05 ng/ml; p=0.002, Mann-Whitney test). Reprinted from Study IV with permission by CC BY NC.

Table 16. Serum REG4 levels in pancreatic ductal adenocarcinoma and in chronic pancreatitis.

	REG4 level (ng/ml)	p-value*
Stage IA (n=6)		**0.197**
Mean	4.45	
Median	3.80	
Range	2.1-8.4	
IQR	3.8 (1.9-5.7)	
Stage IB (n=14)		**0.134**
Mean	5.10	
Median	4.34	
Range	1.5-12.4	
IQR	5.2 (1.7-6.9)	
Stage IIA (n=17)		**0.007**
Mean	5.59	
Median	5.18	
Range	1.3-11.1	
IQR	4.5 (2.9-7.4)	
Stage IIB (n=90)		**0.003**
Mean	5.89	
Median	4.90	
Range	1.0-59.1	
IQR	4.4 (2.7-7.1)	
Stage III (n=3)		**0.293**
Mean	8.41	
Median	4.63	
Range	2.1-18.5	
IQR	-	
PDAC total (n=130)		**0.002**
Mean	5.76	
Median	4.90	
Range	1.0-59.1	
IQR	4.6 (2.6-7.2)	
Chronic pancreatitis (n=34)		**-**
Mean	3.11	
Median	3.05	
Range	0-6.9	
IQR	2.1 (2.0-4.1)	

IQR= Interquartile range. PDAC = pancreatic ductal adenocarcinoma. *Mann-Whitney U test was used for comparing serum REG4 levels in different stages of PDAC and chronic pancreatitis. Serum REG4 levels did not differ significantly between different stages of PDAC (data not shown). Modified from Study IV with permission by CC BY NC.

9.4.2 SERUM REG4 LEVEL AS A DIAGNOSTIC MARKER

To evaluate and compare the diagnostic accuracy of REG4, CA19-9, and CEA, ROC analysis was performed. AUC value for REG4 was 0.675 (95% CI 0.587-0.763, p=0.002), for CA19-9 0.806 (95% CI 0.737-0.874, p<0.001), and for CEA 0.544 (95% CI 0.437-0.650, p=0.460) (Figure 13). With an optimal cut-off value of 5.3 ng/ml, serum REG4 sensitivity was 45% and specificity 91%. Serum CA19-9 had a sensitivity of 81% and a specificity of 74% with an optimal cut-off value of 17 kU/l.

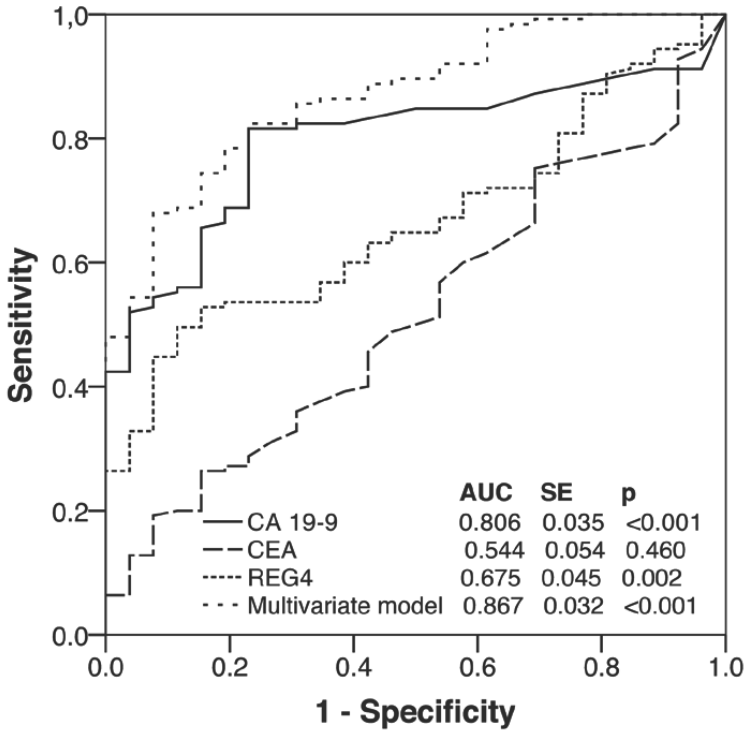

Figure 13. Comparison of serum REG4, CA 19-9, CEA, and calculated probability of cancer with Receiver Operating Characteristic (ROC) analysis in 130 patients with pancreatic ductal adenocarcinoma. 34 patients with chronic pancreatitis served as controls. AUC = area under curve; SE = standard error. Reprinted from Study IV with permission by CC BY NC.

Serum REG4 and CA19-9 were significant and independent risk factors for PDAC in a multivariate logistic regression model adjusted for age (Table 17). This suggests that their combination might be able to improve diagnostic accuracy. The logistic regression model achieved an AUC of 0.867 (95% CI 0.804-0.930, p<0.001), which numerically exceeds the AUCs of REG4 and CA19-9 alone (Figure 13). At the optimal cut-off, sensitivity of 85% and specificity of 79% were achieved.

Table 17. Multivariate logistic regression analysis for risk of pancreatic ductal adenocarcinoma (PDAC).

Variable	OR	95% CI	P value
Age	1.10	1.04-1.16	<0.001
Serum REG4 level	7.88	1.13-54.92	0.037
Serum CA 19-9 level	3.06	1.62-5.81	<0.001

Logarithmic transformation performed for REG4 and CA 19-9 variables. OR = odds ratio; CI = confidence interval. Modified from Study IV with permission by CC BY NC.

9.5 REG4 CORRELATIONS

Tissue REG4 expression and serum REG4 level did not correlate significantly with each other in PDAC (p=0.289, correlation coefficient (r)=0.094, standard error (SE)=0.100). Neither was there a significant correlation between C-reactive protein (CRP) values and tissue REG4 expression (p=0.885, r=0.013, SE=0.096) and serum REG4 level (p=0.318, r=0.089, SE=0.088). Additionally, no correlation was present between CRP values and serum REG4 levels when classified by tumor histological grade. Serum REG4 level correlated with CEA level (p=0.036, r=0.188, SE=0.088), but not with CA19-9 (p=0.975, r=0.003, SE=0.092). CA19-9 and CEA levels did not differ significantly between low and high serum REG4 level groups (data not shown).

10 DISCUSSION

10.1 PODOCALYXIN

Study I showed PODXL to be an independent marker of poor prognosis in PDAC. Positive PODXL expression in PDAC has been demonstrated earlier [236], and it can differentiate PDAC from other adenocarcinomas occurring in the biliary tract [179]. However, immunopositivity of PODXL in these studies was only 44-69%, whereas immunopositivity of 87-92% was achieved in our study. Explanations for the difference may be dissimilar staining techniques, evaluation of specimens, cut-off points, and antibodies used. Immunostaining of PODXL by the new in-house mAb gave similar prognostic results to the commercial pAb (HPA2110).

In cancer, poor prognosis may be associated with membranous PODXL expression rather than with cytoplasmic expression [169,237]. An earlier study supports these findings by demonstrating that aberrant PODXL expression enhances the disruption of cell-to-cell and cell-to-ECM adhesions, leading to tumor dissemination [238]. In PDAC, the staining pattern by the mAb was mainly cytoplasmic, with no clear membranous immunopositivity. The reason for this is unknown. The proportion of tumors with high cytoplasmic or membranous PODXL expression in PDAC was quite large (21.8% and 44.0%, respectively) compared with the corresponding proportions in studies of colorectal, breast, urothelial bladder, and gastric cancer [168-171,174,178,180].

The antibodies used in Study I recognize different epitopes of the PODXL molecule, which may explain the variation of their expression pattern and the case-by-case expression difference. The pAb may recognize an active form of PODXL at the cell membrane, whereas the mAb may recognize overexpression of cytoplasmic PODXL [178]. Patients with both high mAb expression and membranous pAb expression had even worse prognosis in CRC than patients with one of the two [178]. We found a similar trend in PDAC. Only a small difference was present between the two antibodies as prognostic markers. However, PODXL was an independent prognostic factor in multivariate analysis independent of the antibody. Five-year CSS was lower for patients with high PODXL expression by the mAb than for patients with membranous PODXL expression by the pAb (4.4% vs. 14.0%), which supports the role of cytoplasmic overexpression of PODXL as a marker of poor prognosis.

Since our study, two studies have reinforced the hypothesis of positive PODXL expression being a marker of poor prognosis in pancreatic cancer

[181,182]. In one of these studies, membranous PODXL expression was shown to be an independent marker of poor prognosis in intestinal-type periampullary carcinomas, but not in pancreatobiliary-type carcinomas [181]. The problem with comparing the results is that intestinal-type periampullary carcinoma (derived mainly from duodenum or papilla Vateri) is not, de facto, pancreatic ductal in origin. PODXL is known to promote pancreatic cancer cell motility and invasion by binding to the cytoskeletal protein gelsolin [182]. It seems that both intracellular changes enhancing the cancer cell motility and invasion and cell-to-cell interactions leading to metastatic spread are important factors in pancreatic tumor advancement [182,236,238,239].

10.2 PROX1 AND β-CATENIN

In Study II we showed high tissue expression of PROX1 and β-catenin independently to predict better prognosis in PDAC. Loss of PROX1 in the pancreas leads to size reduction [240], to premature acinar cell differentiation, and to increased ductal cell proliferation [241], which makes PROX1 expression vital for pancreatic development. In 2006, pancreatic cancer cells were demonstrated to express less PROX1 mRNA than normal exocrine pancreatic cells, and PROX1 gene expression levels were lower in pancreatic cancer patients with survival less than 6 months [202]. Our study showed a similar tendency by immunohistochemistry, although the difference in survival was not significant. To date, there are no other prognostic studies on PROX1 protein expression in PDAC.

Increased PROX1 expression has been demonstrated to be associated with poor prognosis and with dedifferentiated tumor grade in CRC without being an independent prognostic factor [235]. These findings in CRC were contrary to our results in PDAC. PROX1 is considered to be required in the formation of lymphatic vasculature [199], and overexpression of PROX1 in blood endothelial cells can induce lymphatic endothelial cell gene expression [242]. However, active lymphangiogenesis may not be needed for lymphovascular spread in pancreatic cancer [202]. Recent data show positive PROX1 expression to correlate with positive lymph node metastases in CRC [243]. In gastric cancer, the role of PROX1 expression in patient survival is still under debate since positive or high PROX1 expression has been reported to be a marker of both poor and favorable prognosis [203,244].

We evaluated the cytoplasmic staining of PROX1, while the nuclear staining has been assessed in earlier studies of CRC, hepatocellular carcinoma (HCC), and gliomas [235,245,246]. In recent studies of gastric cancer, also cytoplasmic PROX1 expression by IHC was evaluated and shown to correlate

with PROX1 mRNA amplification [203,247]. Although nuclear staining is present in the normal pancreas, we noted nuclear staining in only two tumor specimens. Decreased nuclear expression and retained cytoplasmic expression of PROX1 in cancerous tissue suggest that PROX1 may not function as an active transcription factor in PDAC. In papillary thyroid cancer (PTC), the role of cytoplasmic PROX1 expression was studied, with the discovery that PROX1 became inactivated through mRNA downregulation by aberrant NOTCH signaling, and cytoplasmic mislocalization of PROX1 increased protein stability in PTC cells [248]. Restoration of PROX1 impaired tumor formation and reduced invasiveness of PTC cells.

Whether downregulation of PROX1 in the nuclei results from the evolved pancreatic cancer or leads to pancreatic cancer formation remains unknown. We can only speculate whether cytoplasmic PROX1 in pancreatic tumor tissue is in active or inactive form because of the limitations of IHC. Two main questions remain to be clarified: the role of cytoplasmic PROX1 expression and the signal leading to relocation of PROX1 into the cytoplasm [248].

β-catenin expression is predominantly localized in the membrane of ductal cells in the normal pancreas. In pancreatic tumors, increased β-catenin expression and protein levels are reported [187]. However, in gene array analysis, inhibition of the Wnt/β-catenin signaling pathway blocked proliferation and induced apoptosis of cultured PDAC cells [189]. β-catenin expression by IHC in PDAC has been more widely studied [188,190-193,249], but there is considerable controversy between the results of these studies. Reduced or abnormal β-catenin expression predicted poor prognosis in PDAC patients in two studies [190,192]. Another study showed that reduced membranous and positive cytoplasmic expression of β-catenin was associacted with poorer survival in PDAC during a one-year follow-up [191]. One study found no prognostic impact of β-catenin cytoplasmic expression on survival in PDAC patients [193]. These results differ from ours. However, two major differences exist when comparing the results. Firstly, the follow-up times in these previous studies are only one or two years, and secondly, the patient cohorts have been small (n= 43 to 48). Our study revealed by IHC that β-catenin expression in PDAC is both membranous and cytoplasmic with no distinct nucler staining and high β-catenin expression predicts better prognosis.

PROX1 and β-catenin expression were also studied together because they are linked in the same signaling pathway and their coactivation or coexpression is increased in CRC [194,235,250]. Moreover, it was recently shown that a transcriptional coactivator deleted in breast cancer (DBC1) acts as a positive regulator and as a key factor of the β-catenin-PROX1 signaling axis in CRC

progression [251]. We demonstrated by IHC that expression of both PROX1 and β-catenin is decreased in PDAC patients, and their expression is correlated significantly. These results are in contrast to those in CRC. It remains unclear whether PROX1 and β-catenin function in a similar, albeit opposite, way in PDAC and CRC. We could not find any further significant prognostic effect with combined PROX1 and β-catenin expression relative to β-catenin expression alone.

10.3 UCHL5

Study III showed nuclear positive and potentially high cytoplasmic UCHL5 expression to be markers of better prognosis in PDAC patients. For patients with positive nuclear UCHL5 expression, differences in survival were discovered in subgroups of patients over 65 years, patients with stage IIB-III disease, and patients with lymph node-positive disease.

In pancreatic cancer samples, low to moderate staining of UCHL5 is distinguishable according to the Human Protein Atlas. Until now, only a few publications have analyzed UCHL5 by immunohistochemistry in cancer. In esophageal squamous cell carcinoma, while higher UCHL5 expression has been associated with decreased survival, UCHL5 has failed to be an independent prognostic factor [208]. In HCC, UCHL5 expression has been reported to be an independent predictive factor for cancer recurrence, but not for overall survival [209]. In addition, immunopositive cytoplasmic UCHL5 expression has been shown to be an independent factor for poor prognosis in epithelial ovarian cancer [210]. In contrast to this data, we have demonstrated that UCHL5 nuclear positivity and probably also high cytoplasmic expression are associated with better survival in PDAC patients. In order to support the survival benefit of UCHL5 immunopositivity, high as well as negative cytoplasmic UCHL5 expression has correlated with increased survival in lymph node-positive rectal cancer [211]. However, nuclear UCHL5 positivity was infrequent in CRC. In PDAC, high cytoplasmic UCHL5 expression appears to be associated with better survival, but the significance of these findings remains unknown due to the small number of patients in this cohort. Confirmation of this survival benefit in another PDAC patient cohort is required.

Interacting with the proteasome, UCHL5 functions as its negative regulator [252]. High levels of nuclear UCHL5 may lead to disproportionate inhibition of the proteasome, and gradually, to accumulation of proteosomal substrates destructive to the cell. Molecular mechanisms of how UCHL5 functions in both cancerous and healthy tissues need to be clarified. Interestingly, PDAC patients with positive nuclear UCHL5 expression tended to be older and to

have smaller tumors. Aging causes disturbances in proteostasis [253], and dysfunction of the proteasome is associated with age-related disorders. Therefore, one can speculate that high UCHL5 expression reduces proteasome activity in the elderly, predisposing tumor cells to apoptosis.

In Study II, we demonstrated that high β-catenin expression is associated with better survival. Because the expression levels of β-catenin are regulated by proteosomal degradation [254], the UCHL5-mediated decrease in proteasome activity might elevate β-catenin levels in tumor cells. PSCs, which are responsible for ECM and tumor-promoting growth factor production in the stroma, are involved in PDAC progression [255]. Smad7 and TGF-β signaling were recently reported to affect PSC-induced cancer cell migration [256]. As UCHL5 is linked to Smad and TGF-β signaling [257-259], it may also play a role in the desmoplastic reaction. However, this hypothesis requires further investigation.

One decade ago, the proteasome inhibitor bortezomib was approved for treatment of multiple myeloma [260,261] and mantle cell lymphoma [262]. Proteasome inhibitors are potent therapeutic agents with a limited therapeutic window, dose-dependent toxicity, and marked drug resistance [263]. There is growing interest in alternative therapeutic agents modulating the UPS, including DUBs [264,265]. Pharmocological inhibition of the two proteasome-associated DUBs, Usp-14 and UCHL5, has been shown to enhance cytotoxicity in cancer cells and to inhibit tumor growth [264,266]. Hence, targeting proteasome-associated DUB activity may provide an alternative strategy to anti-cancer therapy in the future.

10.4 REG4

In Study IV, we demonstrated REG4 expression to predict survival in PDAC patients with early disease (stages IA-IIA) and in those with well-differentiated tumors. Also, significant differences in serum REG4 levels between PDAC and CP were detected. Both REG4 and CA19-9 provided independent diagnostic information, which suggests that REG4 may become useful as a diagnostic marker in PDAC.

Until now, there have been no studies showing the prognostic significance of REG4 expression in PDAC. Recently, low REG1A/B tissue expression was reported as a marker of poor prognosis in PDAC [231]. Typically, REG4 is not expressed in the exocrine pancreas, contrary to REG1A/B [214]. Additionally, the REG4 gene is located on chromosome 1, whereas other REG genes are on chromosome 2 [214,215]. One can hypothesize that the biological functions

of these proteins are distinct from each other because of the location on different chromosomes and encoding by different transcription factors.

REG4 expression has been more widely studied in other gastrointestinal cancers. In CRC, high tissue REG4 expression predicts poor survival by immunohistochemistry [219] and by mRNA expression [218]. However, contradictory results also exist, as positive REG4 tissue expression was shown to predict better survival in non-mucinous CRC [220]. In gastric cancer, high tissue REG4 expression predicts poor survival [221] and peritoneal metastasis [222], while in gallbladder cancer, positive tissue REG4 epression is associated with better prognosis [223]. In our study, PDAC patient survival did not differ significantly according to positive or negative tissue REG4 expression or high or low serum REG4 level.

Interestingly, patients with early stage (IA-IIA) disease show better prognosis with lower serum REG4 levels. Animal models and cell cultures have demonstrated pancreatic tumors to grow larger when REG4 expression is introduced [227-229]. Possibly, negative or low-REG4-expressing pancreatic tumors grow slower and are better differentiated in humans. We also showed positive tissue REG4 expression to predict better prognosis in PDAC patients with histological grade 1 disease. The same tendency has previously been discovered with REG1A/B expression, but not with REG4 expression [231].

REG4 expression is also upregulated in inflammatory processes such as IBD [214,215]. Increased REG4 expression protects against acinar cell necrosis in experimental pancreatitis in mice, which suggests that REG4 may have anti-inflammatory features [267]. We speculate that in well-differentiated pancreatic tumors REG4 expression may have a protective function against cell dedifferentiation. However, we found no correlation between histological grades and REG4 or CRP values. The possible anti-inflammatory role of REG4 warrants further investigation.

We demonstrated that in PDAC and CP serum REG4 levels differ significantly. All patients with CP had undergone extensive surgery in order to exclude malignancy, but histopathology of the surgical samples confirmed the diagnosis of CP. To the best of our knowledge, this was the first study to report such a significant difference. The cut-off value for REG4 in our series was moderately higher than in previous studies. In one study, a cut-off point of 4.53 ng/ml was used, but neither sensitivity nor specificity was reported [226]. In another study, a cut-off point of 3.49 ng/ml with 94.0% sensitivity and 64.0% specificity was described [232]. Nevertheless, in both of these studies, patients with stage IV PDAC were included, and the comparisons of REG4 levels were between PDAC and healthy subjects. We excluded stage IV patients and compared serum REG4 levels between PDAC patients and CP

patients, which is a clinically more relevant comparison. This probably explains the differing results between these studies.

In gastric cancer, REG4 has been shown to be more accurate than CEA or CA19-9 [268]. In ovarian cancer, REG4 expression can serve as a diagnostic serum biomarker for differentiating mucinous ovarian cancer from other epithelial ovarian cancer subtypes, and it may be useful in follow-up [269]. In mucinous ovarian carcinomas, serum REG4 levels were nearly 40 times higher than in serous ovarian carcinomas before surgery, and they declined rapidly after surgery. It would be interesting to study REG4 levels during follow-up of PDAC.

10.5 STRENGTHS AND LIMITATIONS OF STUDY MATERIALS AND METHODS

Patients who were treated with neoadjuvant therapy were excluded from the study cohort because it is largely unknown how neoadjuvant treatment affects the morphology of the tumor tissue. One cannot directly compare immunohistochemistry between patients who have received neoadjuvant therapy with those who have not. Evidence indicates that chemotherapeutic agents have immunologic effects on the tumor microenvironment [270-272], altering the composition of the tumor tissue.

The TMA technique used in all four studies allows analysis of large patient cohorts with relative ease and accuracy. However, only small areas of the tumors are evaluated compared with whole tissue sections. By taking cores from different parts of the tumor, possible sampling error can be diminished. Only a small part of the specimens were lost in the study for technical reasons. The strength of this study is a rather large patient cohort with a long follow-up time. Sadly, due to the long period of data collection (approximately 10 years), some of the crucial clinicopathological parameters and serum samples were unavailable. Also, one of the weaknesses of the study is the lack of knowledge of a reliable resection margin status (R0/R1). This arises from the fact this study being retrospective; only in the last few years have clinicians and pathologists directed attention to this important matter.

The study population was homogeneous as all histological specimens were re-evaluated and only ductal adenocarcinomas were included in the study. In Study IV, the clinical diagnostic dilemma between PDAC and CP was clinically relevant, bearing in mind that CP patients underwent extensive surgery because their diagnosis coud not be determined by preoperative testing.

10.6 CONCLUDING REMARKS

Cancer treatment is a growing healthcare burden, and advances in prevention, diagnostics, and treatment methods are urgently needed. Novel biomarkers are critical for early detection of cancer and allocation of patients to more targeted and individualized therapies [82,273].

This study confirmed PODXL to be a marker of poor prognosis in PDAC by two different antibodies, both of which recognized groups of patients with poor prognosis. We also showed that high tissue expression of PROX1 and β-catenin, both independently, predicts better prognosis in PDAC. PROX1 expression was observed to localize in the cytoplasm, whereas β-catenin expression localized in both the cytoplasm and the cell membrane. Study III demonstrated a novel prognostic biomarker, UCHL5, in subgroups of patients, as positive nuclear expression was associated with better prognosis. This finding must be validated in further studies in other patient cohorts. In Study IV, we demonstrated that REG4 expression in subgroups of PDAC patients had prognostic relevance. In addition, the study showed the possible utility of serum REG4 as a diagnostic marker.

11 CONCLUSIONS

- PODXL was associated with unfavorable clinicopathological parameters and was an independent marker of poor prognosis in PDAC. Both membranous expression of PODXL by a polyclonal antibody and high cytoplasmic expression of PODXL by a monoclonal antibody defined groups with poor prognosis.

- High tissue expression of PROX1 and β-catenin, both independently, predicted better prognosis in PDAC.

- Positive nuclear UCHL5 expression correlated with better prognosis in PDAC.

- REG4 expression may prove useful as a prognostic marker in PDAC patient subgroups, but it may also serve as a diagnostic serum biomarker in addition to CA 19-9.

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14 ORIGINAL PUBLICATIONS