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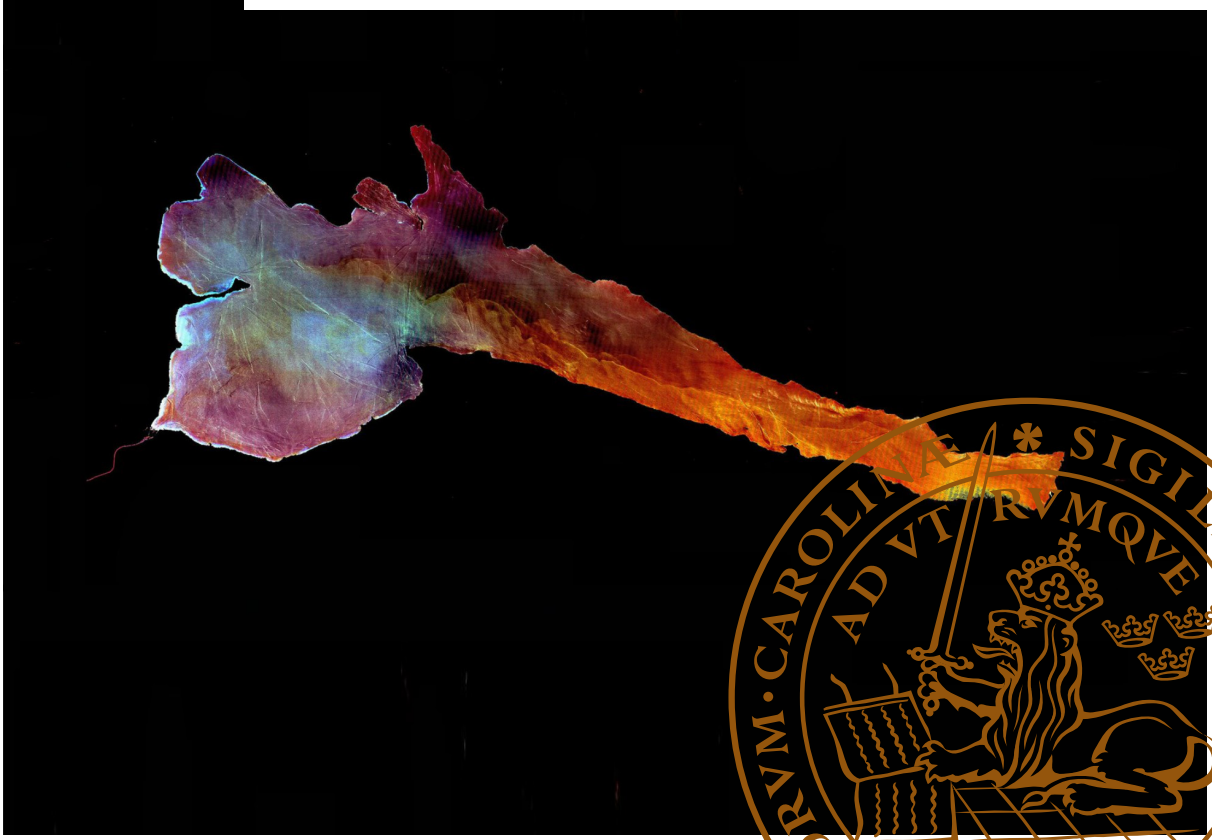

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Axel Bengtsson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University, to be publicly defended on the 26th of May at 09.15 in Lecture Hall F2, Skåne University Hospital, Lund, Sweden

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Title: Clinical Consequences of Tumour Heterogeneity in Pancreatic Ductal Adenocarcinoma

Abstract:

Background: Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers. Improvements in surgical techniques confer short-term benefits, but survival remains poor, due to the heterogeneous biology of the tumour.

Aims: The overarching aim of this thesis was to investigate factors of PDAC tumour heterogeneity at population-, histology- and biomarker-levels. The specific aims were to:

- Characterise the actual long-term survival of PDAC in the recent era, using real-world-data.
- Investigate the prognostic relevance and clinical features of histopathological PDAC subtypes.
- Quantify the stroma in PDAC tumours using digital pathology in a simple, clinically reproducible manner and assess its prognostic significance.
- Systematically evaluate the performance of current prediagnostic blood-based biomarkers for early detection of PDAC

Methods: Paper 1 comprised a retrospective study of the SEER database including patients diagnosed with PDAC between 1975 and 2011, examining actual long-term survival after 5 years. Paper 2 compared all WHO histological subtypes of PDAC simultaneously, using SEER data from 2004 to 2020. Paper 3 used immunohistochemical staining and image analysis to perform binary classification of resected PDAC specimens by their tumour-stroma ratios. Paper 4 was a systematic review and meta analysis of the performance of all available blood-based biomarkers according to time before clinical diagnosis.

Results and conclusions: The retrospective SEER study of all PDAC patients indicated a substantial decrease in actual 5-year survival between tumour stages IA to IB. There has been a clear improvement in survival for surgically treated patients since 1975, but actual long-term survival remains below 5%. The histological SEER study revealed singet-ring cell, colloid and adenosquamous carcinoma to be independent predictors of survival outcome in PDAC. Undifferentiated rhabdoid carcinomas had the lowest survival, while medullary carcinoma conferred the best prognosis. Tumour Stroma Percentage can be calculated in a clinically reproducible manner, and results indicate an improved survival with higher stromal content. The meta-analysis concluded that there is no published biomarker panel that can surpass CA19-9 in diagnostic performance up to 5 years before clinical diagnosis.

Key words: pancreatic cancer, tumour heterogeneity, biomarkers, survival

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Till farfar Lars och vaari Lasse

Table of Contents

Papers included in the thesis

I. Bengtsson A, Andersson R, Ansari D. The actual 5-year survivors of pancreatic ductal adenocarcinoma based on real-world data. Scientific Reports 2020;10:16425.

II. Bengtsson A, Andersson R, Ansari D. Histological variants of pancreatic ductal adenocarcinoma: a survival analysis. Langenbeck's Archives of Surgery 2024;409:312.

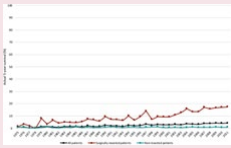
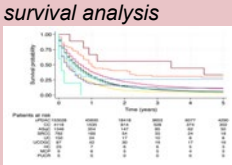
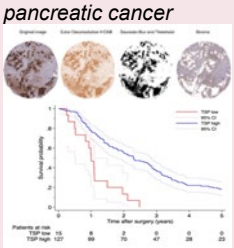
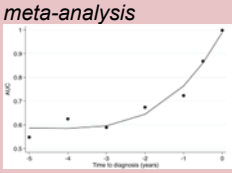
III. Bengtsson A, Andersson R, Andersson B, Ansari D. Digital quantification of stroma percentage enhances prognostic stratification in pancreatic cancer. Surgery Open Science 2026;30:8-13.

IV. Bengtsson A, Draus T, Andersson R, Ansari D. Prediagnostic blood biomarkers for pancreatic cancer: meta-analysis. BJS Open 2024;8:3.

Abbreviations

ADM	Acinar-to-ductal metaplasia
AJCC	American Joint Committee on Cancer
ASqC	Adenosquamous carcinoma
AUC	Area under the curve
CA19-9	Sialyl Lewis A antigen
CAF	Cancer-associated fibroblast
CC	Colloid carcinoma
CHA	Common hepatic artery
cPDAC	Conventional pancreatic ductal adenocarcinoma
CT	Computed tomography
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FFPE	Formalin-fixed paraffin-embedded
GDA	Gastroduodenal artery
HC	Hepatoid carcinoma
HR	Hazard ratio
ICD	International Classification of Diseases
IPMN	Intraductal papillary mucinous neoplasm
ITPN	Intraductal tubulopapillary neoplasm
KRAS	Kirsten rat sarcoma viral oncogene
MCN	Mucinous cystic neoplasm
MCP	Medullary carcinoma of the pancreas
MDT	Multi-disciplinary team
MRI	Magnetic resonance imaging
NCI	National Cancer Institute
OR	Odds ratio
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PSC	Pancreatic stellate cell
PUCR	Pancreatic undifferentiated rhabdoid carcinomas
SEER	Surveillance, Epidemiology, and End Results
SMV	Superior mesenteric vein
SRCC	Signet-ring cell carcinoma
SV	Splenic vein
TMA	Tissue microarray
TSP	Tumour Stroma Percentage
UC	Undifferentiated carcinoma
UCOGC	Undifferentiated carcinoma with osteoclast-like giant cells
WHO	World Health Organization

Thesis at a glance

Paper	Aim	Methods	Results	Conclusions
I. *The actual 5-year survivors of pancreatic ductal adenocarcinoma based on real-world data* 	Characterise the trend and predictors of actual long-term survival of PDAC in the recent era.	84,275 PDAC cases from the SEER database. Trend analysis of survival and multivariable logistic regression according to survival beyond 5 years.	Between 1975 and 2011, actual 5-year survival for all PDAC patients rose from 0.9 to 4.2 per cent. Non-resected patients had no meaningful improvement.	Actual long-term survival in PDAC remains extremely low, improvements in survival are only driven by better surgery.
II. *Histological variants of pancreatic ductal adenocarcinoma: a survival analysis* 	Investigate the prognostic relevance and clinical features of all relevant histopathological subtypes of PDAC.	Multivariable logistic regression and Kaplan-Meier analysis of 159,548 cases with 9 different histological variants from SEER.	A large variation in overall survival between subtypes was observed. Compared to cPDAC, PUCR and MCP carried the best and worst prognosis, respectively.	Histological subtyping confers important prognostic information that should be incorporated into PDAC studies.
III. *Digital quantification of stroma percentage enhances prognostic stratification in pancreatic cancer* 	Quantify the stroma in PDAC tumours by digital pathology in a simple and clinically reproducible manner and assess its prognostic significance.	FFPE tissue microarrays were obtained from 142 individuals resected for PDAC at Skåne University Hospital 1995-2017 Stroma percentage was calculated by digital pathology.	The optimal cut-off point of high vs low TSP was 44.2 per cent. High TSP was strongly correlated with higher overall survival.	High TSP was associated with improved overall survival. The quantification method can be applied consistently across samples and is feasible in large cohorts.
IV. *Prediagnostic blood biomarkers for pancreatic cancer: meta-analysis* 	Investigate the performance of current prediagnostic blood-based biomarkers according to time before diagnosis.	Systematic review and meta-analysis of AUCs in prediagnostic serum/plasma biomarkers.	12 studies reporting AUCs were identified. Only CA19-9 could be meta-analysed. Pooled AUCs for CA19-9 decreased markedly beyond 6 months before diagnosis.	No diagnostic biomarkers, singular or as panels, achieved better performance than CA19-9. The accuracy of CA19-9 is acceptable up to one year before diagnosis.

Populärvetenskaplig sammanfattning

Hur kan vetskap om tumörvariation knäcka koden för bukspottkörtelcancer?

I Sverige insjuknar årligen minst 1500 personer i duktal bukspottkörtelcancer, en tumör som utgår från körtelepitelets gångar. En majoritet av tumörerna fångas sent i förloppet, oftast när cancern har börjat släppa dottertumörer till olika organ i kroppen. Trots det mindre antalet patienter som insjuknar i jämförelse med tjocktarmscancer eller bröstcancer, matchar den årliga dödligheten det årliga insjuknandet nästan helt. Detta beror på att cancern, även om den upptäcks relativt tidigt, är mycket svår att bota, med en snittöverlevnad på bara några månader.

Inom de närmaste åren beräknas bukspottkörtelcancer stå för den näst högsta cancerdödligheten efter lungcancer, delvis på grund av en åldrande befolkning.

Kirurgi och påföljande cellgiftsbehandling är den enda chansen till bot. De kirurgiska tekniker som har förfinats genom årtiondena har gjort att de allra flesta överlever själva bortoperationen av tumören, men vid spridd sjukdom är prognosen dyster, och operation är då inte meningsfull. Den cellgiftsbehandling som finns att erbjuda förlänger överlevnaden och minskar symptomen, men är i de allra flesta fall inte tillräckligt effektiv för att bota sjukdomen. Alltmer uppmärksamhet riktas nu åt att hitta nya alternativ för att uppnå bot. Nya behandlingsmetoder riktar in sig på vanligt förekommande mutationer, tumörens stödjevävnad och kroppens egna immunsystem. Flera metoder verkar lovande men har än så länge inte fått något genomslag i klinisk vardag.

En annan strategi är att fånga tumören i ett så tidigt stadium som möjligt, för att minimera risken för återfall och död. Även detta är svårt eftersom bukspottkörtelcancer oftast ger sig till känna vid spridd sjukdom, som när gallans flöde stoppas, eller när den bryter igenom till bukhinnan och orsakar svåra magsmärtor. Om cancern hade upptäckts i ett tidigare skede, hade överlevnaden ökat markant.

Grundproblemet är att bukspottkörtelcancer uppvisar stor tumörbiologisk variation mellan patienter. Det har även kommit nya rön som visar stora skillnader inne i varje enskild tumör. Detta leder i sin tur till att de tumörmarkörer som kan upptäckas med blodprov varierar stort. Samtidigt producerar tumören så mycket stödjevävnad att den kapslar in sig och gör tumören mycket svårupptäckt via blodprov eller röntgen. För att lösa problemet behövs mer forskning som kan hjälpa till att klarlägga den tumörvariation som inverkar på möjligheten till tidig upptäckt och överlevnad.

Syftet med denna avhandling är att öka förståelsen för hur tumörvariationen för bukspottkörtelcancer inverkar på tidig upptäckt och faktisk överlevnad. Detta har gjorts genom en kombination av registerstudier på populationsnivå, histopatologisk vävnadsanalys, samt en systematisk analys av tumörmarkörers förmåga att hitta bukspottkörtelcancer innan den upptäcks i kliniken.

Arbetet syftar specifikt till att:

I. Undersöka den faktiska överlevnaden hos patienter med duktal bukspottkörtelcancerpatienter i modern tid, genom att använda data på populationsnivå.

II. Jämföra alla kända histologiska subtyper av duktal bukspottkörtelcancer på populationsnivå, och utröna varierande prognos och tumörbiologi.

III. Genomföra en histopatologisk studie med enkel och reproducerbar beräkning av tumörstödjevävnaden hos patienter som opererats för bukspottkörtelcancer.

IV. Systematiskt undersöka och meta-analysera samtliga tumörmarkörer som är detekterbara i blodet, och jämför deras nytta mot den enda kliniskt använda biomarkören CA19-9.

Studie I är en retrospektiv registerstudie som inbegriper alla personer registrerade i den amerikanska databasen SEER mellan 1975 och 2011. Genom att dela upp patienter i de som levde kortare eller längre än 5 år uppnåddes en mer jämförbar och rättvis uppskattning av överlevnaden vid duktal bukspottkörtelcancer över tid. Tumörstadier rapporterades olika, vilket gjorde att surrogatmått som redan fanns tillgängliga i databasen fick användas för analyser över längre tidsspann. I denna studie visar vi att långtidsöverlevnaden efter kirurgi har ökat tack vare centralisering av kirurgin och förfinade metoder, men för icke-opererade patienter har inte långtidsöverlevnaden förbättrats alls på över 30 år. Största tappet i överlevnad sågs redan mellan tumörstadierna IA och IB.

Studie II är också en retrospektiv registerstudie utgående från SEER. Här undersöktes skillnaden i överlevnad mellan olika histologiska subtyper hos duktal bukspottkörtelcancer. Totalt 8 subtyper, erkända av världshälsoorganisationen, kunde identifieras. För direkt jämförelse framställdes överlevnadskurvor, vilka visade på stor variation i överlevnaden. Vissa mycket sällsynta subtyper uppvisade en markant längre medianöverlevnad, andra mer frekvent förekommande subtyper kunde för första gången jämföras på populationsnivå. Resultaten visade att histologisk subtypning spelar stor roll vid prognostisering av tumörsjukdomen.

Studie III använde tumörpreparat från 142 patienter som genomgått operation för bukspottkörtelcancer i Lund och Malmö mellan 1995 och 2017. Små tumörkolvar konstruerades och färgades med CA19-9 för att identifiera tumörcellsvävnad respektive stödjevävnad. Tumörkolvarna genomgick sedan bildanalys för att kvantifiera andelen stödjevävnad. Därefter genomfördes en statistisk analys med fokus på indelning av patienter utefter deras överlevnad och andel stödjevävnad i procent. 44,2 % stödjevävnad var gränsvärdet som genererade maximal skillnad i överlevnad mellan patientgrupper. Överraskande nog var en högre andel stödjevävnad förknippad med bättre överlevnad, vilket utmanar teorin om att mer inkapsling av tumören leder till mer aggressiv och resistent cancer.

Studie IV sammanställde alla biomarkörer som applicerats på blodprov tagna på patienter innan klinisk diagnos av bukspottkörtelcancer. Höga krav ställs på blodprov som ska användas i diagnostiskt syfte. De flesta studier utgick ifrån redan tillgängliga blodprov som hämtats från större hälsostudier, och undersökte oftast paneler av biomarkörer snarare än enskilda protein. Ingen biomarkör som publicerats fram till och med den 30:e juni 2023 kunde mäta sig med CA19-9, trots rigoröst utförda studier.

Sammanfattningsvis kan man konstatera att man förbiser viktiga faktorer, såsom faktisk överlevnad, histologiska subtyper, och även stroma, när man designar studier för bukspottkörtelcancer. Dessutom finns för närvarande ingen tumörmarkör som har tillräckligt bra förmåga att upptäcka sjukdomen tidigt. Trots årtionden av riktad forskning behövs mer för att lyckas bota fler patienter. Forskning gällande faktorer som är gemensamma bland de flesta bukspottskörteltumörerna, såsom vanliga onkogener eller tumörens immunhämmande egenskaper, kan vara en väg framåt.

Introduction

History

The pancreas is first mentioned by Herophilus, one of the founders of the medical school at Alexandria, in 300 BC. The name is derived from Greek (pan = "all", creas = "flesh"). Based on the textual collections of the famous Greek physician Galen, scholars continued to describe the pancreas as a structure with the sole purpose of protecting the larger blood vessels found behind it. The 17th century anatomist Johann Georg Wirsung, from the University of Padua, was the first to properly depict it as a gland. Another physician working in Padua, Battista Morgagni, first described suspected pancreatic cancer in 1761. In his book *De Sedibus et Causis Morborum per Anatomen Indagatis* (On the anatomical locations and causes of diseases), he reports five autopsies where 'hardened pancreata' were found. Included in his work is a case report of a patient likely affected by pancreatic cancer pain, a feeling compared to being torn apart by rabid dogs (Figure 1) [1, 2].

Figure 1. The herdsman Actaeon, transformed into a stag as a punishment by the goddess Artemis, later attacked and slain by his own hounds. Sculpture at the Caserta Royal Palace outside Naples, a city which gave its name to a recent chemotherapeutic trial in pancreatic cancer. Source: Pinterest.com. Image reprinted with courtesy of Joe Adams.

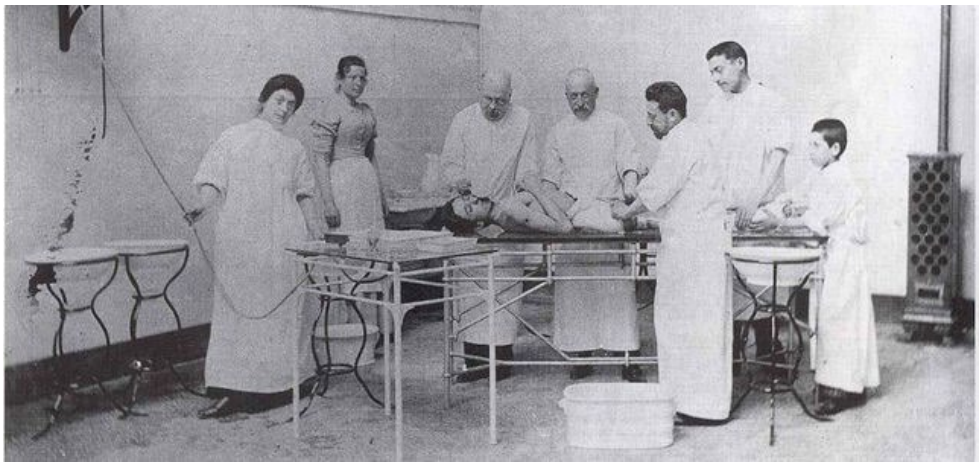


Figure 2. Alessandro Codivilla (middle), together with his staff and a younger colleague. Source: esanum.it. Image published under Public Domain.

Functional pancreatic cancer surgery did not begin until the mid to late 19th century but was confined to simple external and internal surgical drainage of cystic lesions. In 1882, Friedrich Trendelenburg completed the first distal removal of a solid tumour in the pancreatic tail [2]. Six years later, Alessandro Codivilla (Figure 2) performed the first operation of the pancreatic head, a pancreatoduodenectomy performed in two stages. After removal of the tumour with parts of the pancreas, small intestine, distal bile duct and distal stomach, the gallbladder was connected to the small bowel (cholecystojejunostomy) followed by a connection between the small bowel and stomach (Roux-en-Y-gastrojejunostomy). Although the patient died shortly afterwards, it was the first documented removal, i.e. resection, of a primary pancreatic tumour. The first successful resection was completed by Walther Kausch in 1909. The patient survived in the immediate period but succumbed to a gallbladder infection 9 months later [3]. Building on Codivilla's and Kausch's techniques, Allen Whipple refined the procedure in 1940, changing the cholecystojejunostomy to a choledochojejunostomy, and converting it to a one-stage operation: the Whipple Procedure, which remains the gold standard surgical treatment of primary pancreatic cancer to this day (Figure 3).

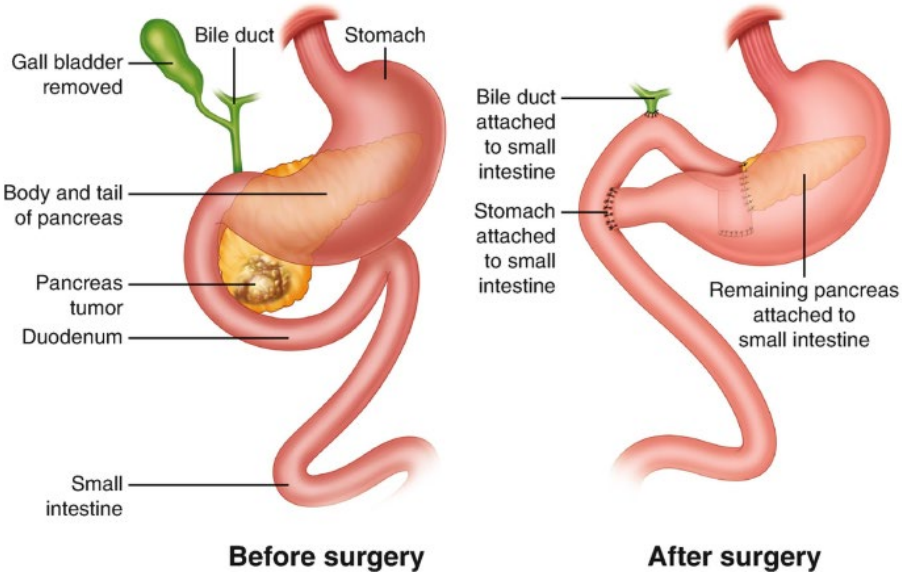


Figure 3. Overview of the pancreatoduodenectomy (Whipple Procedure). Image reproduced from Chouliaras & Kitano, *Common Surgeries Made Easy*: Springer Nature Switzerland AG, © 2020. Reproduced with permission.

Pancreatic ductal adenocarcinoma (PDAC), sometimes simply referred to as pancreatic cancer, is used to describe a gland-forming tumour arising from the epithelium of the pancreatic ducts. Today, PDAC remains one of the deadliest major organ cancers, and is poised to become the second deadliest in the coming decade [4].

Epidemiology

PDAC prognosis is dismal across all regions. Median age at diagnosis is 70 years in Europe [5], and the global 5-year survival estimate is consistently below 10% [6]. By 2030, the projected incidence in Sweden is at least 1,500 patients yearly [7]. Likewise, the incidence of pancreatic ductal adenocarcinoma continues to rise in all high-income regions, with a slight predominance in males [8] and a peak incidence after 80 years of age [9]. The rising incidence is not only attributed to better diagnostics and an aging population, but also to an increase in lifestyle risk factors: tobacco use, alcohol, diabetes, abdominal obesity, and intake of cholesterol and red/processed meat constitute the main modifiable risk factors for pancreatic cancer [10]. Hepatitis B seems to be especially linked with early-onset PDAC [11].

Inherited causes of pancreatic cancer include Lynch syndrome, *BRCA1/2* (BReast CAncer) gene mutation, hereditary pancreatitis, Peutz–Jeghers syndrome, and familial atypical multiple mole melanoma syndrome [12]. 'Familial pancreatic cancer' is defined as two or more first-degree relatives in the same family diagnosed with PDAC, and is an entity separate from the known genetic syndromes, accounting for 5-10% of PDAC cases [13].

Normal anatomy & physiology

Gross anatomy & physiology

The pancreas is located deep within the abdominal cavity, behind the stomach, the transverse colon, and the left liver lobe. It has four main anatomical regions: the head, neck, body and tail. The origin of the primary tumour inside any of these specific regions is relevant to the subsequent spread of the disease. The pancreatic head lies in the C-shape of the first part of the small bowel (duodenum), to the right of the abdominal aorta, and anterior to the inferior vena cava (Figure 4). It extends medially to the neck, which is a small segment that lies anterior to the portal vein. The body lies anterior to the aorta. The tail tapers towards the spleen in the upper left quadrant of the abdomen. The main pancreatic duct (duct of Wirsung) runs the entire length of the pancreas to the ampulla of Vater, where secretions are regulated by the sphincter of Oddi. An accessory duct (duct of Santorini) is present in most cases [14]. The serous secretions contain bicarbonate liquid for neutralising gastric chyme, and digestive enzymes [15].

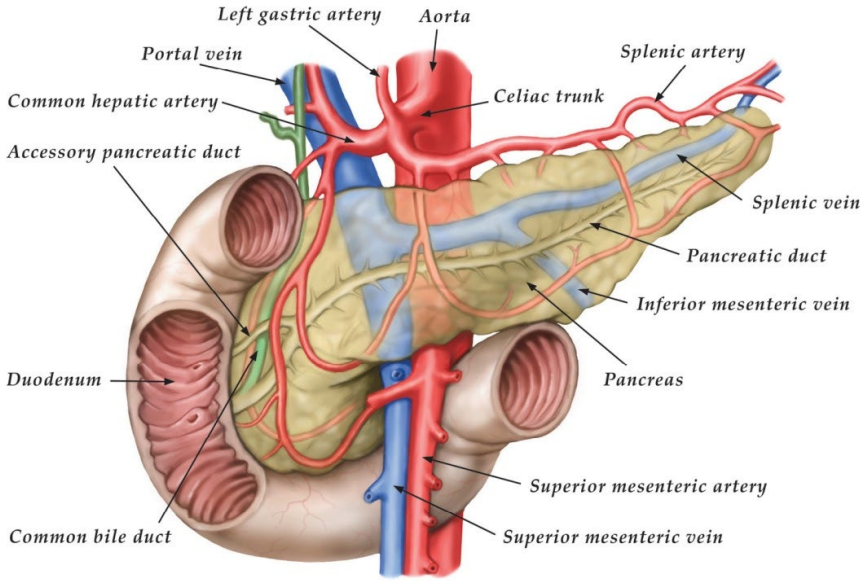


Figure 4. Anatomy of the pancreas and periampullary region. Reprinted with courtesy of dr Daniel Ansari. ©Anders Flood.

The arterial blood supply arises overhead from the coeliac trunk, forming the common hepatic artery (CHA), gastroduodenal artery (GDA) and splenic arteries. A branch of the GDA, called the superior pancreaticoduodenal artery, flows downwards to the duodenum and the pancreatic head. The splenic artery, flowing laterally, is the main supply for the body and tail. From below, the superior mesenteric artery divides into the inferior pancreaticoduodenal artery (IPDA) and the jejunal artery, sharing a common stem. The IPDA combines with the GDA, creating the pancreaticoduodenal arcades [16]. The splenic vein (SV), superior mesenteric vein (SMV), and their connection into the portal vein, represent the main blood drainage system, referred to as the 'portomesenteric axis' [17].

Microscopic anatomy & physiology

PDAC derives from the exocrine system, which constitutes the main pancreatic volume. Acinar, centroacinar, and ductal cells are the most frequent cellular components. The centroacinar cells connect the acinar cells with the intercalated (Figure 5, lower left side), intralobular and interlobular ducts, ending in the main pancreatic duct. Acinar cells (Figure 5, upper left side) are pyramid-shaped with round cell nuclei and reddish points containing digestive enzyme granules. The enzymes are proteases, lipases and amylases for digestion of proteins, fats and

carbohydrates. Centroacinar cells have pale nuclei, while the duct cells are small with pale water-rich cytoplasm for bicarbonate production and secretion into the duodenum [18].

The endocrine system is built up of microscopic clusters of α-, β-, δ-, ε- and PP cells. These clusters (Langerhans islets, Figure 5, right side) produce glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide hormones released into a dense network of blood capillaries. Due to a bidirectional blood flow, exocrine cells can be modulated by the Langerhans islet hormones, and vice versa [19].

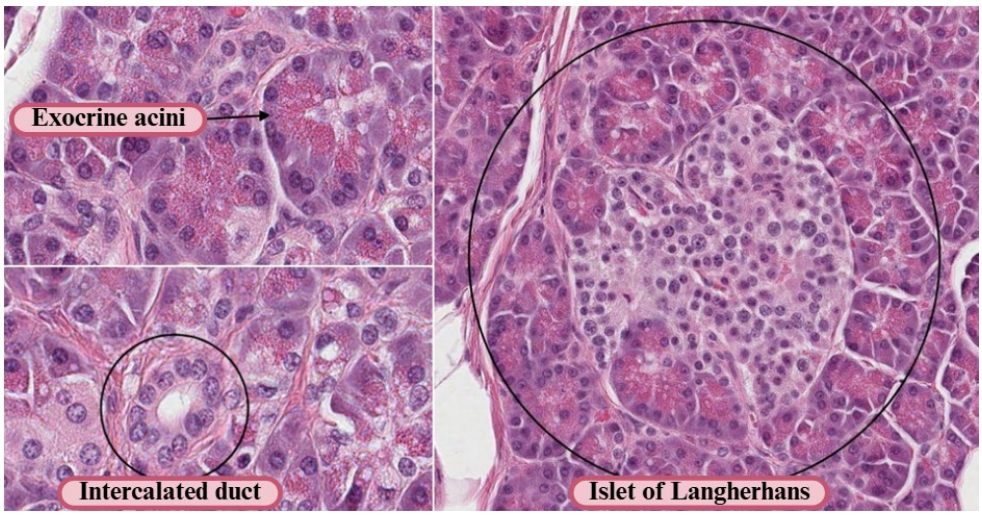


Figure 5. Normal pancreas histology. Adapted from the Human Protein Atlas, proteinatlas.org.

Insulin lowers and glucagon raises blood glucose levels, ghrelin and pancreatic polypeptide regulate appetite and intestinal motility, and somatostatin inhibits the exo- and endocrine secretions [20].

The supportive elements (stroma) of a normal pancreas represent less than one per cent of the tissue, with fibroblast cells being the most frequent cellular components [21]. Immune cells, pancreatic stellate cells (PSCs) [22] and vascular endothelial cells are also present, albeit in small numbers [23]. The extracellular matrix (ECM), is the non-cellular component that contains fibrous proteins (collagens), membrane glycoproteins, and glycosylated proteins (proteoglycans) [24]. These components are mainly produced by PSCs and fibroblasts.

Tumour development

Pancreatic ductal adenocarcinoma develops in multiple sequences, starting with reprogramming of the normal cellular elements. The earliest reprogramming event is termed acinar-to-ductal metaplasia (ADM). ADM is a physiological response to injury, in which acinar cells acquire features like those of ductal cells. When the process becomes irreversible, the cell undergoes dysplasia, the precursor to overt cancer [25]. The most common type of dysplastic lesion is pancreatic intraepithelial neoplasia (PanIN). Lesions occurring outside of the ductal epithelium include intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm (MCN), and intraductal tubulopapillary neoplasm (ITPN) [26, 27]. The *KRAS* gene mutation is seen in early PanIN-1 stage. The PanIN-2 and PanIN-3 stages usually involve mutations in the *SMAD*, *CDKN2A*, and *TP53* genes. These mutations are typically dependent on *KRAS* for subsequent transformation into overt cancer [28] (Figure 6).

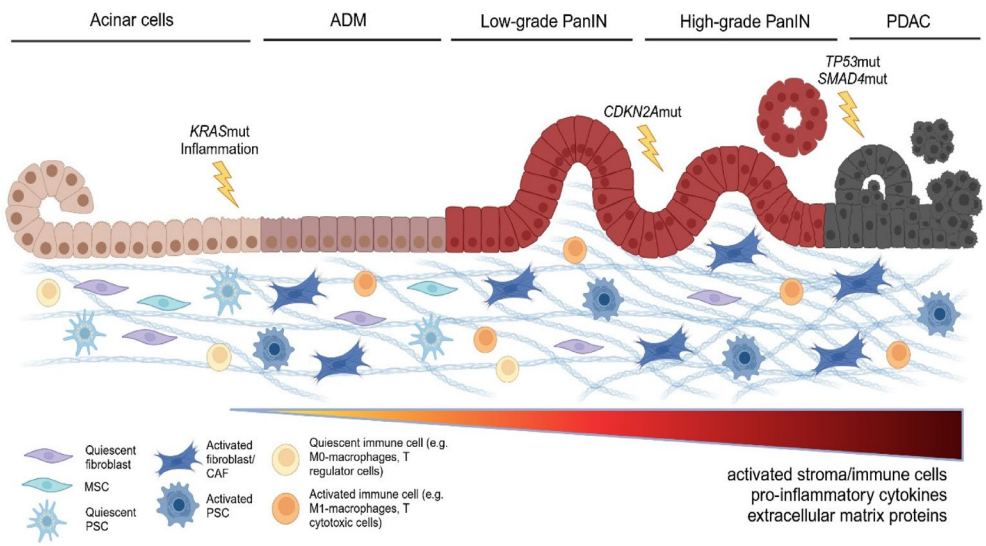


Figure 6. Progression from normal acinar cells to overt PDAC. Reproduced from the open access article by Seidel et al. [29] (2025). Distributed under the Creative Commons Attribution (CC BY) license.

Stromal activation

The *Hedgehog* pathway is one of the most important signalling axes for activating the stromal elements in PDAC. This pathway gives rise to cancer-associated fibroblasts (CAFs) and activated PSCs that maintain the tumour-stroma cross-talk [30]. *Hedgehog* is a target-binding molecule (ligand) that triggers a constant feedback loop that stimulates PanIN progression [31] and secretion of growth

factors and ECM components [30, 32]. A parallel feed-forward loop, where more stroma leads to more paracrine signalling and fibroblast activation, is primarily maintained by the *TGF-β/Smad* pathway. The continual deposition of ECM components is termed 'desmoplasia', a defining feature of PDAC [33].

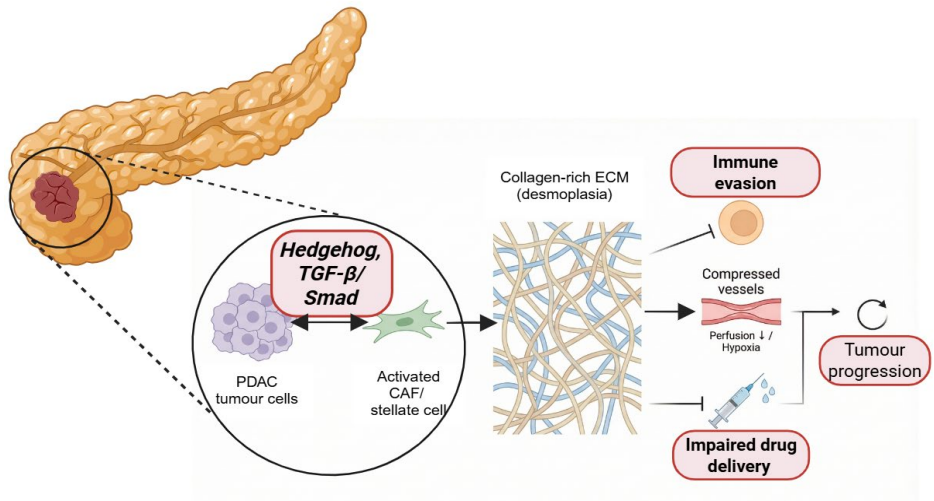


Figure 7. Desmoplastic reaction in PDAC. Created using BioRender.

Cancer establishment

PanIN-3 lesions progress to invasive PDAC through continued stromal remodelling, growth factor expression, and the triggering of the epithelial-mesenchymal transition (EMT) program [34]. Outside the ductal system, late-stage IPMNs, MCNs and ITPNs share similar molecular events to PanINs, including *TP53*, *CDKN2A*, and *SMAD4*, but are more frequently linked to chromatin remodelling (*ARID1A*, *ARID2*) or *PI3K/Akt* pathway genes [35]. Once invasive potential is achieved, further modulation of the stroma continues, culminating in the 'desmoplastic reaction'. This is one of the hallmark events of PDAC: the creation of a dense fibrotic stroma that encapsulates the primary tumour, enhances resistance to chemotherapy, and shields it from the host's immune system (Figure 7).

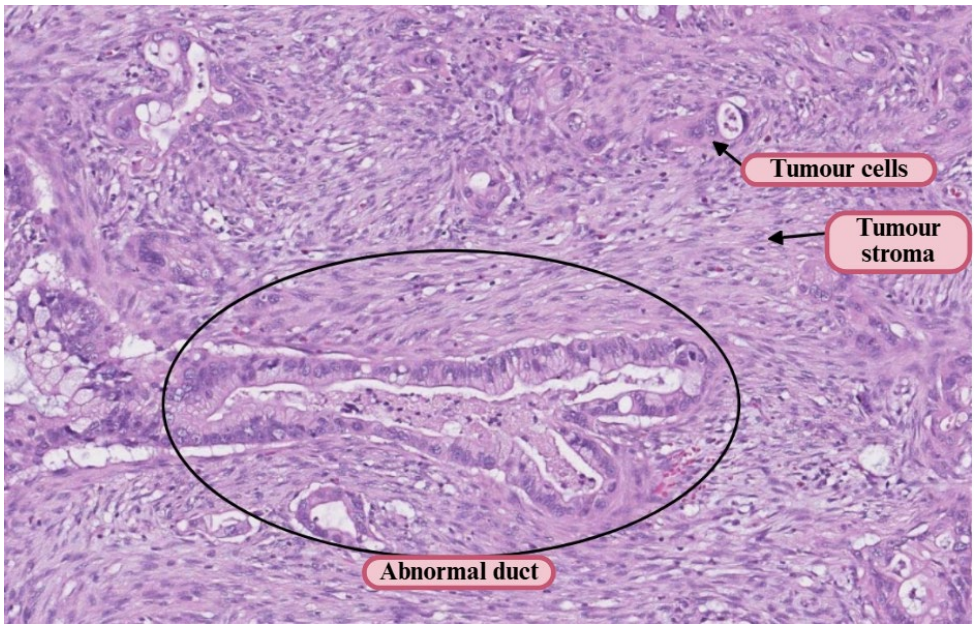


Figure 8. Pancreatic ductal adenocarcinoma histology. Adapted from the Human Protein Atlas, proteinatlas.org.

Diagnostic tools

Clinical presentation

A majority of PDAC cases are diagnosed when the disease has already spread to other organs. Obstruction of the bile fluid, nerve pain, weight loss, gastrointestinal problems and new-onset diabetes (NOD) are common presentations of PDAC [36]. Apart from NOD, and rarely acute pancreatitis [37], all other mechanisms usually manifest at later stages of disease (Figure 9). Biliary obstruction is caused by compression of the distal common bile duct, either by direct tumour extension or lymph node metastasis [38]. Metastasised disease is also associated with venous/arterial thrombosis, commonly presenting as deep vein thrombosis or pulmonary embolism [39]. Tissue factors, released from extracellular vesicles (EVs) containing active *VIIa* and *FX* coagulation factors, play a crucial role in this hypercoagulable state [40].

Across Europe, 'rapid referral pathways' have been, and continue to be implemented [41]. These are comprehensive national and regional guidelines that seek to accelerate diagnosis and treatment. The guidelines emphasise coordination

and multi-disciplinary team (MDT) reviews. Their implementation varies across countries (in Sweden they are called 'standardiserat vårdförlopp', in Norway/Denmark 'pakkeforløp/b') and regions (NHS Scotland has different guidelines compared to NHS England) [42].While general oncology studies demonstrate benefits of these pathways, a long-term benefit specific to PDAC has yet to be shown [43, 44]. Conversely, the impact of perioperative pathways such as ERAS (Enhanced Recovery After Surgery) on the rate of postoperative complications has been confirmed by several analyses [45]. The ERAS pathway has even been linked to a shorter time to chemotherapy [46]. Complications common to the Whipple Procedure are delayed gastric emptying, abdominal infections, pneumonia, acute pancreatitis, postoperative haemorrhage, and the feared postoperative pancreatic fistula. The scoring system used to predict complications, devised by The International Study Group of Pancreatic Surgery (ISGPS), has been instrumental in ensuring standardised classifications and scoring systems to predict the risk of these complications [47].

Figure 9. Clinical signs and symptoms of pancreatic cancer. Created using BioRender.

Imaging and staging

In Sweden, the primary imaging method is computed tomography (CT) of thorax and abdomen and a pancreatic-protocol CT. Other modalities, like MRI Abdomen Protocol, Magnetic resonance cholangiopancreatography (MRCP), or CT Liver Protocol, are usually carried out to prior to surgery [48]. Typical signs on the CT include a diffuse hypoenhancing mass and a dilatation of the common bile duct and main pancreatic duct ("double duct sign"). The involvement of the portomesenteric axis, arteries and regional lymph nodes is also assessed (Figure 10). If liver or venous involvement is indeterminate, or the mass is hard to visualize on CT

(isoenhancing), the MRI Abdomen Protocol can be a suitable complement [49]. In rare instances, FDG-PET-CT/MRI is employed to rule out occult distant metastases in patients planned for curative treatment [50]. Poorly defined tumours that are smaller than 2 cm in diameter are subject to better staging via endoscopic ultrasound, during which a needle biopsy can be obtained to confirm diagnosis before initiation of chemotherapy [51].

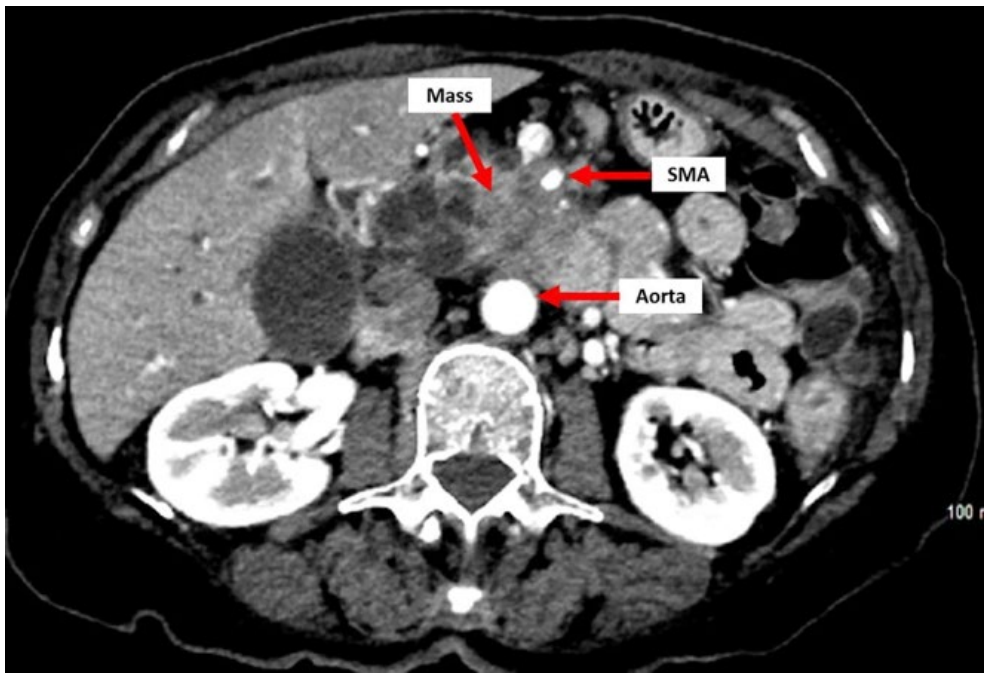


Figure 10. Portovenous phase axial CT of a locally advanced pancreatic ductal adenocarcinoma of the pancreatic body. Reproduced with permission from the article by Kulkarni et al. [52].

The current staging system used in the Western hemisphere is the American Joint Committee on Cancer (AJCC) 8th edition, also known as the TNM system (Table 1) [53]. The AJCC, set up in 1959, is a consortium of experts that agree on a unified cancer staging system. A notable difference to the 7th edition is the focus on size as the sole determinant of T-stage, with the intended goal of a more uniform T-stage distribution in the reported literature. The stage grouping follows the standard four-stage framework where tumours are divided into carcinoma *in situ* (0) localised (I), deeper localised (II), regionally advanced (III) and metastatic disease (IV), usually based on survival outcomes (Table 2).

Table 1. AJCC 8th edition stage [53].

Primary tumour stage (T)	Regional lymph node (N)	Distant metastasis (M)
Tis: Carcinoma *in situ**	N0: No regional lymph node metastasis	M0: No distant metastasis
T1: ≤2 cm in greatest diameter T1a: ≤0.5 cm T1b: >0.5 cm-<1 cm T1c: 1-2 cm		
T2: >2 cm-≤4 cm	N1: Metastasis in 1-3 regional lymph nodes	M1: Presence of distant metastasis
T3: >4 cm	N2: Metastasis in ≥4 regional lymph nodes	
T4: Tumour involves coeliac axis, SMA, or CHA		

*Includes PanIN-3, high-grade IPMN, high-grade MCN and high-grade ITPN.

Table 2. AJCC 8th edition stage grouping [53].

Stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1-3	N1	M0
III	Any T	N2	M0
III	T4	Any N	M0
IV	Any T	Any N	M1

Treatment

Surgery

Surgical technique depends on tumour origin. A pancreatic cancer located in the head warrants a classical pancreatoduodenectomy, whereas a distal tumour calls for a distal pancreatectomy, as pioneered by Friedrich Trendelenburg, along with spleen-preserving measures [54].

Primary resectable, borderline resectable, locally advanced, and metastatic PDAC, are the four stages used in clinical practice. In contrast to AJCC, these resectability criteria are not primarily prognostic but instead rely on the relationship between the tumour and its surrounding blood vessels [52]. As defined by The National Comprehensive Cancer Network (NCCN), the primary resectable stage has no arterial and no or limited venous involvement and is suitable for upfront surgery.

For borderline resectable stage, there is enough vascular contact for an increased risk of a positive resection margin, warranting pre-operative chemotherapy. Consensus guidelines also mention an increased level of Sialyl Lewis A antigen (CA19-9>500 U/mL) as a 'biologically borderline resectable disease' [55]. The locally advanced stage (Figure 10) has vascular invasion to hinder complete resection and surgical repair of vessels. In metastatic PDAC, only palliative systemic therapy or best supportive care is recommended, but surgery for limited metastasis is being explored in clinical trials [56, 57]. In the U.S. and some parts of Asia, staging laparoscopy may also be performed to detect radiographically occult disease in some patients with localised tumours and elevated CA19-9 [58].

The decision on surgical stage is made during MDT conferences, where surgeons, oncologists and radiologists decide on curative or palliative intent. To improve perioperative results, pancreatic cancer surgery has seen extensive centralisation, beginning with Finland and the Netherlands in the 1990s. In the 2010s, many European countries began to implement volume standards for pancreatic cancer surgery, including Sweden, Finland, Denmark and Norway [59]. In practice, it meant that surgery was to be restricted to centres performing more than a certain number of pancreatectomies per year, with 20 operations frequently cited as a minimum [60].

Chemotherapy studies

Medical oncologic therapy is divided into three basic settings: neo-adjuvant (before surgery/local therapy), adjuvant (after surgery) and palliative (symptom relief) therapy. Gemcitabine and fluorouracil are the dominating chemotherapies in the arsenal for PDAC, based on several pivotal clinical trials. Gemcitabine is a nucleoside analogue that inhibits DNA synthesis, and induces programmed cell death (apoptosis) [61]. The efficacy of adjuvant chemotherapy with gemcitabine was confirmed by the Charité Onkologie (CONKO-001) Phase III study [62]. Following the PRODIGE 24 study, gemcitabine was overtaken by fluorouracil-based therapy. Fluorouracil is a pyrimidine analogue that also inhibits DNA synthesis. The current mainstay of chemotherapy in Western countries is fluorouracil combined with folic acid, irinotecan and oxaliplatin, termed mFOLFIRINOX. Although not used in Sweden, combining mFOLFIRINOX with a CD40 agonist has yielded NALIRIFOX, an option for first-line treatment for metastatic PDAC, confirmed by the NAPOLI-3 trial [63]. After results from the 2013 MPACT study were publicised, Gemcitabine with albumin-bound paclitaxel (gemcitabine-nabpaclitaxel) was added to the treatment arsenal as a first-line option for palliative patients [64].

For neo-adjuvant treatment, a FOLFIRINOX- or gemcitabine-based therapy is indicated. In primary resectable pancreatic head cancer, the NORPACT-1 study found no survival benefit with four cycles of pre-operative FOLFIRINOX versus

upfront surgery [65]. For borderline resectable patients, the PREOPANC-1 study found that there was a survival advantage in having preoperative chemotherapy plus radiotherapy, supporting its use in this patient group [66]. In locally advanced PDAC, neoadjuvant chemotherapy is crucial for patients who can tolerate it, which was confirmed by the NEOLAP trial [67]. The evidence is not yet fully clear regarding neoadjuvant therapy in primary resectable PDAC, but results from the PREOPANC-3 study are expected to provide more definitive answers [55]. As for radiation therapy in PDAC, survival results are inconsistent, and its use is not recommended per routine, as shown by the ESPAC-5 phase 2 trial [68].

Palliative care

If the responsible physician and the patient agree on a non-curative approach, treatment shifts focus to symptom relief. As most patients belong to this category, improvement in palliative techniques is essential for better quality of life for the lion's share of PDAC patients. Opioids such as oxycodone, used primarily, or methadone as second-line refractory pain treatment, are often prescribed. Patient-controlled analgesia, endoscopic stenting of biliary or duodenal obstructions, surgical hepaticojejunostomy, or neurolysis of coeliac plexus/splanchnic nerves are some of the more specialised tools available for relief of pain or jaundice [69]. mFOLFIRINOX or gemcitabine-nab-paclitaxel is indicated in palliative patients with good performance status, but a substantial proportion of patients will not tolerate combination regimens and still receive gemcitabine-based therapy [70, 71].

Targeted therapy

Following the POLO II and III trials, olaparib, the poly ADP-ribose (PARP) inhibitor, was FDA-approved for a subset of patients with *BRCA* and *PALB2* DNA damage repair gene mutations. Although only benefits in progression free survival were seen, the non-benefit in overall survival has been attributed to underpowered studies. Nonetheless, absolute differences in survival were measured in weeks, rather than months [72].

With a prevalence of less than one per cent, *NTRK* oncogenic driver gene fusions can be targeted with enctrectinib or larotrectinib [73]. Zenocutuzumab (*NRG1* fusion), selpercatinib (*RET* fusion), trastuzumab (*HER2* amplification), and *KRAS G12C*-inhibitors also see clinical use [74]. However, these therapies are considered only in rare situations, even more rarely employed as first-line treatment, with scarce and varied data on response rates in PDAC [75, 76].

Further experimental PDAC regimens include checkpoint inhibitors for MSI-positive tumours, *KRAS G12D*- or pan-*RAS*-inhibitors [77]. Single-cell RNA sequencing has identified *SERPINE1* as a key target for inhibition [78]. The clinical implications of these medications for PDAC remain to be seen.

Markers of heterogeneity

According to the Merriam-Webster Dictionary, heterogeneity is “the quality or state of consisting of dissimilar or diverse elements”. Some heterogeneity exists across all tumour types. However, in the context of PDAC, dissimilar elements are apparent on all levels: inside each tumour (intratumour heterogeneity), between tumours (intertumour heterogeneity), inside the stroma (tumour microenvironmental heterogeneity) and by varying aggressiveness and response to therapy (functional heterogeneity) [79].

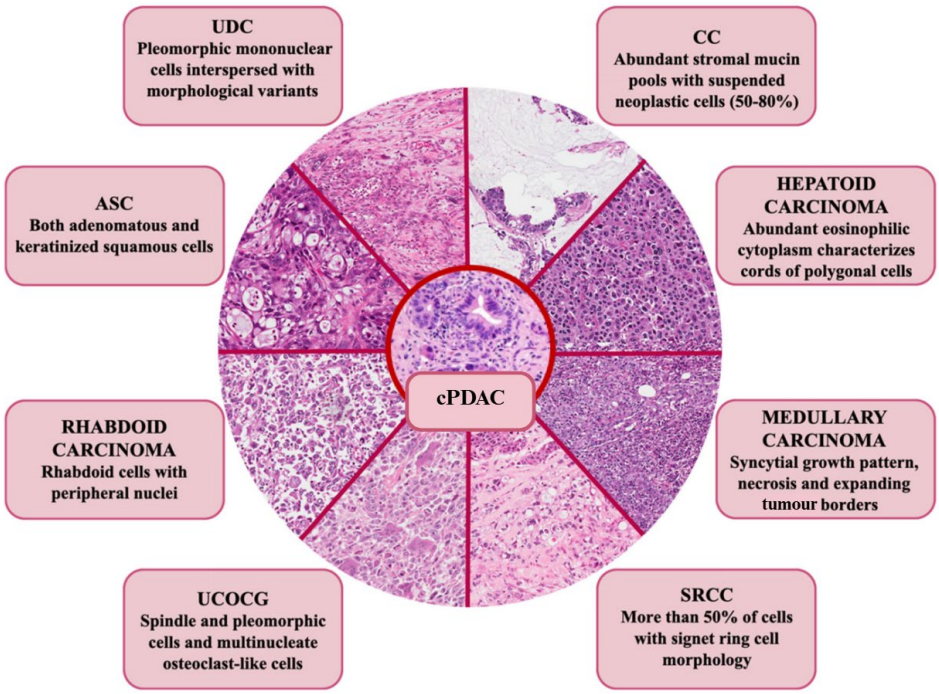


Figure 11. Morphohistological variants of PDAC. Adapted from the open access article by Bazzichetto et al. [80] (2020) under the Creative Commons Attribution (CC BY) license.

Histological subtypes

The classic form of pancreatic ductal adenocarcinoma on a histology slide is a dense desmoplastic stroma coupled with glandular/tubular structures (Figure 8). However, the World Health Organization (WHO) recognises eight other subtypes: colloid carcinoma (CC), adenosquamous carcinoma (ASqC), signet-ring cell carcinoma

(SRCC), undifferentiated carcinoma (UC), undifferentiated carcinoma with osteoclast-like giant cells (UCOGC), hepatoid carcinoma (HC), medullary carcinoma of the pancreas (MCP) and pancreatic undifferentiated rhabdoid carcinomas (PUCR). They are defined by certain morphological features (Figure 11), some exceedingly rare and with limited data on functional heterogeneity [81].

Molecular subtypes

There are many different proposed molecular subtypes of PDAC, all using different methods and refinements of previous works. Table 3 summarises current subtype frameworks. In 2011, Collisson et al. defined three PDAC subtypes: the classical, quasimesenchymal, and exocrine-like signatures. The classical subtype maintains epithelial differentiation and glandular structures, quasimesenchymal is characterised by expression of EMT genes and chemotherapy resistance, while exocrine-like expresses genes important for digestive enzymes [34, 82]. In 2015, Moffit et al. redefined them as two tumour-intrinsic subtypes (basal-like/squamous and classical/progenitor), and two additional stromal subtypes [83, 84]. Further studies by Chan-Seng-Yue et al. in 2020 hinted at a continuum of different expression signatures [85]. In 2022, Hwang et al described seven tumour subtypes coupled with six CAF subtypes, representing the most substantial leap in this field yet [86].

Table 3. Notable studies of PDAC molecular subtypes.

Framework	Identified subtypes	Molecular characteristics
Collisson et al. bulk transcriptomics [82]	Classical, quasi-mesenchymal, & exocrine-like	Classical: Epithelial markers, quasi-mesenchymal: EMT markers
Moffitt et al. bulk transcriptomics [84]	Classical, basal-like, normal & activated stroma subtypes	Classical: High *GATA6, HNF4A, TFF1* expression Basal-like: *KRT5, KRT17, S100A2* expression
Bailey et al. bulk transcriptomics [87]	Squamous; pancreatic progenitor; immunogenic; & aberrantly differentiated endocrine exocrine (ADEX)	Squamous ≈ basal-like, progenitor ≈ classical, ADEX implies exo/endocrine pathways
Puleo et al. bulk transcriptomics [88]	Pure classical, pure basal-like & five stroma subtypes	Validating classical & basal-like subtypes, introducing subtypes based on the tumour microenvironment
Chan-Seng-Yue et al. refinement [85]	Classical A/B, basal-like A/B & hybrid (mixed lineage)	A continuum of subtypes with conversions during PDAC progression [89]
Karasinska et al. correlating bioinformation on glycolysis in PDAC [90]	Quiescent, glycolytic, cholesterogenic & mixed	*KRAS* & *MYC* amplification connected to glycolytic genes
Hwang et al. single-cell/spatial omics [86]	Classical-like, squamoid, basaloid; mesenchymal, acinar-like, neuroendocrine-like & neural-like	Acinar-like & neuroendocrine-like subtypes confirmed to be real malignant states

Biomarkers

According to the National Cancer Institute (NCI) dictionary, a biomarker is "a biological molecule found in blood, body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease". This definition also poses that the molecule can be reliably measured and used for a specific purpose, be it for diagnosis, prognosis or monitoring of a disease. As all described expression markers can be correlated with prognosis from the available data, they can be said to constitute *prognostic* markers. However, in the case of PDAC, a biomarker's ability to monitor and diagnose the disease is one of the most pressing issues.

To date, CA19-9 is the only biomarker approved for clinical practice. It is a carbohydrate antigen produced by epithelial cells located in the pancreas, bile ducts and other glandular structures in the body. It is used for monitoring therapy response and disease progression but is not suited for diagnosis of PDAC. This is because CA19-9 is elevated in many other conditions (pancreatitis, cholecystitis, rheumatoid arthritis) and that 10% of patients lack the ability to synthesise the protein. This in turn means that there is no current method to capture PDAC in the general population or in high-risk cohorts before onset of suspicious symptoms.

What is required of a biomarker in aiding diagnosis of PDAC? While no specific target values have been set, it is implied that a very high specificity would be required (98% or higher) given the low incidence [91, 92]. A plethora of proposed biomarkers exist in the literature, ranging from glycoproteins (mucins, LRR proteins, von Willebrand Factor) [93] and metabolites (proline, creatine, palmitic acid) [94], to cell-free DNA (circulating tumour DNA, vesicular DNA) [95] and microRNAs (Figure 12).

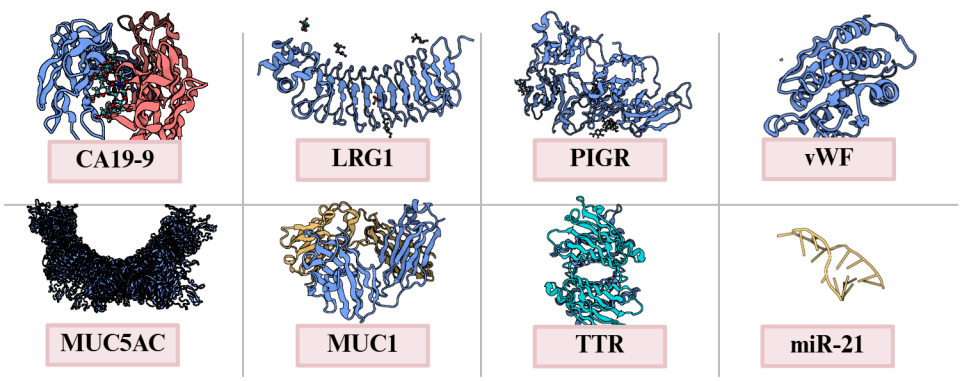


Figure 12. A selection of frequently published biomarkers for PDAC diagnosis. CA19-9: Sialyl Lewis A antigen; LRG1: Leucine-rich α-2-glycoprotein 1 from the LRR protein family; PIGR: Polymeric Immunoglobulin Receptor; vWF: von Willebrand Factor; MUC5AC: Mucin 5AC protein; MUC1: Mucin 1 protein; TTR: Transthyretin transport protein; miR-21: MicroRNA 21, considered oncogenic. Created in BioRender.

Early detection

Since most PDAC patients present with inoperable disease, attention in the last decade has been on new strategies that increase the proportion of patients with earlier stages. Guidelines for familial pancreatic cancer and inherited syndromes exist [92], but to date, there is no established strategy for early detection of sporadic PDAC. Biomarkers play an important role in this strategy, since current radiologic tools (CT, MRI) are either too costly, invasive, or lack sensitivity for small tumours (<2 cm) [96]. Combining several biomarkers into panels can increase sensitivity and specificity, and thereby the diagnostic performance. Diagnostic performance as a function of these values, can be compared directly using the "area under the curve" (AUC) metric. AUC is the preferred way of reporting in biomarker studies, although other measurements have been proposed [97].

Lesions with the most limited spread but with inevitable progression to overt cancer, would be the most logical targets and confer the highest chance of cure. These include high grade PanIN-3, high-grade IPMN, high-grade ITPN and early stages of PDAC. This is complicated by the fact that development of pancreatic cancer from scratch is a slow progress, modelled to take between 7 and 12 years (Figure 6) [92]. Adding to the complexity, simulated progression from PanIN-3 to overt PDAC varies wildly, with estimates ranging from 17 months to 10 years [98]. Nonetheless, it is evident that emerging biomarker panels must find disease long before it presents with clinical symptoms (Figure 13), a requirement usually termed 'prediagnostic accuracy' in the literature [91].

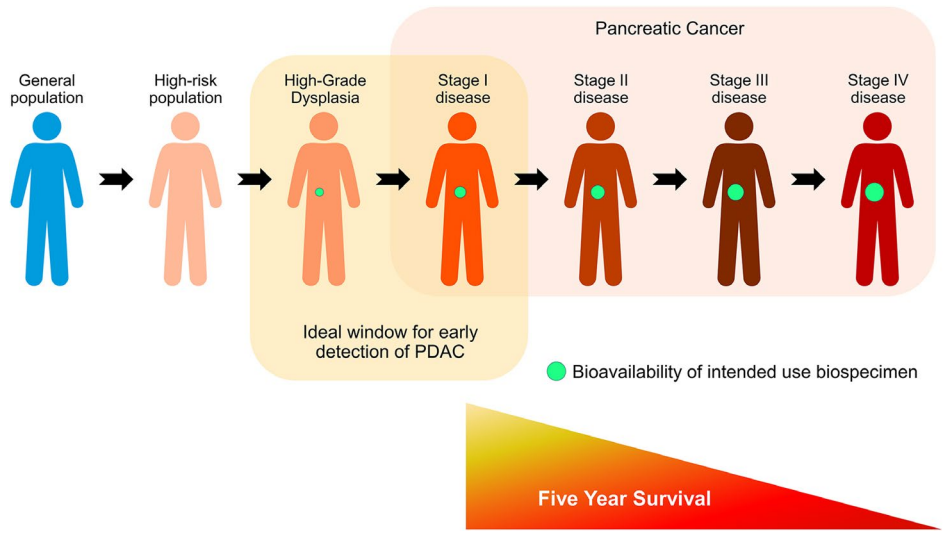


Figure 13. Schematic of optimal time windows for early detection of PDAC. High-risk populations could be elderly patients with new-onset diabetes or suspicious pancreatic cysts on imaging. General population screening remains elusive. Reproduced from the open access article by Smith et al. [91] under CC BY license.

Aims

Effective treatment and early detection strategies remain central challenges in PDAC, owing to the tumoral heterogeneity.

The general aim of the thesis was to improve the understanding of the prognosis and early detection of pancreatic ductal adenocarcinoma by investigating tumour heterogeneity at population-, histology- and biomarker-levels. The aims of the individual studies were as follows:

- Paper I: Characterise the actual long-term survival of PDAC, its trend and predictors in the recent era, using real-world-data.
- Paper II: Investigate the prognostic relevance and clinical features of histopathological subtypes of PDAC in a large population.
- Paper III: Quantify the stroma in PDAC tumours by digital pathology in a simple and clinically reproducible manner and assess its prognostic significance.
- Paper IV: Systematically evaluate the performance of current prediagnostic blood-based biomarkers up to 5 years before clinical diagnosis of PDAC.

Methods

Setting

In Papers I and II, microscopically confirmed cases of pancreatic ductal adenocarcinoma were investigated. For Paper I, actual short-term survivors and long-term survivors of PDAC were compared, based on a complete follow-up of 5 years (60 months) or more. In Paper II, patient groups were divided across the 9 WHO-recognised histological subtypes and compared against conventional PDAC regarding clinical characteristics and survival outcomes.

In Paper III, tissue was collected from a biobank of patients that had undergone resection for PDAC at Skåne University Hospital Lund/Malmö between 1995 and 2017. Data on survival were extracted from health records. Patients were then divided into two groups based on their ratio between tumour cells and stroma.

Paper IV was a systematic review and meta-analysis of prediagnostic serum biomarker studies registered in PubMed, Embase or the Cochrane Library. These databases were screened for studies published between 1 January 2020 and 30 June 2023. A summary is presented in Table 4.

Table 4. Summary of study designs.

Paper	Study design	Material	Cohort origin	No. of individuals	Collection period
I	Population-based retrospective cohort	Microscopically confirmed PDAC cases.	SEER (USA)	84 275	1975-2011 (follow-up until 2016)
II	Population-based retrospective cohort	Microscopically confirmed histological PDAC variants	SEER (USA)	159 548	2004-2020
III	Retrospective single-institution cohort	FFPE tissue microarrays	Skåne University Hospital (Sweden)	142	1995-2017
IV	Systematic review & meta-analysis	Prediagnostic serum or plasma samples	Multiple international prospective cohorts	12 included studies with 23-304 cases + matched controls	2000-2023

Data collection

SEER database (Papers I & II)

For Papers I and II, The Surveillance, Epidemiology, and End Results (SEER) database was used. The database program was started by President Richard Nixon in 1973 and is managed by the NCI in the U.S. Although officially adopting AJCC 3rd edition in 1988, it did not become a routine in the SEER registry until 2004 [99]. In 2010, the introduction of AJCC 7th edition enriched the registry with tumour grades, and better details on tumour site. In 2018, the newly introduced 8th edition shifted towards a focus on tumour size for T-stage and a three-level nodal stage. Today, the SEER database covers nearly 48% of total cancer cases in the US [100].

Data were retrieved in the SEER*Stat version 8.3.6 (Paper I) and 8.4.3 (Paper II) software. Age, gender, tumour location, tumour grade, tumour size, TNM stage, chemo/radiotherapy status and surgery status were extracted. To cover the broadest time frame, Paper I employed SEER historic stage A, which is a coding scheme derived from variables entered in registry. Paper II reported the SEER summary stage, which is directly computed after input of variables by the registrar [101].

Histology codes (Papers I & II)

Patients were selected from the SEER cancer registry based on the WHO ICD-O-3 (International Classification of Diseases for Oncology, 3rd edition) topography codes C25.0 to C25.9. In Paper I, ICD-O-3 histology codes 8140/3 (adenocarcinoma, NOS) and 8500/3 (infiltrating duct carcinoma, NOS) were selected. Paper II included all histological subtypes of PDAC recognised by WHO: 8140/3, 8500/3, 8560/3, 8480/3, 8576/3, 8510/3, 8014/3, 8490/3, 8020/3 and 8035/3.

Tissue microarrays (Paper III)

Paper III utilised a biobank of formalin-fixed paraffin embedded (FFPE) samples from a cohort of patients who underwent resection for PDAC at Skåne University Hospital between 1995 and 2017. Samples were re-evaluated by a pathologist to confirm diagnosis. For each tissue sample, the pathologist marked areas representative of cancer and selected four 2 mm cores. The resulting tissue microarrays (TMAs) were constructed by an automated tissue array instrument (Minicore® 3, Alphelys) (Figure 14, part 1).

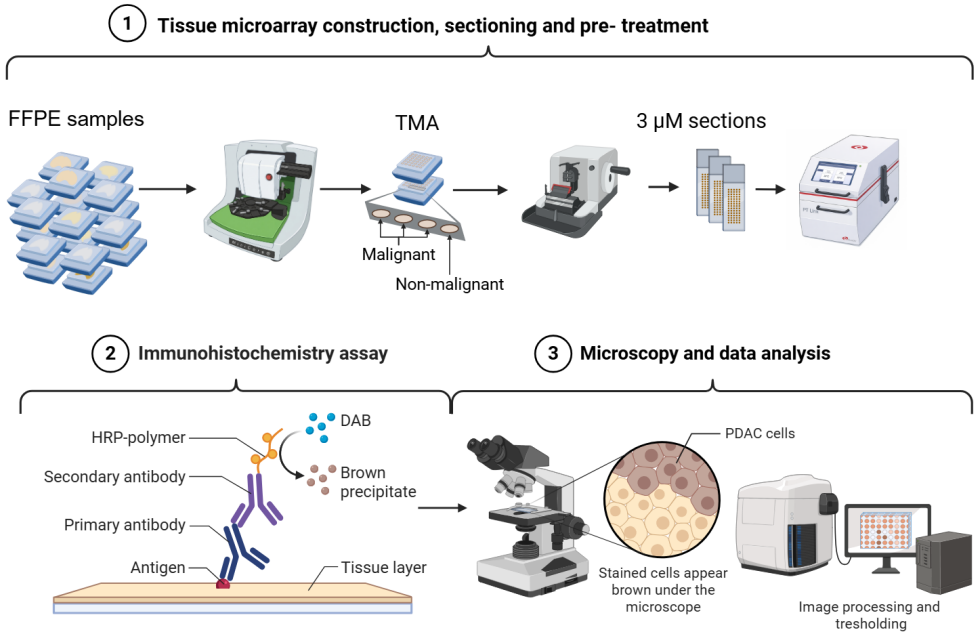


Figure 14. TMA construction workflow. Created using BioRender.

Immunohistochemistry and digital scanning (Paper III)

To label tumour epithelia, TMA slides were subjected to automatic deparaffinisation, rehydration and heat-induced antigen retrieval using the PT-link system (Dako, Agilent Technologies). Slides were placed in the EnVision FLEX Target Retrieval Solution high pH (K800421–2, Dako) and heated. The slides were then incubated with primary antibody CA19-9 (Abcam) at 1:500 dilution. After washing with phosphate-buffered saline, slides were incubated with secondary biotinylated anti-mouse antibody (Vector Laboratories) at 1:200 dilution. This was followed by staining with avidin-biotin-peroxidase complex (Vector Laboratories). Diaminobenzidine chromogen was applied for colour development (Figure 14, part 2).

After immunohistochemical staining, slides were scanned in a Hamamatsu 210 microscope. Tumour tissue was manually annotated in the NDPview software. The regions of interest were automatically extracted with a Python Script. Non-relevant or necrotic regions and section artefacts were visually excluded. In ImageJ software, the colour deconvolution function was used to separate labelled (DAB-brown) from non-labelled (haematoxylin-blue) into different images. DAB intensity significant for tumour cells was determined with a Gaussian blur image-processing filter,

threshold, and particle size analysis in the ImageJ software. The residual stromal area was divided by the total area to get the Tumour Stroma Percentage (TSP) (Figure 14, part 3).

International prospective patient cohorts (Paper IV)

Longitudinal studies specifically designed to investigate cancer aetiology are important for the study of prediagnostic cases of cancer over long time periods. They enable biomarker researchers to employ large well-established cohorts with corresponding biobanks. As one of the largest studies examining nutrition, lifestyle and cancer risk, The European Prospective Investigation into Cancer and Nutrition (EPIC) cohort is frequently used. In EPIC, patients were recruited from ten European countries (including Norway, Sweden and Denmark) between 1991 and 2000, accumulating a total of 153,455 men and 367,993 women (most aged 35-70). A central liquid nitrogen databank, located in Lyon, France, is used to store the collected plasma, serum, red cells, and buffy coat [102]. Today, EPIC has several sub-cohorts focusing on cardiovascular health and aging [103].

Other notable prospective patient cohorts include the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS, United Kingdom excluding Scotland) [104], the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO, United States) [105], and a cohort created specifically for high-risk individuals, called The Pancreatic Cancer Early Detection (PRECEDE) Consortium cohort [106].

Ethical approval & checklists

The Ethics Committee for Clinical Research at LU and the Swedish Ethical review Authority approved the study protocols for Papers I, II and III. Paper I and II utilised the SEER register, a publicly available database containing strictly deidentified patient data. If SEER data is not linked to other datasets, it is usually exempt from institutional review boards. Ethical considerations lie primarily in correct but cautious interpretation and ensuring that results generalise to target demographics. Issues regarding disease classification are discussed later in this thesis.

In paper III, we had to ensure strict anonymisation of survival records linked to biobanked specimens. All patients included had written informed consent regarding tissue donation and return of results. The digital pathology analysis was carried out by the Imagene-IT consultancy company, so proper pseudonymisation and well-characterised specimens were adamant. Only representative and well-preserved tissue was used.

In Paper IV, all included primary studies had obtained ethical approval from their respective institutions. Since only pooled statistics are published, reidentification is next to impossible. Nevertheless, consideration for publication bias and demographics occurs frequently and is also included in the discussion section of this thesis.

The papers of the thesis were constructed based on established checklists. The goal of checklists is to standardise reporting and improve clarity of findings. Papers I, II and III adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. This is a 22-item checklist for the title/abstract, introduction, methods and results sections. The document is applicable to cohort-, case-control-, and cross-sectional studies [107]. Paper III also followed The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines. REMARK was published by an expert panel to address methodological problems in tumour biomarker research, using a 20-item checklist [108]. Paper IV followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement. This checklist contains 27 items covering the abstract, selection process, eligibility criteria, data extraction, reporting and assessment of bias. It also mandates the use of flow diagrams and correct terminology [109].

Statistical methods

Paper I

Patients were divided into short-term or long-term survivors based on actual overall survival (complete follow-up) of 5 years or more. Group comparisons of clinicopathological variables were carried out using Mann Whitney U test for continuous variables and Pearson Chi-square (χ^2) for categorical variables. A trend analysis of survival over calendar years was tested using linear-by-linear association. Each variable (age, gender, tumour location, histological grade, tumour size, AJCC stage, surgical resection, chemotherapy, radiation, T-, N-, and M-stage) was tested separately with univariable logistic regression.

Variables with a p-value less than 0.25 were chosen for multivariable logistic regression. According to the Hosmer–Lemeshow model-building strategy, variables were removed from the multivariable analysis if deemed a non-confounder or non-significant. A main-effects model was used. Missing values were handled by the multiple imputation with chained equations technique [110], with predictive mean matching, including 10 iterations per chain and 10 imputed datasets. Data analysis was conducted in IBM SPSS (version 26) and Stata/MP (version 14) software.

Paper II

The χ^2 test (categorical variables) or Kruskal–Wallis test (continuous variables) was applied for descriptive statistics. Kaplan-Meier survival curves were plotted together with corresponding log-rank tests, to assess survival distributions between subtypes. As with Paper I, each variable's association with overall survival was determined with univariable logistic regression. Any variable with a p-value less than 0.25 was chosen for multivariable analysis. A main-effects model and multiple imputation, as described for Paper I, was chosen. Data analysis was performed in Stata/SE (version 17).

Paper III

The Tumour Stroma Percentage (TSP) was defined as stromal area divided by total tumour area, computed from the processed digital image. Maximally selected rank statistics from the maxstat R package were used to choose the optimal threshold for classifying tumours into high vs low TSP. The association between TSP categories ($\leq$44.2% vs >44.2%) and clinicopathological variables was compared using the χ^2 test. Variables included age, gender, tumour location, tumour size, AJCC stage, tumour differentiation, resection margin status and adjuvant chemotherapy. The survival differences were plotted in Kaplan-Meier curves, and the survival distribution compared with log-rank test. Univariable Cox regression yielded the crude hazard ratio, which was adjusted in two different multivariable analyses: one for age and gender, and another including age, gender, ASA score, tumour location, AJCC stage, tumour differentiation, resection status, and adjuvant chemotherapy. Analyses were performed using Stata/MP (version 18) and RStudio (version 2025.09.2).

Paper IV

The pooled diagnostic accuracy was measured using pooled weighted summary AUC. The Hanley & McNeil formula was used to estimate standard errors not reported in the studies. Heterogeneity across studies was compared with the I^2 and H^2 statistics. A fixed-effects model was applied, since less than five studies were included in the final meta-analysis. A sensitivity analysis was performed by removing one study that had variant time-intervals. Traditional statistical methods for assessing risk-of-bias are usually not valid for diagnostic accuracy studies and were therefore not used. All analyses were carried out in STATA/MP (version 18) and MedCalc (version 22.016).

Results

Paper I

Main findings

- The SEER data (1975-2011, 84,275 patients) demonstrated that actual 5-year survival for all PDAC patients increased from 0.9% in 1975 to 4.2% in 2011 ($p<0.001$) (Figure 16).
- For patients who underwent surgery, actual 5-year survival improved markedly, from 1.5% to 17.4% ($p<0.001$). Non-resected patients had no meaningful improvement in actual 5-year survival from 1975 to 2011 (0.8% vs 0.9%, $p = 0.121$) (Figure 16).
- In surgically resected patients, significantly independent predictors of survival included age, gender, tumour grade, tumour size, TNM stage, and treatment with chemotherapy. In non-resected patients, age, grade and TNM-stage were significant upon multivariable analysis (Table 5).
- Many long-term survivors had traditionally unfavourable clinicopathological characteristics.

Patient characteristics

Median age at diagnosis was 68, with 48.9% females. The proportions of patients with SEER Historic Stage A (localised disease) increased, from 5.4% in 1975, to 7.0% in 2011 (Figure 15). The largest decrease in survival was seen between AJCC stages IA and IB (Table 6).

Table 5. Logistic regression analyses of factors associated with actual long-term survival.

	Univariable		Multivariable	
Variable	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***
Age (years)	0.977 (0.972-0.981)	<0.001	0.984 (0.979-0.989)	<0.001
Female gender	1.16 (1.05-1.29)	0.005	1.13 (1.01-1.27)	0.031
Tumour location				
Head	1 (reference)			
Body	0.434 (0.354-0.532)	<0.001		
Tail	0.590 (0.494-0.703)	<0.001		
Other	0.412 (0.353-0.480)	<0.001		
Grade				
Well diff.	1 (reference)		1 (reference)	
Moderately diff.	0.657 (0.561-0.770)	<0.001	0.557 (0.464-0.667)	<0.001
Poorly diff.	0.319 (0.271-0.376)	<0.001	0.382 (0.318-0.459)	<0.001
Tumour size (cm)	0.637 (0.609-0.666)	<0.001	0.878 (0.837-0.920)	<0.001
T stage				
T1	1 (reference)		1 (reference)	
T2	0.210 (0.172-0.257)	<0.001	0.560 (0.431-0.726)	<0.001
T3	0.282 (0.238-0.336)	<0.001	0.347 (0.275-0.438)	<0.001
T4	0.061 (0.047-0.079)	<0.001	0.247 (0.177-0.344)	<0.001
N stage	0.972 (0.872-1.08)	0.613		
M stage	0.075 (0.062-0.091)	<0.001	0.320 (0.256-0.400)	<0.001
Surgical resection	23.2 (20.2-26.6)	<0.001	10.8 (9.13-12.8)	<0.001
Chemotherapy	2.04 (1.82-2.29)	<0.001	1.45 (1.27-1.64)	<0.001
Radiation	3.06 (2.74-3.40)	<0.001		

OR: Odds ratio.

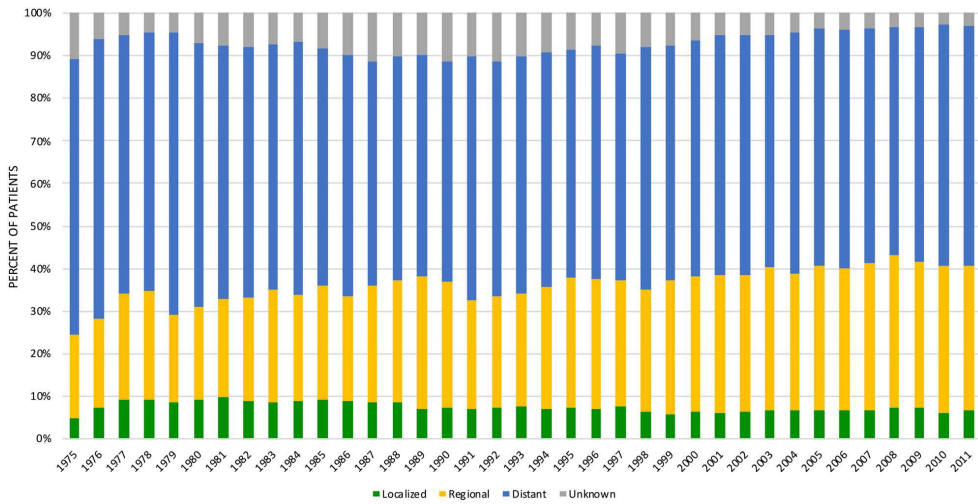


Figure 15. Distribution of PDAC SEER Summary stage at diagnosis.

Table 6. 5-year survival by AJCC stage between 2004 and 2011. Follow-up cut-off date 31 Dec 2016.

Stage	Proportion (%)	Actual 5-year survival (%)
IA	1.3	31.7
IB	4.4	11.8
IIA	11.5	9.0
IIB	16.3	8.7
III	10.6	1.9
IV	56.0	0.5

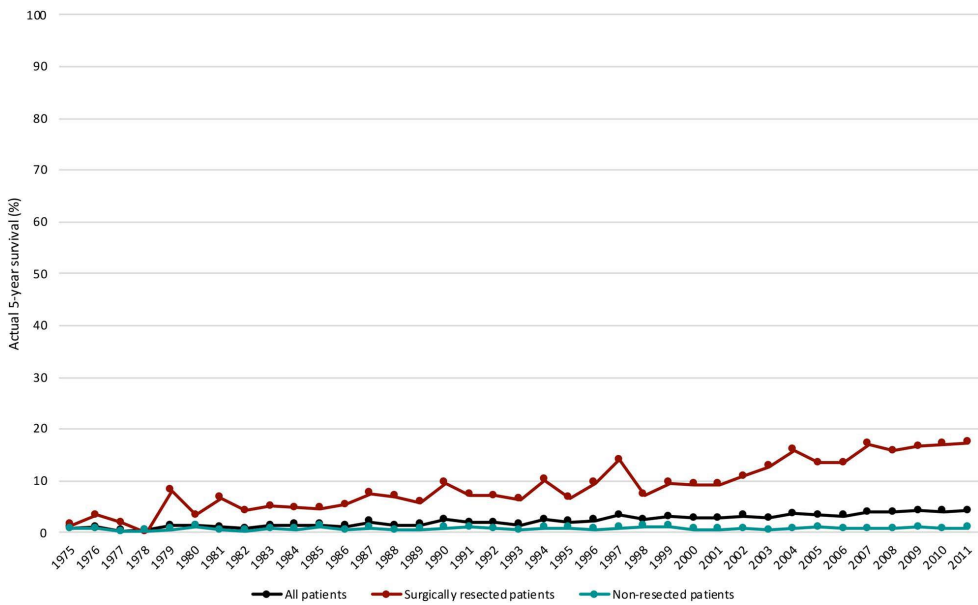


Figure 16. Actual 5-year survival of PDAC by year between 1975 and 2011.

Paper II

Main findings

- There was a large variation in overall survival between subtypes (Figure 17). PUCR had the worst prognosis, with a median survival of 2 months and a 0% 5-year survival rate. MCP had the best prognosis, with a median survival of 41 months and a 5-year survival rate of 33.3%.
- In the multivariable analysis, several histological subtypes were confirmed to be independent predictors of overall survival compared to conventional PDAC: CC (HR 0.78, p<0.001) and UCOGC (HR 0.68, p = 0.002) had a markedly better prognosis compared to conventional PDAC. Conversely, ASqC was associated with worse overall survival (HR 1.16, p<0.001), as was SRCC (HR 1.21, p<0.001). HC and PUCR were not significant, probably due to low sample size (Table 7).

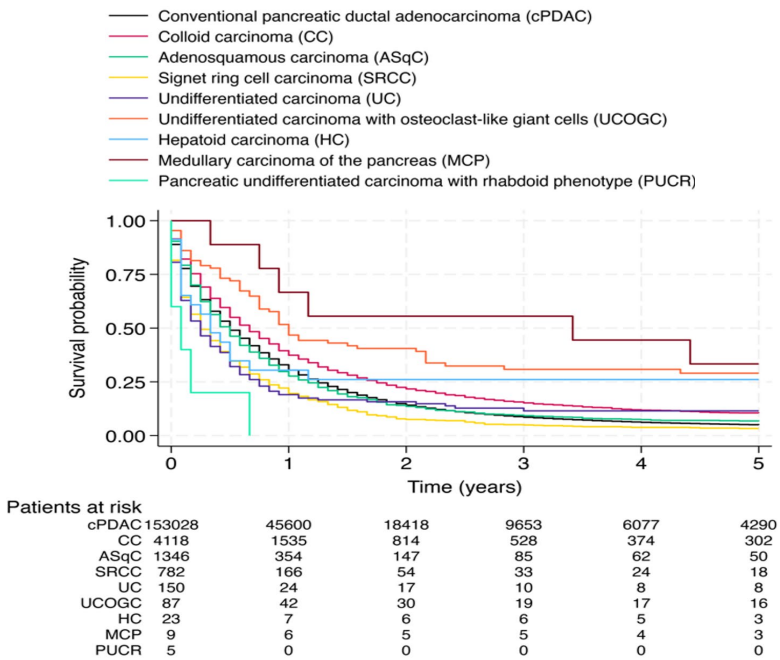


Figure 17. Kaplan-Meier analysis of patient survival according to WHO histological subtype

Table 7. Logistic regression analysis of factors associated with lower survival.

	Univariable		Multivariable	
Variable	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Age (years)	1.01 (1.01-1.01)	<0.001	0.984 (0.979-0.989)	<0.001
Female gender	0.99 (0.98-0.99)	0.005	0.95 (0.94-0.96)	<0.001
Tumour location				
Head	1 (reference)		1 (reference)	
Other	0.75 (0.74-0.76)	<0.001	0.96 (0.95-0.97)	<0.001
Grade				
G1/2	1 (reference)		1 (reference)	
G3	01.49 (1.46-1.51)	<0.001	01.30 (1.28-1.32)	<0.001
Stage				
Localised	1 (reference)		1 (reference)	
Regional	1.04 (1.02-1.06)	0.001	0.560 (0.431-0.726)	<0.001
Distant	2.35 (2.30-2.40)	<0.001	2.26 (2.21-2.31)	<0.001
Surgical resection	0.32 (0.32-0.33)	<0.001	0.43 (0.42-0.44)	<0.001
Chemotherapy	0.55 (0.54-0.56	<0.001	0.47 (0.46-0.47)	<0.001
Radiotherapy	0.61 (0.60-0.62)	<0.001	0.93 (0.91-0.94)	<0.001
Histological subtype				
cPDAC	1 (reference)		1 (reference)	
CC	0.80 (0.77-0.83	<0.001	0.78 (0.76-0.81))	<0.001
ASqC	1.03 (0.97-1.10)	0.316	1.16 (1.09-1.22)	<0.001
SRCC	1.28 (1.18-1.39)	<0.001	1.21 (1.12-1.30)	<0.001
UC	1.07 (0.88-1.31)	0.490	0.99 (0.83-1.18)	0.912
UCOGC	0.52 (0.40-0.67)’	<0.001	0.68 (0.53-0.87)	0.002
HC	0.69 (0.41-1.14)	0.145	0.80 (0.50-1.30)	0.364
MCP	0.32 (0.14-0.71)	0.005	0.48 (0.22-1.07)	0.073
PUCR	2.78 (0.90-8.62)	0.077	1.54 (0.64-3.69)	0.337

HR: Hazard ratio.

Paper III

Main findings

- In the TMAs (Figure 18), High Tumour Stroma Percentage (TSP) strongly predicted improved survival (median 27.8 months vs 12 months, log-rank test: p<0.001) (Figure 19).
- Survival was significantly improved with high TSP (univariable Cox regression: HR = 0.28, 95% CI 0.16-0.49, p<0.001) which was maintained upon multivariable analysis adjusting for 8 covariates (HR = 0.27, 95% CI 0.14-0.52, p<0.001) (Table 8).
- There was a wide variation in stromal content of tumours (median TSP: 66.1%, range: 27.8%-99.9%). The optimal cut-off was 44.2% stroma (Figure 20). 127 patients (89%) had high TSP (>44.2%), 15 (11%) patients had low TSP (≤44.2%). Tumour location in the head (p = 0.043), was the only clinical characteristic significantly more common in high TSP patients.

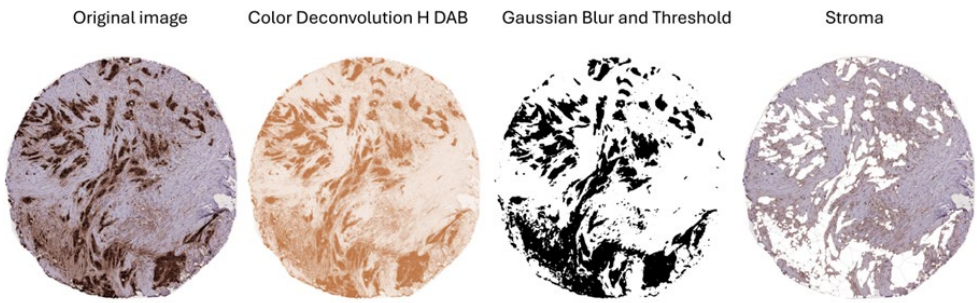


Figure 18. Computer-aided quantification of Tumour Stroma Percentage from CA19-9-labelled TMAs.

Table 8. Multivariable Cox regression analysis of factors associated with overall survival.

Variable	HR (95% CI)	*p*
Unadjusted		<0.001
TSP high	0.28 (0.16-0.49)	
Adjusted for age and gender		<0.001
TSP high	0.27 (0.15-0.48)	
Adjusted for 8 covariates*		
TSP high	0.26 (0.13-0.52)	<0.001

*age, gender, ASA score, tumour location, AJCC, tumour differentation, resection status and adjuvant chemotherapy.

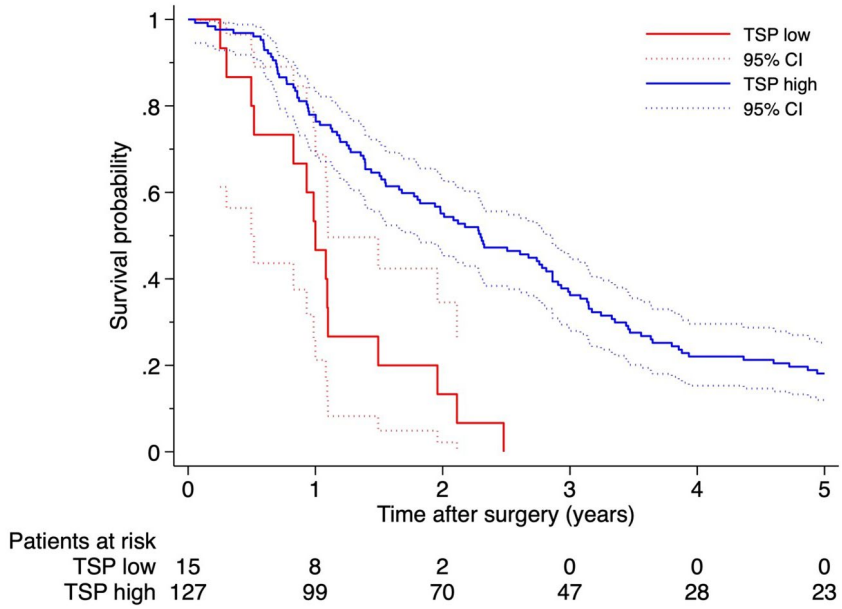


Figure 19. Overall survival of patients classified as TSP low or TSP high.

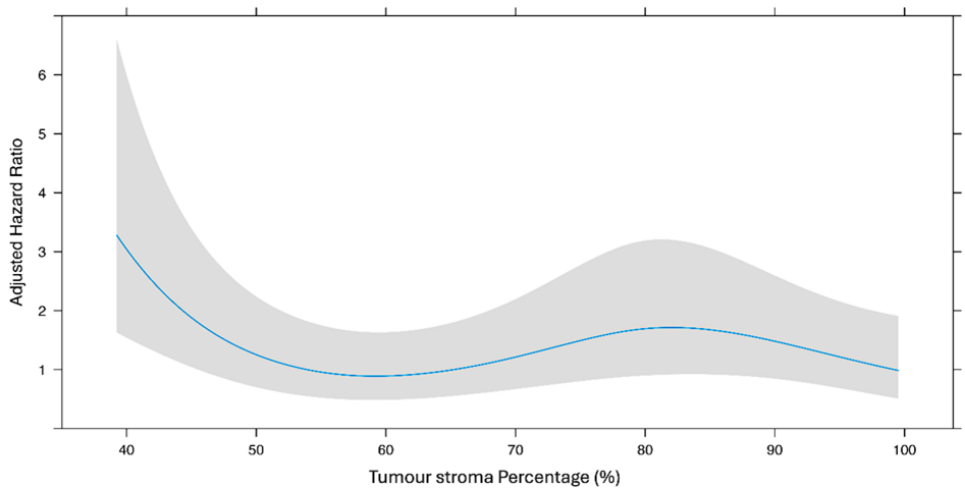


Figure 20. Restricted cubic spline showing adjusted hazard ratio for overall survival as a function of TSP. The graph was plotted to examine a possible nonlinear relationship between TSP and survival. As TSP decreases beyond 44%, survival drastically worsens, confirming our cut-off value.

Paper IV

Main findings

- 12 prediagnostic studies reporting AUCs were identified (Figure 21).
- The primary outcome was pooled AUC. Reviewed biomarkers include CA19-9, CA125, alpha-1-antitrypsin, carcinoembryonic antigen (CEA), THBS1- and 2, type 4 collagen, and several microRNAs (Table 9). CA19-9 was the only biomarker that allowed for a quantitative meta-analysis, based on four studies (Figure 22).
- A sensitivity analysis that excluded one study with differing time intervals did not produce any meaningful changes in AUCs.
- A microRNA (miR-21-5p) presented in a large cohort study by Duell et al. (Table 9, first section) reached an AUC of 0.79 during the whole 5-year lead-up to clinical diagnosis.
- At 4-5 years before diagnosis, endostatin (AUC: 0.57) and type 4 collagen (AUC: 0.60) alone displayed better performance than CA19-9 (Table 9, second section). However, when compared to CA19-9, these biomarkers displayed worse performance over most other timeframes leading up to the 4-year mark.
- For CA19-9, prediagnostic performance was acceptable up to one year before diagnosis (Figure 22A–C, Figure 22H).

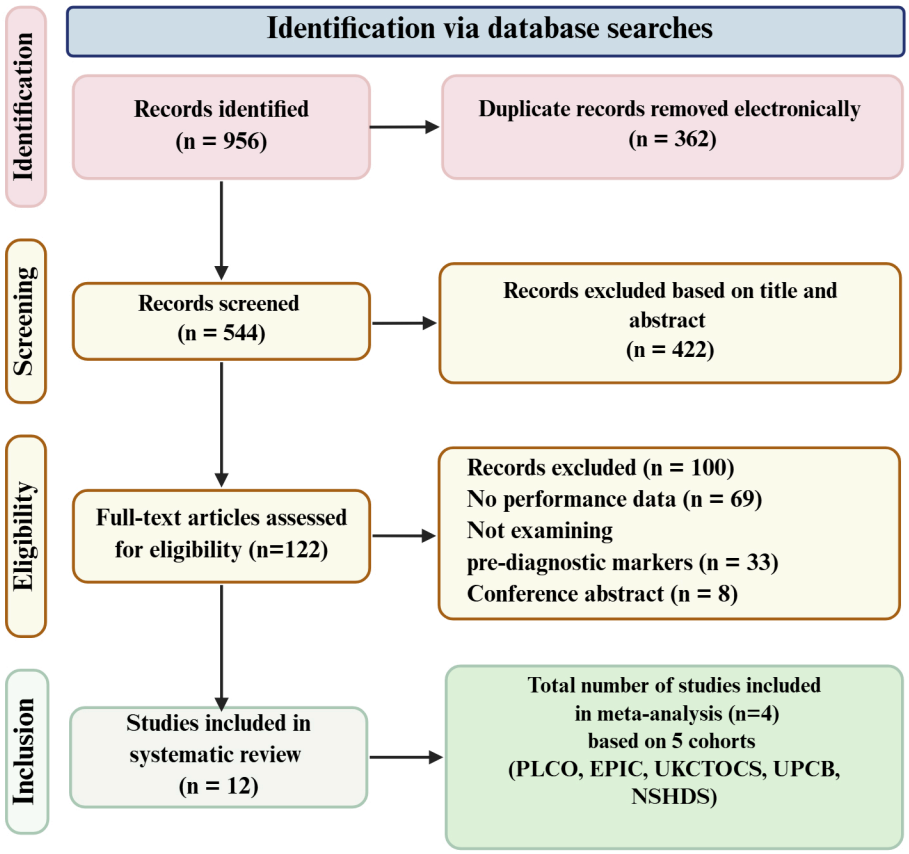


Figure 21. PRISMA diagram of the study selection process. Created using BioRender.

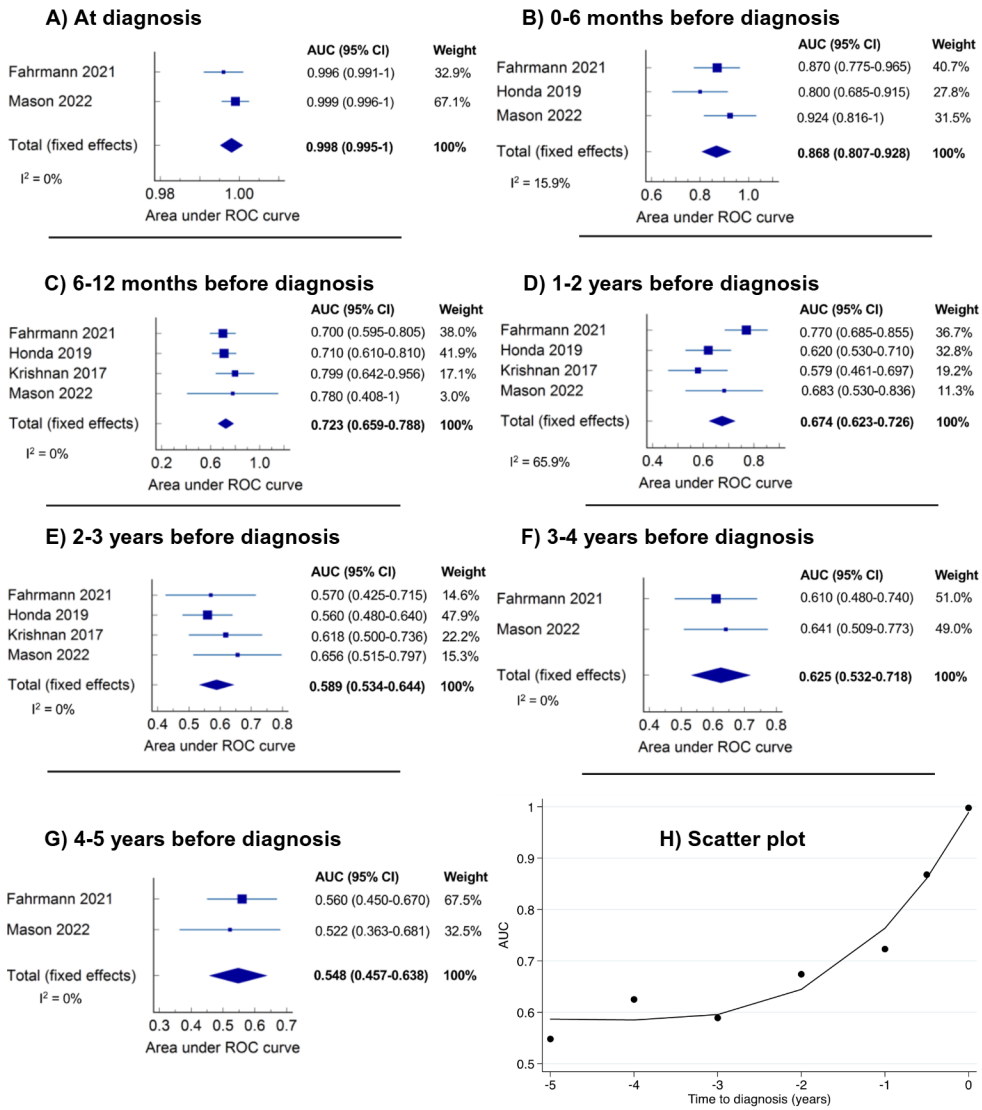


Figure 22. A-G: Forest plots of pooled area under the curves from included studies. H: Scatter plot with overlaid fractional polynomial prediction plot.

Table 9. Studies included in the systematic review.

Reference	Cohort	Biomarkers	AUC (95% CI) before diagnosis						
			At dx	0-5 yrs	0.5-1 yrs	1-2 yrs	2-3 yrs	3-4 yrs	4-5 yrs
Duell et al. [111]	EPIC (225 cases, 225 controls)								***≤5 yrs***
		miR-10a	NA	NA	NA	NA	NA	NA	0.75 (0.66-0.83)
		miR-10b	NA	NA	NA	NA	NA	NA	0.76 (0.68-0.84)
		miR-21-3p	NA	NA	NA	NA	NA	NA	0.74 (0.65-0.82)
		miR-21-5p	NA	NA	NA	NA	NA	NA	0.79 (0.71-0.87)
		miR30c	NA	NA	NA	NA	NA	NA	0.77 (0.69-0.85)
		miR-106b	NA	NA	NA	NA	NA	NA	0.74 (0.65-0.83)
		miR-155	NA	NA	NA	NA	NA	NA	0.74 (0.66-0.83)
		miR-212	NA	NA	NA	NA	NA	NA	0.73 (0.65-0.82)
		miR-score	NA	NA	NA	NA	NA	NA	0.73 (0.65-0.82)
Fahrmann et al. [112]	PLCO (175 cases, 875 controls)	CA19-9	0.996 (0.99-1.00)	0.87 (0.78-0.97)	0.70 (0.60-0.81)	0.77 (0.69-0.86)	0.57 (0.43-0.72)	0.61 (0.48-0.74)	0.56 (0.45-0.67)
		CA19-9-neg*: CA19-9			***0-1 yrs***				
			NA	NA	0.63 (0.53-0.73)	NA	NA	NA	NA
		LRG!	NA	NA	0.63 (0.53-0.74)	NA	NA	NA	NA
		TIMP1	NA	NA	0.52 (0.42-0.63)	NA	NA	NA	NA
		All combined	NA	NA	0.62 (0.52-0.73)	NA	NA	NA	NA
Franklin et al. [113]	UPCB (23 cases, 22 controls); NSHDS (67 cases, 132 controls)								***<5 yrs***
		CA19-9	0.92 (0.84-1.00)	NA	NA	NA	NA	NA	0.62 (0.45-0.79)
		15 miRNAs	0.96 (0.92-1)	NA	NA	NA	NA	NA	0.60 (0.44-0.77)

*=Tumours negative for CA19-9 at baseline blood draw. Greyed areas denote reported AUCs; dx = diagnosis.

Table 9. *(continued)*

Reference	Cohort	Biomarkers	AUC (95% CI) before diagnosis						
			At dx	0-5 yrs	0.5-1 yrs	1-2 yrs	2-3 yrs	3-4 yrs	4-5 yrs
Honda et al. [114]	EPIC (156 cases, 213 controls)				*0-1.5 yrs*		*1.5-3 yrs*	*≥3 yrs*	
		CA 19-9	NA	0.80 (0.68-0.91)	0.71 (0.61-0.81)	NA	0.62 (0.53-0.71)	0.56 (0.48-0.64)	NA
		ApoA2-ATQ/AT	NA	0.62 (0.47-0.77)	0.65 (0.54-0.75)	NA	0.53 (0.43-0.62)	0.52 (0.44-0.60)	NA
		All combined	NA	0.78 (0.66-0.91)	0.74 (0.64-0.84)	NA	0.63 (0.54-0.72)	0.56 (0.48-0.64)	NA
Jenkinson et al. [115]	UKCTOCS (64 cases, 64 controls)					*≤2 yrs*			
		CA 19-9	NA	NA	NA	0.77	NA	NA	NA
		THBS1	NA	NA	NA	0.69	NA	NA	NA
		All combined	NA	NA	NA	0.86	NA	NA	NA
Krishnan et al. [116]	UKCTOCS (304 cases, 304 controls)				*0-1 yrs*	*1-2 yrs*	*>2 yrs*		
		CA 19-9	NA	NA	0.80	0.58	0.62	NA	NA
		CA19-9 /A1AT	NA	NA	0.88	0.66	0.64	NA	NA
Mason et al. [117]	UPCB (16 cases, 40 controls); NSHDS (154 cases, 320 controls)	CA 19-9	0.99 (0.99-1.00)	0.92 (0.78-1.00)	0.78 (0.17-0.95)	0.68 (0.51-0.81)	0.66 (0.50-0.79)	0.64 (0.51-0.77)	0.52 (0.37-0.69)
		CEA	0.79 (0.59-0.92)	0.53 (0.24-0.82)	0.69 (0.41-0.88)	0.59 (0.47-0.72)	0.60 (0.45-0.73)	0.67 (0.46-0.82)	0.44 (0.34-0.56)
		Collagen IV	0.58 (0.42-0.73)	0.41 (0.22-0.67)	0.61 (0.33-0.83)	0.59 (0.45-0.72)	0.56 (0.37-0.72)	0.60 (0.45-0.76)	0.60 (0.49-0.70)
		Endostatin	0.83 (0.64-0.91)	0.73 (0.55-0.89)	0.80 (0.55-0.89)	0.61 (0.46-0.72)	0.73 (0.57-0.84)	0.57 (0.43-0.72)	0.57 (0.42-0.73)
Mirus et al. [118]	CATPAC (24 cases, 24 controls), WHI (87 cases, 87 controls)							*<4 yrs*	
		ESR1/ERBB2/TNC	0.86 (0.76-0.96)	NA	NA	NA	NA	0.68 (0.58-0.77)	NA
		ESR1/ERBB2/TNC/CA19-9	0.97 (0.92-1.00)	NA	NA	NA	NA	0.71 (0.62-0.80)	NA

Greyed areas denote reported AUCs; dx = diagnosis.

Table 9. *(continued)*

Reference	Cohort	Biomarkers	AUC (95% CI) before diagnosis						
			At dx	0-5 yrs	0.5-1 yrs	1-2 yrs	2-3 yrs	3-4 yrs	4-5 yrs
Nené et al. [119]	UKCTOCS (218 cases, 249 controls); ADEPTS (17 cases, 17 controls)				***0-1 yrs***	***0-2 yrs***	***0-3 yrs***	***0-4 yrs***	***0- >4 yrs***
		CA 19-9	NA	NA	0.73 (0.52-0.93)	NA	NA	NA	NA
		CA 125	NA	NA	0.72 (0.62-0.82)	NA	NA	NA	NA
		CEA	NA	NA	0.84 (0.65-0.97)	NA	NA	NA	NA
		THBS2	NA	NA	0.65 (0.55-0.75)	NA	NA	NA	NA
		CA19-9 /CA125/VW -F/THBS2/ IL6ST	NA	NA	0.91 (0.75-1.00)	0.84 (0.72-0.93)	0.79 (0.67-0.88)	0.79 (0.69-0.88)	0.78 (0.69-0.86)
Nolen et al. [120]	PLCO (135 cases, 540 controls)				***0-1 yrs***		***1-3 yrs***		
		CA 19-9	NA	NA	0.70	NA	0.62	NA	NA
		CA 19-9/OPG	NA	NA	0.61	NA	0.52	NA	NA
		CA 19-9/OPG/OPN	NA	NA	0.60	NA	0.49	NA	NA
		CA 19-9/CEA	NA	NA	0.71	NA	0.62	NA	NA
		CA 19-9/CEA/Cyfra-21-1	NA	NA	0.69 (training set)	NA	0.66 (training set)	NA	NA
O'Brien et al. [121]	UKCTOCS (154 cases, 304 controls)	CA 19-9	NA	NA	0.80	NA	NA		
		CA 125	NA	NA	0.73	NA	NA		
		CEACAM1	NA	0.71	NA	NA	NA		
		REG3A	NA	0.73	NA	NA	NA		
		CA19-9/CA125'	NA	0.90	NA	NA	NA		
Udgata et al. [122]	PLCO (179 cases, 475 controls); Mayo Clinic Cohort (37 cases, 140 controls)				***0-1 yrs***	***0-2 yrs***	***0-3 yrs***		
		CA19-9	NA	NA	0.75 (0.68-¨0.82)	0.68 (0.63-0.73)	0.63 (0.59-0.67)	NA	NA
		THBS2	NA	NA	0.54 (0.47-0.62)	0.55 (0.49-0.62)	0.52 (0.47-0.58)	NA	NA
		All combined	NA	NA	0.75 (0.67-0.84)	0.70 (0.61-0.76)	0.62 (0.58-0.70)	NA	NA

Greyed areas denote reported AUCs; dx = diagnosis.

Discussion

Methodological considerations

The thesis included data from patients diagnosed with different subtypes of PDAC and in different eras, so knowledge on the disease classification and annotation methods used over time is important for correct interpretation of outcomes. We also employed immunohistochemistry and image processing, each with its own caveats.

Disease classification

We restricted ourselves to only using microscopically confirmed cancer. It is therefore important to acknowledge that the ICD-O-3 topography codes (C25.0 to C25.9) are only used to broadly categorize the disease as PDAC. To ensure the highest validity possible, we opted to report the same topographic, histologic and confirmatory codes as other studies of SEER in PDAC [123, 124]. Specific histological variants also carry challenges. For example, as referenced in Paper II, a pancreatic undifferentiated rhabdoid carcinoma was misclassified as a solid pseudopapillary neoplasm [125]. WHO also recognises other subvariants of UC/anaplastic, such as pleomorphic/giant cell, sarcomatoid, and spindle-cell subtypes, sometimes referred to as "non-OCUGC subtypes". As for distant disease, SEER is not able to capture the number of metastases, and only includes data on metastatic site from 2010 and onwards, making the data in this patient group less granular [126].

Staging

The studies included in this thesis report summary statistics of cases with AJCC staging from most editions of the manual. A key consideration is that the AJCC 8th edition staging manual was produced from pancreatic head tumours, making it less applicable to tail cancers. In addition, the manual was derived from imaging and clinical studies instead of resected specimens, which has raised questions regarding a discrepancy in T-stage between resected and non-resected patients [127]. Furthermore, by removing extrapancreatic extension in T-staging, previously included in the 7th edition, valuable prognostic information could be lost [128].

Immunohistochemistry and image analysis

Formalin fixation can cause extensive cross-linking between the epitope of CA19-9 and other molecules. Ensuring a good antigen retrieval protocol with uniform optimised pH values and temperatures is crucial, which is why automatic pre-treatment machines are preferred (see Figure 14). Likewise, using a manual approach for choosing thresholds in image processing software introduces user bias. As of now, no automatic thresholding specific to CA19-9 has been employed with ImageJ software, although it has been developed for immunofluorescence images [129].

Another methodological consideration is the limitations with TMAs. By their design, TMAs are evidently unable to capture the whole tissue area [130, 131]. Studies of concordance between TMA and whole-tissue slides have been conducted, but only relating to PD-L1 expression[132]. The TMA workflow is easily reproducible, but as researchers, we must still wait for a formal validation of tissue arrayed CA19-9 compared to whole-tumour tissue.

Prospective cohorts

Three of the four papers were retrospective studies of institutional registries. Papers I and II utilized SEER data, while Paper IV summarised five different large prospective cohorts. Retrospective designs always carry systematic limitations, but a low incidence of PDAC necessitates a larger prospective patient material, which is not available for historical data or data regarding histology. Obtaining specific high-risk cohorts is even more resource intensive than general cohorts, but should be the way forward according to the U.S.-based Early Detection Research Network [133] and PRECEDE consortium [106].

Checklists

Checklists play an important role, not only in respecting the data contributors and research participants, but to support informed decision-making. However, they are frequently misused. The STROBE guidelines explicitly state that their checklists are not to be used for assessing the quality of observational studies identified in the literature. For this purpose, the PRISMA statement is better suited. Checklists should only be used as a tool for the researcher to ensure that all methodological issues have been addressed to their best ability, but not as a mark of quality. They help researchers remember to handle missing data, bias assessment, and complete a detailed report of study selection strategies, but they cannot exclude a deeper methodological flaw in the original study.

Strengths and limitations

One of the greatest strengths in this thesis is the inclusion of real time-to-event outcomes outside the setting of a controlled trial. Using actual survival data reflects real-world outcomes much better than 'actuarial data' in PDAC, where most cases drop-off early in the survival curve, either due to early death or loss of follow-up. Actuarial survival, which is the calculation of Kaplan-Meier curves, tends to grossly overestimate PDAC survival if cases are censored (lost to follow-up). To prognosticate and draw general conclusions about PDAC survival over time, we should mandate methods that measure only the number of patients alive at each time interval, irrespective of censoring [134]. However, plotting survival curves is still required as a means of direct comparison between subgroups, as with Paper II.

By using SEER, we achieved a coverage large enough to directly compare rarer histological subvariants of PDAC. Paper II identified 159,548 microscopically confirmed cases, which is far beyond what single-institution cohorts can achieve. Through multivariable analysis, we were able to confirm the independent prognostic effect of colloid, adenosquamous, and signet-ring cell carcinoma and compare them directly to each other for the first time.

Our TMA workflow allowed for a simple, yet reproducible way to assess stromal content in PDAC. Using four tissue cores per tumour, the number of slides to be digitally analysed is drastically reduced compared to whole-tumour slides. The results from Paper III could enable a high-throughput and cost-effective solution to assess meaningful stromal biology. Similarly, the binary cut-off confers clear survival stratification, which was confirmed by the restricted cubic spine analysis (Figure 20).

A key strength of the meta-analysis is the ability to prioritise biomarkers for further testing. Unfortunately, no biomarker reported in the literature had sufficient prediagnostic performance compared to CA19-9. It systematically assessed all reported blood-based biomarker studies that had started employing prediagnostic cohorts. Rigorous investigation into the patient material was needed to exclude any overlapping studies in the resulting meta-analysis, and to accurately depict the time intervals. Further assessment of risk of bias, using the QUADAS-2 and QUADAS-C tools, revealed low risk of bias in most domains of the included studies.

There are several limitations in the papers included in this thesis. Only including microscopically confirmed cancer cases has inevitably increased the resection rate in the SEER patient material. SEER also tends to underreport the receipt of chemo or radiotherapy, confirmed by multiple studies [135, 136]. This in turn makes it hard to draw conclusions regarding benefits of surgical and other oncological therapies, but the variables should nonetheless be added as confounding factors in the multivariable analyses. Another important aspect is that SEER does not report recurrence for cancers, or specific radio/chemotherapy regimens. Similarly, a

common pitfall in SEER is the failure to distinguish between “no/unknown” and “no” chemotherapy [137]. As a U.S.-based registry, the underreporting of certain demographics (gender, ethnicity, socioeconomical status) due to various reasons, must also be acknowledged.

Ensuring concordance between TMA cores and whole-tumour tissue is important. However, comparison between the expression of TMAs compared to whole-tumour slides is usually assigned to novel markers [138], as CA19-9 has been used as a marker for tumour epithelia in histological PDAC studies since its inception. Careful marking of regions of interests in the tissue core is also critical and could be swapped for automated methods. Such methods are not easily reproducible, and to date, none have been published for PDAC TMAs [139, 140]. Additionally, the results from resected PDAC specimens may not generalise to metastatic PDAC lesions or to the complete microenvironment of the tumour.

In Paper IV, a recurring issue was the different ways of reporting AUC values in the studies, and sometimes the omission of standard errors, which had to be inferred from the available material. Our systematic review omitted extracellular vesicle-derived microRNA, which could be seen as a limitation. However, recent evidence has brought into question the methodological quality of most EV miRNA studies [141]. Furthermore, many meta-analyses of biomarkers identify a clear publication bias for favourable findings [142]. In the case of Paper IV, the systematic review would only be able to identify biomarkers deemed good enough for transition from the case-control study phase to prospective testing, which creates an inherent publication bias for prediagnostic markers. An interesting follow-up could be a comparison between published case-control studies of biomarker panels and published studies performing subsequent testing in prospective cohorts.

Advances in PDAC biomarker discovery

The pace at which bioscience advances depends on the methods available. Since the advent of bulk transcriptomic sequencing in the early 2000s, molecular profiling of PDAC has increased exponentially. Collisson [82], Moffitt [84], and Bailey et al. [87] showed that pancreatic cancers have distinct molecular subtypes with a clear bearing on clinical outcomes. With in-depth single-cell transcriptomics at the whole-tumour level, we can now observe these molecular subtypes within each tumour. This has brought into question the validity of 3D models of pancreatic cancer derived from single cell lines or small biopsies [143]. 3D models are important for the study of PDAC tumour evolution and desmoplasia [144], but it is equally important to recognise their limitations in capturing the clonal and stromal heterogeneity specific to PDAC. In this area, there is still a lot of work to be done.

Today, liquid biopsies represent the fastest growing area of biomarker research. Liquid biopsy is the term used for real-time analyses of tumour constructs, such as circulating tumour DNA, metabolites, extracellular vesicles, and circulating tumour cells (CTCs). The assays for tumour constructs continue to improve at a rapid pace. CTCs in PDAC can now be subdivided according to different origin vessels, and complete genomes of PDAC cells can be extracted from the extracellular vesicles released from the very same cell.

In parallel, high-throughput mass spectrometry of proteins continues to produce promising biomarkers. However, as we demonstrated in Paper IV, prediagnostic capability remains a challenge. For at least a decade, the standard approach has been 'systems proteomics', where researchers try to map as much of the proteome of a tissue as possible and plot the biomolecular function, proteome patterns, and protein-protein interactions of identified compounds. With an ever-growing library of identified proteins, researchers have started to shift towards a top-down approach. Instead of conjuring up new analytes, simultaneous measurements of as many proteins as possible could identify more powerful marker panels more quickly. Affinity-based proteomics, such as OLINK® and SomaLogic®, offer the capability to detect thousands of proteins at the same time in a serum sample, based on existing protein libraries.

If enhanced selectivity in the detection process is also desired, electrochemical sensing could be the next step. The "LAMT-PEC" system, pioneered by Li et al. [145], utilises atom-sized photoelectrodes to bind and detect proteins, minimising background noise while increasing detection rate. It is likely that in the future we will see a combination of electrochemical sensing and affinity-based mass proteomics.

Prognostication and therapeutics

Unsurprisingly, a lot of hope is placed on machine learning models for the analysis of multiple analytes, combining genomic, transcriptomic and proteomic data ("AI-assisted multiomics"). Machine learning also presents an effective way of pairing multiomics with radiological features, for better prognostication. Opisov et al. combined 6,363 clinical and omics features (including tumour stroma) to predict disease survival, but did not report histological subtypes [146]. Similarly, in a paper by Ju et al., a deep-learning model was used to differentiate prognosis-correlated PDAC subtypes, also without mention of variant histology [147]. In Paper II, we showed that the subtypes confer significantly different biology compared to conventional PDAC, so variant histology should be incorporated in predictive PDAC studies going forward.

Oncological ‘precision medicine’ is a field within PDAC research where response to targeted therapy on a case-by-case basis is investigated, and the ongoing molecular subtype research goes hand in hand with the development of new targeted therapies in PDAC. At present, only a handful of expression patterns of PDAC changes therapeutic regimens in the clinic. Interestingly, only the molecular subtypes (see Table 3) have been evaluated for chemotherapeutic response, while WHO subtype studies are lacking [148].

Emerging therapies in PDAC include oncolytic viruses, stroma-targeted modulation, CAR-T (chimeric antigen receptor T cells), and local therapies such as tumour treating fields [149]. Because PDAC desmoplasia causes low cellularity, the tumour is usually immunologically ‘cold’, rendering usual checkpoint-inhibitor therapy ineffective. ‘mRNA neoantigen vaccines’ is a new immunological approach that can trigger an effective personalized T-cell response in up to 50% of patients [150]. Their relationship to histological and stromal subtypes could be a promising area for further research.

The fruits of a heterogeneous PDAC landscape

A most pressing issue in PDAC is the lack of diagnostic tools, in addition to ineffective therapy. Our results suggest there has been no meaningful improvement in the distribution of localised PDAC tumours in the recent era, and that tumours need to be found at a very early stage. The rationale behind early detection tools of PDAC is the multiplexed assay, which must resemble the complex multiclonal evolution and molecular landscape [92]. Unsurprisingly, there is a multitude of ongoing efforts at building multiplexed biomarker assays for clinical practice. Known models include the OLINK®-based ‘Pancreasure’ [151], and a PDAC-associated protease assay called PAC-MANN-1 [152]. In combination with CA19-9, Pancreasure reached a high specificity in pre-symptomatic cases, and showed similar performance to PAC-MANN-1 in the case-control phase. However, none of these early detection tools reach the consensus target of 98% specificity.

In Paper IV, we demonstrated that the biggest difficulty is not the identification of biomarker panels in case-control studies, but to ensure that the diagnostic performance is consistent over time in pre-symptomatic individuals. The transition to prospective testing is both financially and emotionally taxing. To smoothen the transition, researchers could start with high-risk patients harbouring unclear pancreatic lesions on radiology. In an impressive study by Mahajan et al, patients with CT-identified suspicious pancreatic lesions were evaluated using two plasma metabolite signature panels, based on their ability to rule out pancreatic cancer. One of the panels reached an impressive 93.6% specificity in a patient material that was already available for the researchers at the hospital-level [153].

Conclusions

The conclusions from the papers included in this thesis are as follows:

I. Actual long-term survival in PDAC is extremely low, and improvements over time are almost entirely driven by surgically treated patients. Despite advances, real-world long-term survival remains below 5% overall.

II. Histological subtyping provides important prognostic information in PDAC. CC and UCOGC are associated with more noticeably improved survival than conventional PDAC. ASqC and SRCC display worse survival. Rare subtypes such as MCP show significantly favourable outcomes, which is confirmed by case reports. Variant histology should be incorporated into risk stratification and may influence future development of targeted therapies.

III. High stromal content in PDAC, digitally measured in a reproducible manner, is associated with improved overall survival. The method is highly feasible in large cohorts and can be applied consistently across samples. The findings challenge the traditional view that PDAC stroma is uniformly tumour-promoting.

IV. CA19-9 still has the best and most consistent ability to differentiate between future pancreatic cancer cases and healthy controls across all time windows up to 5 years before diagnosis. Accuracy is acceptable up to one year before diagnosis.

Future perspectives

With the enormity of data, and the frustration with PDAC as a disease entity, one cannot help but to sense a “Dunning–Kruger effect” underlying the search for the magic bullet. This effect is a cognitive bias, where less knowledge means more optimism regarding your actual knowledge, and vice versa. Consequently, the more you read up on current PDAC research, the more you realise how little we understand PDAC biology.

Already in 2019, our research team proposed *KRAS*-targeted therapy with special delivery molecules for better efficacy in the fibrotic PDAC environment. Recently, results for *KRAS* therapy in mice models have gained attention in media. With current failing methods, going back to key driver genes present in most tumours is not unsound. Rather than focusing on massively multiplexed techniques, finding simpler but robust common threads in PDAC, should be the new paradigm.

AI (machine learning) does not replace sound judgement and correct priorities, but it is notoriously optimistic in its interaction with humans. This optimism should carry over to PDAC research. PDAC is likely to soon become the deadliest major cancer, meaning that even more resources will be poured into the field. Maybe the magic bullet (or rather golden ticket) is staring us right in the face? All we need is an arm long enough to grab it or be lucky enough to catch a stray one.

If man can go to the moon, man can cure pancreatic ductal adenocarcinoma.

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It has been over eight years since I stumbled into the office at the surgical unit in Lund, wondering where the professor was. Translation: time flies when you are having fun.

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