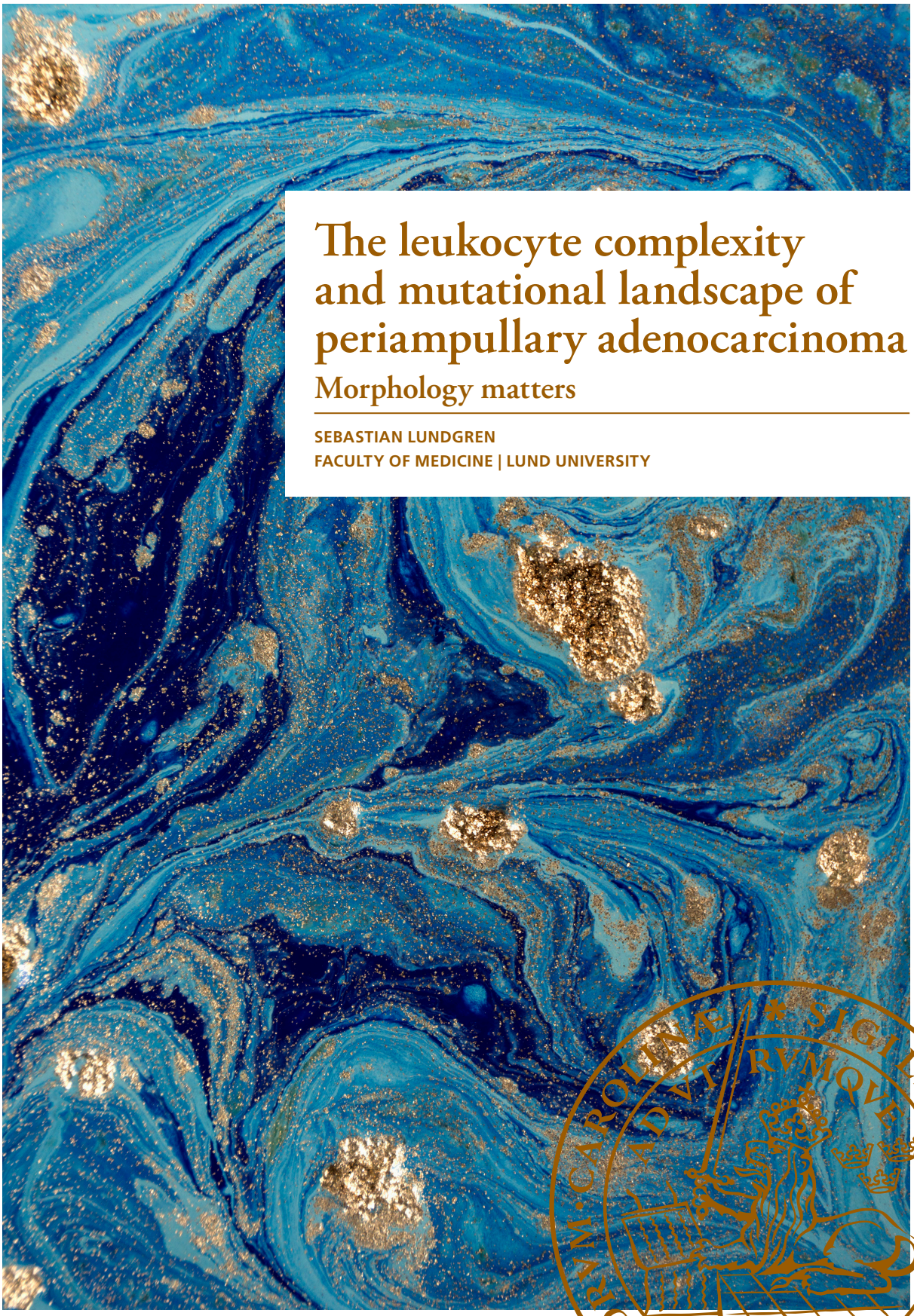
The leukocyte complexity and mutational landscape of periampullary adenocarcinoma



The leukocyte complexity and mutational landscape of periampullary adenocarcinoma

Morphology matters

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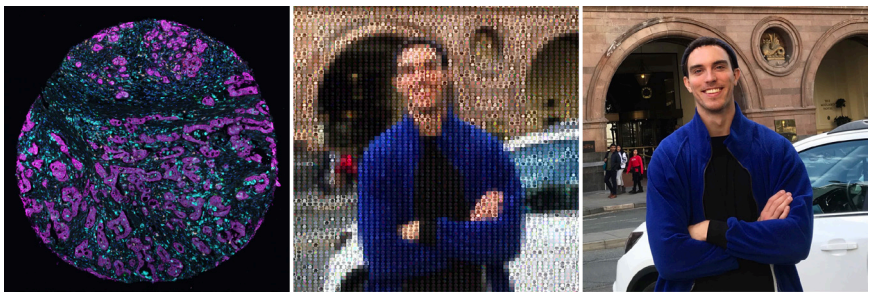

Sebastian Lundgren has during his doctoral studies investigated the inflammatory tumour microenvironment in periampullary adenocarcinoma. The aim has been to characterise the immune landscape in the entire spectrum of periampullary adenocarcinoma, with particular reference to morphology, genetic alterations and clinical outcome.

LUND UNIVERSITY

FACULTY OF MEDICINE

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2019:125
ISBN 978-91-7619-854-4
ISSN 1652-8220

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Morphology matters

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden
To be defended in the Lecture Hall of the Radiotherapy Building, Department of Oncology, Skåne University Hospital, Lund
Friday 13th December 2019 at 09.15 am.

Faculty opponent
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Organization LUND UNIVERSITY Faculty of Medicine Department of Clinical Sciences Division of Oncology and Pathology	**Document name** Doctoral disseration
	Date of issue 13th December 2019
Author: Sebastian Lundgren	Sponsoring organization

Title and subtitle The leukocyte complexity and mutational landscape of periampullary adenocarcinoma: Morphology matters

Abstract

Background: Periampullary adenocarcinomas are a heterogenous group of tumours with poor prognosis that has not improved considerably the last decades. Tumour morphology, i.e. intestinal type (I-type) or pancreatobiliary type (PB-type), has been demonstrated to be a more relevant prognostic factor than anatomical origin. Tumour infiltrating immune cells are vital in shaping the natural progress of cancer. The aim of this thesis was to characterise the landscape of immune cells and common mutations in the entire spectrum of periampullary adenocarcinoma, with particular reference to morphology and clinical outcome.

Methods: All studies are based on tumours from a retrospective, consecutive cohort of 175 patients with periampullary adenocarcinoma who underwent surgical resection in Skåne University Hospital between 2001 and 2011. The infiltration of several immune cell populations was analysed by single-marker immunohistochemistry (Paper I-II) and multiplexed immunofluorescence (Paper IV and V) on tissue microarrays. Targeted next generation DNA seqencing (Paper III) was applied to analyse mutations in 70 common cancer-associated genes in tumours from 102 cases.

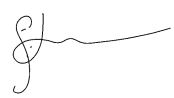
Results: Paper I shows that high infiltration of natural killer NK/NKT cells was associated with prolonged overall survival (OS), particularly in patients with I-type tumours. In patients with PB-type tumours, high infiltration of NK/NKT cell infiltration was only prognostic in cases who did not receive adjuvant chemotherapy, and, notably, there was a significant negative interaction between adjuvant chemotherapy and NK/NKT cell density in relation to OS. Paper II shows that high infiltration of immature dendritic cells was an independent factor of poor prognosis in patients with PB-type tymours. High infiltration of $CD68^+$ and $CD163^+$ macrophages was assocated with reduced OS in the entire cohort, whereas high infiltration of $MARCO^+$ macrophages was a negative prognostic factor only in patients who recived adjuvant chemotherapy, but there was no signficant treatment interaction. Paper III demonstrated that *APC* and *ERBB3* mutations were more common in I-type tumours while *CDKN2A* mutations were more common in PB-type tumours. *KRAS* mutations were associated with reduced OS in patients with I-type tumours. in patients with PB-type tumours, *SMARCA4* mutation was a negative prognostic factor in patients not receiving adjuvant chemotherapy and there was a positive interaction between high expression of BRG1, the protein encoded by *SMARCA4,* and adjuvant chemotherapy in relation to OS. Paper IV demonstrates that the prognostic impact of different lymphocyte subsets, including signatures thereof, differ by morphology, and that high levels of $CD8^+$ T cells interacting with cancer cells and $CD4^+$ T cells, respectively, were associated with a prolonged OS. Moreover, immune cell density differed by the mutational status of several genes. Paper V provides further validation of the beneficial prognostic impact of NK/NKT cells, also in the context of interaction with macrophages. In addition, several prognostic innate immune cell subsets and signatures were defined, that differed by tissue compartment and morphology.

Conclusions: The prognostic and potential predictive impact of tumour infiltrating immune cells and common mutations in periampullary adenocarcinoma differs by morphology, thus highlighting the importance of taking morphology into account in the quest for complementary biomarkers. Moreover, the prognostic value of tumour infiltrating immune cells can be futher refined by analyses of their spatial and compartmental distribution.

Key words: periampullary adenocarcinoma, pancreatic cancer, immune microenviroment, prognosis, macophages, natural killer T cells, natural killer cells, T cells, B cells

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language**
ISSN and key title 1652-8220		**ISBN** 978-91-7619-854-4
Recipient's notes	**Number of pages** 101	Price
	Security classification	

Signature  Date 2019-11-07

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Cover photo by Anna Babii © 123RF.com

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ISBN 978-91-7619-854-4
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2019

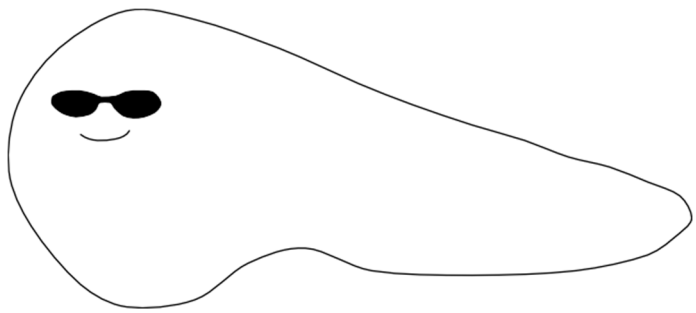

Table of Contents

Papers included in the thesis **10**

Papers not included in the thesis **11**

Abbreviations **12**

Introduction **15**

Background **16**

Periampullary adenocarcinoma 16

Anatomy and histopathology 16

Epidemiology 17

Aetiology, carcinogenesis and molecular aspects 19

Clinical presentation and symptoms 23

Diagnostics 24

Clinicopathological assessment 24

Surgical treatment 27

Chemotherapy 28

Radiation therapy 30

Targeted therapies 30

Immunotherapy 32

The immune system and cancer 34

The role of the immune system against malignant transformation 34

The innate immune system 34

The adaptive immune system 40

Immunoediting and escape 43

Immunophenotypes and their clinical relevance 44

Aims of the present investigation **47**

Specific aims 47

Methodology **48**

Tissue microarray technique 48

Immunohistochemistry 50

Principles 50

Assessment 51

Immunofluorescence 53
Principles 53
Assessment and analysis 53
Next generation sequencing 55
Principles and analysis 55
Study cohort 57
Statistical models 58
Ethical considerations 59
Funding 60
The present investigation 61
Summary and discussion of key results 61
Paper I 61
Paper II 62
Paper III 63
Paper IV 65
Paper V 68
Strengths and limitations 72
Conclusions 74
Future perspectives 76
Populärvetenskaplig sammanfattning 77
Acknowledgements 81
References 82

Papers included in the thesis

The following papers form the basis of the thesis and are referred to by their Roman numerals throughout the text.

I. The Prognostic Impact of NK/NKT Cell Density in Periampullary Adenocarcinoma Differs by Morphological Type and Adjuvant Treatment **Lundgren S,** Warfvinge CF, Elebro J, Heby M, Nodin B, Krzyzanowska A, Bjartell A, Leandersson K, Eberhard J, Jirström K. *PLOS One*. 2016. Doi: 10.1371/journal.pone.0156497

II. The clinical importance of tumour-infiltrating macrophages and dendritic cells in periampullary adenocarcinoma differs by morphological subtype. **Lundgren S**, Karnevi E, Elebro J, Nodin B, Karlsson MCI, Eberhard J, Leandersson K, Jirström K. *Journal of Translational Medicine.* 2017. Doi: 10.1186/s12967-017-1256-y

III. Mutational Landscape in Resected Periampullary Adenocarcinoma: Relationship with Morphology and Clinical Outcome. **Lundgren S**, Olsson Hau S, Elebro J, Heby M, Karnevi E, Nodin B, Eberhard J, Holm K, Staaf J, Jönsson G, Jirström K. *JCO Precision Oncology.* 2019. Doi: 10.1200/PO.18.00323

IV. Quantitative, qualitative and spatial analysis of lymphocyte infiltration in periampullary and pancreatic adenocarcinoma. **Lundgren S**, Elebro J, Heby M, Nodin B, Leandersson K, Micke P, Jirström K, Mezheyeuski A. Submitted manuscript.

V. Spatial interaction and compartmental distribution of innate leukocytes in periampullary and pancreatic adenocarcinoma. **Lundgren S**, Micke P, Elebro J, Heby M, Hrynchyk I Nodin B, Leandersson K, Mezheyeuski A, Jirström K. Manuscript in preparation.

Papers not included in the thesis

- Relationship between mismatch repair immunophenotype and long-term survival in patients with resected periampullary adenocarcinoma. Heby M, **Lundgren S**, Nodin B, Elebro J, Eberhard J, Jirström K. *Journal of Translational Medicine.* 2018. Doi: 10.1186/s12967-018-1444-4
- Discovery of KIRREL as a biomarker for prognostic stratification of patients with thin melanoma. **Lundgren S**, Fagerström-Vahman H, Zhang C, Ben-Dror L, Mardinoglu A, Uhlen M, Nodin B, Jirström K. *Biomarker Research.* 2019. Doi: 10.1186/s40364-018-0153-8
- Clinical significance of stromal ER and PR expression in periampullary adenocarcinoma. Andersson G, **Lundgren S**, Heby M, Nodin B, Elebro J, Jirström K. *Biomarker Research* (forthcoming).

Abbreviations

5-FU = fluorouracil

αGalCer = α-galactosylceramide

ADEX = aberrantly differentiated endocrine exocrine

AJCC = American Joint Committee on Cancer

APC = antigen presenting cells

BCR = B cell receptor

BRG1 = Brahma-Related Gene 1

CART = chimeric antigen receptor

CDK = cyclin dependent kinase

CTLA-4 = cytotoxic T-lymphocyte-associated protein 4

CRT = classification and regression tree

CUP = Cancer of Unknown Primary

DAMPs = damage associated molecular patterns

DAPI = 4′,6-diamidino-2-phenylindole

DC = dendritic cell

CDK = cyclin dependent kinase

DFS = disease free survival

dMMR = deficient mismatch repair

EGFR = epidermal growth factor receptor

EMA = European Medicine Agency

FOLFIRNOX = folinic acid, 5-FU, irinotecan, oxalipatin

GEMOX = gemcitabine oxaliplatin

GemCap = gemcitabine capecitabine

H-score = histoscore

HLA = human leukocyte antigen

I-type = intestinal type

IFNγ = interferon-γ

IHC = immunohistochemistry

IL = interleukin

Ig = immunoglobulin

IPMN = intraductal pancreatic mucinous neoplasm

KIRs = killer cell Ig-like receptors

LOF = loss of function

M-stage = metastatic stage

MDSCs = myeloid derived suppressor cells

mFOLFIRINOX = modified FOLFIRINOX

MFS = metastasis free survival

MHC = major histocompatibility complex

MMR = mismatch repair

MSI = microsatellite instability

N-stage = nodal stage

NCRs = natural cytotoxicity receptors

NGS = next generation sequencing

NKT = natural killer T-cell

NTRK = neurotrophic receptor tyrosine kinase

PanIn = pancreatic intraepithelial neoplasia

PCR = polymerase chain reaction

PB-type = pancreatobiliary type

PDAC = pancreatic ductal adenocarcinoma

PD-1 = programmed death receptor 1

PD-2 = programmed death receptor 2

PDL-1 = programmed death receptor ligand 1

PDL-2 = programmed death receptor ligand 2

OS = five-year overall survival

RFS = recurrence free survival

ROS = reactive oxygen species

SNPs = single nucleotide polymorphisms

T-stage = Tumour stage

TAMs = tumour associated macrophages

TCGA = The Cancer Genome Atlas

TCR = T cell receptor

TGFβ = transforming growth factor β

TMA = tissue microarray

TNFα = tumour necrosis factor α

T_{reg} = T regulatory cells

TRK = tropomyosin receptor kinase

T_H1 = type 1 T helper cell

T_H2 = type 2 T helper cell

T_H17 = type 17 T helper cell

VISTA = V-domain Ig suppressor of T cell activation

wt = wild-type

Introduction

> Before we begin our banquet, I would like to say a few words. And here they are: Nitwit! Blubber! Oddment! Tweak!
>
> Albus Dumbledore

Cancer has been a companion to humankind throughout history, the first documented case that we know of is from 1600 years B.C in ancient Egypt (1). Hippocrates thought cancer had its causes in an unbalance of the four bodily fluids (2). Today we know that cancer is not one disease but rather hundreds of different diseases, all with varying pathogenesis, aetiology and natural course. Some cancers are hereditary, some are related to life style factors such as smoking or obesity, while others are due to infections. However, they all share six characteristics defined in a seminal paper by Hanahan *et al* in 2000 (3), which was updated with four more characteristics in 2011 (4). The first six characteristics include the ability to evade apoptosis, self-sufficiency in growth signalling, insensitivity for anti-proliferative signalling, replicative immortality, sustained angiogenesis and tissue invasion and metastatic capabilities. Progress in our understanding of carcinogenesis during the early 2000s made it clear that the six original hallmarks of cancer did not capture the entire biological basis of malignancy and was therefore amended with two emerging hallmarks; avoidance of destruction by the immune system and deregulation of cellular energetics as well as two enabling hallmarks: genomic instability and tumour promoting inflammation. These 10 characteristics constitute the evolutionary process of cancer and this thesis will focus on several of them including tumour promoting inflammation, genomic instability and avoidance of destruction by the immune system.

Since Hippocrates, our understanding of cancer has developed immensely, however there is yet much we do not understand about the biology of these diseases.

Background

What we know here is very little, but what we are ignorant of is immense.

Pierre Laplace

Periampullary adenocarcinoma

Anatomy and histopathology

Periampullary adenocarcinomas are cancers that arise in the area around the ampulla of Vater. This is a complex anatomical region where many organ systems join together. The ampulla of Vater is the end station of the ductus choledochus, which transports bile from the ductus cysticus and ductus hepaticus, and the ductus pancreaticus, which transports digestive enzymes from the pancreas. The ampulla of Vater empties into the duodenum, the first portion of the small intestine. Therefore, periampullary cancers can have four different anatomical origins: the distal ductus choledochus, caput pancreatis, the duodenum or the ampulla of Vater (Figure 1). Besides having four possible anatomical sites of origin, periampullary cancers can be subdivided into two morphologies, either intestinal type (I-type) or pancreatobiliary type (PB-type). As one might deduct from the terminology, I-type tumours have a morphology resembling that of the small intestine, while pancreatobiliary tumours have a morphology that resembles that of the pancreatic ducts and the biliary tract. Although adenocarcinomas are in general classified by anatomical origin, the importance of morphological subtyping is illustrated when it comes to patient outcome; morphology has repeatedly been shown to be a superior prognostic factor compared to anatomical origin, in that I-type tumours have a more favourable prognosis than PB-type tumours (5-12). Due to the complex anatomical conditions in the region, anatomical site of origin can be hard to determine with radiology (13), and morphology is often difficult to determine in unresected tumours, which constitute the majority of cases. Hence, periampullary adenocarcinomas are often treated as one clinical entity.

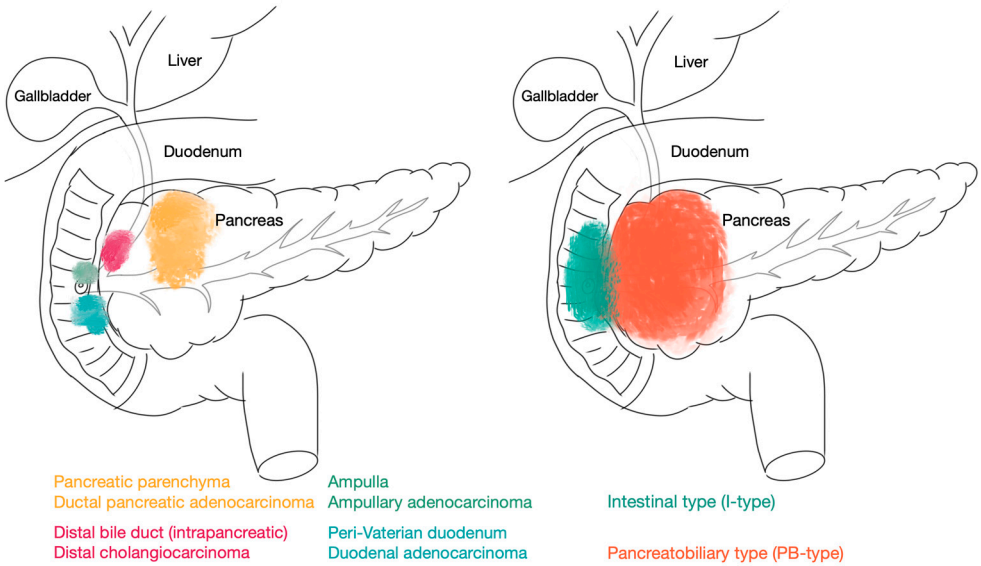

Figure 1:
Schematic illustration of the four different anatomical origins of periampullary adenocarcinoma (left) and distribution of morphological subtypes (right). Courtesy of Dr Gustav Andersson.

In order to determine morphology, biopsies are needed. Morphological diagnosis is determined by fine needle aspiration, core needle biopsy or in some cases surgical biopsy. The aim is to get enough tissue to be able to classify the tumour morphologically and to give a rationale for chemotherapy in a palliative setting. In resectable tumours, morphological and anatomical classification can be carried out more easily. However, one should be aware of that morphological diagnosis can be hard, even for a well experienced pathologist, and there is a considerable inter-pathologist variation. Additionally, tumours might not be clear cut either I-type or PB-type, and therefore there is a spectrum of mixed types or even poorly differentiated periampullary adenocarcinomas (10, 12, 14).

Epidemiology

Pancreatic cancer

Pancreatic adenocarcinoma has its origin in ducts of the exocrine pancreas and is therefore often called pancreatic ductal adenocarcinoma (PDAC). Although other types of cancers with pancreatic origin exist, such as endocrine pancreatic tumours, these are not as common, and they are clinically distinct from PDACs. Throughout this book, the term “pancreatic cancer” will therefore refer to PDAC. Around 76% of pancreatic cancers arise in the caput, with the remaining 24% evenly distributed

in the corpus and cauda (15). Pancreatic cancer has been reported to make up for around 50-75% of all resected periampullary adenocarcinomas (16-20), and for up to 82% when also including non-resected patients (16). Pancreatic cancer is the fourth leading cause of cancer related deaths in Europe (21), hence being responsible for more deaths than for instance breast cancer (22), and the global number of deaths attributed to pancreatic cancer is estimated to be as high as 227 000 (23). Globally, almost 500 000 new cases are reported each year (24), and this number is thought to increase with better diagnostic tools and improvements in reporting infrastructure. Even though many other cancer types have seen dramatic survival improvements in the recent decades, the 5 year overall survival rate (OS) remains very dismal for this group of patients, lingering around as low as 6% (24-26). Since pancreatic cancer accounts for the bulk of periampullary adenocarcinoma, the background of this thesis will mainly focus on pancreatic cancer.

Ampullary cancer

Ampullary cancers have their origin in the ampulla of Vater, the terminal portion of the ductus choleducus and ductus pancreaticus. They are less common than pancreatic cancer, with an incidence of 0.9 per 100 000 people (27). Ampullary cancer makes up for around 10-20% of surgically treated periampullary cancers (16-20). Although being more uncommon, they often have a better prognosis than other types of periampullary adenocarcinoma (28). This may partly be explained by earlier detection, as ampullary cancers tend to become symptomatic at an earlier stage, and by their higher resectability (up to 85%) (29).

Distal bile duct cancer

Distal bile duct cancers (cholangiocarcinoma) arise in the most distal parts of the bile system. Distal cholangiocarcinoma account for approximately 10-20% of resected periampullary adenocarcinomas (16-20). The OS for patients treated with resection in the curative setting has been reported to be around 38% (30, 31).

Duodenal cancer

Duodenal cancers account for about 3-7% of resected periampullary adenocarcinomas (16-20). They have the best prognosis of all periampullary cancers with a reported OS of 30-50%, including patients who do not undergo surgery with curative intent (16, 32). Partly, the less dismal prognosis for this group of patients may be explained by earlier detection.

Aetiology, carcinogenesis and molecular aspects

Risk factors

Pancreatic cancer can develop in three settings with different aetiological backgrounds; sporadic (90%), familial (7%) and hereditary (3%) (33). Several risk factors for pancreatic cancer have been identified including smoking, chronic pancreatitis, age, diabetes mellitus type II, obesity and the metabolic syndrome, diets rich in meat, A, B or AB blood group and family history (23, 34-37). Smoking is a particularly important risk factor, increasing the risk with 75% (38), and estimates attribute 20% of pancreatic cancer to smoking (39). Of note, women seem to be more susceptible to the detrimental effects of smoking when it comes to the development of pancreatic cancer (40).

Family history is an important risk factor. Hereditary pancreatic cancer is most commonly due to germline mutations in the *BRCA* genes (41), however patients with Peutz-Jeghers syndrome (germline mutations in *STK11*) and Lynch syndrome (germline mutations in mismatch repair [MMR] genes) also have an elevated risk of pancreatic cancer (23).

Carcinogenesis

Pancreatic cancer arises from non-invasive precursor lesions, either from pancreatic intraepithelial neoplasia (PanIN) by acinar metaplasia, or from intraductal papillary mucinous neoplasms (IPMN) in larger pancreatic ducts or, less commonly, from mucinous cystic neoplasms (primarily in the cauda). Morphologically, PanINs and IPMNs can be hard to distinguish, but PanINs are typically smaller in size, less than 0.5 cm, while IPMNs are larger than 1 cm. Precursor lesions in the range of 0.5-1 cm can either be large PanINs or small IPMNs (42). Since 2015, there is a consensus that all precursor lesions should be graded into either low grade or high grade depending on the degree of dysplasia (42).

Early in the carcinogenesis, tumour cells acquire *KRAS* mutations and mutations in this gene are almost ubiquitous in pancreatic cancer (43-45). Later on, tumour cells acquire mutations in *CDK2NA*, transforming growth factor β (TGFβ)/*SMAD4* signalling pathways and *TP53* (46-48). There is some divergence in the carcinogenesis of pancreatic cancer, depending on the cellular origin and type of precursor lesion, however the ones listed above are shared and key to progression to cancer. This process is relatively slow, the time from the first mutation until the formation of a non-metastatic parent tumour is estimated to be ten years, and then an additional five years before metastatic disease is established (49). Hence, there is, theoretically, a window of 15 years in which pancreatic cancer can be detected and potentially cured. However, as aforementioned, pancreatic cancer seldom presents at an early stage.

Recent studies have complicated our view on the evolutionary trajectory of pancreatic cancer. The traditional viewpoint that pancreatic carcinogenesis is a gradual evolutionary process is under fire, or at least challenged not to capture the full conceptual evolutionary development of pancreatic cancer It has been shown that the progression of pancreatic cancer is neither gradual nor that the carcinogenesis follows the postulated sequence of gene mutations in *KRAS, TP53*, *CDKN2A* and *SMAD4,* respectively (Figure 2). Notta *et al* demonstrated that most tumours follow the evolutionary trajectory of punctuated equilibrium, meaning that tumours show a burst of evolutionary development but long periods of stability (50). It has been shown in several aggressive cancer types that large catastrophic genetic events such as chromotripsis and polyploidization can drive the acquisition of many somatic mutations relatively fast (51, 52). Notta *et al* showed that for many pancreatic tumours the sequential gradual development proposed by the PanIN model is not true, but that the tumours rather acquire large numbers of mutations in a burst-like fashion, including simultaneous mutations in key driver genes (50). The punctuated equilibrium evolutionary trajectory could also explain why some pancreatic tumours are prone to metastasize very fast, as only one genetic event would be required for one clone to acquire both invasive and metastatic capabilities, and thus a large evolutionary benefit. This is probably also the reason why T1/2N0 tumours causing early obstruction of the bile duct still have very poor prognosis, and why more than one fourth of all Cancer of Unknown Primary (CUP) at autopsy are classified as pancreatic cancer (53).

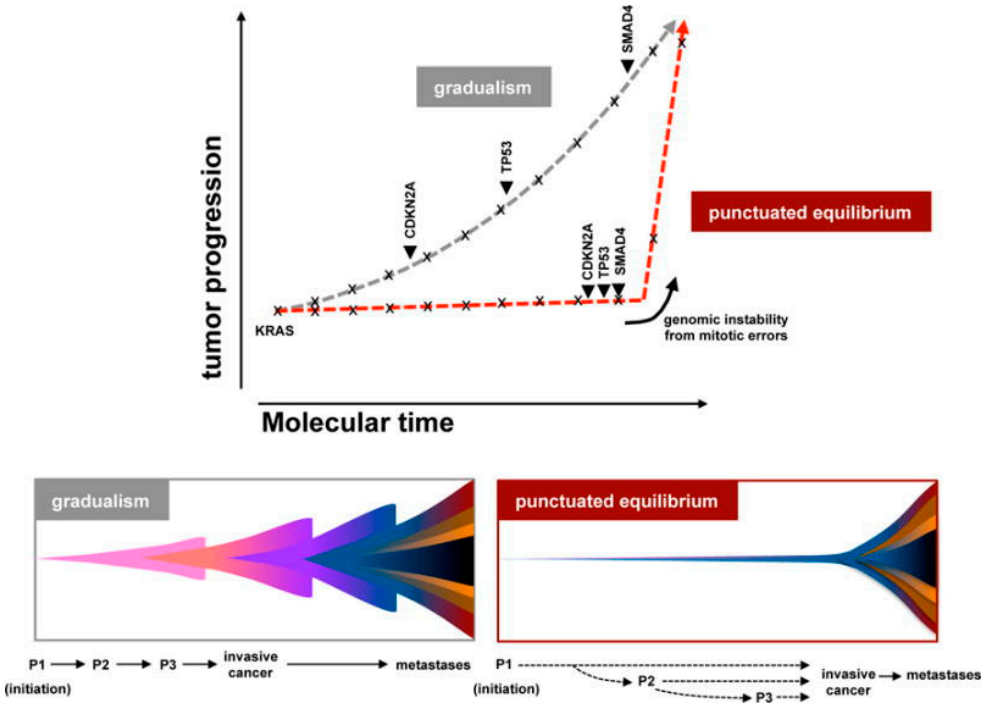

Figure 2:
Conceptual models of pancreatic carcinogenesis. In the gradual model molecular alterations are aquired in an independent and stepwise manner with periods of latency between aqucisiton of mutations in key driver genes. In the puncutated equilibrium model, carcinogenesis is due to two events, an initating event and a transforming event due to genomic instability based on copy number variations leading to a burst of mutations in a large amount of genes. Reproduced with permission from *Nature*.

Molecular aspects

During the 2010s there has been an ongoing effort to identify genetic subgroups of pancreatic cancer so as to better understand the biology of the disease. Bailey *et al*. identified four molecular subtypes based on gene expression; squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX), (54). The study also identified 10 key pathways in which most genetic mutations occur; KRAS, TGFβ, NOTCH, WNT, G1/S cell cycle transition, SWI-SNF, chromatin modification, DNA repair, RNA processing and ROBO/SLIT signalling (54). Waddell *et al* identified four subtypes with whole genome sequencing, based on structural variations in chromosomes including stable, locally rearranged, scattered and unstable. Based on gene expression data, Moffitt *et al* identified two subtypes; basal like and classical (21), and Collinson *et al*, defined three subtypes; classical, exocrine-like and quasimesenchymal. The clinical relevance of these classifications remains disputed, it is however clear that there is a concordance in the separate classifications in these studies (55), indicating that these classifications

tell us something about the underlying biology and evolutionary processes behind pancreatic cancer.

Although some genetic alterations are very common in pancreatic cancer (such as *KRAS* mutations), the number of frequently mutated genes is low but with a large tail of less commonly mutated genes. The implication of this is that the number of potential therapeutic targets is rather low. In the Cancer Genome Atlas (TCGA), the most common mutated genes are *KRAS* (71%), *TP53* (63%), *SMAD4* (23%), *CDKN2A* (20%), *TTN* (18%), *DNM1P47* (14%), *MUC16* (9%), *RNF43* (7%), *RNF213* (6%) and *CSMD2* (6%) (56). Of these, four are considered to be key drivers: *KRAS*, *TP53*, *SMAD4* and *CDKN2A* (57). I-type and PB-type tumours share many common genetic alterations, however, differences in key genetic drivers have also been reported, including some chromosomal deletions exclusive to I-type tumours (58).

Activating mutations in *KRAS* are, as mentioned above, very common. Only a single amino acid substitution is required to obtain an activating *KRAS* mutation; the most common being G12D and G12V. However, other types of codon 12 mutations exist, as well as other less frequent mutations in other codons. *KRAS* is a proto-oncogene that is involved in cellular signal transduction. Physiologically, *KRAS* encodes a GTPase that propagates proliferation signals intracellularly after epidermal growth factor receptor (EGFR) stimulation (59). Activating mutations in the *KRAS* gene lead to EGFR-independent activation of the GTPase encoded by *KRAS* and, thus, excessive proliferation of the cell. Tumours with *KRAS* mutations are not sensitive to anti-EGFR treatment such as cetuximab (59). Hence, *KRAS* mutation is a negative predictive biomarker for response to anti-EGFR treatment. *KRAS* mutations are less frequent in I-type tumours such as ampullary and duodenal cancers (60).

TP53 encodes the protein p53, classically known as “the guardian of the genome”. The protein p53 acts as a tumour-suppressor by maintaining genetic stability (61). The protein is upregulated by cellular stress such as DNA damage, viral infection or oncogene activation, and its upregulation can lead to apoptosis, cell cycle arrest or DNA repair (62). Therefore, p53 is vital in the prevention of malignant transformation of cells. Loss of function (LOF) mutations in *TP53* thus hinder this preventive functionality of p53.

SMAD4 and its gene product SMAD4 are parts of the SMAD pathway which sits downstream of TGFβ signalling (63) SMAD4, like the other proteins in the SMAD family regulate the transcription of a large number of genes. Targeted genes and the resulting transcriptional setting are largely dependent upon the contextual determinants set by TGFβ signalling, including signal transduction conditions, the epigenetic landscape and conditions during transcription (63). The cellular effects of SMAD4 are thus multifold and complex, however, SMAD4 is generally considered to be a tumour suppressor due to its role in cell cycle arrest and apoptosis,

blocking mitogenic signals (64). SMAD4 has a significant crosstalk with other intracellular pathways such as MAPK, PI3K/AKT and WNT/β-catenin (65), and LOF in *SMAD4* can lead to less control of the proliferative effects of these pathways.

CDKN2A encodes two proteins, p14arf and p16. Both of these proteins act as tumour suppressors by negative control of cyclin dependent kinases (CDK) 4 and 6 (66, 67). Inhibition of CDK4 and CDK6 stops the transition of the cell cycle from G1 to S-phase (67).The protein p14arf can also activate p53 (68). *CDKN2A* mutation is an indicator of poor prognosis in pancreatic cancer (69).

Microsatellite instability

Microsatellite instability (MSI) is a condition of genetic hypermutability in tumours due to deficiency in the DNA mismatch repair (MMR) system. This system is a family of enzymes which supervise the DNA replication process and correct errors such as base substitutions, deletions and insertions. LOF in the MMR system is therefore associated with an accumulation of mutations as there is no "proof-reading" of the replicated DNA. The enzymes involved in MMR that are of clinical interest are MSH2, MSH6, PMS2 and MLH1. During the DNA "proof-reading", MSH2/MSH6 and PMS2/MLH1 act in pairs (70). MMR deficient tumours are more prone to accumulate mutations compared to MMR proficient tumours, and therefore have a higher neoepitope burden. In pancreatic cancer, the prevalence of MMR deficient (dMMR) and MSI-high tumours ranges from 13-22% (71-73) and patients with dMMR/MSI-high tumours have been shown to have a prolonged survival (71, 73).

Clinical presentation and symptoms

Due to the anatomical conditions in the periampullary region, tumours in this area tend to be asymptomatic for a long period, giving rise to symptoms only late and in advanced stages. Generally, though, ampullary cancers tend to present themselves earlier than distal bile duct and pancreatic cancers, as ampullary cancers may give rise to biliary obstruction, and thus symptoms, earlier (13). Common symptoms for all periampullary cancers include abdominal discomfort or pain, jaundice, loss of weight, dyspepsia, xerostomia, problems with sleep, diabetes and early satiety (74). Jaundice, weight loss and pain should be considered cardinal symptoms (75, 76). There are few, if any, specific symptoms of periampullary adenocarcinoma although onset of diabetes mellitus type II has been associated with a higher risk of diagnosis of pancreatic cancer later on (77).

Clinically, these tumours have four possible settings at diagnosis: resectable, borderline resectable, locally advanced and metastatic. These four settings have

implications for the clinical management and treatment stratification as will be discussed later on.

Diagnostics

As outlined above, the clinical presentation of periampullary adenocarcinoma is unspecific. Therefore, there is often a considerable delay in diagnosis, which in itself is a factor of poor prognosis. However, even if patients are diagnosed in T-stage IA, the OS is still only 40%, and most patients who undergo surgical treatment with curative intent still die of metastatic disease (78). Thus early, asymptomatic detection is key to raise survival rates in this group of patients.

Screening programmes on a population basis have been proposed but deemed not feasible due to the low incidence of pancreatic cancer (79). There is however now a consensus that individuals with higher risk of pancreatic cancer such as those with a family history should be enrolled into targeted screening programmes (80). According to the Swedish recommendations, families with two or more cases of pancreatic or periampullary cancer in the same family branch should be offered oncogenetic consultation, however usually only individuals with a marked higher risk (15% or more) are enrolled into a screening program (81). There is a lack of consensus regarding whether and if so, individuals with higher non-hereditary risk (such as those with chronic pancreatitis or potential paraneoplastic diabetes mellitus type II) should be enrolled into screening programmes (79). As of today, no such programmes exist in Sweden.

There is also a lack of diagnostic biomarkers. The use of CA19-9, the most extensively studied biomarker in blood is mainly reserved for clinical monitoring of patients with an already established disease and its benefit in diagnostics and early discovery is limited (82). Adding to this, radiology does not have enough sensitivity to detect early pancreatic and periampullary cancer and even less so precursor lesions (78).

Clinicopathological assessment

The TNM Classification of malignant tumours is a universally accepted classification system used to assess the anatomical spread of solid tumours. The TNM system of classification is based on the anatomical origin of tumours and therefore separate TNM classification systems are used in periampullary cancers depending on the anatomical site of origin. Prognostic stage grouping (Stage I-IV) is a combined staging system using the TNM classicisation system as a basis. A summary of the 8th American Joint Committee on Cancer (AJCC) staging system is presented in Table 1 (83).

Tumour stage

Tumour stage (T-stage) describes the anatomical extent of the primary tumour, ranging from TX (not assessable) to T1-T4, with several subgroupings within each stage. In ampullary cancers, T0 stands for no evidence of a primary tumour, and Tis for cancer *in situ* (84). T1-4 stand for tumours with increasing magnitude of invasiveness, where T4 is reserved for tumours that involve arteria mesenterica superior, arteria hepatica communis and/or truncus coeliacus (84). In pancreatic tumours, TX and Tis are similar to ampullary cancer, where Tis includes PanIN and IPMN. T1-3 stand for tumours with increasing magnitude of size and T4 describes tumours with involvement of arteria mesenterica superior, arteria hepatica communis and/or truncus coeliacus regardless of size (84).

Lymph node spread

Lymph node spread or nodal stage (N-stage) is for all periampullary cancers, except for duodenal cancer, classified as either not present (N0), present in one to three regional lymph nodes (N1), or present in four or more lymph nodes (N2). In duodenal cancer, N1 indicates presence in one to two regional lymph nodes and N2 in more than three regional lymph nodes. N-stage is an important prognostic factor to consider in these cancers, as patients with higher N-stage have significantly worse outcomes than those with lower N-stage (17, 85-91). Therefore, the number of lymph nodes that need to be resected during a lymphadenectomy has been standardised to at least ten in order to accurately reflect lymph node spread (92).

Distant metastasis

Metastatic stage (M-stage) indicates whether the tumour has spread to distant sites. M0 indicates no distant metastasis while M1 indicates distant metastasis. It should be noted that for pancreatic cancer, spread to lymph nodes in the cauda or to splenic lymph nodes is considered M1.

Table 1.
Summary of the 8th edition of the AJCC staging system of periampullary cancers.

	Pancreas	Ampulla	Distal bile duct	Duodenum
Primary tumour (T-stage)				
T1	Localised to the pancreas. Largest diameter ≤ 2 cm	Localised to the ampulla or sphincter of Oddi and/or into the duodenal submocusa	Invasion into the bile duct wall with a depth of < 5 mm	Invasion into lamina propria (T1a) or submucosa (T1b)
T2	Localised to the pancreas. Largest diameter > 2 cm or ≤ 4 cm	Tumour invades into the duodenal muscularis propria	Invasion into the bile duct wall with a depth of 5-12 mm	Invasion into the muscularis propria
T3	Localised to the pancreas. Diameter < 4 cm	Invasion extent into pancreas ≤ 0.5 cm	Invasion of the bile duct wall with a depth of > 12 mm	Invasion into the subserosa or non-peritonealised perimuscular tissue
T4	Involvement of arteria mesenterica superior, arteria hepatica communis and/or truncus coeliacus	Involvement of arteria mesenterica superior, arteria hepatica communis and/or truncus coeliacus	Involvement of arteria mesenterica superior, arteria hepatica communis and/or truncus coeliacus	Perforation of the visceral peritoneum or invasion into other organs or structures
Regional lymph nodes (N-stage)				
N1	1-3 regional lymph node metastases	1-3 regional lymph node metastases	1-3 regional lymph node metastases	1-2 regional lymph node metastases
N2	≥ 4 regional lymph node metastases	≥ 4 regional lymph node metastases	≥ 4 regional lymph node metastases	≥ 3 regional lymph node metastases
Distant metastases (M-stage)				
M0	No distant metastases	No distant metastases	No distant metastases	No distant metastases
M1	Presence of distant metastases	Presence of distant metastases	Presence of distant metastases	Presence of distant metastases
Prognostic stage				
IA	T1N0M0	T1N0M0	T1N0M0	T1-2N0M0
IB	T2N0M0	T2N0M0	Not applicable	Not applicable
IIA	T3N0M0	T3N0M0	T1N1M0 or T2N0M0	T3N0M0
IIB	T1-3N1M0	T3bN0M0	T2N1M0 or T3N0-1M0	T4N0M0
III	T1-T4N2M0 or T4N1-2M0	T1-4N2M0 or T1-3N1M0	T1-3N2M0 or T4N0-2M0	T1-4N1-2M0
IV	T1-4N0-2M1	T1-4N0-2M1	T1-4N0-2M1	T1-4N0-2M1

Invasiveness

Invasion into surrounding structures is an important clinicopathological factor to assess during the pathological examination of the surgical specimen, in particular growth into blood and lymph vessels and perineural growth. Growth into these structures is associated with poor prognosis in all periampullary cancers (6, 8, 87, 93, 94)

Differentiation

Differentiation is a measure of how well the tumour resembles normal tissue. Well-differentiated tumours have a high degree of resemblance to normal tissue resemblance while poorly differentiated tumours and undifferentiated tumour diverge substantially from normal tissue. Differentiation grade is highly linked to tumour behaviour and thus patient outcome. Patients with periampullary cancers of poor differentiation have shorter survival compared to those with well-differentiated tumours (17, 90, 91, 93, 95) and differentiation is therefore an important clinicopathological factor to consider.

Surgical treatment

The first documented transduodenal resection of a periampullary cancer was performed over 120 years ago (96). The first *en bloc* resection of the pancreas and the duodenum was described by Codvila (97), which was later improved by Kauch *et al* in a two stage manner (98). Whipple developed the panceatoduodectomy during the 1930s (99), which is still today colloquially called Whipple-operation, and the indication for pancreatoduodectomy was extended to pancreatic cancer by Brunschwig (95).

The only potentially curative treatment for periampullary cancers is complete resection. However, only 15-20% of the patients are eligible for surgery with curative intent when diagnosed (100). Stage T4 disease with involvement of arteria mesenterica superior, arteria hepatica communis or truncus coeliacus, and M1 disease are considered disqualifying for surgery. Tumour operability is usually assessed using computed tomography (CT) scans with dual phase protocols, i.e. pancreatic and portal venous phase (101). Magnetic resonance imaging (MRI) is not first choice of radiology, however MRI can be used to assess liver lesions or if the patient is allergic to iodine contrast (101). Additionally, besides assessment of tumour resectability, assessment of the patients' condition and operability is of uttermost importance. There is no consensus on what constitutes borderline resectable tumours, and the optimal treatment algorithm for these patients is an ongoing debate. Patients with venous engagement are considered eligible for surgery, however, venous reconstruction is technically difficult but possible and the morbidity, survival and mortality rates are similar to those for patients who do not undergo venous reconstruction (102).

Pancreatoduodenectomy is a challenging surgical procedure where the antrum ventriculi, portions of the duodenum, caput pancreatis and ductus choledochus are removed *en bloc*. During the procedure the surgeon will also perform lymphadenectomy. Common post-operative complications include anastomosis leakage, haemorrhage and delayed gastric emptying (103). Even after surgery

survival rates remain low. In Sweden the OS after surgery for periampullary cancers is 60%, but much lower for pancreatic cancer where the median survival rate is only 26 months after surgery (81).

Chemotherapy

Even patients who undergo surgery with curative intent are treated with a systemic approach as pancreatic cancer is considered a high risk disease up front. Common chemotherapy agents used for treatment of periampullary cancer include fluorouracil (5-FU), oxaliplatin, irinotecan, gemcitabine, nab-paclitaxel, capecitabine and tegafur/gimeracil/oteracil (S1). Common chemotherapy regimens include FOLFIRINOX, consisting of folinic acid, 5-FU, irinotecan and oxaliplatin, GEMOX, consisting of gemcitabine and oxaliplatin, and GemCap, consisting of gemcitabine and capecitabine and Gem-nab-paclitaxel.

Neoadjuvant chemotherapy

Neoadjuvant chemotherapy, i.e chemotherapy in the preoperative setting, has several hypothetical benefits such as downstaging, which may increase the number of resectable tumours and improve resection margins, avoidance of surgery in patients with aggressive and rapidly growing tumours, and delivery of treatment to well-oxygenated tissue which may enhance the therapeutic efficacy. However, the main concern with neoadjuvant therapy is that patients may progress to irresectability during treatment. For patients with resectable tumours, neoadjuvant chemotherapy aims to reduce the risk of recurrence and to improve survival rates. In the setting of borderline resectable tumours, neoadjuvant chemotherapy is used for downsizing and improving surgical results (104). However, there has been no prospective, randomised study comparing adjuvant versus neoadjuvant chemotherapy or large studies investigating the effect of neoadjuvant chemotherapy in pancreatic or periampullary cancer. A recent Japanese study (Prep-02/JASAP-05) showed that neoadjuvant gemcitabine/S1 gave significant survival benefits compared to upfront surgery (105), however the results need to be validated in larger studies and other populations. A large retrospective study on 9684 patients with stage 1A and 1B pancreatic cancer could not demonstrate any benefit of neoadjuvant chemotherapy followed by surgery compared to surgery followed by adjuvant chemotherapy (106). Several recent metanalyses have shown that neoadjuvant therapy should not be considered for patients with resectable tumours, however, the benefit for patients with borderline resectability was deemed unclear (107-109). There is an ongoing effort in the research community to identify patients with resectable tumours who might benefit from neoadjuvant chemotherapy, including a phase II study (SWOG S1505) investigating mFOLFIRNOX versus gemcitabine/nab-paclitaxel, where follow-up on survival is ongoing (110).

Obviously, there is a need for prospective and randomised clinical studies that evaluate the benefit of neoadjuvant chemotherapy, especially in the resectable setting. Due to the lack of evidence from prospective clinical studies, the use of neoadjuvant chemotherapy is not recommended for periampullary adenocarcinomas in Sweden. However, neoadjuvant chemotherapy is used routinely in some cancer centres around the world and some Swedish centres enrol patients into such clinical studies.

Adjuvant chemotherapy

There is ample evidence that adjuvant chemotherapy is of benefit both in pancreatic and in other periampullary cancers. Notably, the ESPCAC-3 study published in 2012, showed a significant survival benefit of adjuvant chemotherapy in the entire spectrum of periampullary cancers. In ESPAC-3, patients were randomized into three arms; no adjuvant chemotherapy, gemcitabine or 5-FU. Both chemotherapy arms showed superior survival advantages to no adjuvant chemotherapy (86). In 2017, the ESPAC-4 study demonstrated significant advantages of adjuvant gemcitabine/capecitabine over monotherapy with gemcitabine for pancreatic cancer (111). The results for other periampullary cancers included in the ESPAC-4 study are yet to be published.

More recently, Conroy *et al* presented the results from the PRODIGE-24 study showing that patients who were given a modified FOLFIRINOX regime (mFOLFIRNOX) had significantly longer disease free survival (DFS) and metastasis free survival (MFS) rates than patients given gemcitabine (112). In Japan, S1 has been standard of care in the adjuvant setting since 2013, when a study conducted on a Japanese population showed that adjuvant S1 compared to adjuvant gemcitabine had superior survival advantages (113). Studies are underway to validate these results in Western populations.

The Swedish recommendations are to give gemcitabine/capecitabine to patients with good performance status and who are thought to tolerate the treatment (81). For patients who are not suited for combination therapy, it is recommended to give monotherapy gemcitabine or 5-FU (81).

Palliative chemotherapy

For patients with locally advanced or metastatic disease it is recommended to give systemic chemotherapy as long as they have a good performance status. Palliative chemotherapy leads to both survival benefits and improved quality of life in this group patients (114). In 2010 the first study showing survival benefits of combination therapy (FOLFIRINOX) compared to monotherapy (gemcitabine) was published (115). Two years later, the MPACT study showed that combination therapy of gemcitabine/nab-paclitaxel gave superior survival benefits compared to monotherapy of gemcitabine (116). Since then, combination therapies with either

FOLFIRINOX or gemcitabine/nab-paclitaxel have been recommended as first line treatment of metastatic or locally advanced pancreatic cancer in patients with good performance status. Due to the increased toxicity of combination therapies, the recommendation for patients with low performance status is either gemcitabine, 5-FU or capecitabine, in that order. In other types of periampullary adenocarcinoma it is disputed weather treatment of choice should entirely be based on tumour morphology or not, meaning 5-FU based chemotherapy for I-type tumours and gemcitabine for PB-type tumours. For patients with bile duct tumours and good performance status, gemcitabine with the addition of cisplatin or oxaliplatin should be considered (117).

Second line treatment should be chosen with regard to the first line treatment. The NAPOLI-1 study, first published in 2015 with a follow-up published in 2017, demonstrated that patients with metastatic pancreatic cancer previously treated with gemcitabine-based treatment had prolonged survival when given nanoliposomal irinotecan with 5-FU (118, 119). Additionally, there is evidence from the CONKO-003 trial, that second line treatment with oxaliplatin/5-FU after progression on gemcitabine-based treatment significantly extends survival (120).

Radiation therapy

The role of radiation therapy in pancreatic and other periampullary adenocarcinomas is controversial and there are significant geographical disparities in recommendations and clinical practice. In the neoadjuvant setting, chemoradiotherapy has been shown to lead to locoregional control and improved surgical outcome of pancreatic cancer (121, 122). However, a recent large retrospective study showed that the addition of radiotherapy was associated with higher perioperative mortality and no long term survival benefit (123). In the adjuvant setting there are also conflicting data. The GITSG trial published in 1985 demonstrated a prolonged survival for patients receiving chemoradiotherapy (124), however the EORTC study published in 1999 could not demonstrate any survival benefit of adjuvant chemoradiotherapy (125), and neither could a large metanalysis from 2013 (126). The clinical practice in Europe today is not to give adjuvant chemoradiotherapy routinely. For palliative patients, local radiotherapy is recommended for symptomatic metastases e.g. painful skeletal metastases, in order to improve quality of life (81).

Targeted therapies

Targeted therapy is a modality of treatment where a drug is designed to interfere with molecular pathways essential for tumour growth. Targeted therapies are closely related to the field of personalised medicine, where the aim is to tailor the treatment

to individual patient and tumour characteristics. The only targeted therapy that has shown survival benefit in pancreatic cancer as of date is the EGFR tyrosine kinase inhibitor erlotinib, in the metastatic setting (127). The POLO trial, published in 2019, investigating treatment with the PARP-inhibitor olaparib in patients with metastatic pancreatic cancer and germline *BRCA* mutation demonstrated a longer progression free survival compared to placebo but no significant difference in the interim analysis of OS (128). Olaparib is not yet approved for treatment of metastatic pancreatic cancer.

I-type tumours are less frequently *KRAS* mutated, potentially indicating that patients with I-type *KRAS* wild-type (wt) tumours may benefit from monoclonal antibody anti-EGFR treatment. A phase II study on *KRAS* wt small bowel and ampullary adenocarcinomas treated with panitumumab in the metastatic setting showed no clinical efficacy and the authors argued that this may be due to the embryonic origins of the small intestines and the ampulla of Vater (129). The small intestine and the ampulla of Vater have the same embryonic origin as the right sided colon. Right sided colon cancer has previously been shown to be less responsive to anti-EGFR treatment compared to left sided colon cancer, which is thought to, at least partly, be due to different embroyonic origins (130). A small study on only a few patients with *KRAS* wt small bowel adenocarcinoma showed promising results (131), although the small number of patients makes it hard to draw any definite conclusions. Anti-EGFR treatment has also been studied in pancreatic cancer, and a recent study including 127 patients who received cetuximab in the adjuvant setting could not demonstrate any significant survival benefits (132). These results are in contrast to a study published in 2016 that showed an improved survival for patients who received combination treatment of cetuximab and bevacizumab (a vascular endothelial growth factor receptor [VEGFR] inhibitor) compared to patients who only received gemcitabine. More research is needed both in I-type and PB-type tumours to further elucidate the possible benefit of anti-EGFR treatment for these patients.

Larotrectinib, a tropomyosin receptor kinase (TRK) inhibitor, was approved in the United States in 2018 for treatment of all solid metastatic tumours with neurotrophic receptor tyrosine kinase (*NTRK*) gene fusions in the United States for all solid metastatic tumours with TRK gene fusions in 2018 (133). In 2019 the European Medicine Agency (EMA) granted larotrectinib market authorisation on similar indications (134). However, the prevalence of *NTRK* gene fusions has been reported to be lower than 1 % in the majority of solid tumours (135). A study on several types of metastatic solid cancer types, including one patient with pancreatic cancer showed durable response (136). There are also case reports of patients with pancreatic cancer who have benefited from treatment with entrectinib, a combined TRK and ROS1 inhibitor treatment (137). Larger prospective studies are needed to validate the use of TRK inhibitors in pancreatic and periampullary cancers.

Immunotherapy

Immunotherapy is emerging as the fourth pillar of cancer treatment, the other three being surgery, radiation and chemotherapy. Immunotherapy is a modality of treatment where the endogenous immune response to malignant cells is manipulated to achieve tumour control and/or eradication. Several types of immunotherapy exist, including monoclonal antibodies (most noteworthy checkpoint inhibitors for which the Nobel Prize in Medicine or Physiology was awarded in 2018), cellular immunotherapy (such as dendritic cell approaches or adoptive T cell transfer such as chimeric antigen receptor [CAR] T cells) and cytokine therapies.

Checkpoint inhibition

Checkpoint inhibitors such as ipilimumab (cytotoxic T-lymphocyte-associated protein 4 [CTLA-4] inhibitor), nivolumab and pembrolizumab (programmed death receptor 1 [PD-1] inhibitors) and durvalumab (programmed death receptor ligand 1 [PDL-1] inhibitor) work by blocking co-receptors on T-cells that regulate their activity. Specifically, CTLA-4, which is a co-inhibitory receptor, binds to B7-1 (CD80) and B7-2 (CD86), subsequently leading to inhibitory signals in T-cells (138). PD-1 is also a co-inhibitory receptor expressed on T-cells, B-cells and natural killer T (NKT) cells. The ligand of the PD-1 receptor, PDL-1, is expressed on antigen presenting cells (APC) and on tumour cells (139). By blocking the PD-1/PD-L1 axis, the activity of T-cells is upregulated (140).

Monotherapy with checkpoint inhibitors in unselected pancreatic cancer patients has thus far been disappointing as it has not yielded any survival benefits (141, 142). Therefore, focus has shifted to trials with different combinations of immunotherapy. Several studies are currently investigating different immunotherapy combination regimes in pancreatic cancer, including durvalumab/tremelimumab (clinical trial number: NCT02558894) and pembrolizumab/nivolumab (clinical trial number: NCT02309177 and NCT02268825). As for biliary tract cancer, a phase II study published in 2018 showed promising results of the activity of nivolumab in patients with refractory disease (143). Several other studies are now underway investigating the impact of PD-1/PD-L1 axis inhibition in biliary tract cancer (clinical trial number: NCT03101566 and NCT02829918).

In 2017, a study investigating the effect of pembrolizumab on MSI-H non-colorectal gastrointestinal cancers, including pancreatic, ampullary and bile duct cancers showed promising results and potential single agent activity (144). In the United States, pembrolizumab was the first drug ever to be approved in a tissue agnostic setting, i.e. in all MSI-H tumours, thus making it available for a small subset of patients with periampullary cancer (145).

Cancer vaccines

Cancer vaccines have been thoroughly studied in pancreatic cancer. Two main approaches have been investigated; whole cancer cell vaccines and peptide-based vaccines. Whole cancer cell vaccines have the benefit of hypothetically targeting a much wider array of epitopes compared to peptide based vaccines (146).

The ECLIPSE trial, a large prospective clinical study in metastatic pancreatic cancer, investigating the impact of GVAX, a type of whole cell cancer vaccine, was disappointing and did not demonstrate any survival benefits after showing promising results in early phase studies (147). Additional studies are investigating the use of GVAX in combination with radiotherapy and in combination with other types of immunotherapy agents, both in the neoadjuvant and in the adjuvant setting (clinical trial number: NCT00727441, NCT02451982, NCT01896869, NCT02243371 and NCT02648282). The IMPRESS trial, investigating the impact of cancer cell derived algenpantucel-L failed to demonstrate any survival benefits in a phase III study after having shown promising results in early phase trials (148). There is a plethora of peptide based cancer vaccines (targeting for example *KRAS*^{G12D} mutations) currently being studied in early phases (141, 146), however, these trials need to progress into phase III studies before any conclusions can be drawn regarding their clinical relevance.

Adoptive T-cell transfer

CAR T-cell therapy, where the patients' own T-cells are engineered *ex vivo* to target neoepitopes on cancer cells have shown great clinical success in haematological malignancies. Early phase I and II studies are ongoing after pre-clinical models demonstrated anti-cancer activity, however this approach is still in the proof of concept stage in pancreatic cancer and other periampullary cancers (141, 146).

All in all, although immunotherapy has been a great clinical accomplishment in many cancer types, these achievements have not translated directly to pancreatic and other periampullary cancers. Checkpoint blockade has shown some promise in MSI-H tumours, but this is a very small group of patients. Larger, consistent and better designed studies are needed in order to properly define the best timing and combinations of immunotherapies.

The immune system and cancer

The role of the immune system against malignant transformation

The immune system is a multi-layered defence against foreign pathogens such as bacteria, viruses, parasites and, importantly, also against cancer. Already in 1863, Virchow observed leukocyte infiltration into tumours and formulated the idea that cancer and inflammation are intimately related (149). The relationship between the immune system and cancer is reflected in the Hallmarks of Cancer, defined by Hanahan *et al,* where immune evasion and tumour promoting inflammation are key characteristics of all cancer types (3, 4). The immune system should be considered the second line of defence against neoplastic transformation, when the first line, inherent intracellular mechanisms leading to apoptosis has failed. The main hurdle for the immune system in preventing neoplastic transformation is that malignant cells are, unlike foreign pathogens, native to our own body. Furthermore, cancer antigens may be considered chronic, leading to T cell exhaustion, a state of hyporesponsiveness (150). This poses a problem when immune cells and other components of the immune system are to identify rogue cells. A brief overview of selected unidirectional actions of various immune cells toward cancer cells is shown in Figure 3.

The innate immune system

The innate immune system consists of several parts. First anatomical barriers such as the skin and mucosal membranes, and chemical barriers such as gastric acid. These barriers are however of little importance for immunosurveillance against cancer. The cellular component of the innate immune system is however of great importance and consists of mast cells, macrophages, neutrophils, dendritic cells, basophils, eosinophils, natural killer (NK) cells and semi-innate lymphoid cells such as natural killer T (NKT) cells and γδ T cells. This chapter will focus on the cell populations of relevance for this thesis. These cells have widely different roles in immunosurveillance and eradication of cancer cells but share the ability to recognise aberrant molecular patterns expressed by transformed cells without prior sensitisation.

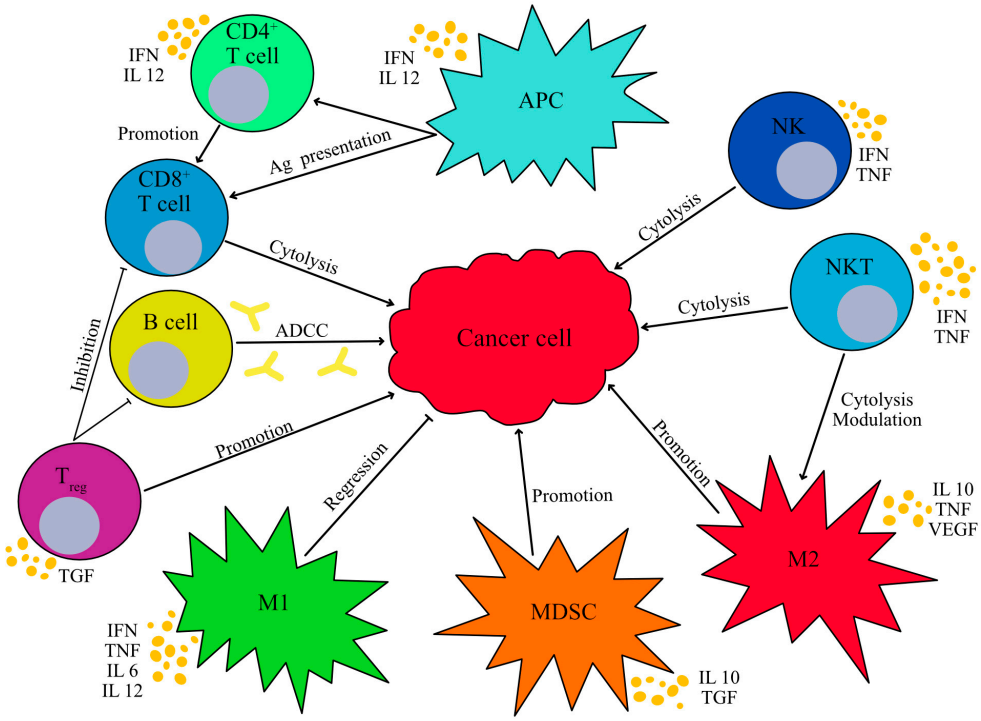

Figure 3:
A very brief and simplified overview of selected unidirectional actions of the innate and adaptive immune system toward cancer cells. Key cytokines secreted by the immune cells are indicated as well due to their importance to the immune response.

Natural killer cells

NK cells are lymphoid cells, sharing the same lymphoid progenitor origin as T cells, B cells and NKT cells and make up for about 10-15% of all circulating lymphocytes (151). However, in contrast to the lymphoid cells that are part of the adaptive arm of the immune system, NK cells lack antigen specific receptors. Additionally, NK cells do not express CD3 (like T cells) but CD56 and are thus usually phenotypically defined as $CD3^{-}CD56^{+}$. Two main subpopulations of NK cells have been identified, $CD56^{bright}$ and $CD56^{dim}$ (151, 152). The expression levels of CD56 are tied to NK cell effector functionalities, which mainly fall into two areas: cytolytic and immune modulating. NK cells with lower CD56 expression have a higher cytotoxic capacity while NK with high CD56 expression have a higher ability to secrete cytokines, thus being thought to play a larger role in regulating the immune response (153).

The activation state of NK cells is controlled by a combination of inhibitory and activating signals and is thus determined by the balance of incoming inhibitory and activating signals. Inhibitory signals are transferred via killer cell Ig-like receptors

(KIRs) while activating signals are transferred via natural cytotoxicity receptors (NCRs) that recognise stress-induced ligands on target cells. Examples of activating receptors are NKp30, NKp44 and NKp46 and NKG2D.

NK cells are generally activated when they encounter target cells with reduced levels of human leukocyte antigen (HLA) molecules. Reduction of HLA levels is a common mechanism of cancer cells to evade the adaptive arm of the immune system. Normally, inhibitory receptors such as KIRs on NK cells are activated when binding to major histocompatibility complex (MHC) I molecules however this inhibition is lost when target cells express reduced levels of MHC I molecules. This activation model is called "missing-self" (154, 155) .

When activating signals outweigh the inhibitory signals, NK cells are activated and can kill targets cells via cytotoxicity. NK cell mediated cytotoxicity is carried out in three ways: after recognition of a target cell, NK cells release cytotoxic proteins such as perforin and granzymes from cytoplasmic granules into the immunological synapse (152) and granzymes subsequently induce apoptosis in the target cell. NK cells can also recognize target cells through FasL, or by antibody dependent cellular cytotoxicity (ADCC) if the target cell is coated with antibodies, as NK cells also express CD16, a Fc receptor (152, 154).

In addition to important cytolytic capacities, NK cells exert regulatory functions on several other immune cell populations including DCs, macrophages, T cells and B cells. NK cells secrete interferon-γ (IFNγ) and tumour necrosis factor α (TNFα). These cytokines are important in the maturation and activation of DCs (156). Activated DCs then in turn secrete interleukin (IL) 12, a potent NK cell activator (156). NK cell derived INFγ can also activate T cells in lymph nodes. Negative regulation of the immune response is carried out by NK cells for example by killing of T cells and DCs with reduced levels of MHC molecules (157-159). The NK cell mediated cytolytic editing of immature DCs is thought to be of particular importance for the subsequent activation of the adaptive arm of the immune system (160).

Natural killer T cells

NKT cells are lymphoid cells that express invariable αβ T cell receptors (TCR), and are thus much less diverse in their TCR repertoire than conventional T cells (161). NKT cells are mostly CD1d restricted, meaning that NKT cells recognise lipid antigens presented by CD1d molecules. CD1d molecules are related to MHC class I molecules, a type of protein able to present peptide antigens, and are expressed by antigen presenting cells (APC). They share effector characteristics of both NK cells and conventional T cells and, thus, blur the line between the innate and the adaptive immune system. Morphologically, NKT cells resemble NK cells, both being large granular lymphocytes (162). NKT cells express CD3, a pan-T cell marker, and costimulatory receptors usually associated with conventional T cells such as CD4,

CD8, CD45RO and CD28 (163-165), although some populations, unlike conventional T cells, can be $CD4^-$ and $CD8^-$. Additionally, NKT cells express receptors usually observed on NK cells such as the CD56 and natural killer receptors (NKR) such as NKG2D, NKp30, NKp44 and NKp46 (163-166).

Based on their TCR repertoire, NKT cells can be divided into Type I NKT cells or Type II NKT cells (161). Type I NKT cells are less diverse than Type II NKT cells and mainly recognise α-galactosylceramide (αGalCer) while Type II NKT cells are less restricted in their recognition of antigens and can recognise non-αGalCer molecules (167-170). Adding to this, NKT cells are further subdivided by their functionality based on their cytokine secretion (171). This subclassification is similar to the one used for T helper cells.

NKT cells are typically activated after recognition of aberrant glycolipids presented by CD1d on APCs (172, 173). However, NKT cells can also be activated in a NK cell like fashion (174, 175), wherein the balance of inhibitory and activating signals through KIRs and NKRs are weighed together as outlined above. NKT cells, due to their expression of NKRs such as NKG2D are thus not entirely CD1d restricted (176). Activated NKT cells have several functions; first, as modulators of the tumour microenvironment by secreting large amounts of cytokines, second by direct cytotoxic effects on tumour cells and other cells in the tumour microenvironment and third by mediating ADCC (177-181).

The modulating role of NKT cells is carried out by cytokine secretion and by direct interaction with other immune cell populations. The cytokine secretion profile is dependent upon the functional and phenotypical subset of the NKT cell (161). Through CD1d-TCR interaction, NKT cells are able to induce maturation of dendritic cells (DC). By secretion of cytokines such as IFNγ, NKT cells are able to induce activation of NK cells, DCs and macrophages (161, 182).

The cytotoxic role of NKT cells is thought to be minor compared to their regulatory role, but is nonetheless important. Type I NKT cells kill tumour cells in a CD1d dependent manner and through NKR activation or absence of MHC molecules (183-185). NKT cell cytotoxicity has also been implicated in shaping of the tumour microenvironment as they have been shown to co-localise with M2 polarised macrophages leading to cytolysis of M2 polarised macrophages or skewing of macrophage polarisation to the more favourable M1 phenotype (186, 187). Adding to this, as NKT cells are able to skew DCs into a mature phenotype, the impact of tolerogenic DCs on T cell populations (importantly the skewing of T-cells into T regulatory [T_{reg}] cells) might be reduced (161).

Dendritic cells

DCs are essential for the mounting of an effective anti-tumour response. Their function is to bridge the innate immune response to the adaptive immune response by posing as APCs. DCs can be divided into four major subpopulations: plasmacytoid DCs, conventional type 1 DCs, conventional type 2 DCs and monocyte derived DCs (188) based on functionality and expression of surface receptors (189). Plasmacytoid DCs are potent producers of IFNγ, which activates cytolytic lymphocytes (189). Both conventional type 1 and type 2 DCs are especially good at cross-presenting antigens to T cells and secreting cytokines to promote polarisation to a type 1 T helper cell (T_H1) and secretion of IL 12 which promotes lymphocyte effector functions (189). Monocyte derived DCs mature at sites of inflammation and exert their function there. Their influence on other immune cells is bound by context and they can promote both T_H1, type 2 T helper cell (T_H2) and type 17 T helper cell (T_H17) polarisation (188, 190).

In a sterile environment like cancer, DCs recognize damage associated molecular patterns (DAMPs) on dead or stressed cells (191). They phagocyte cells and debris, process antigens and present these on MHC molecules to T cells in order to activate the adaptive and the humoral immune system. DCs activate T cells in a three-step manner. First, tumour infiltrating DCs acquire antigens and then they present them to on MHC II molecules to T cells in lymph nodes while at the same time providing costimulatory signalling through CD80/CD86. Lastly, they secrete cytokines (such as IFNγ and IL 12) to further potentiate the activation of T cells (188, 191).

However, there is another aspect to DCs as well in that they can hinder an efficient anti-tumour response under pathological conditions. Activated DCs can express the checkpoint inhibitors PDL-1 and programmed death receptor ligand 2 (PDL-2) leading to suppression of T cell functionality (192). Tumour derived cytokines such as TGFβ can manipulate the functionality of DCs, inducing an immature, tolerogenic phenotype and thus facilitating tumour progression (192, 193). Immature DCs cannot activate T cells, but rather encourage T cell exhaustion and anergy (194).

Macrophages

Macrophages are mature forms of monocytes in tissues. The primary roles of macrophages are to phagocyte cells and to acts as APCs (195). Macrophages reside in a wide range of different tissues where they carry out highly specialised physiological roles, depending on their tissue of residence. Macrophages are important as first responders at sites of inflammation by secreting large amounts of cytokines such as TNFα and IL 1 as well as nitric oxide which has antimicrobial effects (196). However, these mechanisms are also highly tissue damaging and, thus, macrophages, in order to control tissue damage, macrophages switch to an anti-inflammatory phenotype after initial response (196). This dynamic illustrates

the highly plastic nature of macrophages which is important to understand when it comes to their role in tumour control.

There is an established conceptual model of tumour associated macrophages (TAMs) in which they are classified as either M1 or M2. The M1 type phenotype is associated with a high capacity to produce IFNγ, IL 6, IL 12 and TNFα, a high capacity to present antigens and high capacity to produce reactive oxygen species (197, 198). M1 type macrophages are thus regarded as being proinflammatory. On the other hand, the M2 type phenotype is associated with production of IL 10 which acts to curtail the immune response and remodelling of tissues as well as the production of VEGF and TGFβ (197, 198).

However, this dichotomised model, although useful in a pedagogical sense, does not capture the full plastic, spatiotemporal nature of macrophages. *In vivo*, macrophages exist on a functional spectrum, either skewed more towards an inflammatory phenotype or more towards an anti-inflammatory phenotype that is highly fluid and dependent on the context of the microenvironment (199).

As such, the role of TAMs in the tumour microenvironment is two faced; by recruiting and activating cytolytic cells such as NK cells and cytotoxic T cells they can contribute to tumour regression (197). However, they can also be integral parts of cancer driven inflammation, leading to tumour escape. Macrophage derived IL 10 inhibits the anti-tumour response of cytotoxic T cells, skew T helper cell towards a T_H2 phenotype and stimulates T_{reg} function. TAMs also support tumour neoangiogenesis and tumour invasion (197).

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) are cells with myeloid origin. Although sharing a common progenitor with macrophages and DCs, MDSCs are phenotypically and functionally distinct from their relatives. Two main subgroups of MDSCs have been defined: polymorphonuclear MDSCs (granulocytic) and monocytic MDSC with some divergence in phenotype, morphology and functionality (200). Polymorphonuclear MDSC are usually defined as $CD33^+CD11b^+CD14^-CD15^+$ while monocytic MDSCs are usually defined as $CD11b^+/CD33^+CD14^+HLADR^{-/low}CD15^-$ (200).

The role of MDSCs in the tumour microenvironment is mainly to promote tumour progression by suppressing the antitumor immune response and create a favourable environment for cancer cells to thrive. Immunosuppression is carried out by secretion of nitric oxide synthase, TGFβ, IL 10, arginase and reactive oxygen species (ROS), leading to detrimental effects on NK cell, B cell and T cell functionality (201-205). MDSCs create a favourable microenvironment for cancer cells by supporting neoangiogenesis, tissue invasion and epithelial-mesenchymal transition (204).

Basophils

Basophils are the least common type of granulocytes, comprising less than one percent of all leukocytes (206). They were discovered in 1879 by Paul Ehrlich and named basophils due to their sensitivity to basic dyes (207). Paul Ehrlich received the Nobel prize in Medicine or Physiology in 1908 for his discoveries, which he shared with Ilya Mechnikov, who was the first to describe macrophages. Basophils are powerful mediators of inflammation, mostly known for their effector functions in allergy and hypersensitivity (208), however they are also potent immune regulators, exerting influence on the adaptive arm of the immune system (209). Their role in cancer has not received much attention and remains debated. They are capable of secreting cytokines such as IL 4 and IL 13, leading to promotion of T_H2 and M2 phenotypes (206, 210). In pancreatic cancer, infiltration of basophils into tumour draining lymph nodes has been shown to be associated with T_H2 skewing and reduced survival (211). However, mouse models have also shown that basophils are critical for recruitment of cytotoxic T cells by chemokine secretion, although under T_H1 conditions (212).

The adaptive immune system

The adaptative (or acquired) immune system mainly consists of T and B cells. These two cell types are lymphoid cells with wide ranging effector functions depending on the subtype. The adaptive immune system has three major functions: recognising aberrant antigens among endogenous antigens, to form receptors and antibodies with high specificity for these antigens and to generate immunological memory.

T cells

T cells are characterised by a large clonal diversity of TCRs. These receptors are not bound to what is encoded in the germline but are created by somatic recombination and thus show a remarkable clonal variation of antigen recognition. TCR recognise antigens presented on MHC I and II molecules. MHC I molecules are expressed by all nucleated cells whereas MHC II molecules are primarily expressed by APCs. Three major forms of T cells have been defined: cytotoxic T cells, helper T cells, and T_{regs}. Cytotoxic T cells are usually defined as $CD3^+CD8^+$, helper T cells as $CD3^+CD4^+$, and T_{regs} as $CD3^+FoxP3^+CD4^+$.

Naïve T cells are produced as common lymphoid progenitor cells in the bone marrow and mature in the thymus. In the thymus they undergo both positive and negative selection in order to minimise autoreactivity. They then migrate to secondary lymphoid organs where they are exposed to antigens by APCs. As outlined in the previous section, the activation of T cells is a three-step mechanism. First, TCR on T cells need to interact with an antigen on an MHC molecule (priming), then the T cell needs to be provided with co-stimulatory signals from

APCs such as CD80. In the third and final step, the $CD8^+$ T cells needs to be stimulated by cytokines from APCs such as IL 2, IL 7 and IL 15 (213). The third step is necessary to induce cytotoxic capacities. APCs (and tumour cells) can also provide coinhibitory signalling through for example PD1/PDL1 and PD2/PDL2, leading to inhibition of T cell response. During the activation of cytotoxic T cells, they reciprocally stimulate DCs to secrete IL 12 and provide survival signals (214).

Activated cytotoxic T cells enter the circulation after activation in secondary lymphoid organs and home to sites of inflammation (213). Activated cytotoxic T cells also undergo clonal expansion, rapidly expanding the number of individual cells that can target a specific tumour neoantigen. Recognition of neoantigens presented by tumour cells by T cells will lead to the release of perforin and granzyme from the cytoplasm into the immunological synapse to the target cell. This will subsequently lead to apoptosis in the target cell (215). Cytotoxic T cells and NK cells thus use the same mechanism to induce target cell death, although using different mechanisms to identify target cells.

Helper T cells ($CD4^+$ T cells) enhance the response of $CD8^+$ T cells. After maturation in the thymus they home to secondary lymphoid organs where they await presentation of the antigen they are programmed to respond to. The activation of helper T cells is a multistep process requiring three separate signals. The first is provided when the TCR binds to an MHC II molecule on an APCs with the correct antigen. The second signalling is provided with co-stimulatory signalling through the binding of CD28 on the helper T cell and B7 molecules on APCs. Without co-stimulation, the helper T cell will become anergic. The third signal is provided by IL 2, which will stimulate proliferation of the helper T cell. The helper T cell and APC interaction will also have effects on the APC, such as increased expression of MHC molecules and costimulatory molecules for $CD8^+$ T cells to bind to (216). Activated $CD4^+$ T cells secrete large amounts of IFNγ, IL 2 and chemokines to attract and enhance the response of $CD8^+$ T cells (216). Lastly, recently a subpopulation of $CD4^+$ T cells were shown to also be capable of cytotoxicity through Fas/FasL and TCR mediated cytotoxicity (216).

Three major effector subpopulations of helper T cells have been defined: T_H1, T_H2 and T_H17. T_H1 cells primarily produce IFNγ, IL 2 and TNFα/β (217). T_H2 cells primarily produce IL 4, IL 5 and IL 13, stimulating the response of eosinophils, mast cells and immunoglobulin (Ig) production. In general, the T_H2 phenotype promotes tumour progression by inhibiting $CD8^+$ T cell cytotoxicity and increasing M2 polarisation (217, 218). T_H17 cells primarily produce IL 17, IL 21 and IL 6, and their effect on the tumour microenvironment is highly contextual (217, 218)

Sometime after the initial response, T cells will undergo further differentiation forming memory T cells. This cell population is essential for a renewed, rapid

response of the adaptive immune system and forms the basis for immunological memory (219, 220).

T_{regs} are T cells with regulatory, immunosuppressive functions. These cells promiscuously interact with all components of the tumour microenvironment including cancer cells, other lymphocytes and innate immune cells. The regulatory functions of T_{regs} can be divided into four mechanisms of action: secretion of inhibitory cytokines, cytolysis, metabolic disruption and DCs targeting (221). They inhibit the function of NK and cytotoxic T cells by TGFβ and IL 10 secretion (222). T_{regs} can also kill B cells, T cells and NK cells by granzyme and perforin mediated cytolysis (221). T_{regs} are capable of metabolic disruption of other effector lymphocytes by taking up IL 2, effectively starving other lymphocyte populations of proliferation signals (221). Lastly, T_{regs} are capable of skewing DCs into phenotypical and functional states where they are less effective of activating cytotoxic T cells (221). On the other hand, they have positive crosstalk with MDSCs, M2 polarised macrophages and cancer cells, and this positive crosstalk is often bidirectional (222).

B cells

B cells are in control of the humoral arm of the adaptive immune system. Like T cells, B cells are derived from the bone marrow. The development of B cells is very similar to that of T cells as they both undergo positive and negative selection during their maturation. They are activated after B cell receptor (BCR) interaction to antigens presented by APCs in secondary lymphoid organs (223, 224). The antigen-BCR complex is then internalised by the B cell and processed in order to be presented on MHC II molecules on the B cell surface for presentation to $CD4^+$ T cells (224). The subsequent B cell and $CD4^+$ T cell interaction fully activates B cells. Two fates then await B cells; either they differentiate into antibody producing plasma cells or they differentiate into memory B cells (225). B cells can also be activated in a T cell independent manner, typically to antigens with highly repetitive epitopes (226), however the B cell response tends to be weaker through this mechanism of activation.

The role of B cells in cancer has been relatively overlooked compared to the collective research effort put into the role of T cells. Antibodies produced by B cells can coat tumour cells leading to their complement mediated killing, phagocytosis by macrophages or ADCC by NK cells or $CD8^+$ T cells (227). There is also evidence of direct cytotoxic activity by B cells mediated by Fas/FasL (228). However, B cells can also adopt an immunosuppressive phenotype, secreting IL 10 and TGFβ which inhibits an efficient antitumor response by the immune system (227).

Immunoediting and escape

Immunoediting is the process by which cancer cells undergo changes in their immune activating capacity due to pressure exerted by immunosurveillance. This process has three phases: elimination, equilibrium and escape (229). In the first phase, immune cells are able to identify tumour cells and efficiently kill them by immune surveillance mechanisms. In the second phase, due to evolutionary pressure, some cancer cells have developed resistance mechanisms protecting against the immune response. During this phase there is an active moulding of the immunogenicity of cancer cells and cancer cells might even acquire mechanisms to escape elimination entirely. During the last phase, cancer cells have escaped the control of the immune system and their growth in no longer controlled. There are a multitude of mechanisms in which tumours cells can breach the control of the immune system (Table 2).

Table 2.
Brief summary of immune escape mechanisms deployed by cancer cells.

Type of evasion	Mechanism	Escaping from
Conceal identity	Loss of MHC I molecules, loss of neoantigens, loss of NKG2D ligands	T cells and NK cell
Supress immune cells	Promoting an immunosuppressive environment, upregulation of checkpoint inhibitors	Effector immune cells, DCs, macrophages
Hide in the tumour microeniroment	Promoting a dense stroma, hypoxia and neoangiogenesis	Effector immune cells, DCs, macrophages
Defence	Secreting FasL and MICA	T cells and NK cells
Avoid apoptosis	Resistance to Fas/FasL signaling, resistance to complement mediated cytotoxicity, resistance to cellular cytotoxciity	Cytotoxic immune cells and the complement system

By reducing surface expression of MHC I molecules and NKG2D ligands cancer cells are able to evade recognition by cytotoxic T cells and NK cells (230). Cancer cells suppress the activity of the immune system in two principal ways; firstly, by promoting an immunosuppressive microenvironment by skewing macrophage polarisation to M2, skewing helper T helper cell polarisation towards a T_H2 phenotype and recruiting T_{regs} and MDSCs (231, 232). Secondly, by expressing inhibitory molecules on their surface such as checkpoint inhibitors and chronic exposure to cancer antigens leading to T cell exhaustion (150, 231).

Tumour cells also promote a chaotic tumour microenvironment with dense stroma, hypoxia and neoangiogenesis, making it difficult for the immune system to mount an efficient response (231, 232). This is done directly by cytokine secretion by the cancer cells but is also achieved indirectly by recruiting immunosuppressive cells. Cancer cells can also acquire resistance to cell mediated cytotoxicity, complement mediated cytotoxicity and Fas/FasL cytotoxicity (233). Lastly, cancer cells can secrete FasL to induce apoptosis in effector immune cells after they themselves have achieved resistance to Fas/FasL signalling (234).

Immunophenotypes and their clinical relevance

In the advent of immunotherapy and the deluge of interest in immune-oncology, it has become clear that the complexity of the inflammatory tumour microenvironment cannot be understood by looking at one cell type at a time i.e. sometimes it hard to see the forest because of all of the trees. Thus, it has become evident that it is of great importance to characterise not only single immune cell infiltration but to put immune cell infiltration into a context. In this spirit, several general immunophenotypes have been defined. These signatures fall into three broad categories: inflamed, excluded and desert (Figure 4).

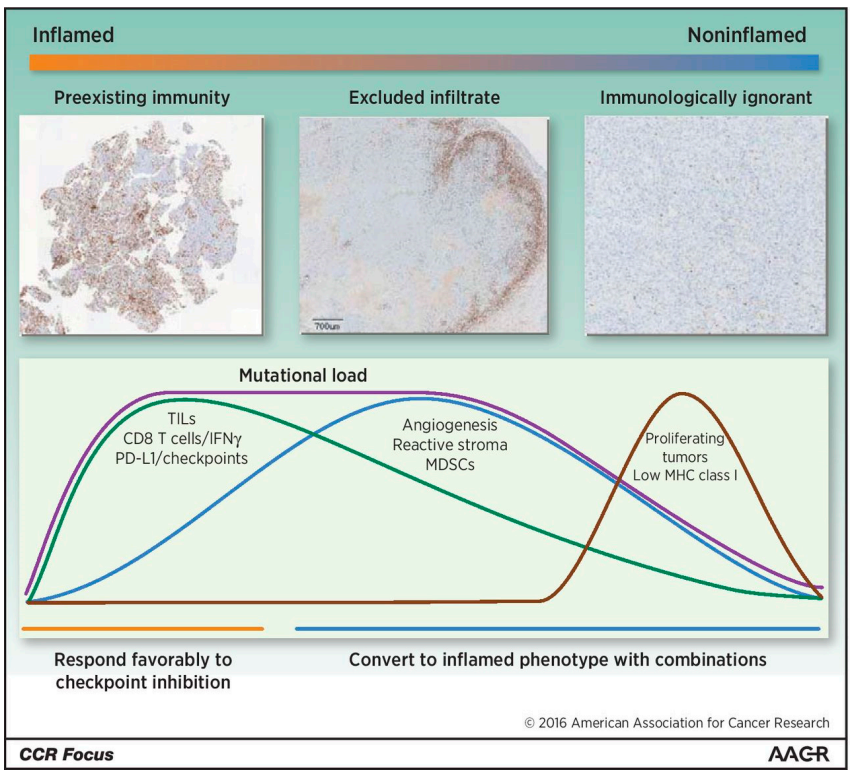

Figure 4:
The tumour-immunity continuum. Upper panel showing immunohistochemical images of $CD8^+$ lymphocyte infiltration of the immune inflammed (left), the immune exluded (middle) and the desert phenotype (right). The lower graph shows how immune cell infiltration,mutational burden, expression of surface proteins and stroma differs between the three immunophenotypes. Reproduced with permission from *American Association for Cancer Reseach.*

The concept of inflamed tumours and immune excluded tumours is also illustrated in Figure 5 with representative immunofluorescence images from paper IV.

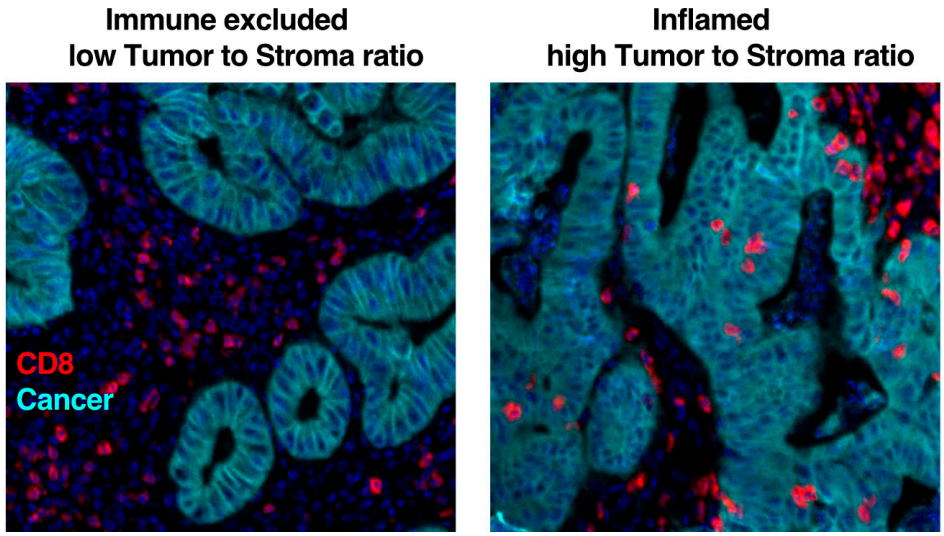

Figure 5:
Representative immunofluorescence images illustrating the concept of immune excluded (left) and inflamed (right) tumours. In the left panel, CD8+ cells are predominatly located in the stromal compartment, with great paucity of these cells in the tumour compartment, while in the right panel CD8+ cell infiltration extends into the tumour compartment.

The underlying biological mechanisms for the different immunophenotypes are outlined in Figure 6. Inflamed tumours are characterised by high levels of $CD8^+$ and $CD4^+$ T cells, high levels of inhibitory co-receptors, high levels of myeloid cells and genomic instability (235, 236). Immune excluded tumours are characterised by immune cells in the periphery of the tumour rather than in the tumour itself (237). The desert immunophenotype is characterised by a paucity of effector immune cells overall, though not necessarily myeloid cells and with a low neoantigen load (as a consequence of relative genomic stability) (235, 237). Both the excluded and the desert immunophenotype should be considered to be noninflamed tumours and these tumours are typically non-responders to immunotherapies used in the clinic today (237). Although many biological mechanisms have been identified that contribute to different immunophenotypes, we have few, if any, tools to manipulate these as of today.

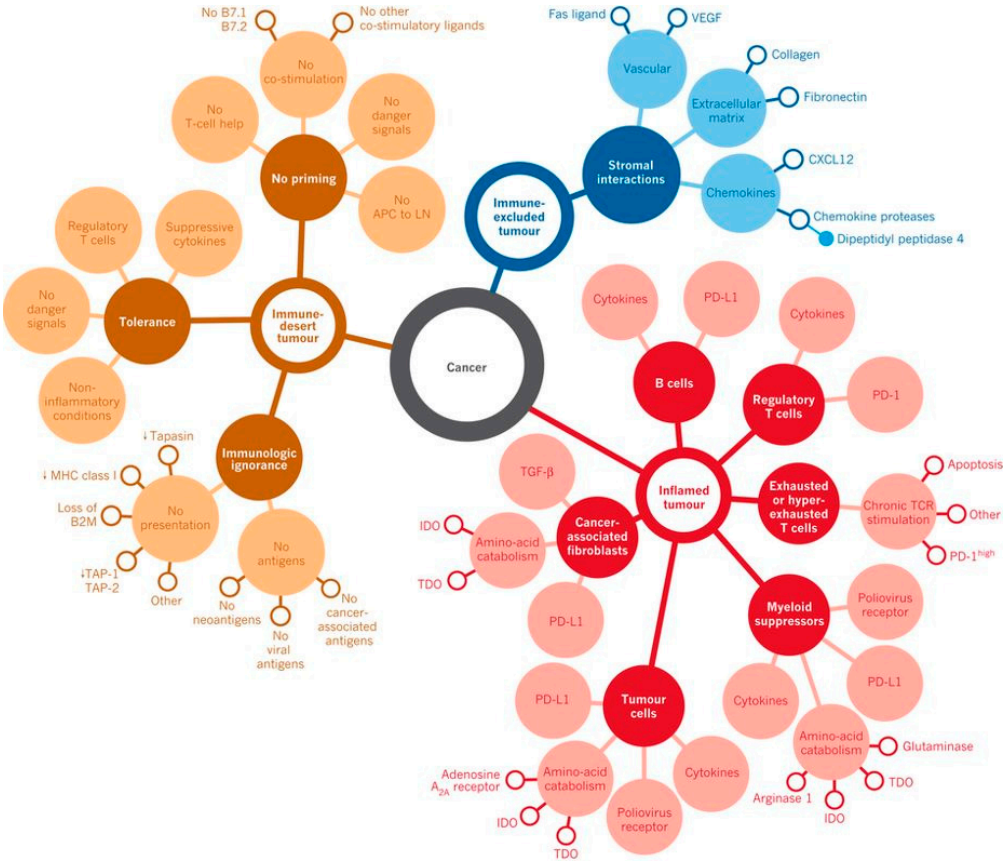

Figure 6:
Overview of different immunephenotypes. Inflamed tumours (red), immune excluded tumours (blue) and immune desert tumours (brown). The tree chart illustrates the underlying biological mechanisms of immunological ignorance or efficient antitumour immune response. These biological mechanisms are intimately tied to immuneevasion strategies dicussed in the previous section. Reproduced with permission from *Springer Nature*.

Extending beyond immunophenotypes, ambitious immunogenomic analysis of more than 10 000 cancers across 33 cancer types, using data from TCGA, defined six immune subtypes; wound healing, INFγ dominant, inflammatory, lymphocyte depleted, immunologically quiet and TGFβ dominant (238). These six subtypes were demonstrated to be independent of cancer type, although pancreatic cancer was overrepresented in the inflammatory subtype. The six immune subtypes were also shown to have clinical implications, as they were associated with OS. The inflammatory subtype was associated with the most favourable OS while lymphocyte depleted and TGFβ were associated with the poorest OS (238).

Aims of the present investigation

> In so far as a scientific statement speaks about reality, it must be falsifiable: and in so far as it is not falsifiable, it does not speak about reality.
>
> Karl Popper

The overarching aim of this thesis was to characterise the prognostic and predictive impact of immune cell infiltration in the entire spectrum of periampullary adenocarcinoma, with particular reference to morphology and genetic alterations.

Specific aims

- Paper I aimed to investigate the prognostic and predictive impact of NK and NKT cells with particular reference to morphology and potential response to adjuvant chemotherapy response
- Paper II aimed to investigate the prognostic and predictive impact of several subpopulations of macrophages and DCs with particular reference to morphology and potential response adjuvant chemotherapy response.
- Paper III aimed to investigate the mutational landscape of periampullary adenocarcinoma and to identify any significant differences in the mutational spectrum of I-type and PB-type tumours.
- Paper IV aimed to more comprehensively map lymphocyte infiltration in periampullary adenocarcinoma, to define lymphocyte infiltration signatures and to map the spatial interaction of cancer cells and lymphocytes, with particular reference to different tissue compartments.
- Paper V aimed to more comprehensively map innate immune cell infiltration, to define innate immune cell infiltration signatures and to map the spatial interactions of cancer cells and innate immune cells, with particular reference to different tissue compartments.

Methodology

All models are wrong, but some are useful.

George Box

Tissue microarray technique

The tissue microarray (TMA) technique is a high throughput method for analysis of protein expression in tissues. The technique is compatible with both immunohistochemistry (IHC) and immunofluorescence. The method was introduced in 1998 by Kononen *et al* and has since become a pillar of biomarker research in oncology (239). The method allows for rapid analysis of protein expression across many tumours simultaneously. At the same time, the TMA technique is tissue and reagent saving, compared to whole section analysis (240). Adding to this, less antibodies are needed as less tissue is stained. Initially, however, the method is somewhat labour intense, requiring retrieval of tumour specimens from pathological archives, pathological review and annotation of viable and non-necrotic areas.

After histopathological evaluation of the donor block, selected tissue cores with a diameter of 0.6-2 millimetres are mounted on a recipient block in a matrix (Figure 7). The receiver block is then subsequently sliced into 4 μm thin sections and mounted on glass slides.

Since the tissue cores sampled are small, there is a possibility that the cores might not be representative due to tumour heterogeneity and thus not reflect the biology of the tumour. However, with generous sampling with several cores this issue is mitigated. Adding to this, even whole sections do not accurately reflect the spatial heterogeneity of tumours.

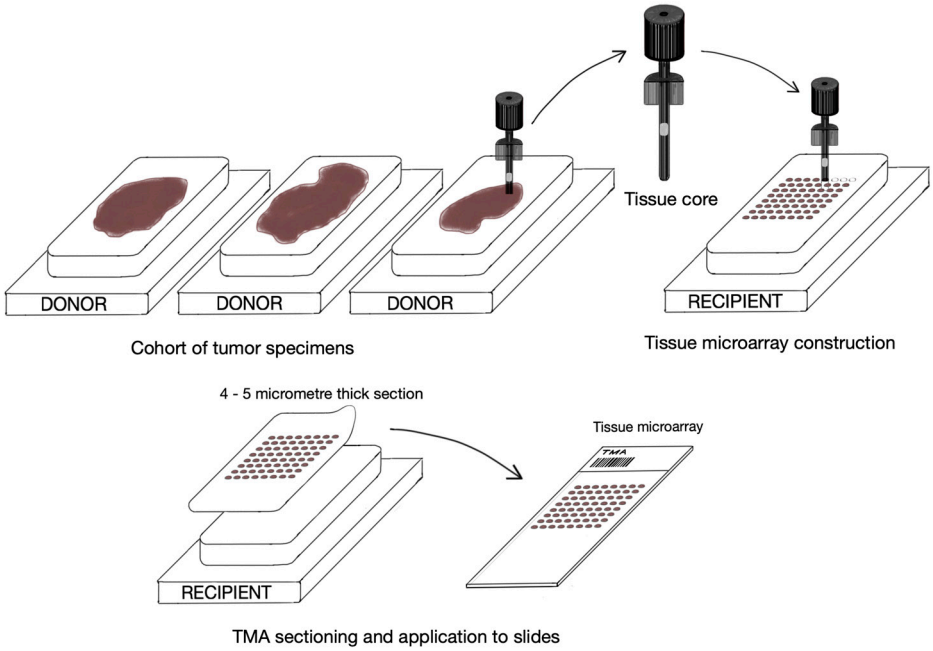

Figure 7:
Construction of a tissue microarray. Several tissue cores are collected from viable, non-necrotic areas of the formalin fixed paraffin embedded donor block. The cores are then mounted in a recepient block from which thin sections can be obatined to create tissue microarray slides. Courtesy of Dr Gustav Andersson.

Immunohistochemistry

Principles

IHC is a method to visualise the expression and localisation of proteins or other molecules in tissues. The method was developed in the 1940s by Coons *et al* and is today a widely used for analysis of protein expression in tissues, both in research and clinical laboratories (241). The method involves several steps as outlined below and shown in Figure 8.

First, formalin fixed tissues are prepared as discussed in the previous section. After this, to compensate for loss of antigenicity due to formalin fixation, antigen retrieval is necessary which can be done by several methods but for the present thesis heat induced epitope retrieval was used in all investigations. After this a primary antibody solution is applied to the glass slide on to which the TMA sections were mounted. The primary antibody binds to an epitope on the protein of interest. After some incubation time, the glass slide is washed to remove unbound antibodies. A secondary antibody solution is then applied to the glass slide, incubated and washed away. The secondary antibody binds to the primary antibody and is labelled with an enzyme providing a visible stain that can subsequently be evaluated in a light microscope.

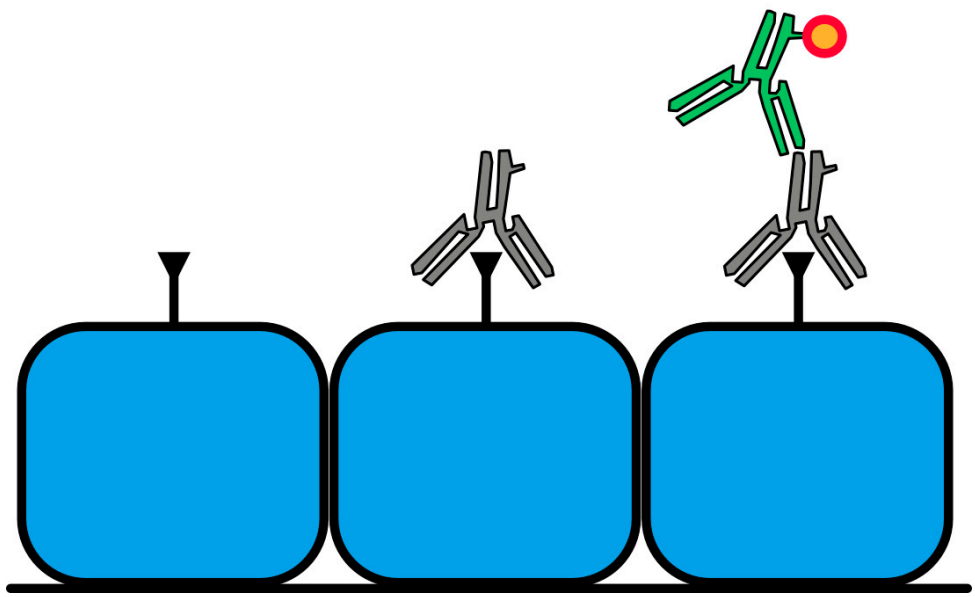

Figure 8:
Graphical representation of the principles of immunohistochemistry. After antigen retrival an antibody can bind to an antigen present on the protein of interest (black). A primary antibody (grey) binds to the antigen. A secondary antibody (green) binds to the primary antibody. The secondary antibody is labled with an enzyme (coloured circle) that contributes with a visuable staining after a chemical reaction.

Assessment

The second phase of IHC is the assessment of the staining. This can be done in two principal ways; manually or digitally. The advantages and disadvantages of each method are outlined in Table 3.

Manual assessment

Manual assessment is by nature qualitative (242). Staining positivity can be assessed both in terms of intensity and percentage. Before starting the assessment however, one needs to consider the expression patterns of the protein of interest, for example the localisation of the target molecules which can be cytoplasmic, membrane bound or nuclear. Adding to this, one needs to consider which type of cellular expression of a particular protein that is relevant, since it might not be specific for a particular type of cell. An example of this is the adhesion molecule CD56 which is expressed by both innate lymphocyte subpopulations, neural cells and cancer cells with neuroendocrine differentiation. Thus, morphological evaluation of cells is also of uttermost importance. Also, one should be aware of the considerable intra- and interobserver variation in manual IHC staining assessment (243).

The most common way to assess IHC staining is with histoscore (H-score), whereby the intensity (graded as 0-3) and the percentage of area of the most predominant staining is multiplied (244, 245). H-score is therefore a semi-quantitively means to evaluate IHC staining. A modified version of the H-score takes into account all the staining intensities and the percentage of the area stained with the following formula:

$$[1 \times (\%\ \text{cells } 1+) + 2 \times (\%\ \text{cells } 2+) + 3 \times (\%\ \text{cells } 3+)]$$

This version of the H-score gives relatively higher weight to the high intensity staining, even if it might be less frequent than lower intensities. When it comes to immune cells, the number of cells present might be more interesting than staining intensity and area stained. Therefore, simple quantification of the number of positive cells with the appropriate morphology might be best suited for analysis of immune cell infiltration into tumours, although this might be difficult to do when the number of infiltrating immune cells is high.

Digital image analysis

Digital assessment of IHC staining may mitigate the qualitative and time-consuming nature of in manual assessment of IHC. Adding to this, it may be tricky to quantitatively assess IHC staining in a reliable way in cases with rich immune cell infiltration. In these cases, digital assessment can be helpful, providing fast and reliable quantification. Digital image analysis can also decrease the intra and interobserver variation in IHC assessment seen in manual evaluation (243, 246).

The digital image analysis workflow has three principal phases; scanning, reviewing and quantification. The TMA slide is digitally scanned in high resolution. This is followed by a manual curation of individual TMA cores to exclude necrotic areas and inadequate cores. During this phase an algorithm is developed to optimally quantify the biomarker of interest. Several factors need to be considered here, such as staining intensity threshold, cellular morphology and subcellular location of the biomarker of interest. When the curation and the algorithm is developed, the software automatically quantifies the expression levels of the biomarker of interest. The main hurdles in digital assessment are validation and the development of a reliable algorithm. Staining can vary in quality and intensity from slide to slide and thus, might create problems for an algorithm which by nature is static and cannot as easily compensate for this as a pathologist.

Digital image analysis was used to quantify immune cell infiltration in some of the papers included in the current thesis using the Halo Image analysis software (Indica Labs, Corrales, United States of America) together with the Object Colocalization module. The module is able to assess object number, object density, object area and object diameter therefore making quantification of the positive number of cells with appropriate morphology easy (247).

Table 3.
Comparison of advantages and disadvantages of manual assesment and digital image analysis.

Manual assessment	Digital image analysis
Requires no digital infrastructure	Requires considerable digital infrastructure and training in digital systems
Considerable inter- and intraobserver variation	No variation in assessment if the algorithm is validated and reliable
Variations in staining intensity and quality are easily compensated for	Digital image analysis does not compensate as easily for variations in staining quality
Core curation can be done simultaneously with assessment	Requires a separate curation step
More difficult to quantify a large number of cells	Easier to quantify a large number of cells

Immunofluorescence

Principles

The immunofluorescence technique utilizes the same basic principles as IHC, but instead of creating a visible stain through an enzymatic process, the secondary antibody is bound to a fluorophore, creating a fluorescent dye which can be measured.

Assessment and analysis

For the papers included in this thesis, multispectral immunofluorescence protocols were used with an automated quantitative imaging system from Akoya Bioscience (Marlborough, United States of America) as part of the Phenoptics workflow.

The Phenoptics workflow has three phases: immunofluorescence staining, imaging and image analysis. In the first step, a panel of up to seven different biomarkers of interest is designed including 4′,6-diamidino-2-phenylindole (DAPI) in order to visualise cell nuclei as described previously (248). In the next step, TMAs are scanned and annotated for regions of multispectral analysis using the Vectra Polaris System (Akoya Bioscience) at a 2 pixel per 1 μm resolution. During the image analysis step, each core is manually curated in order to exclude areas of non-tumour tissue, necrosis or artefacts.

Following this, tissue segmentation is done as schematically outlined in Figure 9. A set of cores is manually categorized into three types of tissue compartments: tumour, stroma and blank areas. A machine learning algorithm is then created and carries out the tissue segmentation on the remaining number of cores. In this step, individual cell segmentation can be done as well using DAPI and, thus, it is possible to assess positivity and negativity of each antibody on a single cell resolution.

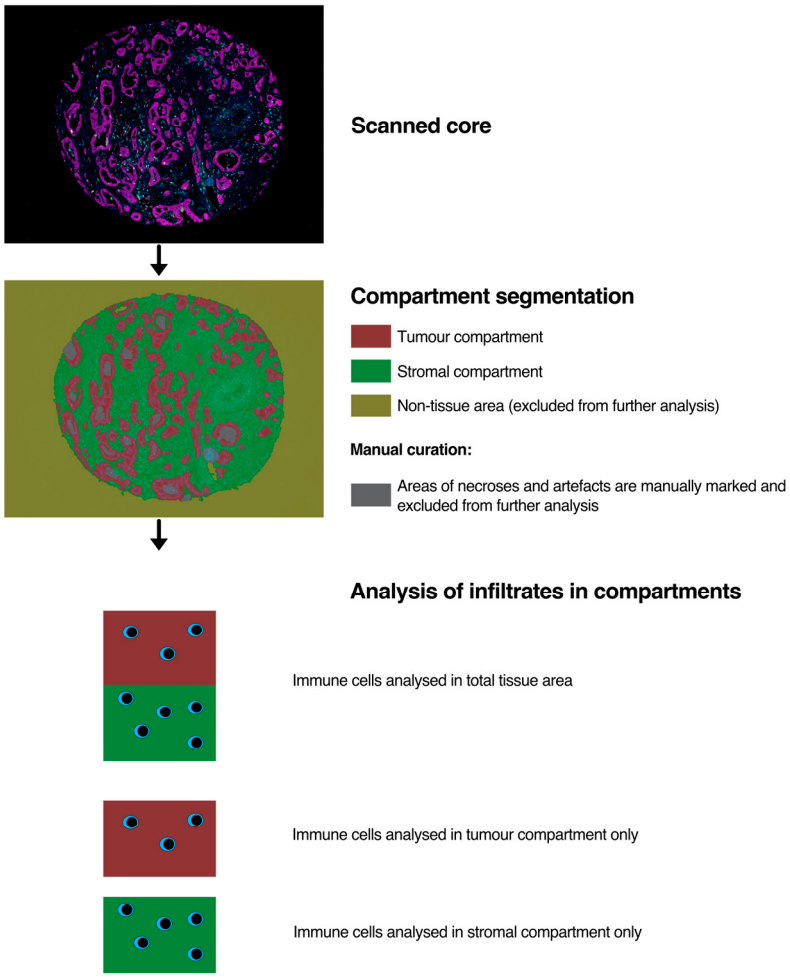

Figure 9:
Overview of the digital tissue segmentation and definitions of compartments used in tissue specific analyses.

Next generation sequencing

Principles and analysis

Next generation sequencing (NGS) is a high throughput, massively parallel method of sequencing the genome (DNA) or the transcriptome (RNA). With the previously used technique for DNA sequencing, Sanger sequencing, it took years to sequence the whole human genome, with NGS the same analysis can be done in a workday. Thus, the introduction of NGS has revolutionised the field of genomics and enabled massive and fast sequencing of genomes both in the research community and in clinical practice. NGS can be done in several contexts, for example whole genome sequencing, whole exome sequencing or targeted gene sequencing (249). In this thesis, targeted sequencing of 70 genes was performed. Additionally, sequencing can be done at different depths (or coverage), i.e the number of reads of a given nucleotide (30x coverage means that each nucleotides, on average, was sequenced 30 times) (249). With deeper coverage, there are more overlaps of sequenced fragments and thus making alignment of the sequencing easier.

In principle, the first step is to retrieve DNA from tissues. This can be done in numerous ways and the choice of method depends upon the way in which the tissue has been preserved, i.e. formalin fixed or fresh frozen. For the paper included in the present thesis, cores were retrieved from the primary tumours and then DNA extraction was carried out with the Qiagen GeneRead kit (Qiagen, Hilden, Germany).

Following DNA extraction the samples can be sequenced with a NGS workflow which typically consists of four steps: library preparation, cluster generation, sequencing and data analysis as outlined in Figure 10 (249). During the library preparation, the DNA samples are randomly fragmentised and then ligated with adapters on the 5’ and 3’ ends (Figure 10A). The ligated adapters act as reference points during the following steps of DNA amplification, sequencing and alignment. The ligated DNA fragments are amplified in a polymerase chain reaction (PCR). Following the first step, the ligated DNA fragments are loaded into a flow cell or chip where the ligated DNA fragments bind to complementary synthetic oligonucleotides of the library adapters, creating templates for subsequent sequencing (Figure 10B). The oligo-DNA clusters are then amplified again in what is called cluster generation. Next, sequencing can be done in several different ways. For the papers included in the present thesis, sequencing was done using a proprietary reversible terminator-based method by Illumina (Illumina Inc, San Diego, United States of America) (249). Sequencing reagents including fluorescent nucleotides are added to the flow cell (Figure 10C). The modified nucleotides will bind to the DNA and emit light at pre-specified wavelengths. At each sequencing

round, the emitted light is recorded and thus the nucleotide sequence on the DNA helix can be determined. The last step is alignment where the sequenced data is aligned to a reference genome (Figure 10D). By referencing a 'standard' genome, variations in the sequenced DNA can be detected. Further referencing to databases with more genomic data can be done in order to exclude single nucleotide polymorphisms (SNPs).

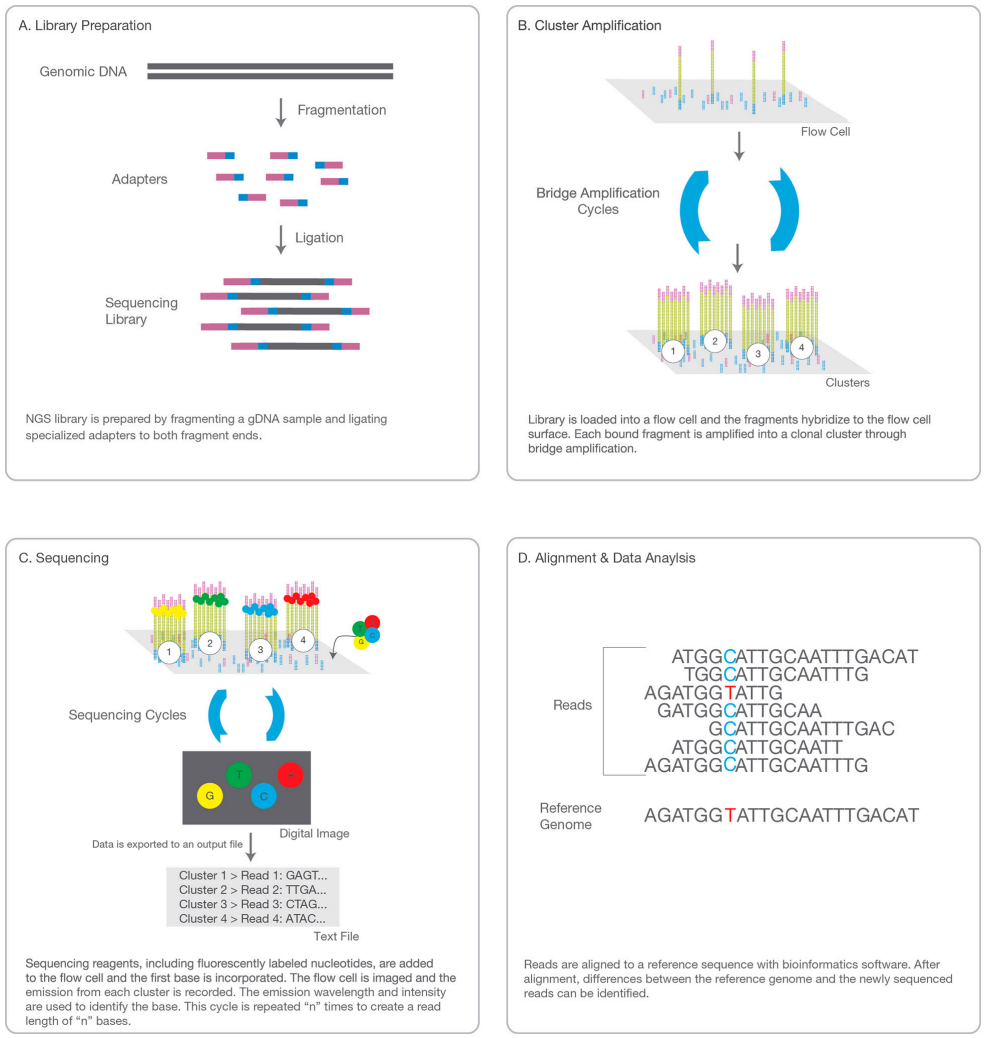

Figure 10:
Overview of the four steps involved in next generation seqencing: A. Library preparation B. Cluster amplification C. Seqencing and D. Aligment and analysis of data. Reproduced with permission from *Illumina Inc.*

Study cohort

The study cohort used in all papers included in the present thesis consist of all 175 patients who underwent resection of periampullary adenocarcinoma with pancreatoduodenectomy at Skåne University Hospital, Sweden, between January the 1st 2001 and December 31st, 2011. The clinical characteristics of the cohort are presented in Table 4. All cases underwent strict pathological reassessment by a board-certified pathologist blinded to clinical outcome, whereby 65 were classified as I-type and 110 as PB-type tumours (250). Data on treatment with neoadjuvant and/or adjuvant chemotherapy, recurrence and cause of death were obtained from patient charts retrospectively. Clinicopathological data were obtained from pathological records and patient charts retrospectively. Data on survival were retrieved from the Swedish National Civil Register, with the most recent follow up made on March 31st, 2017. It was possible to obtain matched lymph node metastases in 63 cases and matched benign tissue in 34 cases.

Table 4.
Summary of clinical characteristics of all 175 patients included in the cohort.

	Entire cohort	Intestinal type	Pancreatobiliary type
Number of patients	175	65	110
Median age (range)	67 (38-83)	67 (38-83)	67 (44-81)
Women/Men	86/89	35/30	51/59
Pancreatic origin	46		46
Ampullary origin	70	51	19
Distal bile duct origin	45		45
Duodenal origin	14	14	
Matched lymph node metastases	63	21	43
Matched benign tissue	34	9	25
Adjuvant chemotherapy	98	18	59
No adjuvant chemotherapy	77	47	51
Gemcitabine	52	7	45
GemCap	4	1	3
GEMOX	3	1	1
5-FU	13	5	8
Oxaliplatin	5	4	1

Out of the 175 cases included in the cohort, two received neoadjuvant treatment and were therefore excluded from all statistical analyses as the neoadjuvant treatment might have influenced the tumour microenvironment, thus potentially introducing

bias (Figure 11). Additionally, two patients with PB-type tumours died due to complications of the pancreatoduodenectomy within 30 days of the surgical procedure and they were therefore excluded from the survival analyses (Figure 11). One patient with a PB-type tumour who emigrated was also excluded from the survival analyses as it was not possible to obtain survival data (Figure 11).

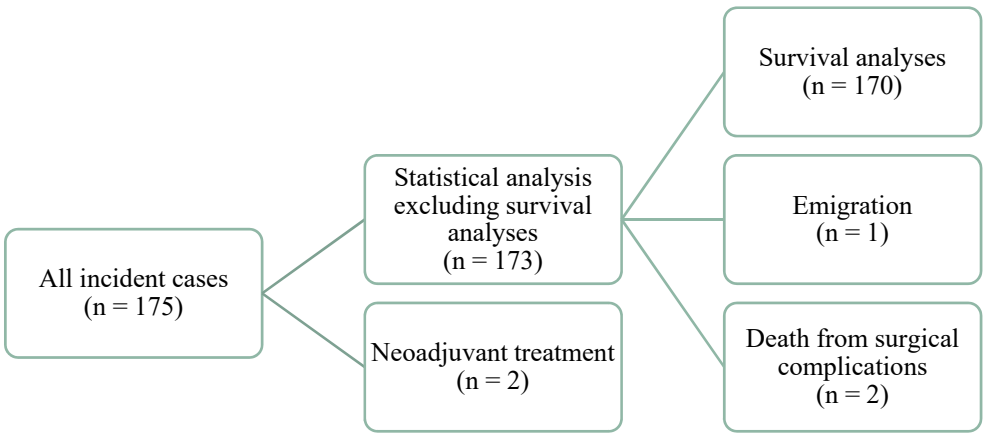

Figure 11:
Flow chart of inclusion and exclusion in the statistical analyses of the present cohort.

Statistical models

In paper I and II, Mann-Whitney U test was used to examine differences in immune cells distribution according to clinicopathological factors. Paired T test was used to assess differences in immune cell infiltration. Classification and regression tree (CRT) analysis was used to find prognostic cut offs. Kaplan-Meier analysis and log rank test were used to assess differences in OS and recurrence free survival (RFS), according to immune cell infiltration. Cox proportional hazards regression was applied to estimate hazard ratios in both univariable and multivariable analysis.

In paper III, the $\chi 2$ test was applied to demonstrate any differences of clinicopathological factors between the original cohort and in those cases where it was possible to perform DNA sequencing. Paired T test was used to assess any differences in clinicopathological features according to mutation status. Kaplan-Meier analysis and log rank test were used to assess differences in OS in relation to

mutational status. Cox proportional hazards regression was applied to estimate hazard ratios in both univariable and multivariable analysis. The proportional hazards assumption was tested with Cox regression with a time-dependent covariate analysis, whereby the proportional hazards assumption was considered to be satisfied when the factor × time interaction did not reach significance. The proportional hazards assumption was also evaluated graphically using log-minus-log plots. Genes mutated in less than 10% of the cases were not included in any statistical analyses as this was deemed not to be statistically meaningful.

In paper IV and V, Wilcoxon signed rank test with Pratt modification was used to assess differences in two groups with related samples. Kruskal-Wallis test was applied for comparison of multiple groups. Mann-Whitney U test was used to assess differences in immune cell infiltration in relation to clinicopathological features. Unsupervised hierarchal clustering was applied to identify patterns in immune cell infiltration. Kaplan-Meier analysis and log rank test were applied to assess differences in OS in relation to immune cell infiltration. Cox proportional hazards regression was applied to estimate hazard ratios in both univariable and multivariable analysis.

All statistical tests were two sided and p-values < 0.05 were considered significant. All calculations were performed with SPSS version 22.0 (SPSS Inc. Chicago, United States of America) or with R software version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria) together with the integrated development environment RStudio version 1.0.143 (RStudio Team, Boston, United States of America).

Ethical considerations

The studies included in this thesis were approved by the Ethics committee at Lund University (ref nr 445/07). The committee waived no need for consent other than the option to opt-out of the studies. All data that could be used to identify patients were anonymised prior to any analyses. Further, all regulations and requirements set by national, the European Union and international organs such as decision no. 1110/94/EC of the European Parliament and of the European Council (OJL126 18,5,94), the Helsinki Declaration on ethical principles for medical research involving human subjects, and the European Union Council Convention on Human rights and Biomedicine were complied with.

Funding

The research in this thesis was supported by grants from the Swedish Cancer Society, the Swedish Research Council, the Swedish Government Grant for Clinical Research, the Mrs Berta Kamprad Foundation, the Sjöberg Foundation, BioCARE, the Faculty of Medicine at Lund University, Skåne University Hospital and the AT/ST Committee at Region Skåne.

Travel grants from the John och Augusta Perssons Foundation and the Royal Physiographic Society in Lund has enabled this research to be presented at international conferences.

The present investigation

I'm very into science. Or at least I'm very into someone who is very into science.

Cecil Baldwin

Summary and discussion of key results

The detailed results are presented in the original papers. The principal findings are presented and discussed here only in brief.

Paper I

The study is the first to provide a detailed description of the prognostic impact of $CD56^+$ NK/NKT cells in the full spectrum of periampullary adenocarcinoma. In I-type tumours, high $CD56^+$ NK/NKT cell infiltration was significantly associated with favourable clinicopathological factors such as low N-stage, absence of growth into perineural and lymphatic structures and peripancreatic fat. In PB-type no significant associations could be established between $CD56^+$ NK/NKT cell infiltration and clinicopathological factors. $CD56^+$ NK/NKT cell infiltration was found to be significantly associated with $CD3^+$ T cell infiltration, $CD1a^+$ DCs infiltration and $CD68^+$ macrophage infiltration.

High $CD56^+$ NK/NKT cell infiltration was associated with a prolonged OS and RFS in the entire cohort as well as in I-type tumours. This association was seen in both Kaplan-Meier analysis and in unadjusted Cox regression analysis but did not remain significant after adjustment for clinicopathological factors in the multivariable analysis. Similar results were found in the entire cohort and in I-type tumours using a CD56/CD3 lymphocyte ratio. These findings are in line with previous studies on other gastrointestinal malignancies such as oesophageal, gastric and colorectal cancer (251-254).

Next, we investigated the impact of $CD56^+$ NK/NKT cell infiltration in strata according to adjuvant chemotherapy. This revealed that the beneficial prognostic impact of high infiltration of $CD56^+$ NK/NKT cells was only evident in patients who

had not received adjuvant chemotherapy, and a significant negative treatment interaction was found between high infiltration of CD56+ NK/NKT cells and adjuvant chemotherapy in the entire cohort and in PB-type tumours, but not in I-type tumours. This association has been described previously in breast cancer (255), and, speculatively, tumours with high infiltration of NK/NKT cells may have an efficient anti-tumour response that is impeded by standard chemotherapy.

The study also highlights the immunomodulatory role of NK/NKT cells, as a high ratio of NK/NKT cells to DCs and macrophages was found to associated with a prolonged OS in the entire cohort and in PB-type tumours, although not in adjusted analysis. This finding is particularly interesting since these tumours have a highly immunosuppressive inflammatory microenvironment, characterised by high levels of myeloid immune cells. All in all, paper I highlights the fact that further research is needed to elucidate the relationship of NK and NKT cells with other immune cells in the inflammatory tumour microenvironment.

Paper II

In this paper four different immune cell populations were investigated: $CD1a^+$ immature tolerogenic DCs, $CD68^+$ macrophages (a pan-macrophage population), $CD163^+$ macrophages (M2 phenotype polarised) and $MARCO^+$ macrophages. Paper II provides a first description of the prognostic impact of tumour infiltrating DCs and macrophages in the full spectrum of periampullary adenocarcinoma.

First, we examined the intercorrelations of the investigated immune cell populations. Infiltration of $CD68^+$ macrophages was significantly associated with infiltration of $CD163^+$ macrophages and $MARCO^+$ macrophages, infiltration of $CD68^+$ and $MARCO^+$ macrophages was significantly associated with $CD3^+$ lymphocyte infiltration, and infiltration of $CD1a^+$ immune cells and $CD68^+$ macrophages was significantly associated with $CD56^+$ NK/NKT cell infiltration. These results indicate that most of the macrophages in the tumour microenvironment of periampullary adenocarcinoma are M2 polarised.

Kaplan-Meier analysis revealed that high infiltration of $CD1a^+$ immune cells was an independent predictor of shorter survival in PB-type tumours, but was not prognostic neither in I-type tumours nor in the entire cohort. Previously, a varying prognostic impact has been reported for tumour infiltrating DCs in pancreatic as well as other solid cancers, being both beneficial, unfavourable or of no prognostic importance (256-258). However, one needs to be cautious when comparing these results directly, as most studies use different markers for DCs and, hence, possibly refer to different DC subsets. Conceivably, single marker studies of DCs are not optimal for the investigation of their biology due their plastic phenotypical nature, the diversity of subpopulations and the fact that they can be derived from both

myeloid and lymphoid progenitors. Moreover, it is possible that the TMA technique is not well suited for evaluation of DC infiltration, due to their relative paucity of tumour infiltration.

High infiltration of both $CD68^+$ and $CD163^+$ macrophages was significantly associated with shorter survival in the entire cohort but not in strata according to morphology, but these associations did not remain significant in adjusted Cox regression analysis. These findings are in line with previous reports in pancreatic cancer (259, 260). in which both the anti-inflammatory population ($CD163^+$ macrophages) and the pan-macrophage population were associated with poor prognosis. Although typically only anti-inflammatory macrophages are thought to participate in tumour progression it is likely that this population makes up for the bulk of tumour infiltrating macrophages, which is then reflected in the prognostic impact of $CD68^+$ macrophages. This hypothesis is supported by the fact that there was a strong intercorrelation between $CD68^+$ and $CD163^+$ infiltration in this study cohort.

High infiltration of $MARCO^+$ macrophages was significantly associated with shorter survival only in I-type tumours, but this association did not remain significant in adjusted analysis. However, high infiltration of $MACRO^+$ macrophages emerged as an independent predictor of shorter survival in the entire cohort after adjustment for clinicopathological factors. Additionally, among patients with I-type tumours with high tumour infiltration of $MARCO^+$ macrophages, those who received adjuvant chemotherapy had a significantly shorter survival compared to those who did not receive adjuvant chemotherapy. The study could however not establish any significant interaction between infiltration of $MACRO^+$ macrophages and adjuvant treatment in I-type tumours. MARCO, which is a scavenger receptor, has been found to be expressed by a subset of macrophages aggregating close to tumour nests in lung cancer (261). In mouse models, treatment with antibodies against MARCO has shown to lead to reduced tumour progression and to promote into more a more inflammatory phenotype (262). $MARCO^+$ macrophages, in addition to creating a physical barrier protecting tumour cells, also express high levels of checkpoint inhibitors such as PDL-1, V-domain Ig suppressor of T cell activation (VISTA) and CTLA-4 (261). They are therefore an interesting target for immunotherapy, and maybe of particular relevance in periampullary cancers given their exceptionally immunosuppressive microenvironment.

Paper III

This study is the first to describe the mutational landscape in the full spectrum of periampullary adenocarcinoma, with particular reference to tumour morphology. We designed a targeted gene panel of 70 cancer associated genes. DNA of sufficient quality could be obtained in 102 cases. There were no statistically significant

differences regarding the distribution of clinicopathological factors between these 102 cases and the original cohort. Out of the 70 characterised genes, only nine were mutated in more than 10% of the patients, as shown in Table 5. Mutational burden in the 70 characterised genes did not differ significantly between I-type and PB-type tumours, and did not confer any prognostic value. The mutational burden that could be calculated from this targeted gene panel may however not be representative, and, thus, one should be careful when interpreting these results.

Table 5.
Summary of the frequency of the most common mutated genes in intestinal and pancreatobiliary type tumours.

Gene	Intestinal type	Pancreatobiliary type
TP53	43%	55%
KRAS	43%	48%
RNF43	15%	13%
SMAD4	13%	15%
SMARCA4	10%	15%
CDKN2A	3%	19%
APC	28%	0%
ERBB3	20%	5%
NF1	10%	11%

We found significant differences in the mutational landscape between the two morphologies; *APC* mutations were found exclusively in I-type tumours, *ERBB3* mutations were significantly more common in I-type tumours, and *CDKN2A* mutations were significantly more common in PB-type tumours. These results are in line with one previous study, where it was concluded that ampullary cancers with I-type morphology have significantly more mutations commonly associated with colorectal cancer, while ampullary cancers with PB-type morphology have significantly more mutations commonly associated with pancreatic cancer (263).

Out of the nine most frequently mutated genes, only four conferred a prognostic value. Mutations in the *APC* and *ERBB3* genes were significantly associated with prolonged survival in the entire cohort in univariable but not in multivariable analysis. When stratifying for morphology neither *APC* nor *ERBB3* mutations were significantly associated with survival. As both *APC* and *ERBB3* mutations were significantly more common in I-type tumours, this may well explain their associations to a prolonged survival in analysis of the entire cohort.

KRAS mutations were associated with shorter OS in the entire cohort and in I-type tumours, both in unadjusted and adjusted Cox regression analysis. Previously, two studies on ampullary cancer have shown similar results (264, 265), though these

studies did not consider morphology. Anti-EGFR treatment has, as discussed in the background section, not shown efficacy in pancreatic cancer and the efficacy in small bowel cancer is still being debated (129, 131, 132). As of date, no investigation has studied the efficacy of anti-EGFR treatment in periampullary tumours in relation to morphology (266). One could theorise that *KRAS* wt I-type tumours may well benefit from this type of treatment and, hence, the results from this study highlight both the importance of taking morphology into consideration and that molecular profiling of these tumours might be of clinical relevance.

SMARCA4 mutations were significantly associated with shorter OS in patients with PB-type tumours who did not receive adjuvant chemotherapy. Due to the implicated relationship between *SMARCA4* and chemotherapy response we decided to also investigate the prognostic and potential predictive impact of the protein Brahma-Related Gene 1 (BRG1), which is encoded by *SMARCA4*. High expression of BRG1 was found to be significantly associated with shorter OS in patients with PB-type tumours who did not receive adjuvant chemotherapy and, adding to this, a significant interaction was found between high BRG1 expression and adjuvant chemotherapy in relation to OS in PB-type tumours. BRG1 is an ATP-dependent chromatin remodelling protein (267), and it has been shown to have oncogenic properties, promoting formation and progression of the pancreatic cancer (268). Previously, *in vitro* models have shown that BRG1 contributes to chemoresistance, especially resistance against gemcitabine (269). This stands in contrast to our results and therefore further research into the role of BRG1 and chemotherapy response is warranted.

Paper IV

Paper IV is the first study to provide a detailed and comprehensive mapping of the topography and composition of lymphocyte infiltration in the full spectrum of periampullary adenocarcinoma. For this purpose, a multiplex immunofluorescence panel was designed with antibodies against the following proteins: CD4, CD8α (henceforth referred to as only CD8), CD20, CD45RO, FoxP3, E-cadherin and pan-cytokeratin. It was possible to phenotype the following lymphocyte subsets: $CD4^+$ single cells (negative for all other markers), $CD4^+CD45RO^+$ cells (activated or memory $CD4^+$ cells), $CD4^+FoxP3^+$ cells ($CD4^+$ T_{regs}), $CD8^+$ single cells (negative for all other markers), $CD8^+CD45RO^+$ cells (activated or memory $CD8^+$ cells), $CD8^+FoxP3^+$ cells ($CD8^+$ T_{regs}), $FoxP3^+CD45RO^{low}$ cells, $FoxP3^+CD45RO^{high}$ cells, CD20 single positive cells (B cells) and cytokeratin and E-cadherin positive tumour cells. As $FoxP3^+CD45RO^{low}$ cells were hard to distinguish from $FoxP3^+$ cancer cells in the tumour compartment, analysis of this subpopulation was restricted to the stromal compartment only. A summary of the different lymphocyte subsets characterised can be viewed in Table 6.

Table 6.
Summary of lymphcyte subsets characterised in paper IV.

	CD4	CD8	CD45RO	FoxP3	CD20
Single $CD4^+$ cells	+	-	-	-	-
$CD4^+CD45RO^+$ cells	+	-	+	-	-
$CD4^+$ T_{regs}	+	-	-	+	-
Single $CD8^+$ cells	-	+	-	-	-
$CD8^+CD45RO^+$ cells	-	+	+	-	-
$CD8^+$ T_{regs}	-	+	-	+	-
$FoxP3^+CD45RO^{low}$	-	-	low	+	-
$FoxP3^+CD45RO^{high}$	-	-	high	+	-
B cells	-	-	-	-	+

Lymphocyte infiltration was found to vary depending on morphological subtype, with I-type tumours having significantly higher infiltration of several lymphocyte populations including $CD8^+CD45RO^+$ cells, $CD8^+$ T_{regs}, $FoxP3^+CD45RO^{high}$ cells, $FoxP3^+CD45RO^+$ cells and B cells. Similar results were seen when stratifying for anatomical origin, with particularly low levels of tumour infiltrating lymphocytes in pancreatic tumours. This association was even more evident in the tumour compartment, indicating that there is a link between morphology and infiltration patterns of lymphocytes. Low infiltration of most of the studied lymphocyte subsets was significantly associated with adverse clinicopathological features such as perineural growth, invasion into vascular and lymphatic structures and growth into the peripancreatic fat.

There were also significant differences in the infiltration patterns into the tumour and stromal compartments in that all $CD8^+$ lymphocyte subsets, $CD4^+CD45RO^+$ cells and B cells were significantly more abundant in the stromal compartment, while single positive CD4 cells and $CD4^+$ T_{regs} were significantly more abundant in the tumour compartment. These findings might indicate different trafficking patterns of lymphocytes in different tissue compartments and highlights the importance of analysing these compartments separately. Similar results were seen when we compared infiltration patterns in malignant versus benign tissue, where lymphocyte infiltration was found to be higher in the former. This pattern has been observed in a previous report on pancreatic cancer (270), and suggests that there is an active immunological response against cancer cells. Interestingly though, this response might not be efficient as the lymphocyte population with the highest predominance in the malignant versus benign paired tissue were the $FoxP3^+$ lymphocyte subsets. Hence, it may well be so that there is an induction of FoxP3 expression in T cells in the tumour compartment.

The density of tumour infiltrating lymphocytes was found to be significantly associated with some genetic alterations. Most noteworthy, the densities of $CD8^+$ cell subsets and B cells were significantly lower in *KRAS* mutated tumours. Furthermore, infiltration of $CD8^+$ T_{regs} was significantly lower in *CDKN2A* mutated tumours, which is in line with previous studies across many solid tumours, including pancreatic cancer (271). As expected, lymphocyte infiltration was also found to be higher in dMMR I-type tumours than in pMMR I-type tumours. Taken together, these results suggest that specific genetic alterations might modulate the immunogenicity of tumours.

Survival analyses were done by several metrics, firstly, according to each lymphocyte subset, secondly by classifying cases as inflamed or immune excluded, thirdly by identification of lymphocyte infiltration patterns by using unsupervised hierarchal clustering and, lastly, by single cell spatial analysis.

When analysing the prognostic impact of each lymphocyte subset, single positive CD4 cells, $CD8^+CD45RO^+$ and B cells were demonstrated to be of particular prognostic importance, even after adjustment in several sub-analyses. Further, by dividing the tumour lymphocyte densities with the stromal lymphocyte densities, we aimed to identify inflamed tumours and immune excluded tumours whereby inflamed tumours had high infiltration into the tumour compartment while immune excluded tumours had lymphocyte infiltration predominantly into the stromal compartment. In adjusted Cox regression analysis of the entire cohort and in PB-type tumours, single positive $CD8^+$ cells were independently associated with a prolonged OS, whereas no significant associations with survival were seen in I-type tumours.

Several immune cell signatures were identified using unsupervised hierarchal clustering. The clusterings were performed separately for the total count, tumour compartment count, and stromal compartment count. Several of these signatures shared traits such as high levels of $CD4^+CD45RO^+$, $CD8^+CD45RO^+$ cells and $FoxP3^+CD45RO^{high}$ cells. Notably, a stromal immune cell signature characterised by high levels of $CD4^+CD45RO^+$ cells, $CD8^+CD45RO^+$ cells, B cells and $FoxP3^+CD45RO^{high}$ cells but low levels of $FoxP3^+CD45RO^{low}$ cells, was significantly associated with a prolonged OS compared to several other identified stromal infiltration patterns in the entire cohort, also in adjusted analysis. A similar association was seen in I-type tumours as well, but did not reach significance in adjusted analysis. Since this stromal immune signature was characterised by low levels of $FoxP3^+CD45RO^{low}$ but high levels of $FoxP3^+CD45RO^{high}$, it might indicate a difference in functionality between these two lymphocyte populations. $FoxP3^+CD45RO^{low}$ cells could, at least partly, stand for a naïve T_{reg} population. However, previous research has shown that both naïve, activated and memory T_{regs} have the same immunosuppressive functionality (272). Thus, the difference in

functionality might not lie in the T_{reg} populations themselves but rather in the interplay with other immune cell populations, which have previously been reported to be of prognostic importance (270, 273, 274).

Adding to this, in PB-type tumours, another stromal infiltration pattern, characterised by high levels of $CD8^+CD45RO^+$ cells, B cells and single positive CD4 cells but low levels of $FoxP3^+CD45RO^{high}$ cells, was significantly associated with a prolonged OS even in adjusted Cox regression analysis. This may suggest that there is a difference in what constitutes an efficient anti-tumour response between the two morphologies. Taken together, these results imply that there are subgroups of patients with immunogenic tumours with high levels of effector lymphocytes but low levels of immunosuppressive lymphocytes, which is in line with previous studies (270, 275).

In the next step, the importance of the spatial topography on a single cell resolution was examined. Proximity of single positive CD4 cells, single positive CD8 cells, $CD8^+CD45RO^+$ cells and B cells to cancer cells was prognostically favourable in the entire cohort. Similar trends were observed in I-type tumours, whereas in PB-type tumours, only single positive CD8 cells were associated with a prolonged OS. These findings stress the importance of the interaction of $CD8^+$ T cells with tumour cells, a finding that has been described previously (270, 275). Lastly, *in situ* cell mapping also allowed us to identify potential cell to cell interactions. We could demonstrate that the presence of single positive CD4 cells in the interaction zone of single positive CD8 cells was associated with a prolonged OS. Possibly, the biological mechanism behind this observation is $CD4^+$ T cell activation of $CD8^+$ T cells, via IFNγ and IL 2 (276-278), or possibly due to bystander killing of cancer cells, where these two T cell populations cooperate (279). This paper provides a first description of such a potential mechanism in the cancer microenvironment *in situ*.

Paper V

Paper V can be viewed as an extension of paper I and II, aiming to further describe innate immune cell infiltration patterns, and to deeper understand their functionality in the tumour microenvironment, an unmet need identified in the conclusion of both of these papers. For this purpose, two multiplex immunofluorescence panels were designed. The first panel consisted of antibodies against CD68, CD163, CD56, NKp46, CD3, E-cadherin and pan-cytokeratin. The second panel consisted of antibodies against CD1a, CD123, CD208, CD15, CD68, E-cadherin and pan-cytokeratin. This allowed us to characterise the following immune cell populations in the first panel: $CD68^+$ macrophages (negative for all other markers), $CD163^+$ anti-inflammatory myeloid cells (negative for all other markers), $CD68^+CD163^+$ macrophages, $CD56^+NKp46^+$NK cells, $CD56^+$ NKT cells, $NKp46^+$ NKT cells and $CD56^+NKp46^+$ NKT cells. A summary of the phenotypical characterisations of the

immune cell populations in the first panel is provided in Table 7. With the second panel, the following immune cells could be characterised: $CD1a^+$ immature tolerogenic DCs (negative for all other markers), $CD208^+$ mature DCs (negative for all other markers), $CD123^+$ plasmacytoid DCs (negative for all other markers), $CD1a^+CD15^+$ granulocytes, $CD123^+CD15^+$ granulocytes, and $CD208^+CD15^+$ granulocytes. CD68 was used to exclude macrophage populations. Table 8 summarises the phenotypical characteristics of the immune cell populations in the second panel.

Table 7.
Summary of immune cell subsets characterised in the first panel of paper V.

	CD68	CD163	CD56	NKp46	CD3
$CD68^+$ macrophages	+	-	-	-	-
$CD163^+$ myeloid cells	-	+	-	-	-
$CD68^+CD163^+$ macrophages	+	+	-	-	-
NK cells	-	-	+	+	-
$CD56^+$ NKT cells	-	-	+	-	+
$NKp46^+$ NKT cells	-	-	+	+	+
$CD56^+NKp46^+$ NKT cells	-	-	+	+	+

Table 8.
Summary of the immune cell subsets characterised in the second panel of paper V.

	CD1a	CD208	CD123	CD15	CD68
Immature DCs	+	-	-	-	-
Mature DCs	-	+	-	-	-
Plasmacytoid DCs	-	-	+	-	-
$CD1a^+$ granulocytes	+	-	-	+	-
$CD208^+$ granulocytes	-	+	-	+	-
$CD123^+$ granulocytes	-	-	+	+	-

As in paper IV, immune cell infiltration differed between the two morphologies in that infiltration of NKT cells and macrophages was significantly higher in I-type compared to PB-type tumours. These results, taken together with those presented in paper IV, indicate that PB-type tumours harbour mechanisms that suppress immune cell infiltration or that I-type tumours are capable of provoking a more vivid immune response. Also, in line with the findings in paper IV, infiltration of several immune cell populations, most noteworthy the NKT cell subsets, were associated with favourable clinicopathological factors. NKT cell infiltration was lower in the tumour compartment compared to the stromal compartment, and in the malignant

compared to benign tissue. Together, these findings suggest that cancer cells have mechanisms of hindering NKT cell infiltration into tumours.

As in paper IV, *KRAS* mutated tumours had lower infiltration of effector immune cells in the tumour compartment, but *KRAS* mutations were also associated with higher stromal infiltration of $CD56^+$ NKT cells and $CD68^+$ macrophages. Mutations in *SMARCA4*, *APC, SMAD4, ERBB3* and *RNF43* were also significantly associated with different infiltration levels of various immune cell subsets. Regarding *APC* and *ERBB3* mutations, the higher levels of immune cell infiltration into these tumours may well be due to the fact that these tumours are predominantly of I-type morphology. Adding to this, the abundance of NKT cells, $CD68^+$ macrophages and $CD123^+CD15^+$ granulocytes was significantly higher in dMMR tumour of I-type morphology, findings that are in line with those presented in paper IV.

Survival analyses were performed in a similar manner to paper IV and revealed a consistent positive prognostic impact of high infiltration of NKT cells. First, the impact of each individual immune subset was investigated. In the entire cohort, high tumour infiltration of $NKp46^+$ NKT cells was found to be an independent predictor of a prolonged OS, also in separate analysis of PB-type, but not I-type, tumours. Inverse associations were seen for $CD1a^+CD15^+$ granulocytes and $CD123^+CD15^+$ granulocytes, that were independently associated with a shorter OS, also in separate analysis of PB-type, but not I-type, tumours. In I-type tumours, high infiltration of $CD163^+$ macrophages into the tumour compartment was an independent predictor of a shorter OS.

Next, we dichotomised cases into immunologically inflamed or immune excluded. This revealed that a high tumour to stroma count ratio of both $CD1a^+$ DCs and $NKp46^+$ NKT cells were independently associated with a prolonged OS in the entire cohort and in PB-type tumours, respectively.

As in paper IV, we aimed to identify immune cell infiltration patterns and to assess their prognostic impact by applying unsupervised hierarchal clustering. An immune infiltration pattern in the tumour compartment characterised by high levels of $NKp46^+$ NKT cells, $CD123^+$ DCs, NK cells and $CD56^+NKp46^+$ NKT cells, and low levels of $CD68^+$ macrophages, was an independent predictor of a prolonged OS, but only in I-type tumours, not in the entire cohort or in PB-type tumours. As such, these findings further indicate that there is a difference in what constitutes an efficient anti-tumour response between the two morphologies, a conclusion that could also be drawn in paper IV. The finding that the prognostic impact was only evident in compartment specific analysis further underlines the importance of taking the immune cell topography into consideration.

All in all, the relationship of NKT cells with favourable clinicopathological features and prolonged survival highlights the importance of this understudied immune cell

population. Previous research into the role of NKT cells in cancer has been sparse. One previous study using a pancreatic cancer model showed that depletion of NKT cells paved the way for tumour progression (280), which could explain the results presented in paper V. The results also show that infiltration of DCs and macrophages was higher in the tumour compartment compared to the stromal compartment and higher in the malignant compared to benign tissue. These cell populations were also in several cases independent factors of a reduced OS, including $CD1a^+CD15^+$ DCs and $CD163^+$ macrophages, all in line with previous research (281-283). In this paper, we could not identify any prognostic impact of single positive CD1a DCs, which is in contrast to the findings in paper II. It is however possible that the population studied in paper II included $CD1a^+CD15^+$ granulocytes. This highlights the fact, as also pointed out in paper II, that it is of uttermost importance to characterise the phenotypical and functional subsets of DCs and, if possible, not rely on single markers, as this analysis might, at least in some cases, be too crude.

Lastly, we performed spatial analysis on a single cell resolution. A long distance between $CD56^+$ NKT cells and cancer cells was demonstrated to be an independent prognostic factor of a reduced OS in the entire cohort. In I-type tumours, a long distance between $NKp46^+$ NKT cells and several myeloid cells, including $CD68^+$ macrophages, $CD123^+$ DCs and $CD123^+CD15^+$ granulocytes, and cancer cells was associated with a reduced OS in adjusted Cox regression analysis. In PB-type tumours, only remoteness of $CD123^+CD15^+$ granulocytes to cancer cells was significantly associated with a reduced OS, also in adjusted analysis.

We therefore focused on $NKp46^+$ NKT cells and $CD68^+$ macrophages in the subsequent analyses of potential cell-to-cell interactions. In these analyses it was demonstrated that the presence of $CD68^+$ macrophages and $CD163^+$ macrophages in the interaction zone of $NKp46^+$ NKT cells was an independent prognostic factor of a prolonged OS. Previous reports have shown that NKT cells co-localise with M2 polarized macrophages, leading to NKT cell mediated cytotoxicity of these macrophages (161, 186, 187). In addition to direct cytotoxicity, NKT cells are also able to modulate macrophage functionality, skewing polarisation into M1 phenotypes (284). The results from this study together with previously published reports, support the hypothesis of an intricate but beneficial crosstalk between NKT cells and macrophages in the inflammatory tumour microenvironment.

Strengths and limitations

The present study cohort is clinically well characterised, and a particularly unique trait of the cohort is that around half of the patients did not receive adjuvant chemotherapy. Therefore, although being retrospective in its nature, it is possible to draw at least some preliminary conclusions also on the potential predictive value of the investigated biomarkers. Additionally, the cohort is comparatively large, encompassing a total of 175 patients, although some subgroup analyses, especially in paper I, II, IV and V, rendered a rather small number of cases. Therefore, some findings need validation in larger and prospective studies.

The TMA technique is today a standard in the field. However, due to the paucity of some immune cells, particularly DCs, analyses of these immune cells would potentially be more accurate if whole sections are used. Also, the tissue cores were primarily sampled from areas enriched for tumour tissue, and not from the adjacent stroma. Although periampullary adenocarcinomas in general have a high stroma to tumour ratio, it is possible that sampling from both compartments would yield more reliable results. However, since each primary tumour was sampled with at least three cores, this issue should be negligible.

There might also be issues with the validity of the definitions of different immune cell populations. Some immune cell populations have unique markers which can be used for their detection, for example lymphocytes. However, myeloid cells are famously (or infamously) plastic, and several myeloid subpopulations can express the same markers. We have tried to get around this problem by using several markers to more accurately describe the different phenotypes. An example of this is the use of multiplexed immune panels in paper IV and V. The confirmation in paper IV and V of the results from paper I and II, wherein single marker stainings were used, is however comforting, and indicate that single marker techniques, despite being crude, are reliable and can be used in a clinical setting or for exploratory studies.

There are of course more cells in the tumour microenvironment than immune cells. The studies included in this thesis have for example not taken into account other cells that are vital in forming the tumour microenvironment such as cancer associated fibroblasts. Future studies, however, could take a more holistic approach and also analyse the interplay between immune cells, cancer cells and other type of cells in the tumour microenvironment.

In all of the included papers, only one patient cohort has been studied. However, many different methodologies have been applied such as manual and digital IHC assessment and multispectral immunofluorescence.

Since many statistical tests have been performed during the course of this thesis work, there is a risk of type I errors. No multiple testing correction has been performed in any of the papers, as all of the studies have been exploratory. Also, the use of CRT for finding an optimal cut-off in papers I and II might introduce a bias in overfitting of the model. However, as mentioned above, most of the results in papers I and II were confirmed in papers IV and V, where CRT analysis was not used to identify prognostic groups, and therefore the influence of this particular bias should be deemed as low.

Conclusions

> Success in research needs four Gs: Glück, Geduld, Geschick und Geld.
>
> Paul Ehrlich

High infiltration rates of NK and NKT cells was associated with a prolonged OS, possibly depending on adjuvant treatment. This association was demonstrated to be consistent through several metrics and methodologies, including densities of individual subpopulations, immune cell signatures, immune inflamed phenotypes and in relation to other immune cells in the tumour microenvironment, especially macrophages. The beneficial prognostic impact of NK and NKT cell infiltration was particularly evident in I-type tumours, while the potential modulatory effect of adjuvant treatment was particularly evident in PB-type tumours. These results highlight the beneficial role of NK and NKT cells in the tumour microenvironment and in immunomodulatory functions.

Macrophages and immature DCs were associated with a reduced OS, but $CD123^+$ DCs and granulocytes were associated with a prolonged OS, particularly in PB-type tumours.

In general, high infiltration of lymphocytes was associated with a prolonged OS. The strongest associations were seen for several subpopulations of $CD8^+$ cells, in particular $CD8^+CD45RO^+$ cells. This association was also consistent through several metrics, including densities of individual subpopulations and immune cell signatures. Noteworthy, $CD8^+$ lymphocyte interaction with cancer cells and $CD4^+$ cells, respectively, was associated with a prolonged OS, indicating that the spatial interactions of $CD8^+$ cells in the tumour microenvironment are important.

Immune cell infiltration patterns were found to differ significantly not only between the two morphologies, with I-type tumours in general having a higher abundance of immune cells, but also between the tumour and stromal compartments. Topography is therefore an important factor to consider.

I-type tumours had significantly more mutations in *APC* and *ERRB3,* while PB-type tumours had significantly more mutations in *CDKN2A. KRAS* mutations were significantly associated with reduced OS in I-type tumours only.

Both *SMARCA4* mutation and high expression of the *SMARCA4*-encoded protein BRG1 was associated with a reduced OS in patients with PB-type tumours who did not receive adjuvant chemotherapy. A potential predictive role of *SMARCA4*/BRG1 is supported by the significant interaction between BRG1 and adjuvant treatment.

Considering all of this, it can be concluded that morphology matters.

Future perspectives

> Science is always wrong. It never solves a problem without creating ten more.
>
> George Bernard Shaw

Although this thesis answers many questions on the role of the immune system in periampullary adenocarcinoma, and the importance of taking morphology into account, the studies also raise further questions that need to be answered.

A prospective, clinical, observational study, the Chemotherapy, Host Response and Molecular Dynamics in Periampullary Adenocarcinoma (CHAMP) study (clinical trial number: NCT03724994) has been initiated by Professor Jirström at the Department of Oncology at Skåne University Hospital, partly based on the results from the studies in the present thesis.

The aims of the CHAMP study are to validate the prognostic and predictive potential of immune cell populations and key mutations in a prospective setting, as well as to delineate the spatial and temporal heterogeneity of periampullary adenocarcinoma. Several sophisticated methodologies will be applied to further elucidate the functional interplay between the local and systemic immune response against the tumours, as well as the clonal evolution of cancer cell genomes under selective pressure from chemotherapy. The results from the CHAMP study are highly anticipated.

Populärvetenskaplig sammanfattning

> It would be so nice if something made sense for a change.
>
> Lewis Carrol

Periampullär cancer är ett samlingsbegrepp för cancer som uppstår kring bukspottkörteln, gallgångarna och tolvfingertarmen. Namnet kommer från det grekiska ordet peri som betyder kring och Vater ampulla, en anatomisk struktur där gallgångarna och bukspottkörlen mynnar ut i tolvfingertarmen. Periampullär cancer kan därför har fyra olika anatomiska ursprung men behandlas oftast som en sjukdom på grund av att det kliniskt kan vara svårt att skilja dem åt. Det går att dela upp periampullära tumörer i två subgrupper baserat på tumörernas mikroskopiska utseende (morfologi), antingen intestinal typ (I-typ) eller pankreatobiliär typ (PB-typ) Morfologi är en viktig faktor att ta i beaktning eftersom patienter med I-typ tumörer har markant bättre prognos än patienter med PB-typ tumörer. Tyvärr har patienter med periampullär cancer, oavsett morfologi, en mycket dålig prognos. Femårsöverlevanden är omkring 5% och medianöverlevnaden är endast 6 månader. På grund av vaga symtom upptäcks periampullär cancer oftast i sena stadier där botande behandling, som idag enbart utgörs av kirurgi, inte är möjlig. Vidare så svarar periampullär cancer dåligt på sedvanlig cellgiftsbehandling och nya målstyrda behandlingar och immunterapi har tyvärr inte uppvisat någon effekt. Det finns därför ett stort behov av djupare förståelse av biologin bakom periampullär cancer och av att identifiera nya s.k. biomarkörer för att bättre kunna skräddarsy behandlingen för enskilda patienter.

Under 2010-talet har det gjorts stora framsteg inom fältet immunonkologi, ett fält som kombinerar studier av immunsystemet med studier av cancer. Det har lett till att flera behandlingar som utnyttjar mekanismer i det kroppsegna immunsystemet mot cancer har godkänts. Dessa behandlingar har dock, som ovan nämnts, än så länge inte förbättrat överlevnaden för patienter med periampullär cancer. En av orsakerna till detta är att den inflammatoriska mikromiljön i och omkring dessa tumörer är immunhämmande. I en tumör finns det en uppsjö av celler, inte bara cancerceller, som interagerar med varandra. Bland dessa finns många olika typer av immunceller och bindvävsceller. Tillsammans interagerar de på ett sätt som närmast liknar ett miniekosystem.

Vi vet sedan många år tillbaka att immunsystemet är av stor betydelse i kampen mot cancer. Många olika immunceller har förmågan att motarbeta cancerceller med högt specialiserade mekanismer. Exempel på olika immuncellspopulationer som är viktiga i kampen mot cancer är:

- Mördar-T-celler som kan döda cancerceller.
- T hjälparceller som stödjer mördar-T-cellers funktion.
- Regulatoriska T-celler som agerar som en broms för att förhindra ett för starkt immunsvar.
- Minnes-T-celler som skapar det immunologiska minnet.
- B-celler som producerar antikroppar som kan binda till cancerceller och göra dem synliga för andra immunceller.
- Makrofager som kan äta upp cancerceller, men också understödja en hämmande miljö för andra immunceller, vilket underlättar spridningen av tumörceller.
- Dendritceller som hjälper T celler att bli effektiva, men precis som makrofager även under vissa förhållanden kan hämma andra immunceller.
- Naturliga mördarceller som har liknande förmågor som mördar-T-celler men som kan agera snabbare och utan stöd av andra T-celler.
- Naturliga mördar-T-celler som har egenskaper av både mördar-T-celler och naturliga mördarceller, men framför allt regulatoriska egenskaper.

Immunsystemet kan delas in i två olika huvudsystem, det medfödda immunförsvaret och det förvärvade immunförsvaret. Det medfödda immunförsvaret utgörs av bland annat makrofager, dendritceller, naturliga mördarceller och naturliga mördar-T-celler. Det är dessa immunceller som initierar immunsvaret mot cancerceller. Det förvärvade immunförsvaret kommer igång lite långsammare, men kan bilda många celler som attackerar specifika ytproteiner på cancerceller. Om cancerceller försöker undkomma en arm av immunförsvaret betyder det oftast att den gör sig sårbar för den andra armen. På så sätt har det medfödda och förvärvade immunförsvaret kompletterande mekanismer för avdödande av cancerceller.

Mot denna bakgrund har syftet med detta avhandlingsarbete varit att närmare kartlägga den inflammatoriska tumörmikromiljön vid periampullär cancer, med särskilt fokus på relationen till tumörmorfologi, genetiska förändringar samt överlevnad. För detta ändamål studerades tumörer från 175 patienter, varav 65 var av I-typ och 110 av PB-typ. Samtliga 175 patienter har behandlats med kirurgi vid Skånes Universitetssjukhus mellan 2001 till 2011. Av dessa fick ungefär hälften cellgiftsbehandling efter operationen.

I det första avhandlingsarbetet undersöktes hur nivåerna av naturliga mördarceller och naturliga mördar-T-celler påverkade överlevnaden och svar på cellgiftsbehandling. Detta gjordes genom en metod som kallas immunohistokemi, vilket kortfattat innebär att man preparerar vävnadsprover från tumören med antikroppar som binder till de immunceller man vill studera. När antikropparna har bundits in blir det en färgreaktion som gör att immuncellerna kan studeras i ett mikroskop. Vi fann ett samband mellan höga nivåer av naturliga mördarceller och naturliga mördar-T-celler i tumörerna och längre överlevnad. Detta samband var dock mindre tydligt eller obefintligt hos patienter som fått cellgiftsbehandling, särskilt hos dem med tumörer av PB-typ.

I det andra delarbetet undersöktes, med samma metod som i det första delarbetet, hur nivåerna av omogna dendritceller och tre olika subpopulationer av makrofager påverkar överlevnaden och svar på cellgiftsbehandling. Vi fann samband mellan höga nivåer av såväl dendritceller som makrofager och kortare överlevnad, särskilt i tumörer av PB-typ.

I det tredje avhandlingsarbetet undersöktes förekomsten av mutationer, dvs genetiska förändringar, i tumörerna med hjälp av s.k. nästagenerations djupsekvensering. Vi fann flertalet skillnader i mönstret av mutationer mellan de två olika tumörmorfologierna. Tumörer av I-typ hade fler mutationer i generna *APC* och *ERBB3*, medan tumörer av PB-typ hade fler mutationer i genen *CDKN2A.* Vi fann också ett samband mellan mutationer i genen *KRAS* och kortare överlevnad, särskilt hos patienter med tumörer av I-typ. Slutligen fann vi ett samband mellan mutationer i genen *SMARCA4* och sämre överlevnad hos patienter med tumörer av PB-typ som inte fick cellgiftsbehandling. Därför gick vi vidare och undersökte även med hjälp av immunhistokemi hur proteinet BRG1, som kodas av *SMARCA4*-genen*,* uttrycks i tumörerna. Resultaten visade att även patienter vars tumörer hade höga nivåer av BRG1 levde kortare om de inte fick cellgiftsbehandling, men att de hade en påtaglig överlevnadsvinst om de fick behandling. Sammantaget poängterar resultaten att genetisk profilering av periampullär cancer kan vara av nytta i den kliniska vardagen.

I det fjärde avhandlingsarbetet undersöktes den rumsliga fördelningen av olika immunceller i tumörerna mer i detalj. För detta ändamål använde vi oss av mer avancerad teknologi, som baseras på s.k. immunofluorescens, som i princip liknar immunohistokemi, men istället för att ge en färgreaktion avger antikroppen ett fluorescerande ljus. Denna teknik möjliggör infärgning med flera olika antikroppar samtidigt och totalt kunde vi med vår panel av antikroppar undersöka nio olika populationer av T och B celler med automatisk bildanalys

Vi fann samband mellan flera av dessa immuncellspopulationer, främst olika typer av mördar-T-celler, och längre överlevnad. Vi fann också att patienterna levde längre om avståndet mellan mördar-T-cellerna och tumörcellerna var kortare. Vi

kunde vidare identifiera ”signaturer” med olika sammansättning av T- och B-celler som påverkade överlevnaden, med skillnader även mellan de två tumörmorfologierna.

I det femte och sista avhandlingsarbetet använde vi oss av samma teknik som i det fjärde delarbetet, för att på samma sätt närmare studera olika immuncellspopulationer i det medfödda immunförsvaret. Genom att sätta ihop två paneler med olika antikroppar kunde vi karakterisera totalt 13 populationer av naturliga mördarceller, naturliga mördar-T-celler, makrofager och dendritceller. Vi fann återigen ett samband mellan höga nivåer av naturliga mördar-T-celler och förlängd överlevnad. Vi identifierade dessutom en immuncellssignatur bestående av höga nivåer av naturliga mördar-T-celler, naturliga mördarceller samt låga nivåer av makrofager, som var kopplad till förlängd överlevnad, men endast hos patienter med tumörer av I-typ.

Vi fann också att patienter med tumörer i vilka naturliga mördar-T-celler låg nära makrofager hade en förlängd överlevnad. Resultaten i denna studie stödjer därmed fynden i delarbete 1 och 2 och understryker de naturliga mördar-T-cellernas viktiga roll i att styra immunförsvaret i positiv riktning.

Sammanfattningsvis har vi visat att patienter med periampullär cancer uppvisar olika typer av mutationer och variationer i immunförsvaret. Vi visar att vi genom att dela upp patienter avseende tumörmorfologi vid bedömning av den inflammatoriska tumörmikromiljön kommer ett steg närmare att kategorisera denna brokiga grupp och dess behandlingssvar. Därmed är vi också närmare en mera skräddarsydd behandling som krävs för att öka överlevanden för denna svårt drabbade patientgrupp.

Acknowledgements

> Nanos gigantum humeris insidentes.
>
> Bernard of Chartres

First and foremost, Karin Jirström, my main supervisor. If I have seen further, it is because I have been standing on your shoulders!

My three co-supervisors, Jakob Eberhard, Karin Leandersson and Emelie Karnevi, for invaluable discussions and advice.

Former and present fellow PhD students in the Jirström group: Gustav Andersson, Jonna Berntsson, Karolina Boman, Jacob Elebro, Richard Fristedt, Margareta Heby, Charlotta Hedner, Anna Larsson, Sofie Olsson Hau, Alexandra Petersson, Bahar Rastegar, Christina Siesing, Maria Svensson, and Sara Wahlin, for many memorable hours of scientific discussions, fun and dancing.

Björn Nodin, for making many, many hours in the microscope more fun.

Co-authors Anders Bjartell, Göran Jönsson, Karolina Holm, Ina Hrynchyk, Mikael CI Karlsson, Agnieszka Krzyzanowska, Artur Mezheyeuski, Patrick Micke and Johan Staaf for fruitful and interesting collaborations.

Former and present Head of the Department of Clinical Sciences, Bo Baldetorp and Mikael Bodelsson, for providing a stimulating research environment.

The Human Protein Atlas and Atlas Antibodies for their helpful support and collaboration.

Susanne André, Anette Saltin and Magnus Zätterström for helpful administrative support.

My dear and loved friends for their never-ending encouragement.

My family, mamma och pappa, siblings and many nieces and nephews for their love and support.

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I hate quotations. Tell me what you know.

Ralph Waldo Emerson

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