Pancreatic cancer evolution across space and time: From diagnosis to terminal disease

Petersson, Alexandra

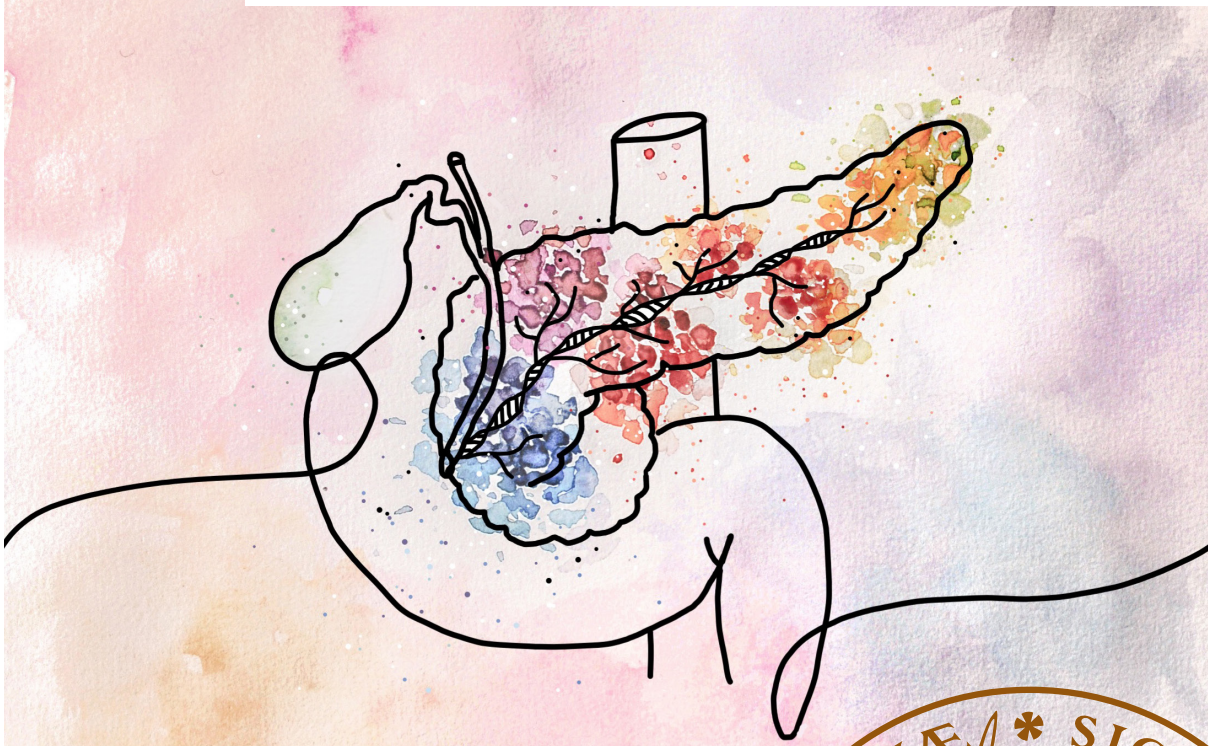
2024

Document Version:
Publisher's PDF, also known as Version of record

Pancreatic cancer evolution across space and time

From diagnosis to terminal disease

ALEXANDRA PETERSSON
DEPARTMENT OF CLINICAL SCIENCES LUND | FACULTY OF MEDICINE | LUND UNIVERSITY



Pancreatic cancer evolution across space and time

Pancreatic cancer evolution across space and time

From diagnosis to terminal disease

Alexandra Petersson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 6th of September at 09.00 in the Belfrage Hall, BMC D15, Klinikgatan 32, Lund, Sweden

Faculty opponent
Laura Delong Wood, MD, PhD
Johns Hopkins University School of Medicine, Baltimore, MD, USA

Organization: LUND UNIVERSITY, Faculty of Medicine, Department of Clinical Sciences Lund

Document name: Doctoral dissertation **Date of issue** September 6, 2024

Author: Alexandra Petersson **Sponsoring organization:**

Title and subtitle: Pancreatic cancer evolution across space and time: From diagnosis to terminal disease

Abstract:

The grim prognosis for patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) may be explained by a combination of late detection, a desmoplastic tumour stroma hindering therapeutic penetration, and a sparse repertoire of treatment options. The overall low availability of tumour tissue has further stalled research advances and most likely contributed to delayed clinical improvements and the persistent poor patient outcome. The work of this thesis aimed to evaluate the genetic evolution of PDAC across all disease stages, together with the accompanying local and systemic immune response. Adding to that, liquid biopsies were explored as a complement and/or a substitute to tissue biopsies for better tumour characterisation, prognostication and disease monitoring. Most of the analyses were performed within the prospective clinical CHAMP study, as outlined in paper II (study protocol).

Spatial genetic heterogeneity was analysed in resected tumour areas antemortem in paper I and tumour specimens primarily collected postmortem in paper IV, by mapping relationships between genetically defined subclones. Particularly, copy number alterations (CNAs) were found to divide the clonal landscape into phylogenetic networks with clinical implications, further stressing their importance in PDAC progression. The findings from paper I and IV also validate the previously noted early metastatic dissemination pattern of PDAC. Moreover, in paper I, the evolutionary complexity of each patient-specific phylogeny was negatively linked to overall survival, indicating that the clonal landscape before treatment may predict the predisposition to rapid recurrence. This hypothesis was further strengthened by varying CNA heterogeneity in pre-treatment plasma samples in paper IV.

Analyses of the local and systemic host immune response in paper I and paper III, respectively, indicated that immunoediting processes, such as tumour expression of regulatory molecules, may be linked to the burden of genetic alterations and circulating tumour DNA (ctDNA). In addition, a few circulating immune cells and soluble proteins were found to carry prognostic potential in paper III.

In paper III and IV, a novel probe panel was designed for sequencing of ctDNA. Analyses of plasma-derived ctDNA before and during chemotherapy administration were found suitable for prognostication, monitoring of disease progression and identification of markers suggestive of opportunities for precision medicine. In particular, the findings of paper III propose that an upfront dichotomisation of palliative patients, i.e. patients not eligible for curative surgery, into a $ctDNA^{high}$ and $ctDNA^{low}$ category, respectively, could be of value in clinical decision making.

In conclusion, this thesis provides insights into PDAC evolution, highlights its complex relationship with the surrounding host response and proposes future directions for disease management.

Key words: Pancreatic cancer, evolution, copy number alterations, heterogeneity, liquid biopsy, ctDNA

Language English **ISSN and key title**: 1652-8220

ISBN: 978-91-8021-593-0

Recipient's notes **Number of pages**: 141

Pancreatic cancer evolution across space and time

From diagnosis to terminal disease

Alexandra Petersson

LUND
UNIVERSITY

Cover art by Alexandra Petersson

Faculty of Medicine

Department of Clinical Sciences Lund

ISBN 978-91-8021-593-0

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University

Lund 2024

Till Lilly

Table of Contents

Thesis at a glance

Paper I

- **1st author**
- Published 2022
- *Molecular Cancer Research*

In summary
- Nine resected tumours
- Spatial heterogeneity
- Local immune response

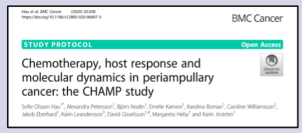

MOLECULAR CANCER RESEARCH | CANCER "-OMICS"

Branching Copy-Number Evolution and Parallel Immune Profiles across the Regional Tumor Space of Resected Pancreatic Cancer

Paper II

- **2nd author**
- Published 2020
- *BMC Cancer*

In summary
- Study protocol
- The CHAMP study


BMC Cancer

STUDY PROTOCOL Open Access

Chemotherapy, host response and molecular dynamics in periampullary cancer: the CHAMP study

Paper III

- **Shared 1st author**
- Submitted manuscript

In summary
- 60 CHAMP patients
- Longitudinal blood sampling
  - ctDNA
  - Immune cells
  - Serum proteins
- Tissue comparisons

Paper IV

- **1st author**
- Manuscript in preparation

In summary
- Five first autopsied cases
- Heterogeneity of terminal disease
- Preceding events
- Post mortem tissue
- Longitudinal ctDNA

Abbreviations: ctDNA = circulating tumour DNA, CHAMP = Chemotherapy host response and molecular dynamics in periampullary cancer

Abstract

The grim prognosis for patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) may be explained by a combination of late detection, a desmoplastic tumour stroma hindering therapeutic penetration, and a sparse repertoire of treatment options. The overall low availability of tumour tissue has further stalled research advances and most likely contributed to delayed clinical improvements and the persistent poor patient outcome.

The work of this thesis aimed to evaluate the genetic evolution of PDAC across all disease stages, together with the accompanying local and systemic immune response. Adding to that, liquid biopsies were explored as a complement and/or a substitute to tissue biopsies for better tumour characterisation, prognostication and disease monitoring. Most of the analyses were performed within the prospective clinical CHAMP study, as outlined in **paper II** (study protocol).

Spatial genetic heterogeneity was analysed in resected tumour areas antemortem in **paper I** and tumour specimens primarily collected postmortem in **paper IV**, by mapping relationships between genetically defined subclones. Particularly, copy number alterations (CNAs) were found to divide the clonal landscape into phylogenetic networks with clinical implications, further stressing their importance in PDAC progression. The findings from **paper I** and **IV** also validate the previously noted early metastatic dissemination pattern of PDAC. Moreover, in **paper I**, the evolutionary complexity of each patient-specific phylogeny was negatively linked to overall survival, indicating that the clonal landscape before treatment may predict the predisposition to rapid recurrence. This hypothesis was further strengthened by varying CNA heterogeneity in pre-treatment plasma samples in **paper IV**.

Analyses of the local and systemic host immune response in **paper I** and **paper III**, respectively, indicated that immunoediting processes, such as tumour expression of regulatory molecules, may be linked to the burden of genetic alterations and circulating tumour DNA (ctDNA). In addition, a few circulating immune cells and soluble proteins were found to carry prognostic potential in **paper III**.

In **paper III** and **IV**, a novel probe panel was designed for sequencing of ctDNA. Analyses of plasma-derived ctDNA before and during chemotherapy administration were found suitable for prognostication, monitoring of disease progression and identification of markers suggestive of opportunities for precision medicine. In particular, the findings of **paper III** propose that an upfront dichotomisation of palliative patients, i.e. patients not eligible for curative surgery, into a $ctDNA^{high}$ and $ctDNA^{low}$ category, respectively, could be of value in clinical decision making.

In conclusion, this thesis provides insights into PDAC evolution, highlights its complex relationship with the surrounding host response and proposes future directions for disease management.

Populärvetenskaplig sammanfattning

Bukspottkörtelcancer, eller *pankreascancer* på latin, representerar en av modern onkologis svåraste utmaningar. Det är en relativt ovanlig sjukdom, med omkring 1500 fall årligen i Sverige, men svarar för den tredje vanligaste orsaken till cancerrelaterad död. Drygt var tionde patient lever fem år efter diagnos, men majoriteten av patienterna dör inom ett år. Denna dystra prognos beror delvis på bukspottkörtelns anatomiska position djupt inne i buken, vilket oftast endast medför vaga och ospecifika symptom i ett sent skede och som i sin tur försvårar tidig upptäckt. Detta gör att tumören oftast har spridit sig utanför bukspottkörteln redan vid diagnostillfället, det vill säga bildat *dottertumörer* i omgivande kärl eller i andra organ såsom i den närliggande levern. I nuläget betyder det att cancern inte längre går att bota.

Ungefär var femte patient diagnosticeras dock med lokal sjukdom, vilket innebär att tumören går att operera bort i botande syfte. Dessa patienter behandlas sedan med cellgiftsbehandling efter operation, så kallad *adjuvant* behandling, för att minska risken för återfall. Tyvärr återinsjuknar de flesta patienter ändå inom ett år. För dessa patienter, tillsammans med dem som diagnosticeras med *avancerad* (ej opererbar) sjukdom redan från början, ges i stället cellgifter i livsförlängande syfte, också kallad *palliativ* behandling. I majoriteten av fallen blir tumören dock snabbt motståndskraftig mot behandling, vilket utgör en av de största utmaningarna med pankreascancer.

Hur tumören ändrar sig över tid har dock endast blivit sparsamt studerat då sådana analyser har varit beroende av tillgång till tumörvävnad. Fram till idag har många studier därför fokuserat på de 20 % av patienterna där tumören gått att operera bort, vilket medför en skevhet mot patienter med bättre diagnos och möjligtvis mindre aggressiva tumörer. Från palliativa patienter, dvs den absoluta majoriteten, finns i bästa fall endast små vävnadsbiopsier, som tas för att ställa diagnosen, vilka oftast inte ger tillräcklig information om tumörens egenskaper.

En viktig aspekt för ett effektivt behandlingssvar är hur *heterogen* en cancer är, det vill säga hur lika eller olika de enskilda cancercellerna är sinsemellan avseende olika biologiska egenskaper. Detta kan till exempel jämföras med en flock med hundar där en grupp med enbart golden retrievers är betydligt mer *homogen* än en grupp med blandade raser, så som både golden retrievers, taxar och någon pudel. Likaså, en tumör med stor variation, hög *heterogenitet*, medför oftast en ökad risk för att någon grupp cancerceller, härefter refererat till som *tumörklon*, är resistent mot cellgiftsbehandling. Dessa skillnader, så kallade *genetiska* skillnader i klonernas *DNA* (liknande de förändringar i arvsmassan som bidrar till skillnaderna mellan en pudel och en tax) kan ofta, men inte alltid, upptäckas i de olika tumörklonernas arvsmassa.

För att kartlägga genetisk heterogenitet kan man använda sig av *fylogenier*, vilka typiskt sett utnyttjas för att illustrera evolutionära släktträd mellan arter. Om vi återgår till

hundar som exempel så kan fylogenier demonstrera deras härstamning från vargar, där olika förgreningar representerar utvecklingen av de olika hundraser vi ser idag. På samma vis kan man beskriva de evolutionära förhållandena mellan olika tumörkloner och undersöka tumörens utveckling.

En annan uppmärksammad faktor är hur patientens egna *immunförsvar* bidrar till behandlingssvar och prognos. Immunförsvaret arbetar normalt dygnet runt med att döda celler som är skadade och som uppvisar avvikande mönster i celldelning, vilket hindrar tumörer från att bildas i kroppen. I ytterst ovanliga fall lyckas dock vissa skadade celler undvika immunförsvarets bevakning, och en tumör bildas. Tumören kan sedan utnyttja och manipulera olika spelare inom immunförsvaret att arbeta till dess fördel, vilket ligger till grund för många av de *immunterapier* som tagits fram.

Syftet med den här avhandlingen har således varit att undersöka hur pankreascancer utvecklas över tid, och hur detta påverkas av både cellgiftsbehandling och patientens egna immunförsvar. Störst fokus har lagts på *genetisk heterogenitet* i olika stadier av sjukdomen, och hur denna i sig bidrar till behandlingsresistens och överlevnad. Ett annat mål med studierna har även varit att undersöka om, och i vilken utsträckning, blodprover kan komplettera, eller ersätta, vävnadsbiopsier.

Avhandlingen omfattar fyra *delarbeten* (**I**-**IV**) där arbete **I** innefattar patienter med opererbar sjukdom i en retrospektiv patientkohort. Arbete **II**-**IV**, å andra sidan, bygger på en klinisk prospektiv observationsstudie, *CHAMP-studien*, som startade i Malmö 2018 och som utökades till Lund under 2019. Studien bjuder in alla patienter med pankreascancer som genomgår cellgiftsbehandling, vilket beskrivs i detalj i arbete **II**, som utgör ett protokoll med studiemål, analyser och kliniska ändpunkter. CHAMP studien involverar bland annat upprepad blodprovstagning för varje patient både innan, under och efter avslutad behandling, vilket legat till grund för majoriteten av analyser i arbete **III**. År 2021 gjordes även ett tillägg till studien med introduktionen av riktade *obduktioner*, vilket möjliggör omfattande analyser av tumörvävnad i sjukdomens slutskede. Vävnad från obduktioner användes huvudsakligen i arbete **IV**.

Fokus i arbete **I** och **IV** var att undersöka genetisk heterogenitet mellan geografiskt olika tumöromården, så kallad *spatial* tumörheterogenitet. I **arbete I** gjordes detta på den bortopererade *moder*tumören, det vill säga den ursprungliga tumören i bukspottkörteln. I arbete **IV** gjordes det dels på vävnad från modertumören, dels på de dottertumörer, *metastaser*, som hittades vid obduktion. I båda delarbetena användes fylogenier för att kartlägga olika tumörkloners släktskap och genetiska evolution. I arbete **IV** ställde vi oss också frågan om vilka tumörkloner från bukspottkörteln som lyckats överleva i cirkulationen och sedan fått fäste i andra organ. Resultat från båda studierna visade att de tumörkloner som gett upphov till metastaser verkar ha uppstått tidigt och att de i vissa fall förmodligen spridit sig till andra organ redan innan operation. I sådana fall är dock metastaserna så små att de inte kan upptäckas med

röntgen, så kallade *mikrometastaser*, vilket är anledningen till att cellgiftsbehandling ges även efter operation.

I arbete **I** studerade vi de olika tumörklonssläktträden vidare avseende storlek och mängden förgreningar. Det visade sig att det i vår patientgrupp fanns ett samband mellan hur extensivt trädens förgreningsnätverk är och hur länge patienterna överlevde efter operation. Detta väckte en hypotes om att genetiskt heterogena tumörer, med komplexa släktträd, är mer *evolutionsbenägna* vilket innebär en högre risk för snabbt återfall efter operation. Sådana tumöregenskaper borde således motivera en mer frekvent monitorering för att upptäcka återfall i tid.

De många utmaningarna med att utföra vävnadsbiopsier motiverade utforskandet av så kallade *flytande biopsier*, i det här fallet blodprover, i arbete **III** och **IV**. När en cell i kroppen dör utsöndras ofta delar av fragmenterat DNA till blodet, så kallat *cellfritt* DNA, och en växande tumör utsöndrar ofta sådana fragment i högre utsträckning. Detta gör att DNA från tumören, härefter refererat till som *cirkulerande tumör-DNA* (ctDNA), ofta uppnår tillräckligt höga nivåer i blodet för att vi ska kunna upptäcka det. Dessutom, till skillnad från ett vävnadsprov som representerar en bild av just det specifika tumörområdet, kan ctDNA ofta spegla en översikt av flertalet tumörlokaler i kroppen, såsom olika metastaser vid spridd sjukdom. Analyser av ctDNA är alltså ett relativt enkelt sätt, som inte kräver något kirurgiskt ingrepp, att övervaka hur tumören utvecklas under behandling.

Inför arbete **III** och **IV** utvecklades en specifik och känslig analysmetod för att både kunna uppskatta mängden ctDNA i blodet och upptäcka specifika tumöregenskaper som kan indikera att en patient är lämplig för *målinriktad* behandling. Ett annat mål var även att se ifall ctDNA kunde hjälpa till att identifiera de tumörer som är mer evolutionsbenägna.

Resultaten från arbete **III** visade att monitorering av ctDNA-nivåer, före och under cellgiftsbehandling, ofta gav en god fingervisning om terapisvar och tumörutveckling. Det betyder till exempel att ctDNA-analyser skulle kunna användas som ett komplement i kliniken för att upptäcka återväxt av tumören i ett tidigare skede jämfört med olika typer av röntgenundersökningar. Vår egendesignade metod visade sig även kunna identifiera specifika tumörmarkörer som hade kunnat motivera administrering av målinriktad behandling.

Ett annat viktigt fynd i arbete **III** var att palliativa patienter kunde delas upp i två grupper, *ctDNA^{höga}* eller *ctDNA^{låga}*, beroende på mängden ctDNA i deras blod innan behandlingsstart. Patienter i den ctDNA^{höga} gruppen visade sämre svar på behandling och hade betydligt kortare överlevnad än de i den ctDNA^{låga} gruppen. Således, medan cellgiftsbehandling enligt nuvarande klinisk praxis och frekvent ctDNA-monitorering konstaterades vara effektivt i den ctDNA^{låga} gruppen kunde vi inte se någon övergripande behandlingsnytta för patienter i den ctDNA^{höga} gruppen. Vårt förslag

inför framtida vård av patienter med höga nivåer av ctDNA i blodet är därför att tidigt undersöka möjligheten till behandling med målinriktade terapier, och i annat fall erbjuda bästa möjliga understödjande vård med det primära syftet att lindra symptom och förbättra livskvalitén.

I kombination med de genetiska släktträden i arbete **I** och de olika blodnivåerna av ctDNA i arbete **III** undersökte vi även det lokala immunförsvaret i själva tumören samt det systemiska immunförsvaret, dvs immunceller och immunrelaterade proteiner, som cirkulerar i blodet. Våra resultat i arbete **I** visade att tumörer med *långa* träd, vilket representerar många genetiska förändringar, verkar visa upp speciella proteiner på sina cellytor som kan ”tysta” det omgivande immunförsvaret. Dessa kallas för *regulatoriska* molekyler då de i vanliga fall används av specifika immunceller som ser till att vårt immunförsvar inte löper amok och börjar attackera våra friska normala celler. I arbete **III** såg vi även liknande mönster i blodet för de patienter som hade höga nivåer av ctDNA. Dessa resultat ger en fingervisning om hur tumörer kan använda olika medel för att undkomma immunförsvaret och till och med vända det till sin fördel.

I arbete **III** identifierade vi även tre blodmarkörer vars nivåer före, eller under, cellgiftsbehandling var tydligt kopplade till patientens överlevnad. Dessa kan komma till nytta i framtida monitorering och hjälpa till att avgöra när man bör byta behandlingsstrategi.

Slutligen, i linje med fynden i arbete **I**, så visade vi i arbete **IV** att skillnader i genetisk tumörheterogenitet kunde upptäckas även i blodet. I två obducerade patienter med höga ctDNA nivåer före behandlingsstart var ctDNA-mönstret för den ena patienten likt det som sågs i vävnadsbiopsin vid diagnos, medan det i den andra patienten speglade en mix av förändringar från olika metastaser. Överlevnaden skilde sig också mellan dessa patienter, såtillvida att patienten med ett mer komplext ctDNA-mönster hade en jämförelsevis aggressiv tumör och en kort överlevnad på knappa tre månader. Även om det inte går att dra några säkra slutsatser från analyser av endast två patienter överensstämmer resultaten med vår hypotes om att en heterogen tumör är mer evolutionsbenägen och motståndskraftig mot behandling.

Sammanfattningsvis har studierna i den här avhandlingen bidragit med nya insikter till den genetiska utvecklingen hos pankreascancer och dess komplexa samspel med immunförsvaret, som skulle kunna användas i framtida behandlingsstrategier. De uppmuntrar också användandet av icke-invasiva flytande biopsier, i detta fall blodprov, som komplement till vävnadsbiopsier i kliniken.

List of Papers

Paper I

Petersson A, Andersson N, Hau SO, Eberhard J, Karlsson J, Chattopadhyay S, Valind A, Elebro J, Nodin B, Leandersson K, Gisselsson D, Jirström K. Branching copy number evolution and parallel immune profiles across the regional tumor space of resected pancreatic cancer. *Molecular Cancer Research* 2022;20:749-761

Paper II

Olsson Hau S, **Petersson A**, Nodin B, Karnevi E, Boman K, Williamsson C, Eberhard J, Leandersson K, Gisselsson D, Heby M, Jirström K. Chemotherapy, Host response and Molecular Dynamics in Periampullary cancer: the CHAMP Study. *BMC Cancer* 2020;20:308

Paper III

Petersson A*, Svensson M*, Hau SO, Sincic V, Lundberg K, Bergström R, Lindberg J, Mayrhofer M, Chattopadhyay S, Jönsson G, Eberhard J, Heidenblad M, Leandersson K, Gisselsson D, Jirström K. Temporal dynamics of circulating tumor DNA and the systemic host response during chemotherapy in patients with newly diagnosed pancreatic cancer. *Submitted manuscript*

Paper IV

Petersson A, Jacobsen H, Hau SO, Svensson M, Karlsson J, Andersson N, Bergström R, Lindberg J, Mayrhofer M, Eberhard J, Heidenblad M, Gisselsson D, Jirström K. The genetic landscape of terminal pancreatic cancer and preceding temporal events: An initial CHAMP autopsy study. *Manuscript in preparation*.

** Equal contribution*

List of publications not included in the thesis

Siesing C, **Petersson A**, Ulfarsdottir T, Chattopadhyay S, Valind A, Nodin B, Eberhard J, Brändstedt J, Syk I, Gisselsson D, Jirström K. Delineating the intra-patient heterogeneity of molecular alterations in treatment-naïve colorectal cancer with peritoneal carcinomatosis. *Modern Pathology* 2022;35:979-988

Pally D, Banerjee M, Hussain S, Kumar R.V, **Petersson A**, Rosendal E, Gunnarsson L, Peterson K, Leffler H, Nilsson U.J, Bhat, R. Galectin-9 Signalling Drives Breast Cancer Invasion through Extracellular Matrix. *ACS Chemical Biology* 2022;17:1376-1386

Rastegar, B, Andersson N, **Petersson A**, Karlsson J, Chattopadhyay S, Valind A, Jansson C, Durand G, Romerius P, Jirström K, Holmquist Mengelbier L, Gisselsson D. Resolving the Pathogenesis of Anaplastic Wilms Tumors through Spatial Mapping of Cancer Cell Evolution. *Clinical Cancer Research* 2023;29:2668-2677

Hau SO, Svensson M, **Petersson A**, Eberhard J, Jirström K. Trajectories of immune-related serum proteins and quality of life in patients with pancreatic and other periampullary cancer: the CHAMP study. *BMC Cancer* 2023;23:1074

Abbreviations

APC	Antigen presenting cell
BAF	B-allele frequency
BRCA	Breast cancer gene
CA19-9	Carbohydrate antigen 19-9
CA125	Cancer antigen 125
CAF	Cancer associated fibroblast
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cfDNA	Cell free DNA
CH	Clonal haematopoiesis
CHAMP	Chemotherapy, host response and molecular dynamics in periampullary cancer
CIN	Chromosomal instability
CNA	Copy number alteration
CNNI	Copy number neutral imbalance
CT	Computed tomography
CTC	Circulating tumour cell
ctDNA	Circulating tumour DNA
DAMP	Damage associated molecular pattern
DC	Dendritic cell
ddPCR	Digital droplet PCR
ECM	Extracellular matrix
FF	Fresh frozen
FFPE	Formalin fixed paraffin embedded
FOLFIRINOX	Folinic acid, fluorouracil/5-FU, irinotecan, oxaliplatin
gDNA	Genomic DNA
GemCap	Gemcitabine, Capecitabine
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HRR	Homologous recombination repair
IHC	Immunohistochemistry
InDel	Insertions and deletions
IPMN	Intraductal papillary mucinous neoplasm
KRAS	Kirsten rat sarcoma
LOH	Loss of heterozygosity
MCN	Mucinous cystic neoplasm
mFOLFIRINOX	Modified FOLFIRINOX
mGE	Mutated genome equivalent
MHC	Major histocompatibility complex
MIP	Molecular inversion probe

ML	Maximum likelihood
MMP	Modified maximum parsimony
MMR	Mismatch repair
MP	Maximum parsimony
MRCA	Most common recent ancestor
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MUC-16	Mucin 16
NGS	Next generation sequencing
NK cell	Natural killer cell
NOS3	Nitric oxide synthase 3
OS	Overall survival
PanIN	Pancreatic intraepithelial neoplasia
PARP	Poly(ADP-ribose) polymerase
PEA	Proximity extension assay
PDAC	Pancreatic ductal adenocarcinoma
PD-1/PD-L1	Programmed death 1/ Programmed death 1 ligand
PCR	Polymerase chain reaction
PMI	Postmortem interval
SMAD4	Mothers against decapentaplegic homolog 4
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SPTC	Single patient tissue chip
scRNAseq	Single cell RNA sequencing
sWGS	Shallow WGS
T_c cell	Cytotoxic T cell
TCF	Tumour cell fraction
TDS	Targeted deep sequencing
T_h cell	Helper T cell
TMA	Tissue microarray
TMB	Tumour mutational burden
TME	Tumour microenvironment
TNM	Tumour, nodes and metastasis
TP53	Tumour protein 53
T_{reg} cell	Regulatory T cell
UMI	Unique molecular identifier
VAF	Variant allele frequency
WES	Whole exome sequencing
WGD	Whole genome doubling
WGS	Whole genome sequencing

Before we begin

"Nothing in biology makes sense except in the light of evolution"

Theodosius Dobzhansky, 1973

Cancer – an evolutionary consequence

During a human lifetime, trillions of cell divisions take place in different parts of the body to maintain proper organ health and function. In some rare cases, these processes give rise to cellular errors which eventually initiate cancerous tumour growth. Hence, cancer may be seen as an evolutionary consequence, the cost for being a multicellular organism able to evolve over time[1]. In light of this, it is actually quite remarkable how seldom cancer develops[2]. For instance, one could think that a greater number of cells in an organism and a longer lifespan, leading to an increased cell division frequency, would be directly associated with overall cancer risk. That is, however, incorrect. In fact, one of the largest animals on earth, elephants, rarely develop cancer, whereas mice frequently do – referred to as *Peto's paradox*[3,4].

Historical perspective

So how long have we been studying *cancer*? Its name originates from the Greek word for crab or crayfish (*karkinos*), and was first suggested by the Greek physician Hippocrates (460-370 BC) owing to its resemblance to the crab's jointed outward-stretching legs[5]. But the earliest note on cancer dates all the way back to ancient Egypt, with the description of a breast cancer tumour in the Edwin Smith Papyrus, written around 3000 BC[5]. Since then, countless studies on cancer have revealed its multifaced nature, collectively including over a hundred types of diseases, seen in the majority mammals[2], and defined as uncontrolled cell growth and spread.

Carcinogenesis

The formation of cancer, *carcinogenesis*, builds upon the theory that all cells arise from pre-existing cells. This was initially proposed by Robert Remak in the 19th century but

has mainly been attributed to Rudolf Virchow, also referred to as "the father of modern pathology"[6]. Prior to each cell division (**Figure 1**), the genome must undergo a complete duplication, tightly regulated by cell cycle check points ensuring that each component has been appropriately replicated. If not, the normal cell will undergo apoptosis, also known as *programmed cell death*. However, some of the fundamental characteristics of cancer cells include their ability to bypass these regulatory circuits, i.e. tumour-suppressive mechanisms, allowing for abnormal cell division[7]. On that note, considering multicellularity, Peto's paradox suggests a link between increased mammal body size, longer lifespan, and a more effective cancer defence, counterbalancing cancer risk[2]. Returning to the abovementioned example, elephants have inherited at least 20 DNA copies of the gene *tumour protein 53* (*TP53*)[8], encoding the p53 protein also known as the "*guardian of the genome*" due to its importance in hindering the proliferation of damaged cells. For comparison, humans only have two *TP53* gene copies.

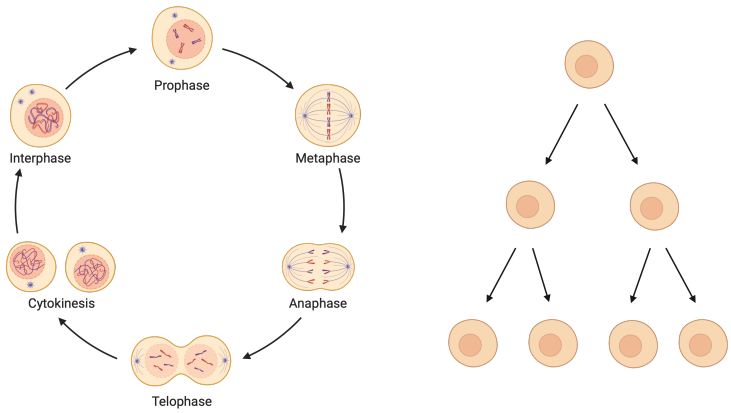


Figure 1. The cell cycle and theory. Illustration of a cell division (left) and the theory that all cells are derived from a pre-existing cell (right).

The exact mechanisms leading up to tumour initiation are, however, still under debate. All the way back in 1914, long before James Watson and Francis Crick discovered the double helix structure of DNA[9], the German zoologist Theodor Boveri proposed the first notion of cancer as a genetic disease[10]. He postulated that cancer forms through multipolar mitosis, resulting in an abnormal genotype and uncontrolled proliferation[10]. This acknowledgement later formulated the basic principles of the *somatic mutation theory* of cancer, stating that cancer initiation is due to the accumulation of genetic mutations starting in a single cell. This theory has however met criticism for being too simplistic, primarily for not taking the tissue context into account, as in the *tissue organization field theory* proposed by Soto and Sonnenschein[11]. The definitive answer, if one such exists, is however yet to be found. The perplexing nature of cancer,

supported by decades of research within its different branches, indicates that tumour initiation arises from a complex interplay between genetic, epigenetic and environmental factors, varying between different cancers and tissues of origin[12].

The hallmarks of cancer

Building on the foundation laid by the above-mentioned pioneers and others, cancer research is today a multifaceted field, stretching from genetics to the host response and microbiome[13]. In the year of 2000, Robert Weinberg and Douglas Hanahan published "*The Hallmarks of cancer*", encompassing the delineation of six fundamental traits of cancer cells in comparison to normal cells[7]. The six hallmarks included the ability of malignant cells to (i) activate replication immortality, (ii) evade apoptosis, (iii) ignore growth inhibitory signals, (iv) sustain proliferation independent of stimulatory signals, (v) induce angiogenesis (formation of new blood cells) and (vi) invade both neighbouring tissues and distant sites[7]. In the two succeeding editions, one 2011[14] and one 2022[13], eight new hallmarks, both emerging and enabling, were discussed, four at a time. These additional properties of cancer included genomic instability, the capability to evade immune recognition and the acknowledgement that the surrounding tumour stroma, featuring players such as various immune cell populations, might in fact be tumour-promoting[13,14].

Acknowledging the complex nature of cancer biology and evolution, this thesis primarily focuses on evaluating the latter of the aforementioned hallmarks, namely the genetic underpinnings of cancer and certain aspects of the surrounding *tumour microenvironment* (TME) and infiltrating immune cells.

Oncogenes and tumour suppressors

One pivotal genetic aspect of cancer initiation and progression involves genes that, when mutated, may directly confer a growth advantage to the cell, so called *driver genes*. Cancer-related driver genes are often divided into two main categories: *proto-oncogenes*, involved in normal cell proliferation and growth; and *tumour suppressor genes*, regulating different steps of the cell cycle. Alterations associated with proto-oncogenes result in gain-of-function effects, turning it into an activated *oncogene* which stimulates abnormal cell growth. On the other hand, alterations in tumour suppressors often lead to loss-of-function effects, inactivating their regulatory actions which also results in uncontrolled cell proliferation. In the 1970s, Alfred Knudson further presented the *two-hit hypothesis*, stating that tumour suppressor genes require inactivation of both alleles to lead to deleterious effects[15]. Thus, whereas there are limited ways to activate or amplify the effect of proto-oncogenes, inactivation of tumour suppressors may be induced in numerous ways, as will be discussed in the forthcoming sections[16].

Genetic alterations

The human genome and its variations

Every normal human cell typically contains a set of 46 chromosomes encapsulated in the nucleus: 22 pairs of autosomal (chromosome 1-22) and one pair of sex chromosomes (**Figure 2**). Cells are often further described according to their *ploidy level*, i.e. the total number of full chromosome-sets. The ploidy of human cells with one parental and one maternal allele, denoted as having the allelic composition "1+1", is referred to as *diploid* (2N).

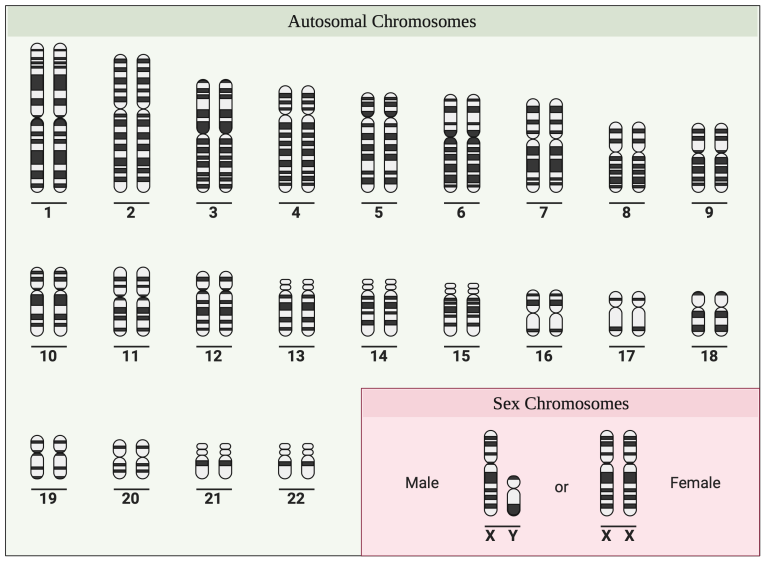


Figure 2. The human karyotype. The human set of chromosomes, with 22 autosomal chromosome pairs and one pair of sex chromosomes.

Chromosomes are built up by tightly packed linear DNA, wrapped around proteins called *histones*. The condensed chromosomes are primarily seen during the metaphase of mitosis after a complete DNA replication, resulting in the classic "X-shape" structure (**Figure 3**). Each chromosome can further be divided into a shorter *p-arm* and longer *q-arm*, attached to each other by the *centromere*, which facilitates the separation of the duplicated DNA content during cell division.

The DNA itself is composed of a double helix, consisting of two strands with a sugar-phosphate backbone and four nucleotide bases – adenosine (A), thymine (T), cytosine (C) and guanine (G) (**Figure 3**). These bases pair specifically to each other, i.e. A to T

and G to C, via hydrogen bonds, and the order of the nucleotides encodes the *genetic information*. Nevertheless, despite protective cell cycle mechanisms, a low number of genetic errors, i.e. *mutations*, are often introduced during cell division and incorporated into the DNA of the resulting daughter cells. However, although these alterations occur frequently in the cells throughout our bodies, they rarely affect our health[17].

Different types of genetic alterations

Genetic alterations may be divided into either small-scale or large-scale changes. Small-scale mutations include base pair substitutions, also known as *single nucleotide variants* (SNVs), and base pair *insertions and deletions* (InDels). On the other hand, large-scale changes include for instance *chromosome translocations*, *copy number alterations* (CNAs) and *whole genome doubling* (WGD) events. An illustration visualizing the different types of alterations is shown in **Figure 3**, together with a demonstration on the principle by which genetic information is translated from DNA to a chain of amino acids. Of note, a non-mutated gene is often referred to as *wild type* (WT). Chromosomal translocations have not been studied within the current thesis and will, thus, not be further discussed.

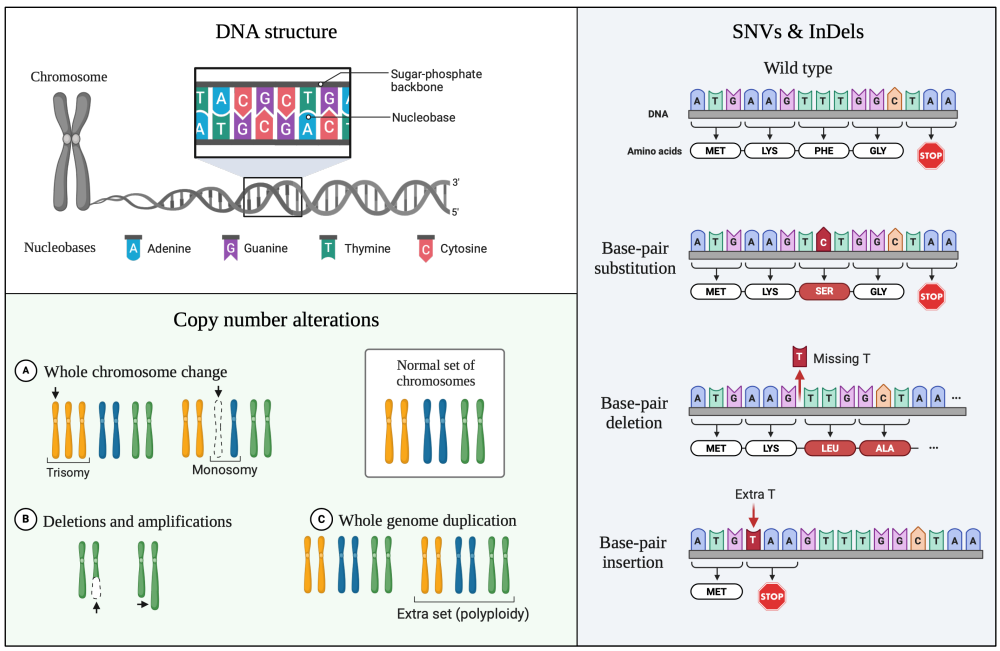


Figure 3. DNA alterations. Generic DNA structure (**upper left**), along with illustrative examples of different types of genetic alterations: copy number alterations (CNAs, **lower left**), base pair substitutions (single nucleotide variants, SNVs) and base pair insertions and deletions (InDels) (**right**).

Small scale genetic alterations

Small scale genetic variants can either be *synonymous*, not causing any effect, or *non-synonymous* and thus, in different ways, alter the resulting gene product. Non-synonymous variants are most often found in the coding region of genes (*exons*), whereas synonymous variants are generally found in non-coding regions (*introns*). In addition, a *driver mutation* is further characterized as inferring an advantage to the cell which can be selected for during cancer evolution[18].

SNVs altering the encoded amino acid, and particular those found in conserved regions of the resulting peptide, are the ones most probable to change the function of the protein and, hence, to be of importance in cancer progression[19-22]. In addition, InDels often lead to frameshifts in the nucleotide sequence, which may cause a premature stop codon in the amino acid sequence which may either lead to degradation of the resulting transcript or to a truncated protein[23]. Lastly, variants detected in splice regions of the RNA transcript (intronic DNA gene regions), may also cause deleterious effects through mechanisms such as alternative splicing, which may in turn cause premature stop codons and aberrant proteins[24]. On the other hand, *passenger variants* may be of importance in neoantigen presentation and immune recognition, even if they do not have a substantial effect on cancer progression, as discussed below in the section "*The immune system and cancer*".

The most common germline variant – single nucleotide polymorphisms

Mutations can further be classified as either *somatic* or *germline*, referring to variants acquired in specific cells, or those inherited and thus present in all cells, respectively. Although some germline variants may lead to a predisposition to develop certain diseases, including cancer, the majority are simply bystanders[25]. The most common type of germline variants are *single nucleotide polymorphisms* (SNPs), which are germline base pair substitutions detected in more than 1% of the population[25]. These have been extensively studied and some have been shown to increase the susceptibility for certain cancers[26].

Large scale genetic alterations

Copy number alterations

CNAs, referring to somatic changes in copy number of larger genome segments (**Figure 3**), are a common feature across cancers and was observed by David Hansemann already in the 19th century[27,28]. CNAs can affect either whole or parts of chromosomes. They include copy number gains (e.g. 2+1) and copy number losses, which can either be *hemizygous*, if affecting only one of the two alleles (1+0), or *homozygous* if referring to a deletion of both alleles (0+0). *Loss of heterozygosity* (LOH) is also a widely used term for

describing the loss of one allelic segment, and whenever the remaining allele gets duplicated (2+0) it is denoted as a *copy number neutral imbalance/LOH* (CNNI/cnLOH). In addition, whenever a whole chromosome is gained or lost, the term *aneuploidy* is often used to describe the incomplete set of chromosomes[29].

Chromosomal instability

Accumulation of CNAs and consequent aneuploidy are often a result of *chromosomal instability (CIN)*, which refers to an increased chromosomal missegregation rate during cell divisions. CIN has been a frequently detected feature across malignancies, especially in late-stage disease, and has been classified as a hallmark of cancer[14,30]. Disruption of several cancer-associated pathways has been shown to induce CIN by altering mitotic chromosome segregation in different ways[31]. The mechanisms include disruption of different key aspects of cell division such as: premitotic *replication stress* during DNA duplication; disturbances in *sister chromatid cohesion*; defects in *spindle assembly checkpoint* (SAC) signalling, causing premature anaphase onset; elevated numbers of centromeres leading to multipolar mitosis; and disturbances in the dynamics of *kinetochore-microtubule attachment*[31,32]. In addition, defects in proteins regulating the cell cycle, such as p53, or those associated with *homologous recombination repair* (HRR), e.g. *breast cancer gene 1/2* (BRCA1/2), and *telomere dysfunction* followed by breakage-fusion-bridge cycles, have also been shown to correlate with an increased number of CNAs and CIN[31,33-36].

A prevalent genomic phenomenon associated with CIN, found to both promote tumorigenesis and to correlate with a poor prognosis, is whole-genome doubling (WGD) and the resulting *polyploid* cell state[37-39]. Interestingly, although WGD has been considered to generate unstable genotypes, research has shown that cells surviving WGD may also have an elevated tolerance for subsequent mitotic errors, highlighting its role in tumour evolution[39,40]. These phenomena have further been shown to characterize a subset of enlarged and particularly stress tolerant cancer cells, referred to as *poly-aneuploid cancer cells* (PACCs)[41,42]. The presence of PACCs in tumour tissue across cancer types have further been associated with metastatic disease, and their treatment resistant nature is thought to be an effect of their ability to enter polyploid non-dividing cell states[42-46].

Lastly, in contrast to CNAs affecting whole or large parts of chromosomes, mitotic errors might also induce focal but complex genomic rearrangements as a result of one single catastrophic event, known as *chromothripsis*. In chromothripsis, parts of a chromosome, or the whole chromosome, is shattered in up to hundreds of pieces, followed by random assembly of the segments, and this phenomenon has been shown to play a role in cancer progression and evolution[47,48].

Cancer cell evolution

Cancer from an evolutionary perspective

Cancer can, and should, be viewed through the lens of evolution, where the accumulation of traits in a lineage of cells may infer proliferative and survival benefits to surrounding cell lineages. The concept of evolution by *natural selection*, opting for traits leading to a fitness advantage, was described in the book "On the origin of species" by Charles Darwin in 1859[49]. This book is now considered the cornerstone of evolutionary biology. Darwin postulated that natural selection is based on the competition of limited resources, spatial constraints and adaption to the surrounding environment, where species with advantageous adaptive traits produce more offspring with inherited beneficial features[49]. He further postulated that evolution based on natural selection requires (i) heritable variation, (ii) diversity in fitness within the population and (iii) competition between organisms[49,50].

These evolutionary concepts can be adapted to cancer development as tumours, in many ways, resemble natural ecological systems with distinct cell subpopulations present within a complex and dynamic environment[51]. Clonal evolution of cancer, i.e. the iterative acquisition of alterations, followed by clonal expansion and selection, was suggested by Peter Nowell already in 1976[52] (**Figure 4**). His publication now represents a landmark in the field, and its description of the evolving clonal landscape, with various distinct cell populations defined as cancer *subclones*, remains central to this day. These subclones might, for instance, possess differences in their ability to metastasise and in their sensitivity towards chemotherapy[51,52].

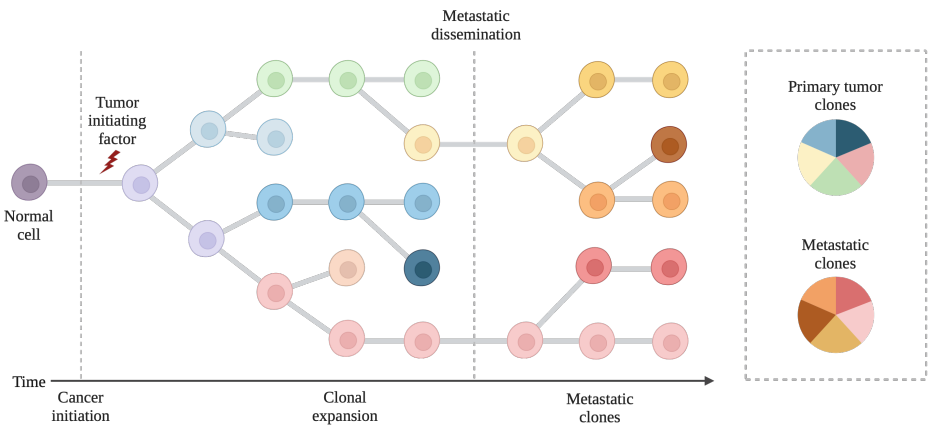


Figure 4. Cancer cell evolution. Clonal expansion of cancer cells over time. Created according to Birkbak et al.[53].

Tumour heterogeneity

As stated by Darwin, the presence of distinct subpopulations that differ from each other *phenotypically*, i.e. regarding their physical characteristics[49], comprises the substrate on which selection pressures may act. The cancer equivalence, commonly known as *tumour heterogeneity*, signifies the existence of unique subclones both in different tumour areas (*spatial*) and at various time points (*temporal*). Furthermore, spatial heterogeneity is often divided into two main types: *intratumoural* heterogeneity, referring to differences within one single tumour mass, and *intertumoural* heterogeneity, classified as disparities between separate tumour sites in the same patient or between patients (**Figure 5**).

Tumour heterogeneity has been denoted in nearly all malignancies, including both genetic and phenotypic variability, and is known to impact both tumour progression and patient survival[30,54-59]. Anti-tumoral treatments, e.g. chemotherapies targeting proliferative cells, act as exogenous selective pressures and will in many cases inevitably encourage the emergence of resistant cells[60,61], contributing to the low success rate of systemic treatment in metastatic disease. Thus, to overcome the oncological challenge of therapy resistance, one must also consider the evolutionary aspect of cancer and exploit its weaknesses.

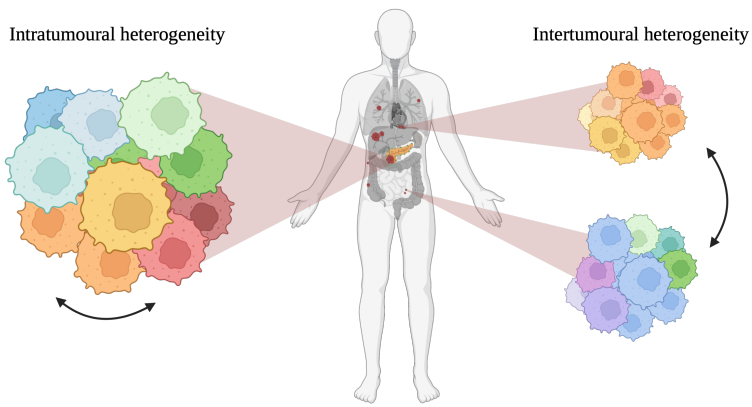


Figure 5. Tumour heterogeneity. Illustration of spatial tumour heterogeneity, including both intratumoural (left) and intertumoral (right) heterogeneity.

Such novel dynamic therapies are already on the way, and include for instance *extinction* therapy, *evolutionary double bind* therapy and *adaptive* therapy[62-64]. Extinction therapy, also known as *first-strike-second-strike*[64], is a two-step treatment based on the idea of first intensively decreasing the tumour mass using one drug agent, resulting in a smaller exposed cell population more sensitive to exogenous stress. Then, a second, different drug agent is administered, aiming to exploit the vulnerable state and, thus, forcing the

tumour cell population into an extinction cortex, which it cannot recover from. The evolutionary double bind therapy also utilizes the consecutive administration of two different drug agents, but in this case aiming to use the initial drug to provoke sensitivity to the second[62]. Lastly, adaptive therapy refers to evolutionary strategies focusing on tumour control, rather than a cure of the disease[63,65], and is further discussed in the section "*Treatment resistance and evolutionary based strategies*".

Phylogenetic analyses

In evolutionary biology, it is common to illustrate the ancestral relationship between different species in a branching diagram called *phylogenetic tree* or *phylogeny*. By phylogenetic analysis, it is possible to visualise and distinguish at which point during evolution two species deviated from one another and to evaluate similarities and differences between them. Similarly, through detailed analyses of the cancer genome in various tumour areas, enabled by the successful development of *next generation sequencing* (NGS) during the past decades, ancestral relationships between cancer clones can also be constructed (**Figure 6**)[54,66]. A tumour's evolutionary history is inferred by estimating the most probable order of events, based on the proportion of each alteration in the various samples, following deconvolution of detected subclones[67]. Phylogenies depicting the relationships between subclones is also often referred to as a *clone tree*[68], and may provide important information regarding specific subclones and their ability to metastasise, survive antitumoral agents and cause recurrent disease[30,54,56,69-71].

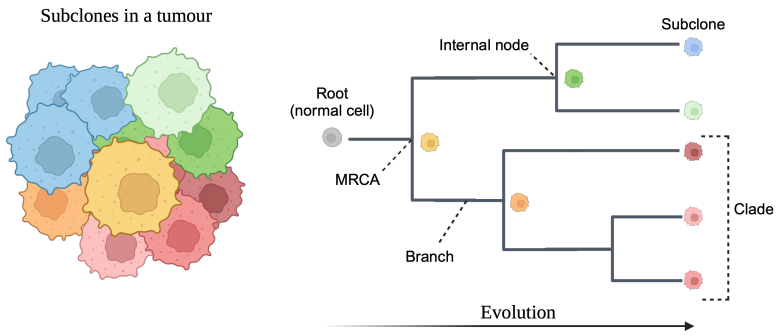


Figure 6. Phylogenetic tree based on tumour subclones. A phylogeny depicting the ancestral relationships between the subclones detected in a tumour, along with associated phylogenetic tree components. Abbreviations: MRCA; most recent common ancestor.

In **Figure 6**, depicting a typical phylogeny based on tumour subclones, horizontal lines are referred to as *branches*, the lengths of which are directly proportional to the associated number of genetic events. The phylogeny may be rooted or unrooted, where

the rooted tree displays a direction from an assigned *most recent common ancestor* (MRCA), whereas the unrooted tree lacks direction[72]. For relationships between tumour clones, the phylogeny is often rooted in a normal cell, free of somatic mutations. Genetic alterations that have been estimated to be present in all cells of the tumour comprise the *trunk* of the tree. These mutations are also denoted as *stem* alterations. *Internal nodes* depict speciation events, i.e. points of divergence, and lastly, a *clade* refers to a group of species (subclones) which all have descended from a common ancestor.

Within the studies of this thesis, phylogenetic reconstruction was utilised to evaluate tumour heterogeneity and trace the evolutionary points of metastatic dissemination. However, as stated above, it should be noted that clonal phylogenies only estimate the order of genetic events, without providing information on the precise timeline. To evaluate the exact time point for a specific genetic event, one could instead use clock-like mutational processes, the steady mutation rate of which serves as a biological chronometer[73].

Liquid biopsy

Historically, radiology and tissue biopsies, together with a few blood biomarkers, have been the go-to methods for detection, diagnosis and monitoring of various cancers. In addition, molecular profiling of tumour tissue has further facilitated the emergence of individualized treatments, such as targeted therapies, which have had a considerable impact on disease management and patient survival in several cancer types[74-76]. However, these methods are limited by factors such as imaging resolution, tumour tissue accessibility, biomarker specificity, cost and discomfort for the patient. In addition, single tissue biopsies often fail to capture tumour heterogeneity and are, thus, seldom able to provide a representative overview of the tumour's molecular landscape[54]. In addition, considering cancer evolution, repeat biopsies are needed to monitor the clonal dynamics over time, a procedure that would come with an increased risk of complications for the patient without removing the challenges of tumour heterogeneity. In the specific case of pancreatic cancer, the location of the tumour often makes it difficult to obtain tissue biopsies even for diagnostic purposes. Hence, to achieve improved cancer management, from early detection to disease monitoring, there is a critical need for less invasive, cost-effective and sensitive methods, with the ability to capture molecular tumour dynamics.

To meet this urgent demand, the field of *liquid biopsy* has emerged during the past twenty years. A liquid biopsy is, as the name reveals, the study of biofluids, such as blood, urine and cerebrospinal fluid[77], as a minimally invasive versatile tool able to complement clinical decision making[78]. Exemplifying evidence on the advantages of

liquid biopsies include for instance the aid of blood based genetic profiling in clinical management of prostate cancer[79], multiple myeloma[80], colorectal cancer[81] and non-small-cell lung cancer[82]. The ability of liquid biopsies to provide enhanced early detection, and facilitate repeated biopsy sampling, promotes their future role as a tool in all parts of the cancer management spectrum, including monitoring of cancer evolution and therapy resistance[83,84].

Blood based analytes

Blood based liquid biopsies have been particularly investigated in malignant diseases, offering a broad range of various tumour-derived analytes to study (exemplified in **Figure** 7). These include, but are not limited to, circulating nucleic acids, i.e. *cell free* DNA (cfDNA) and RNA, *extracellular vesicles* (EVs), cancer associated proteins, tumour educated platelets, and *circulating tumour cells* (CTCs)[85]. Specifically, a broad repertoire of studies exploring clonal cancer evolution have focused on circulating CTCs and cfDNA shed from tumour cells, referred to as *circulating tumour DNA* (ctDNA)[86].

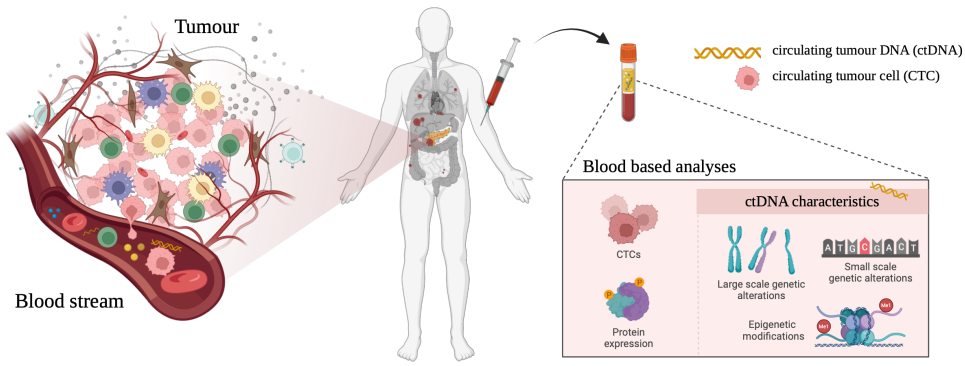


Figure 7. Blood based liquid biopsies. Illustration of blood based liquid biopsies and examples of analytes.

CTCs have been shown to represent distinct subclones of tumour cells with metastatic capacity[87], but their low frequency in the peripheral blood poses a challenge for clinical use[88]. However, studies have shown that CTCs provide important input for both early detection, disease prognosis and prediction of therapeutic response in various malignancies[89-94]. Contrastingly to CTCs, ctDNA represents fragments released into the bloodstream from various parts of the tumour and may, hence, provide a more holistic perspective of the tumour landscape[86]. The studies in this thesis have primarily focused on different aspects of the utility of ctDNA in PDAC management, and the following sections will thus mainly address liquid biopsies in terms of circulating DNA.

Cell free DNA

Varying levels of cfDNA can be detected in the blood stream of all people, with an average cfDNA concentration of approximately 10 ng per ml plasma in healthy individuals[95-97]. In general, cfDNA represents greatly fragmented pieces of DNA from numerous organs in the body, although the majority has been observed to come from white blood cells[78,98,99]. Levels and composition of cfDNA may however be altered in certain physiological conditions, such as pregnancy or malignancies[86,100]. Through sensitive methods, cfDNA from the foetus may be analysed in the blood of pregnant women using non-invasive prenatal testing (NIPT)[101], and similarly, ctDNA from the tumour may be quantified and characterised in the blood of cancer patients[86].

The presence of cfDNA in blood was detected already in 1948[102], and since then, numerous studies have been evaluating its mechanisms for release, molecular characteristics and impact for clinical medicine, not least oncology[98]. Proposed release mechanisms include both passive shedding, through *apoptosis* or *necrosis*, or *active secretion*[103-105], although the proportion of cfDNA originating from active release mechanisms remains to be determined[106]. Shedding by necrosis has been shown to result in DNA with higher molecular weight[103], whereas, in comparison, apoptotic release produces cfDNA fragments with a size of around 167bp. This size corresponds to that of a *nucleosome* (~147bp), i.e. a DNA segment wrapped around a histone protein complex, and an additional linker region (~20bp)[107,108].

The logical take home message from the phenomenon of passive release, through apoptosis or necrosis, would be that cfDNA primarily appears to represent dead cell populations, such as a treatment sensitive tumour population. This does, however, not seem to be the case. In fact, the opposite appears to be true, and various studies have observed that aggressive cancers, with high proliferative rates, seem to shed more cfDNA to the blood stream[98,109,110]. The reason behind this is not entirely understood, although it is known that the intricate balance between proliferation and apoptosis, important for maintaining tissue homeostasis, plays a vital part[98]. Cells undergoing apoptosis may for instance stimulate proliferation of surrounding cells, and in the other way around, a higher number of cells may undergo apoptosis in a fast-growing tumour due to increased stress and competition rates[106]. Higher levels of necrosis in late-stage disease have also been associated with an increased release of nucleic acids[111].

Moreover, clinical studies have shown that an increased total concentration of plasma-derived cfDNA, regardless of its origin, is a negative prognostic marker for patients with metastatic solid tumors[112]. A recent preprint also showed increased total levels of cfDNA shed from *non-tumour tissue* in early-stage cancers and proposes that this increase could be due to the combination of a higher turnover of normal cells and a decreased overall cfDNA clearance[113]. Similar results were presented in another study where the observed increase of cfDNA in cancer patients could be traced back to a

primarily neutrophilic origin, also highlighting a possible slower clearance rate[78]. Of note, cfDNA clearance is mainly performed by the liver, and the cfDNA half-life has been estimated to range between minutes to a couple of hours[114,115]. Taken together, although it may be tempting to focus foremost on ctDNA as this is shed by the tumour itself, cancer, and especially metastatic disease, is a systemic entity, and one should thus keep a holistic view in mind.

Circulating tumour DNA

Diving into the field of *plasma derived ctDNA*, this was first acknowledged already in 1989[116]. Nevertheless, it would take until 1994 before the first discovery of mutated ctDNA, matching the mutations found in tumour tissue, was published, namely the detection of mutant *KRAS* in plasma from patients with pancreatic cancer[117]. Since then, thanks to a remarkable development of sensitive techniques such as *digital polymerase chain reaction* (dPCR) and NGS, ctDNA has been extensively studied within vastly all cancers, both analysing specific genomic loci to evaluate *hotspot* mutations, and genome-wide features to capture large scale alterations[86,118-122]. The clinical utility of ctDNA can broadly be divided into two fields of study: quantitative measures, such as the *plasma fraction* or *concentration*, and qualitative analyses, such as the presence or emergence of *specific* genetic alterations.

ctDNA plasma burden and clinical implications

Quantitative analyses have revealed that the plasma ctDNA concentration correlates with both a larger tumour size, metastatic disease burden and later disease stage in various malignancies[110,118,123]. Numerous studies have also demonstrated the prognostic value of ctDNA levels in cancer, and its mere presence in plasma has been shown to correlate with a decreased overall cancer survival[124-127]. It should however be noted that the cfDNA plasma fraction varies depending on the type of malignancy. ctDNA analyses have also been shown to aid in disease subtyping, leading to a more accurate prognosis[128]. Another metric of interest includes the fragment size of ctDNA, as this has been observed to be shorter than cfDNA originating from normal cells[96,129,130]. Consequently, this has further spurred evaluations of the clinical value of cfDNA fragment size distribution, which has shown utility as a prognostic biomarker in various cancer types[131,132].

The short half-life of cfDNA also makes it especially suitable for real-time tumour monitoring, as validated by studies exploring the correlation of ctDNA dynamics and treatment response[120,133-136]. Hence, there are several advantages of ctDNA monitoring compared to expensive repeated imaging methods or invasive repeat tumour biopsies. Along that line, another area of interest includes the detection of *minimal residual disease* (MRD) following surgery or other curative treatments, as this is known to

impact recurrence rates[137]. For this purpose, ctDNA detection in plasma has been proposed to be of particular value, as it has been shown to detect disease relapse several months earlier than standard clinical parametres[138-142], as illustrated in **Figure 8**.

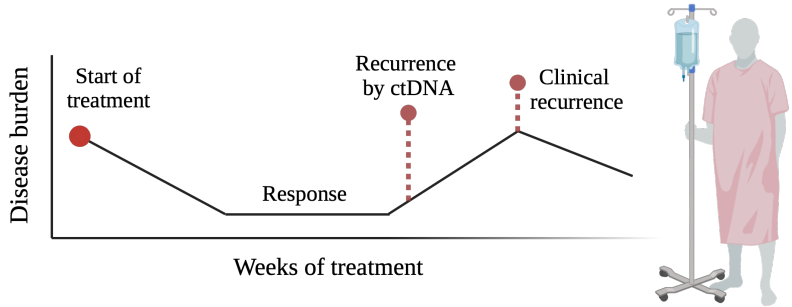


Figure 8. Disease monitoring by ctDNA. Illustration of real-time monitoring of the disease burden using ctDNA analyses, and its superiority in detecting recurring disease in comparison to standard clinical methods.

An important aspect in order to improve overall survival (OS) is also early diagnosis, for which ctDNA analyses have been observed to enable presymptomatic detection of disease long before diagnosis[143,144]. One should however approach the applications of these results with caution, as screening of asymptomatic individuals may lead to overdiagnosis and a high rate of false positives.

ctDNA characteristics in cancer evolution

Qualitative measures of ctDNA, such the aforementioned detection of specific hotspot mutations, have also been shown to provide significant information for clinical practice[120,127,145], and especially for identifying patients who may be eligible for targeted therapy[146,147]. Additionally, in contrast to single tumour tissue biopsies, ctDNA has been observed to capture a holistic overview of the genetic tumour landscape, both in terms of intratumoural and intertumoural heterogeneity[148-151]. Of further significance, it has also been argued that ctDNA analyses are superior to tumour biopsies for evaluating clonal evolution and emerging resistance[81,152,153]. In a study by Parikh et al.[152], a direct comparison between tumour and post progression cfDNA biopsies identified discrepancies in 76% of cases, where resistance alterations detected in cfDNA were not found in the matched tumour tissue. Likewise, several studies focusing on larger chromosomal ctDNA variants have also revealed opportunities for clinical guidance[122,154-156]. In a recent study investigating 45 cases of paediatric cancers, where CNAs are known to comprise the majority of genetic drivers, monitoring of ctDNA provided important insight for a more accurate diagnosis and in tracking response to treatment[155].

Hence, various clinically relevant applications have been observed for the use of plasma derived ctDNA, and the future will likely further reveal its added value in all aspects of clinical oncology.

Detection limits of ctDNA analyses

The probability of detecting ctDNA in plasma is dependent on several different factors, ranging from clinical blood handling procedures to the bioinformatic data pipeline, and obtaining qualitative information from ctDNA fractions below 1% remains challenging to this day[157]. For instance, as ctDNA corresponds to the tumour proportion in a "bulk" of cfDNA, shedded from different organs, increased levels of non-tumour cfDNA inevitably dilutes the ctDNA fraction. Moreover, the detection limit also depends on the actual number of ctDNA molecules present in the analytical sample and, hence, the total input plasma volume is important for sufficient sensitivity. Lastly, different sources of background noise, such as mutations from normal cells, complicate ctDNA analyses. For example, mutations acquired through *clonal haematopoiesis* (CH), i.e. the clonal expansion of blood cells, are also often detected in plasma with similar frequencies as ctDNA, increasing with age[158]. Distinguishing ctDNA specific mutations from those associated with CH is thus important to enhance ctDNA sensitivity, and can be performed by comparisons to mutations detected in matched peripheral white blood cells[159].

Historically, to enable detection of low burden disease by sequencing, such as in the search of MRD, the predominant paradigm has been to utilize cost-effective ultra-deep NGS of a limited number of genomic target regions[160-163]. However, although providing state-of-the-art accuracy at a low ctDNA burden for the selected target regions, these methods are particularly limited by the number of ctDNA fragments in the plasma sample[164]. This has spurred the exploration of detection breadth instead of depth, i.e. utilizing information from thousands of random mutations across the genome instead of focusing extensively on only a few. This may for example be carried out by performing *shallow whole genome sequencing* (sWGS), which has been shown to be less vulnerable to the presence of specific ctDNA fragments[86], and recent research proposes that this method is superior for ultra-sensitive ctDNA detection in plasma[86,164,165].

Taken together, various factors affect the ability to detect plasma derived ctDNA at low frequencies. Yet, while noise reducing actions should always be implemented, it is crucial to meticulously evaluate the choice of sequencing method based on the current research question.

The immune system and cancer

A tumour mass is not only constituted by cancer cells, but rather comprises a complex ecosystem with various *immune cells* (ICs), *blood vessel cells*, *stromal cells* and *extracellular matrix* (ECM), together forming the TME as illustrated in **Figure 9**[166]. In 2011, the role of the host immune response was recognized as a cancer hallmark for tumour development, following a decade of evidence and insights into their perplexing interplay[14]. The dual function of the immune system in cancer had been described already in 1968 when Dvorak and colleagues compared tumours to a never-healing wound[167]. Today, we know that inflammation significantly contributes to tumour progression across various malignancies, and that the tumour, in turn, may further spur inflammation[14]. Thus, while the immune system serves as one of the body's main defences against neoplastic formation, together with various cell cycle regulating processes, cancer cells may evade immune surveillance by using various strategies, also referred to as *immunoediting*[14,168]. In other words, the TME can work both in favour of and against the tumour[166].

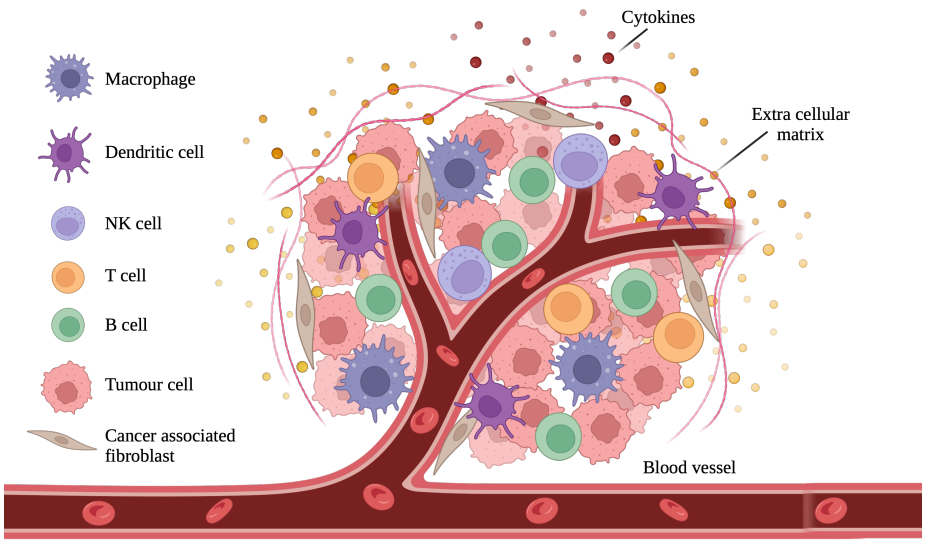


Figure 9. The tumour microenvironment. Illustration of the main components in the tumour microenvironment.

The following sections will provide a brief introduction to the most common types of ICs and their role in cancer, both in terms of *anti-tumour* and *pro*-tumour responses, with particular focus on general populations relevant for this thesis.

Immune players in the innate and adaptive response

The basic principle of the human immune system is to be able to distinguish self from non-self, i.e. the body's own components from foreign intruders. It can further be divided into two major parts: the *innate* and the *adaptive* immune system. The *innate* response refers to the part that is present already from birth, a less specific immediate defence especially suited to combat foreign pathogens. Contrastingly, the *adaptive* response, also referred to as the *acquired* response, denotes a highly specific line of defence which develops an immunological memory over time, enabling an accelerated second encounter with a specific pathogen.

To activate the specific adaptive response, innate cells with professional mediating properties, so called *antigen presenting cells* (APC), are needed[166]. These cells are specialized in capturing different foreign molecules, which they then present as *antigens*, i.e. processed peptides, on their cell surface for adaptive cells to recognize. Antigens are presented on cell surface proteins called *Major Histocompatibility complex* (MHC) molecules, also known as *human leukocyte antigen* (HLA) in humans. There are two main types of MHC molecules, class *I* and *II*, where the former is expressed on all nucleated cells, and the latter is mainly expressed by APCs for antigen presentation to adaptive lymphocytes[166]. By this bridge of information between innate and adaptive ICs, a directed specialized defence is activated, which eliminates the specific foreign invader.

Innate immune cells

Immune cell players in the innate response include cells such as *monocytes*, *macrophages*, *mast cells*, *dendritic cells* (DCs), *natural killer* (NK) cells, *eosinophils*, *basophils* and *neutrophils*, with various distinct subpopulations within each cell population. Innate cells specialized in antigen presentation mainly include macrophages and DCs, which are crucial for activation of the specialized adaptive defence. Of note, although originally acknowledged as an innate IC, NK cells have during the past decades been shown to have characteristics of both innate and adaptive immunity, for instance by being capable of forming an immunologic memory[169].

Monocytes are innate cells which circulate the bloodstream. Depending on the stimuli, they may differentiate into a range of various cell types, such as DCs and macrophages, and have shown distinct functions both in inflammation, homeostatic maintenance and pathogen response[170]. In the context of cancer, different subtypes have been associated with antitumour processes such as induction of cell death and phagocytosis of tumour cells, but also pro-tumorigenic properties such as the differentiation into tumour promoting macrophages, suppression of lymphocyte functions, recruitment of regulatory T cells, and mediation of wound healing, angiogenesis and ECM remodelling[170].

One of the descendants of monocytes are tissue-resident *macrophages*, although other progenitors have been proposed as well[171]. As described, macrophages have an important role in both the innate and adaptive defence, with immunity functions including phagocytosis of pathogens and antigen presentation. However, they also have critical roles in other physiological processes such as tissue development, homeostasis and repair[172]. Their anti-tumour defence includes killing of cancer cells by for instance *antibody-dependent cellular cytotoxicity* (ADCC) and phagocytosis, and the activation of adaptive IC mechanisms[173]. Macrophages with these properties are often referred to as having an inflammatory *M1*-like phenotype[173]. Contrastingly, tumour associated macrophages (TAMs) often have a pro-tumour phenotype, also known as *M2*-like, which is immuno-suppressive and more specialized in wound healing[173]. The M2-phenotype may for instance be induced by a hypoxic environment, or by specific cytokines and mediators in the TME, and supports cancer progression[166]. It should, however, be mentioned that M1-like TAMs are also frequently present in the TME. Of note, although recent studies of TAMs have shown that this previously established dichotomized model is oversimplified and thereby fails to capture the true plasticity of macrophages, especially for the M2-like subtype[174], it will be used as a discussion stand point throughout this thesis.

DCs represent the perhaps most important mediator between the innate and the adaptive immune response. Whenever they encounter an antigen, they engulf it and travel towards secondary lymphoid organs to present it to awaiting T cells, thereby activating an adaptive response[166]. They can be divided into four subtypes with varying functions and surface markers; *plasmacytoid* (pDCs), *conventional* type 1 (cDC1s) and type 2 (cDC2), and *monocyte derived* (moDCs) DCs[175]. DCs can further detect stressed cells, such as tumour cells, by their expression of *damage associated molecular patterns* (DAMPs) on the cell surface, which may initiate an anti-tumour response[176]. However, as long as DCs remain in their immature state they promote tumour cell tolerance, which the cancer may exploit[177].

NK cells are cytotoxic lymphocytes which have a dual role in the immune system and carry both innate and adaptive qualities. They are typically defined as $CD3^{-}CD56^{+}$, although various subtypes exist, and are specialized in killing cells that have downregulated their MHC expression. This could be cells infected with different viruses, but also tumour cells, and they are most often found patrolling the circulation[166]. Explained in a simple way, NK cells scout other cells for cell surface MHC class I molecules, and whenever they encounter a cell with reduced expression, the NK cells get activated. This activation model is important for tumour surveillance, as cancer cells often downregulate MHC class I expression on their cell surface to avoid exposing tumour specific antigens, *neoantigens*, that may otherwise be recognized by adaptive ICs[178].

Adaptive immune cells

Immune cells involved in the adaptive response broadly include lymphocytes called T cells and B cells, which carry out antigen-specific immune responses. In general, these cells patrol secondary lymphoid organs of the body, such as lymph nodes, where they await different activation signals.

T cells harbour specific T cell receptors (TCRs) which are specialized in recognizing specific MHC bound antigens, such as neoantigens on tumour cells or antigens from various pathogens presented by APCs. Thus, the TCRs cannot bind directly to soluble antigens. T cells are broadly defined as $CD3^+$ cells and are often divided into two major subtypes, cytotoxic $CD8^+$ T cells (T_c) and $CD4^+$ T helper cells (T_h). Whenever activated T_c cells, also called *killer* T cells, recognize their specific antigen on MHC class I molecules on a cell surface, such as that of a tumour cells or a cell infected by virus, they eliminate their target cells by releasing perforin and granzymes which induce apoptosis of the target cell.

The main function of T_h cells is to assist in the activation of other immune cells, such as T_c cells and B cells, although there are several subtypes, each with distinct functions[179]. One of the subtypes is called *regulatory* T cells (T_{regs}) and is highly important for preventing other immune cells from becoming overly active[179,180]. Thus, T_{regs} have the ability to suppress other active immune cells in order to maintain a balanced immune response, which is crucial to avoid autoimmune reactions[180]. This is, however, unfortunately also characteristics that are beneficial to tumour cells. Thus, in the TME, tumour cells may utilize T_{regs} to inactivate other immune cells, and, hence, let the tumour cells escape destruction[181].

B cells represent the humoral part of the adaptive immune response and are the cells responsible for *antibody* production. Antibodies, also called *immunoglobulins* (Ig), are the soluble form of the membrane bound *B cell receptor* (BCR)[182]. These are proteins specialized in binding to different pathogenic antigens, and are released from the effector form of B cells, also called *plasma* cells and *plasmablasts*[183]. Furthermore, although mature B cells are traditionally characterized as $CD20^+$ cells, plasma cells and plasmablasts represent a $CD20^-$ population. The role of B cells in cancer has been vastly understudied in comparison to the abovementioned T cells, but their abilities to both promote and inhibit tumorigenesis, depending on the specific B cell subtype, is now commonly known[184]. Their anti-tumour activities include priming of detected tumour cells, by coating them with antibodies, so that other cells such as phagocytes, NK cells or T_c cells, may find and destroy them[184]. There are however suppressive phenotypes of B cells as well, which may inhibit an active immune response and act in favour of the tumour[184].

Checkpoint molecules

As previously stated, to prevent unwanted autoimmune reactions, it is important to maintain a balance between immune stimulatory and inhibitory signals. Regulatory proteins known as *immune checkpoints* are central for this equilibrium[185]. Depending on the specific immune checkpoint protein, stimulation can either dampen or enhance the immune activity of the cell[185]. Among the many immune checkpoint signalling pathways which inhibit T cell activity is the programmed death 1 (PD-1) and its ligands (PD-L1/2)[186]. PD-1 is a cell surface receptor and a marker of exhaustion, normally expressed by T cells after continuous stimulation[187], but it is also found on other ICs such as B cells, NK cells and macrophages. However, as illustrated in **Figure 10**, cancer cells may leverage these inhibitory pathways by expressing PD-L1 on their surface, which inhibits T cell activation[186]. This is further utilized in immunotherapies such as PD-1 blockade, where specific antibodies bind to PD-1 proteins, preventing inhibitory signals and maintaining anti-tumour T cell activity[185].

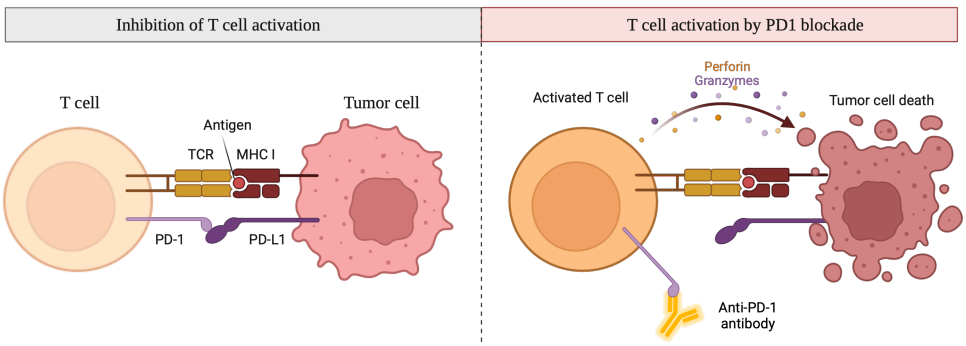


Figure 10. Immune checkpoint inhibition. Left) Cytotoxic T cell recognition of a tumour cell by binding of the T cell receptor (TCR) to the specific antigen MHC class 1 complex. The PD-L1 expressed by the tumour cell binds to the T cell bound PD-1, which inhibits T cell activation. **Right)** Immune checkpoint blockade using anti-PD-1 antibodies, which prevents inhibitory signals and allows for T cell induced apoptosis of the tumour cell.

Genetic instability and immune recognition

A paradoxical relationship has been demonstrated between chromosomally unstable tumour cells and immune evasion. On one hand, studies have shown that the large number of CNAs generated by CIN may serve as a substrate for Darwinian selection of cells with increased fitness in terms of, for instance, immune escape[188-190]. On the other hand, mitotic errors often generate cell cycle arrest and activates damage signalling, which consequently attracts immune players that eliminate the damaged cell[191,192]. Extensive research has been performed to untangle the various methods utilized by chromosomally unstable tumour cells to evade the different elimination

procedures carried out by both innate and adaptive cells. Reported immune evasion mechanisms, besides the above-mentioned expression of checkpoint molecules[186], include changes in stress signalling patterns, such as those downstream of the cGAS-STING pathway[189], and downregulation of antigen presenting molecules such as HLA[193,194]. The complete picture of how CIN in tumour cells shapes the surrounding immune environment, and in the end impacts responses to various therapies, however, remains to be unveiled[189].

Pancreatic ductal adenocarcinoma

Pancreatic and other periampullary cancer

The patient group studied within this thesis includes patients diagnosed with pancreatic or other periampullary carcinomas, which refers to malignancies arising in the pancreatic head, ampulla of Vater, duodenum or distal bile duct (**Figure 11**). Owing to the anatomic position, situated deep into the upper part of the abdomen, the disease is often detected at an advanced stage and surgery is seldom possible. For most patients, diagnosis thus relies on radiology and either a core needle biopsy or fine needle aspiration and, consequently, the precise origin of the tumour often remains uncertain.

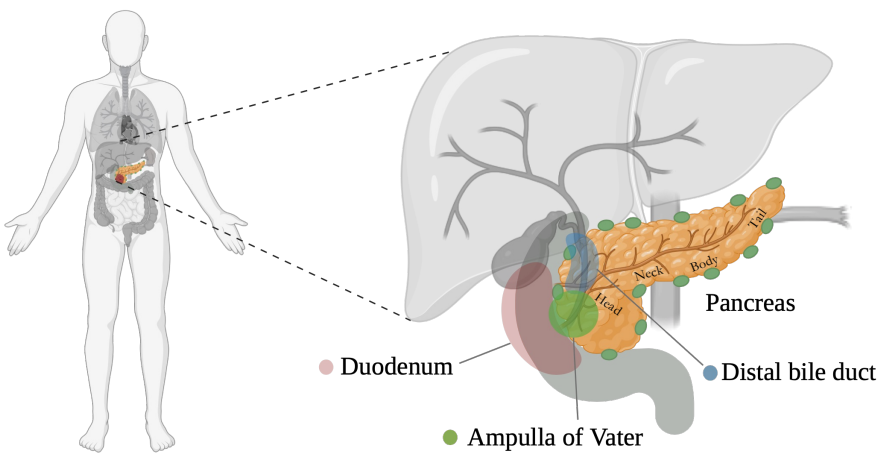


Figure 11. The pancreas and periampullary region. Sketch illustrating the anatomic location of the pancreas and its different parts. Ir further highlights the different periampullary regions.

Most neoplasms in the periampullary region, however, arise within the pancreas, where pancreatic ductal adenocarcinoma (PDAC) represents about 80% of all cases[195]. Hence, although this thesis and the affiliated clinical study includes patients with tumours originating in all of the above-mentioned sites, the remaining part of the thesis will primarily address PDAC.

The pancreatic organ is constituted by two main parts: the exocrine and endocrine pancreas. Pancreatic cancer may arise within either of them, although PDAC, originating from the exocrine ducts, accounts for the majority of tumours[196]. On the other hand, neuroendocrine tumours, originating from the endocrine pancreas, are rare and represent a clinically distinct subtype. Hence, these will not be further addressed within this thesis.

Epidemiology

PDAC is a relatively rare but deadly malignancy. It is the third leading cause of cancer related death in the United States, associated with a five-year OS of only 12% and increasing incidence rates[197]. Focusing on Sweden alone, around 1500 people were diagnosed with PDAC in 2021, and almost 2000 people died from the disease (Cancer i siffror 2023). In addition, although one out of five cases presents with localized disease, enabling surgery with curative intent, the cancer almost always recurs[198]. Thus, although the oncological advancements during the past decades have led to significantly improved survival rates for many cancer diagnoses, the prognosis of patients with PDAC has remained dismally unchanged.

The majority of PDACs are diagnosed in people above 70 years old, with men representing a slight majority[198,199]. Globally, incidence rates vary (**Figure 12**), with the highest rates of 2022 detected in Europe and Northern America, and with almost four times increased rates in regions with high human development index (HDI) compared to regions with low HDI[199]. This discrepancy may, however, partially be confounded by differences in diagnostic modalities and registry qualities between regions[200]. Nevertheless, despite the large regional discrepancy in incidence, mortality has been shown to be equal. In addition, although racial differences have been significantly associated with incidence rates, this has been explained by lifestyle risk factors such as dietary habits, alcohol consumption and smoking[200].

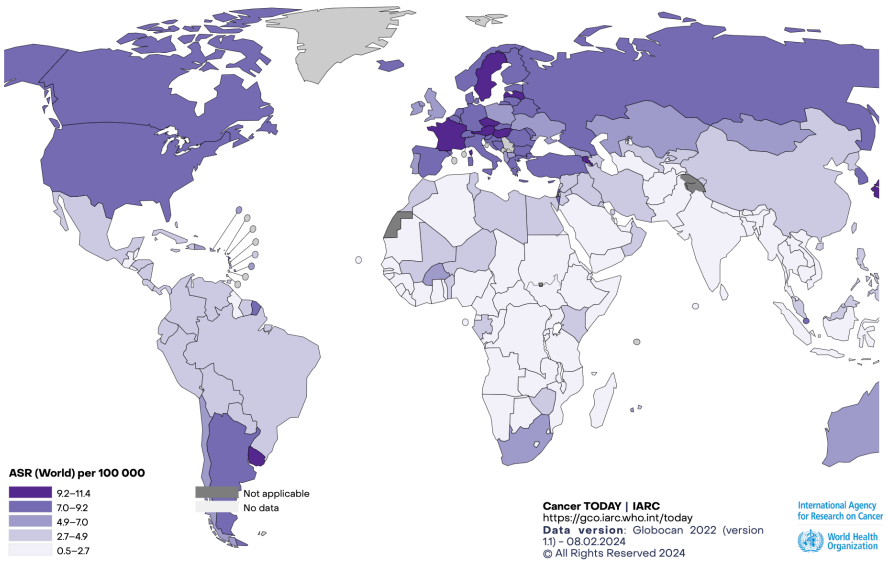


Figure 12. Global incidence rates of pancreatic cancer. Global map with estimated age-standardized incidence rates (ASR) per 100 000 people for pancreatic cancer in 2022. The graphs show both sexes and all ages. Reproduced from https://gco.iarc.fr/ [199].

Risk factors

Lifestyle and chronic disease

Besides increased age and male sex, the risk of developing PDAC has been associated with several other chronic disease-related and environmental risk factors, including cigarette smoking[201,202], obesity[203,204], alcohol consumption[205,206], chronic pancreatitis[207,208], oral microbiota[209,210], and diabetes, especially new-onset[211-213]. On the other hand, allergy has been shown to infer a decreased risk of PDAC[214,215].

Familial and hereditary PDAC

People with first-degree relatives with PDAC also have an increased risk of developing the disease[216], referred to as *familial PDAC*. Studies have however shown that familial PDACs are indistinguishable from *sporadic* disease, i.e. PDAC in patients without a family history, and are shown to have comparable somatic mutation profiles[217,218]. On the other hand, familial PDAC has been associated with an increased prevalence of precursor lesions, which may develop into invasive cancer[219].

Moreover, it is estimated that up to 35% of PDAC can be attributed to genetic heritability[216], although causative pathogenic constitutional variants have been identified in a mere 3-10%[198]. The most common variants found to increase PDAC

risk primarily affects DNA damage response genes[220-223] (**Table 1**), and have been associated with inherited syndromes such as hereditary breast and cancer syndrome, Lynch syndrome, familial atypical mole melanoma syndrome and Peutz-Jeghers syndrome, with implications for clinical treatment[220,222,224-231]. Genetic testing for these germline gene variants has, however, not yet become a routine part of clinical practice in Sweden.

Table 1. Genes associated with increased risk of PDAC, their prevalence (%) and associated syndromes216.

Gene	Prevalence (%)	Associated syndrome	Refs
BRCA2	2 - 7	Hereditary breast and ovarian cancer syndrome	222,232,233
BRCA1	< 1 - 2.2	Hereditary breast and ovarian cancer syndrome	222,233
PALB2	< 1	Familial breast cancer	233-235
ATM	< 1- 2	Familial breast cancer	220,223,236
STK11	< 1	Peutz-Jeghers syndrome	224,225
CDKN2A	< 1- 2.5	Familial atypical mole melanoma syndrome	220,226,227
MLH1, MSH2, MSH6, PMS2	< 1	Lynch Syndrome	220,228
PRSS1	< 1	Hereditary pancreatitis	236-239

Presentation, diagnostics and clinicopathological assessment

In general, clinical presentation and prognosis of PDAC depends on the anatomic site of the disease. Approximately 70% of all PDAC cases originate in the pancreatic head or neck (HN) (**Figure 11**) and are associated with a better prognosis compared to tumours arising in in the pancreatic body or tail (BT)[240]. This discrepancy may be explained by the fact that BT tumours are more frequently associated with advanced disease compared to HN tumours, due to the late onset and characteristics of symptoms as further described below[198,240].

Symptoms

The general presentation of PDAC includes diffuse and non-specific symptoms such as a decrease in appetite, dyspepsia, altered bowel habits, anorexia and fatigue, which too often delays diagnosis[241]. HN tumours frequently present with jaundice due to biliary obstruction, sometimes leading to earlier detection[198,242]. In contrast, BT tumours more commonly present with vague pain-related symptoms, such as abdominal and/or back pain[198]. In addition, newly diagnosed diabetes may also be an indicator of PDAC and always warrants further examination[241,243].

Diagnosis

PDAC is diagnosed by computed tomography (CT), utilizing intravenous contrast to enhance visualization of the pancreas and its surroundings[198]. This is of particular importance to enable evaluation of the operability of the tumour. In advanced cases, a core needle biopsy or fine needle aspiration from either the primary tumour or a metastatic site, depending on the accessibility of the tumour, is needed to confirm the diagnosis by histopathological or cytological assessment. Complementary radiology, including magnetic resonance imaging (MRI) or endoscopic retrograde cholangiopancreatography (ERCP), may be utilized whenever necessary to discern benign from malignant lesions in the pancreas or liver, and to improve evaluation of secondary lymph nodes[198,244,245].

Clinicopathological assessment

As illustrated in **Figure 13**, PDAC can be divided into the following four categories: resectable disease (20 %), borderline resectable or locally advanced disease (35%), and metastatic disease (55%)[246]. Classification is performed according to the tumour, nodes and metastasis (TNM) system, a universally used system from the American Joint Committee on Cancer (AJCC, 8th edition[247,248]). In summary, characterization is performed based on tumour size and involvement of nearby arteries and veins (T1-T4), tumour spread to regional lymph nodes (N0-N2) and presence of distant metastases (M0-M1).

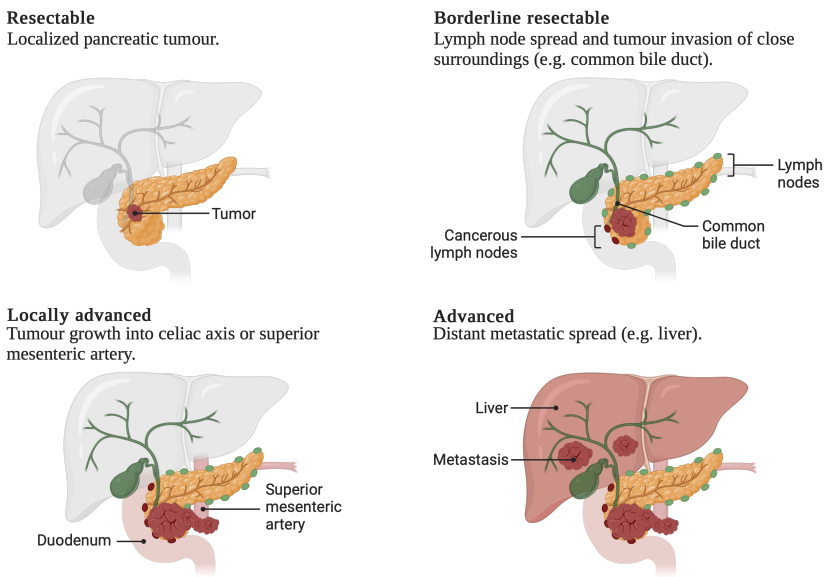


Figure 13. PDAC categorization. The four different PDAC categories based on the tumour, nodes and metastasis system from the American Joint Committee on Cancer (8th edition)[247,248].

Routine biomarkers

In spite of the plethora of conducted biomarker research on PDAC, the serum glycoprotein *carbohydrate antigen 19-9* (CA19-9) remains the main biomarker in current clinical use for PDAC management, even 40 years after its implementation[249]. CA19-9 is produced by ductal cells in several organs such as the pancreas, colon and biliary system, although 6% of Caucasians and 22% of non-Caucasians have been shown to be non-producers[250]. However, whilst frequently used for both diagnosis and disease monitoring of PDAC, CA19-9 has not yet proven to be specific or sensitive enough to be used for disease screening purposes[249].

Carcinogenesis

Precursor lesions and subtypes of PDAC

Benign neoplasms of the pancreas are common and include pancreatic intraepithelial neoplasias (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs)[251,252]. Most PDACs emerge from PanINs, which are neoplasms smaller than 5mm arising in the pancreatic ducts[253]. It should, however, be noted that the majority of PanINs will never develop into cancer[251]. The second most common precursors of PDAC are IPMNs, which are larger and may thus even be detected by imaging[254]. The different precursor lesions are further classified into one out of two morphologically distinct subgroups, characterised by either low-grade or high-grade dysplasia[253], where especially the latter have been associated with invasive carcinoma[252,255].

Microscopic investigation of PDAC, as exemplified in **Figure 14**, most often unveils randomly distributed malignant glands, surrounded by a desmoplastic stroma commonly composing more than 90% of the tumour mass. A great heterogeneity of morphological patterns is frequently seen, including different grades of cell differentiation within the same tumour[256]. In addition, there are several morphological subtypes besides conventional PDAC, such as *adenosquamous carcinoma*, *colloid carcinoma* and *undifferentiated carcinoma* with *osteoclast-like giant cells*, further highlighting the complexity of the disease[257].

Many studies have further evaluated transcriptional subtypes of PDAC with clinical impact in terms of drug response and patient outcome[258-263]. Taken together, considering the consensus from these studies, two major subtypes have been described, the *classical* and *basal-like* PDAC, with some examples of mixed types (**Figure 15**)[264]. Of importance, in the Canadian COMPASS trial, these subtypes have been shown to be therapeutically relevant, where the basal-like subtype has been associated with lower response rates to first line 5FU-based therapies[265].

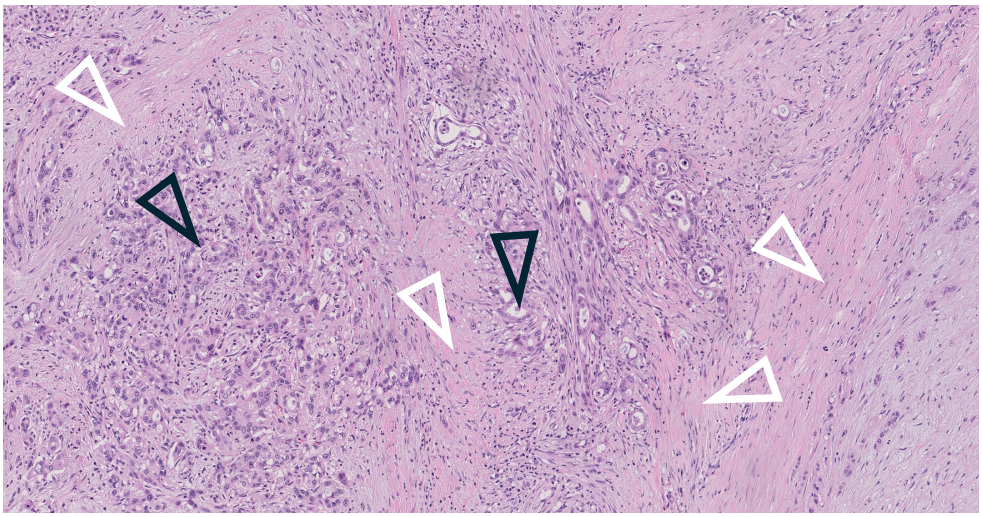


Figure 14. Microscopic image of PDAC. A microscopic image of PDAC stained with haematoxylin and eosin. Black arrows indicate tumour cells and white arrows indicate tumour stroma.

SNVs and InDels in PDAC development

During the past decades, thorough investigations of PDAC and associated precursor lesions have characterized a cumulative PDAC progression model[258,266-273]. Four genes have been found to be the most commonly mutated, i.e. *Kirsten rat sarcoma* (*KRAS*, > 90%), *TP53* (70%), *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*, 30%), and *mothers against decapentaplegic homolog 4* (*SMAD4*, 30%)[272,274]. Adding to these, a tail of infrequent gene mutations has been detected across different cohorts, with a high proportion of passenger mutations[19,258,270-275]. Within the detected low-frequency variants, seen in less than 10% of cases, alterations affecting genes involved in chromatin modification and DNA damage repair, among others, are observed[276].

Previous research has also proposed that mutations in the above-mentioned four genes occur early in PDAC development, as they have also been found to a varying extent in precursor lesions, several years before the development of invasive carcinoma (**Figure 15**)[255,277]. *KRAS* variants in particular have been detected in low-grade precursors, whereas variants in *TP53, CDKN2A* and *SMAD4* have been more associated to high-grade precursor lesions[251]. Moreover, IPMNs and MCNs are often associated with variants in the *ring finger protein 43* (*RNF43*) gene, and variants detected in the *guanine nucleotide binding protein alpha stimulating* (*GNAS*) gene are almost exclusive for IPMNs[278]. Hence, detection of variants in these genes may indicate that the PDAC emerged from a cystic precursor lesion.

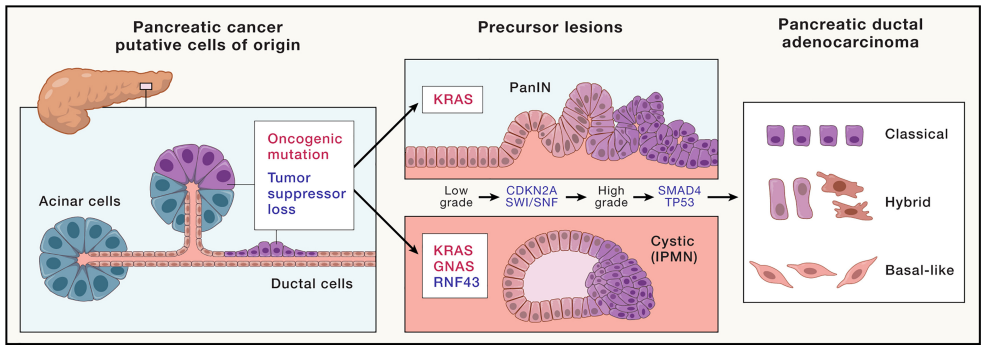


Figure 15. PDAC carcinogenesis. Illustration of PDAC carcinogenesis with the most commonly mutated genes and increasing grades of dysplasia. Reprinted from Halbrook et al.[264] which is licensed under CC BY-NC-ND 4.0 (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Evolutionary analyses of PDAC and associated precancerous lesions have further revealed their shared ancestral relationship, with individual precursors preceding the invasive carcinoma[255,277]. However, a relatively high genetic heterogeneity has been detected, even for known driver variants, proposing that more than one cell has given rise to the emergence of individual precursor lesions (polyclonal origin)[255,279]. A recent publication investigating the microanatomy of precancerous lesions from the normal pancreas, by 3D genomic mapping, also revealed a high load of PanINs with a remarkable multifocality and genetic heterogeneity[280]. Thus, these results further validate the early accumulation of known PDAC drivers and provide future hope for PDAC prevention.

KRAS

KRAS belongs to a group of oncogenic proteins that bind guanosine triphosphates (GTP) called *GTPases*[281]. KRAS is activated by upstream protein tyrosine kinase receptors, such as the *epidermal growth factor receptor* (EGFR)[281]. Upon activation, KRAS mediates several signalling pathways, such as the *MAPK-ERK* or *PI3K-AKT-mTOR* signalling pathway, which initiates cellular processes such as cell division, growth and survival (**Figure 16**)[282]. In PDAC, mutated *KRAS* (SNVs) results in EGFR-independent activation, and almost always affects one out of the following three codons; 12 (90%), 13 (2%) and 61 (7%)[276].

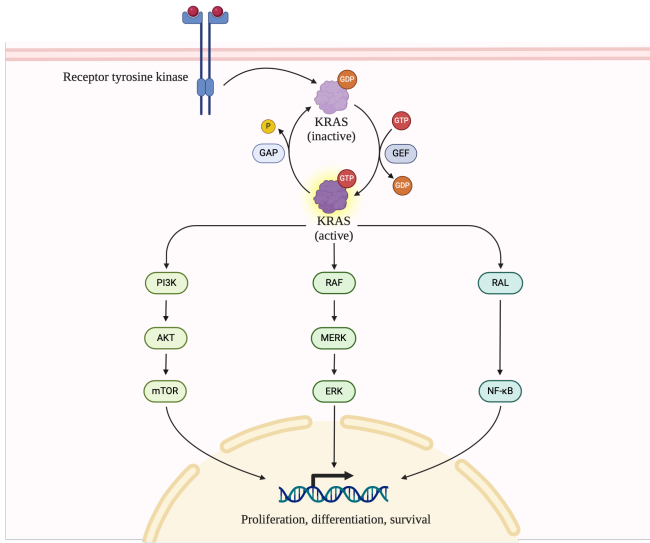


Figure 16. KRAS signalling pathway. Illustration of the KRAS signalling pathway according to Huang et al.[283].

TP53

As previously mentioned, the p53 protein encoded by the *TP53* gene is often referred to as "*the guardian of the genome*" due to its crucial role in cell cycle control[284]. It was also the first gene to be denoted as a tumour suppressor and has been known to be a key player in cancer ever since it was first described in 1979[284]. It can be inactivated through various different mechanisms, including both small-scale and large-scale alterations, and also by binding to viral proteins[284]. In PDAC, however, the most commonly seen mechanisms are missense mutations followed by allelic loss of the second allele, although frameshift variants and homozygous deletions also occur[276]. The default state of p53 is *off*, but whenever activated, its main function is to halt cell division of stressed or damaged cells, followed by either stimulation of repair mechanisms or induction of apoptosis, if the damage is irreversible[284]. It can also inhibit angiogenesis, a crucial factor in expanding tumours[284]. Inactivating mutations in *TP53* in PDAC have also been shown to be correlated with polyploidy and to occur late in PDAC development[277,285,286].

CDKN2A

The two proteins encoded by the *CDKN2A* gene, the *INK4 family member p16* ($p16^{INK4a}$) and $p14^{ARF}$, are both involved in cell cycle regulation and *CDKN2A* is, hence, also considered a tumour suppressor[287]. The specific roles of the resulting proteins include inducing cell cycle arrest in the *G1* phase ($p16^{INK4a}$), by inhibiting the binding of CDK4/6 to cyclin D1, and inducing p53-mediated cell cycle arrest ($p14^{ARF}$)[287].

Studies of PDAC have further shown biallelic *CDKN2A* inactivation in 90% of cases through different mechanisms, including homozygous deletion and mutation in one allele together with either loss or inactivation by hypermethylation of the other allele[276].

SMAD4

Like *TP53* and *CDKN2A*, *SMAD4* is also a tumour suppressor, known to be involved in the multifaceted *transforming growth factor β* (TGF-β) signalling pathway. This pathway is crucial for tissue homeostasis, and whenever malfunctioning, it may lead to tumour growth, tumour cell migration and immune evasion[269,288]. Inactivation of *SMAD4*, resulting in the abovementioned consequences, is often also achieved by a combination of mutations and LOH events, or a homozygous deletion of both alleles[276]. It has also, just as for TP53, been correlated to late-stage disease, and particularly with invasion events[277,286,289].

Copy number alterations

Adding to small scale mutations, a high burden of chromosomal alterations such as CNAs, structural rearrangements and chromothripsis events have also been observed to characterize the PDAC genome[271,273,285]. Frequent WGD events have also been observed, followed by additional alterations covering large chromosomal segments[37,285], adding to the complex genomic profile. Chromosomal segments commonly altered by CNAs in PDAC include, but are not limited to, LOH events of *9p* (including *CDKN2A*), *17p* (including *TP53*) and *18q* (including *SMAD4*), together with homozygous focal loss of segments covering *CDKN2A*, again pointing to the importance of these genes in PDAC carcinogenesis[271,273,290]. Based on genome wide profiling of large-scale chromosomal changes in a now considered landmark study of PDAC, four genomic subtypes were proposed[271]. These were named the (i) *stable*, with less than 50 alterations, (ii) the *locally rearranged* with at least one significant focal rearrangement event, (iii) the *scattered* with a moderate burden of alterations but below 200 changes, and (iv) the *unstable* with over 200 detected alterations[271]. Of clinical value, PDAC tumours with a defined unstable genome and/or signs of a BRCA mutational signature[59], were associated with sensitivity to platinum-based therapy[271]. Furthermore, a recent study showed that the quantity of detected CNAs appears to carry prognostic value in resected cases, where a higher burden was associated with a lower OS[290]. Whether the CNA burden is prognostic across all PDAC stages remains to be explored, proposedly with the use of ctDNA based analyses, as has recently been demonstrated to be feasible[291-293].

Moreover, a genome-wide study by Notta et al.[285] challenges the previously described stepwise and slow development of PDAC, by showing evidence of punctuated catastrophic events, where a large proportion of all alterations appeared to have been acquired during a short period of time. It should, however, be noted that the exact

timeframe for PDAC development remains unknown till this day. Nevertheless, consistent with the observations made by Notta, additional evidence from precancerous pancreatic lesions demonstrates that CNAs and other large-scale alterations are a more prevalent feature in high grade compared to low grade precancerous lesions[280,285,294]. Hence, this promotes the notion of CNAs as evolutionary late events in PDAC carcinogenesis.

Metastatic spread of PDAC

The majority of PDACs inevitably spread to distant sites, unfortunately often sooner than later, and many patients present with advanced disease already at diagnosis. Building on that, several studies have shown evidence indicating that metastasis formation is an evolutionary early event in PDAC[70,295], as even resectable PDAC tumours, and those with sizes smaller than 2 cm, have been associated with metastatic dissemination[296-298]. The most common sites for metastases, detected at autopsy, are, in order of their occurrence: the liver, peritoneum and lungs, although PDAC has been shown to be capable of disseminating to vastly all organs in the human body[299-301].

Dissemination may occur by several mechanisms such as neural, intraductal or lymphovascular invasion[302,303], and different routes have been associated with specific distant metastatic sites[304]. Future research is, however, needed to establish their individual impact on the metastatic burden of PDAC[304]. Interestingly, genomic studies have shown remarkably similar genetic profiles in terms of known driver events, such as *KRAS* and *TP53* mutations, when comparing different tumour lesions in metastatic treatment naïve PDAC[305]. Contrastingly, it has also been noted that platinum-based therapy, following surgery, appears to enrich for genetic drivers activating the MAPK/ERK and PI3K/AKT signalling pathways in recurrent disease[70].

To summarize, metastatic dissemination is the main cause of death from PDAC. Further research is, hence, needed to provide additional insights into the impact of specific invasion routes and how to utilize prior knowledge about treatment induced bottlenecks, to improve survival outcomes both in the adjuvant and palliative patient groups.

Treatment modalities

According to European standard of care, treatment options for PDAC primarily include surgery and chemotherapy, although radiation, targeted therapies and immunotherapy are administrated in a minority of cases. Radiation therapy is however rarely performed in Sweden, and will, thus, not be covered in this thesis.

Surgery

All the way back in 1858, Jacob Mendez Da Costa was the first to describe PDAC based on a microscopic evaluation[306]. After 40 years, in 1898, the first successful resection of a periampullary tumour was performed by William Stewer Halsted from Johns Hopkins Hospital[307]. It would however take until 1935 before Allen Oldfather Whipple set the scene for the currently used *pancreaticoduodenectomy*, also referred to as the *Whipple procedure*[308]. This procedure involves the *en bloc* removal of the pancreatic head, the distal part of the stomach, the duodenum, the common bile duct and the gallbladder[309].

Today, surgery is the only treatment of PDAC allowing for a chance of cure. The most common procedure for periampullary cancers and PDAC originating in the pancreatic head is to perform a pancreaticoduodenectomy, either by a classic Whipple or a *pylorus preserving Whipple* (PPW)[309]. For tumours arising in the pancreatic body or tail, although the proportion of operable tumours remain sparse, resection of the distal part of the pancreas is performed. The remaining surgical option is to perform a *total* pancreatectomy, which is carried out whenever the size or location of the tumour renders the other options impossible. However, this procedure, although showing comparable mortality rates to pancreas-preserving techniques, is associated with a drastic adverse impact on quality of life and development of severe diabetes[310,311].

Chemotherapy

Chemotherapy has been the standard systemic treatment of PDAC since the 1980s, although with moderate success and effect on patient survival. However, two studies in particular have laid the foundation for the currently used regimens and significantly improved the survival outcome of PDAC patients, namely the introduction of *gemcitabine* in 1997 and the triplet combination chemotherapy regimen *FOLFIRINOX* (oxaliplatin, irinotecan, fluorouracil/5-FU, folinic acid) in 2011[312,313]. Today, chemotherapy treatment is given in both the neoadjuvant, adjuvant and palliative setting. The most commonly used treatment regimens are administration with either *modified* FOLFIRINOX (mFOLFIRINOX, omitting 5-FU bolus and lower dosage of Irinotecan), gemcitabine alone or a combination of gemcitabine and *nab*-paclitaxel. The main agents of these therapies all exert anticancer activity by disrupting cell proliferation and inducing cell death. More specifically, the drugs oxaliplatin, 5-FU, irinotecan and gemcitabine are primarily targeting DNA synthesis and repair, whereas *nab*-paclitaxel predominantly binds to microtubules, which blocks mitosis and induces cell cycle arrest[314-318].

Adjuvant treatment

Adjuvant chemotherapy, that is treatment given *postoperatively* to reduce the risk of relapse, has been shown to significantly improve OS for patients with PDAC[319-321]. In

addition, studies comparing survival outcomes for patients receiving either gemcitabine or mFOLFIRINOX in the adjuvant setting showed a substantial increased survival for patients receiving mFOLFIRINOX[322], and it is therefore now standard of care for eligible patients. However, according to individual performance status and comorbidities, other regimens are also common, including standard FOLFIRINOX, single gemcitabine, and gemcitabine in combinations with either *nab*-paclitaxel or Capecitabine (GemCap), which is an oral fluorouracil prodrug[323].

Neoadjuvant treatment

Neoadjuvant chemotherapy, i.e. *preoperative* treatment, has not been a common strategy in PDAC historically. However, it has recently been revisited as it shows potential both for managing micro-metastatic disease and for tumour downstaging prior to surgery and, hence, an increased probability of achieving a negative resection margin[324,325]. It further enables a larger group of patients to benefit from systemic treatment, including patients who would not be fit enough for adjuvant treatment, and spares patients who would most likely develop rapid recurrence from undergoing a complex surgical procedure[325]. In Sweden, neoadjuvant treatment is primarily administrated using standard FOLFIRINOX and within clinical trials.

Palliative treatment of metastatic disease

As previously stated, the majority of all PDAC patients are diagnosed with advanced disease, for which palliative chemotherapy remains the sole treatment option. Treatment at this stage is mainly given to prolong OS and to sustain an acceptable quality of life[326]. After many years of using solely gemcitabine as first-line treatment for metastatic disease[313], improvements in survival were shown both for FOLFIRINOX (2010) and for the combination of gemcitabine and *nab*-paclitaxel (2012) in comparison to single gemcitabine, which changed the priority of regimens[312,327,328]. At present in Sweden, patients are offered one of the following first-line treatments, depending on their performance status: standard or modified FOLFIRINOX or gemcitabine with *nab*-paclitaxel for patients with high performance status, and usually either single gemcitabine or single 5-FU for more fragile patients.

Personalised medicine

At present, personalised medicine has only been observed to benefit a minority of PDAC patients. For instance, tumours with pathogenic germline variants detected in HRR genes, e.g. *BRCA1/2* and *partner and localizer of BRCA2* (*PALB2*), have been shown to be associated with therapeutic sensitivity to *poly(ADP-ribose) polymerase* (PARP) inhibitors, such as olaparib[229,230,329], which may be administrated subsequent to first-line platinum-based therapy. Of note, these tumours have also been associated with sensitivity to platinum-based treatment and patients have shown an improved OS[330-332].

Comparisons of PDAC to other cancers have revealed a fairly modest *tumour mutational burden* (TMB), and thus a resulting low number of neoantigens[59,333]. Patients with variants in *mismatch repair* (MMR) genes, leading to a hypermutated cancer genome and a significant increase in neoantigen burden, represents a specific PDAC subgroup (~1%) with *microsatellite instability high* (MSI-high) tumours[334], such as in Lynch syndrome. These tumours have been associated with sensitivity to immune checkpoint therapy, in particular to the PD-1 inhibitor pembrolizumab[231], which may be administrated as second-line treatment.

The pursuit of therapies targeting *KRAS* mutations has been going on for decades, but has previously been deemed extremely challenging, or even impossible, due to limited binding pockets and exceptionally high affinities for GTP and GDP at the nucleotide-binding sites[282]. Yet, a recent breakthrough with inhibitors targeting $KRAS^{G12C}$, i.e. sotorasib[335], has shown promise for PDAC patients, although the prevalence of $KRAS^{G12C}$ is low (1-2%). In addition, other KRAS targeting approaches, such as pan KRAS inhibitors[336], vaccines[337], G12D specific inhibitors[338] and T-cell receptor therapies[339], are currently being investigated, which could prove to be of great importance for PDAC management and treatment in the future.

In addition to the above-mentioned subgroups, which represent the most relevant for the studies within this thesis, individual patients may be eligible for other targeted treatments based on specific molecular traits. For example, a small subgroup has been found to harbour either *neurotrophic receptor tyrosine kinase* (*NTRK*) fusions or *neuregulin 1* (*NRG1*) fusions, which have shown sensitivity to the tropomyosin receptor kinase inhibitor larotrectinib and the tyrosine kinase inhibitor afatinib, respectively[340,341].

Treatment resistance and evolutionary strategies

Chemotherapy resistance

As for malignancies in general, treatment resistance represents the greatest oncological challenge in PDAC management[342]. There is also a lack of biomarkers in clinical use that are able to predict sensitivity to chemotherapy, thus leading to a high number of patients experiencing adverse side effects without having any treatment benefit. General resistance mechanisms include, but are not limited to: increase in drug efflux, decreased drug uptake by reduction of transporters, blocking of the apoptosis pathway, changes in metabolism of the drug, and changes in the pathway leading to *epithelial to mesenchymal transition* (EMT)[60]. However, further research on agent specific modes of resistance is important to understand and overcome the low therapy response rates in PDAC.

Adaptive therapy

But what if cancer eradication is not the only option for disease management? As previously mentioned, other researchers have proposed the introduction of non-curative oncological strategies similar to those used in pest control[343]. Similarly to cancer cells, pests belong to a genetically heterogenous population and pesticide resistance is an inevitable problem[344]. But in contrast to the majority of oncological treatments, farmers have put their main focus on controlling the pest population, and minimizing negative side effects, rather than trying to get rid of them all[344]. The evolutionary heuristics used in pest management, based on the principles of Darwinian natural selection, have thus paved the way for what has become the field of evolutionary treatment and *adaptive therapy*[63,65,345-348].

The fundament of adaptive therapy is built upon the exploitation of competition between treatment sensitive and resistant cells, where resistance poses a potential fitness cost[63,347]. Hence, by monitoring individual tumour dynamics, cancer control is achieved by balancing the reduction of sensitive cells whilst avoiding a complete eradication which would allow resistant cells from taking over (**Figure 17**)[63,349]. In practice, this means administrating treatment until noting a sufficient reduction in tumour volume, and then turning it off, to permit for the re-growth of sensitive cell populations[63,65]. In contrast, the goal of traditional systemic therapy is to maximize treatment and to kill as many tumour cells as possible[350], although the chance of complete eradication is dismally low and rapid resistance almost unavoidable.

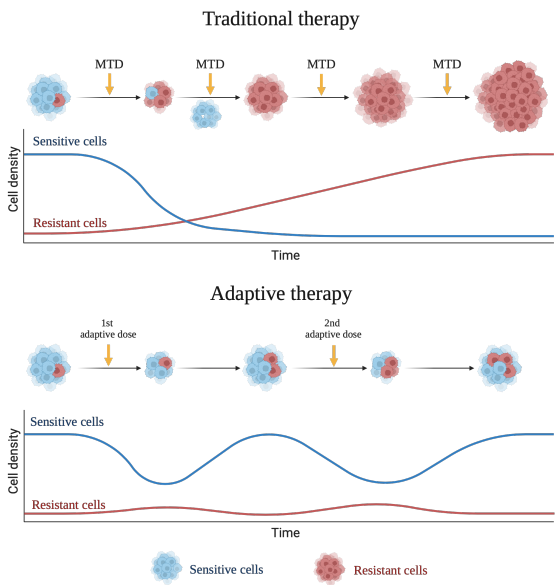


Figure 17. Adaptive therapy. Illustrative comparison of treatment administration in traditional and adaptive therapy strategies, according to Zheng et al.[65]. MTD; Maximum tolerated dose.

Although still in early phases[349], adaptive therapy and mathematical evolutionary modelling of disease dynamics have recently shown promising results in metastatic castrate-resistant prostate cancer, with increased time to progression compared to standard protocols[65,351], and studies are ongoing in, for example, ovarian cancer[352]. Whether adaptive therapies will provide future prolonged disease control in PDAC remains however to be seen.

Circulating tumour DNA in PDAC

Despite a high proportion of patients diagnosed with metastatic disease, pan-cancer analyses have shown that plasma levels of ctDNA in PDAC are relatively low compared to other malignancies[118,353]. In addition, discrepancies between ctDNA detection rates have been observed in the different publications during the past decade, most likely due to the overall low ctDNA burden[354]. Furthermore, ctDNA in PDAC has primarily been evaluated using *targeted deep sequencing* (TDS) and *digital droplet PCR* (ddPCR), and untargeted approaches such as whole genome exome sequencing have seldom been utilized[146,291,355-361].

Due to its high prevalence, *KRAS* has been the most common target in studies evaluating ctDNA in PDAC, where mutations detected in plasma have been shown to provide prognostic significance both in patients with operable and advanced disease[356,357,359]. Further associations have also been observed between detection of ctDNA in plasma before treatment and the presence of liver metastases[362-364]. However, whether this is due to an increased shedding from liver metastases in particular, or to an overall increase in ctDNA levels due to a malfunctioning clearance of ctDNA by the liver, remains to be ascertained.

Moreover, longitudinal monitoring of PDAC has been proposed to provide important clinical guidance. Analyses carried out before, during and after treatment, in both surgically treated patients and patients treated with palliative intent have shown capability to predict relapse and progression, respectively, even earlier than imaging data and clinical routine biomarkers[360,365-367]. In addition, due to the frequently limited availability of PDAC tumour tissue, studies have also acknowledged the utilization of ctDNA analyses to detect genetic alterations which may in turn predict response to targeted therapies[293,355,368-370]. A few recent studies have also investigated the possibility and clinical impact of monitoring CNAs in ctDNA of PDAC with promising results, including its potential in future studies on PDAC evolution[291-293].

In summary, ctDNA analyses show great potential in aiding clinical management of PDAC patients in all different disease stages, especially as current routine biomarkers are not specific or sensitive enough. However, due to the generally low ctDNA burden, efforts should be made to customize clinical methodologies to the specific task at hand.

The desmoplastic tumour microenvironment

The pancreatic TME is uniquely characterized by a dense desmoplastic stroma with the presence of diverse, and often suppressive IC populations, cancer associated fibroblasts (CAFs) and increased ECM formation[371,372]. In fact, as stated in the aforementioned sections, in most cases, the TME constitutes the main part of the tumour mass, and the actual cancer cells are in minority[371]. CAFs are the primary providers to the prominent stromal compartment of PDAC, sometimes constituting up to 80% of the tumour[373]. Recent studies have also demonstrated that CAFs may contribute either too a "good" stroma, with anti-tumour activity, or to a "bad" desmoplastic stroma, promoting tumour progression and invasiveness, depending on their functional state[374]. The desmoplastic reaction further leads to a hypoxic environment, reduced vasculature and an increased interstitial pressure, all contributing to an impeded infiltration of therapeutic agents[372]. In addition, the hypoxic environment attracts immunosuppressive IC populations, such as T_{regs} and *M2*-polarized macrophages[372]. Lastly, various soluble factors, for example TGF-β and *matrix metalloproteinase 9* (MMP-9), also facilitate PDAC invasion by assisting in ECM remodelling, immunosuppression and angiogenesis[372].

Present investigation

"Without data, you're just another person with an opinion"

by W. Edwards Deming

Aim

The overarching aim of the present investigation was to study both spatial and temporal genetic heterogeneity across various stages of PDAC, to uncover novel insights into the disease evolution in the context of both treatment and host response.

Specific aims of paper I-IV

Paper I

To identify geographical genetic traits associated with OS in resected PDAC, following adjuvant treatment, in combination with spatial characteristics of the surrounding immune microenvironment.

Paper II

This paper outlines the study protocol for the prospective, observational, single-arm clinical trial CHAMP.

Paper III

To assess the temporal dynamics of ctDNA, in conjunction with the parallel systemic immune response, considering potential treatment effects and patient outcome.

Paper IV

To investigate the clonal landscape of terminal metastatic PDAC in relation to spatial heterogeneity, dissemination routes and OS. Preceding clonal events in matched liquid biopsies were also assessed regarding their potential to reflect the terminal genetic tumour landscape.

Patient cohorts

Retrospective patient cohort

In **paper I**, the study material encompassed resected tumour specimens from 11 PDAC patients. These patients were selected, based on multiregional tissue availability, from a retrospective consecutive cohort of all patients who were surgically treated at Skåne University Hospital in Lund between the 1st of January 2012 and the 31st of December 2014. By multiregional sampling, different areas of the primary tumour and nearby lymph node metastases were investigated to explore genetic and immune cell heterogeneity.

The CHAMP study

The main cohort investigated in this thesis comprised patients enrolled in the prospective, observational, and ongoing clinical trial CHAMP, short for "Chemotherapy, Host response And Molecular dynamics in Periampullary cancer"[375]. The study was initiated in Malmö in November 2018 and is available at clinicaltrials.org, registered as NCT03724994. The study was also expanded to Lund in 2019. CHAMP invites all patients diagnosed with PDAC or other periampullary adenocarcinoma who are being treated with neoadjuvant, adjuvant or first-line palliative chemotherapy at Skåne University Hospital in Lund and Malmö, with the only exclusion criteria being patients with another concurrent lethal condition. In 2021, the CHAMP study was also extended to include research autopsies, where patients in a later, palliative phase are asked by their palliative care physician if they want to participate. The autopsies are then performed as soon as possible after passing by a senior pathologist, where multiple tumour samples are collected from the primary tumour, if still present, and all metastases of sufficient size.

An overview of the CHAMP study design is shown in **Figure 18**, including descriptions of the sampling procedures and following analyses, although some are outside the scope of the thesis. In brief, tumour analyses are conducted on specimens obtained from surgical resections, biopsies, or tissues collected during autopsies, after histological re-examination by a senior pathologist. Blood sampling, on the other hand, is performed longitudinally, i.e before (baseline), during and after the last treatment cycle, if feasible. Blood components, including plasma, peripheral immune cells (buffy coat) and serum, are isolated by centrifugation within two hours of blood draw and stored in -80°C. Analyses of blood and tumour tissue further include, but are not limited by, DNA sequencing, immunohistochemistry, proximity extension assay of circulating soluble proteins, flow cytometry and spatial transcriptomics.

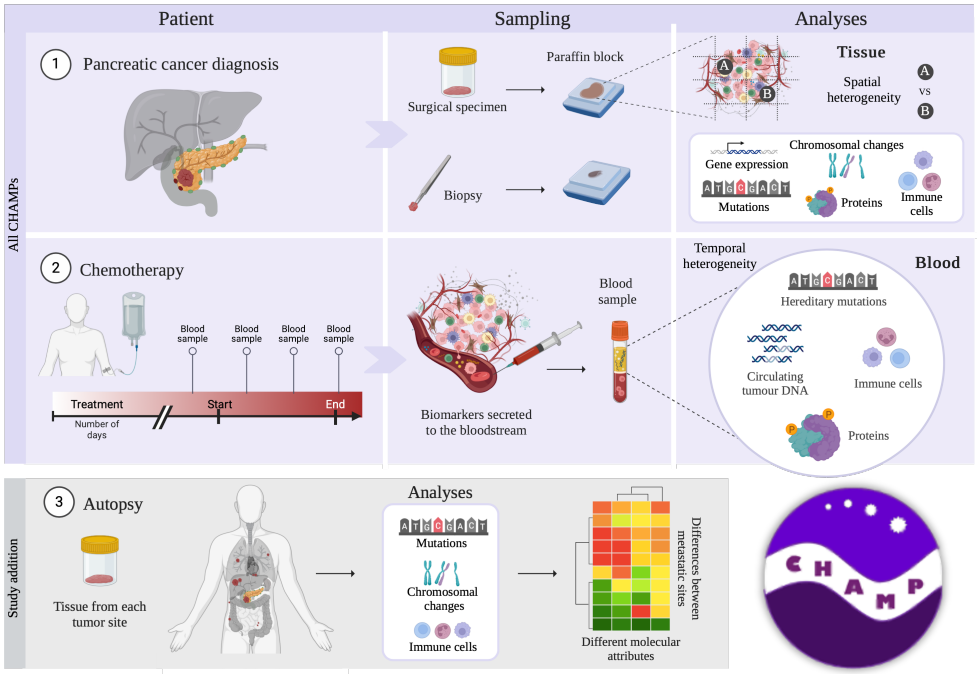


Figure 18. The CHAMP study design. A schematic overview of the clinical study CHAMP, including blood and tumour tissue sampling procedures and subsequent analyses. The third panel demonstrates the additional autopsy study with comprehensive sampling of the terminal tumour burden.

Paper II comprises the published CHAMP protocol, stating overarching aims and methods of the study. **Paper III** is a multimodal investigation of the disease dynamics during treatment, and the concomitant host response, including all patients included up until the 31st of December 2020 (n=60). Fifteen patients were treated with adjuvant and 45 patients with palliative chemotherapy, and the main focus was on the latter. **Paper IV** is an in-depth case study of the five first autopsied CHAMP patients, providing a detailed exploration of the genetic heterogeneity in the terminal disease burden, along with on-treatment tumour dynamics.

Ethical considerations

Paper I

For the retrospective cohort included in paper I, study approval was received from the Regional Ethical Review Board (LU 445/07 with amendments LU 2008/35, LU 2011/670 and LU 2014/748), permissioned with an opt-out consent approach. The study also complies with the Declaration of Helsinki.

Paper II through IV

Ethical approval of the CHAMP study was initially granted by the Regional Ethical Review Board (LU 2018/13) and additional amendments, including for instance the autopsy study, were later approved by the Swedish Ethical Review Authority (2021-00166 and 2021-06065). All treatment regimens and the clinical workup follow national guidelines, without any modifications due to trial participation. The CHAMP study also complies with the Declaration of Helsinki.

Individuals diagnosed with PDAC who meet the inclusion criteria receive information about the study from their treating oncologist or a research nurse. If they choose to participate, they sign an informed written consent, declaring that they have understood the information and that the purpose of the CHAMP study is primarily to improve the outlook of future patients. Importantly, the only additional procedure for these patients includes blood sampling during and after treatment, although this is performed in conjunction with their clinical treatment visits and through an already established central venous catheter. Access to antemortem tumour tissue is also only provided if the specimen has already been collected for clinical purposes. No additional tumour sampling is performed due to study participation.

Worth noting is, however, that although the principal aim of the CHAMP study is not to benefit the participants themselves, I have through our close collaboration with the oncology department understood that study participation may add some sense of purpose, in an otherwise often hopeless situation.

Methodological considerations

"*I have never tried that before, so I think I should definitely be able to do that.*"

Pippi Longstocking, by Astrid Lindgren

This section includes a brief introduction to the main methods used in the present investigations along with the reasoning behind their selection. In-depth details are available within the paper specific methodology sections (**paper I, III** and **IV**).

Introduction to key concepts and techniques

Sample handling for clinical diagnostics

For tumour tissue analyses throughout the different studies in this thesis, *formalin fixed paraffin embedded* (FFPE) specimens have been utilized. Preserving tissue material in paraffin wax after fixation in formalin is the gold standard for clinical diagnosis and pathology assessment. This method also enables long term storage of specimens in room temperature, while maintaining tissue architecture[376]. Hence, by cutting thin slices of the paraffin block, downstream microscopic analyses may be performed, including diagnostic evaluations of cell and tissue characteristics, together with protein expressions and other biomarkers. The drawback with FFPE preservation, however, includes DNA and, particularly, RNA degradation[377]. Both formalin itself, but also agents required for paraffin removal, prior to DNA extraction, are known to cause DNA fragmentation and introduction of artifacts in the nucleotide sequence. Hence, this poses a challenge for downstream NGS analyses.

The field of genomics has instead promoted the use of *fresh frozen* (FF) tissue specimens for sequencing purposes, which often provides an increased yield and less degraded DNA[377]. Nevertheless, FF material comes with other challenges, such as the need for storage at ultra-low temperatures. In addition, although FF specimens have shown to be superior to FFPE for sequencing analyses, studies show that the majority of FFPE material provides sufficient quality to aid clinical decision making[377,378]. The frequent use of FFPE specimens in the clinic have also spurred the installation of tissue specimen biobanks. Thus, it can be argued that the disadvantages of FFPE specimens in terms of DNA quality might be balanced by the immense availability of material and the enabling of larger and more robust cohort studies, while still allowing for clinical routine diagnostics[377].

The TMA technique

To effectively analyse large numbers of tissue samples from different patients, or different tumour regions within one patient, construction of *tissue microarrays* (TMAs) is often utilized[379]. Since its introduction in the late 90s, the TMA technique has proven indispensable for oncological biomarker investigations and has had a profound impact on our knowledge of tumour biology[379,380].

TMAs are constructed by collecting cylindrical punches from archival FFPE tissue blocks, which are then added to a recipient paraffin block, forming a tissue core matrix (**Figure 19**). Obtaining a thin slice from the TMA paraffin block and mounting it on a glass slide further enables analyses of, for instance, different protein expressions by immunohistochemistry (described below) or evaluations of different nucleic acid segments by fluorescence *in situ* hybridization (FISH)[380]. In **paper I**, TMAs were used for immunohistochemistry analyses to evaluate the distribution of different immune cell populations infiltrating the TME.

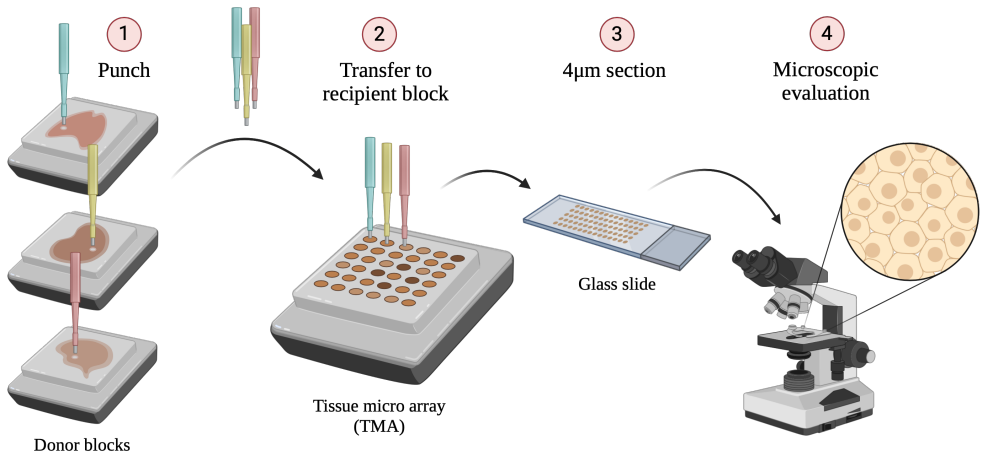


Figure 19. Tissue microarray technique. Illustration of the construction of tissue microarrays (TMAs), followed by microscopic analysis.

Extraction and purification of DNA

Already in 1979, Vogelstein and Gillespie found that DNA molecules have a high binding affinity to silicates[381]. This discovery laid the foundation for the silica-based methodologies utilized in modern DNA extraction and purification[382]. Current procedures most often include a stepwise spin column process (**Figure 20**) which starts with nucleic acids selectively binding to a silica membrane, whereas other sample analytes do not. This is then followed by a washing step, which purifies the DNA, and lastly, the DNA is eluted by an aqueous solution.

Throughout this thesis, silica-based technologies have been used for DNA isolation from both tissue and plasma by using automated and manual methods, respectively. However, in comparison to levels of *genomic DNA* (gDNA) available after tumour cell lysis, cfDNA concentrations are very low and require large volumes of plasma to be added to the spin column, thus posing a practical challenge. To overcome this, a specialized but widely established protocol was used, in which a vacuum pump generates a negative pressure gradient that drives large volumes of plasma through the membrane, allowing for continuous cfDNA binding. This method has shown superior yields compared to other, including automatic, protocols[383].

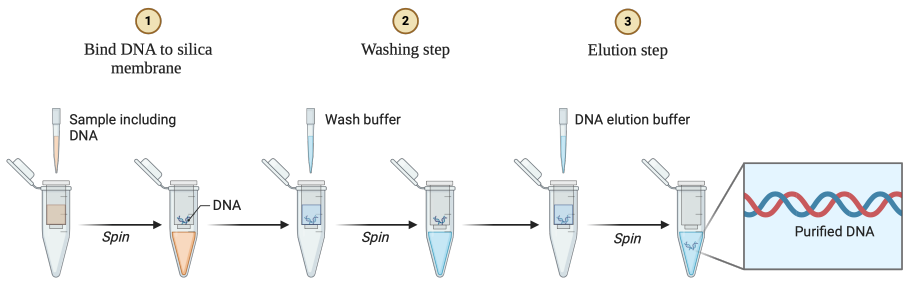


Figure 20. DNA extraction. General principle of silica-based DNA extraction using spin columns.

Next generation sequencing

The consecutive order of nucleotides in DNA and RNA can be determined by *genetic sequencing*. The precursor of modern techniques was described in 1977 by Nobel prize winner Frederick Sanger[384], and represented the start of an impactful sequencing era to come. The first generation of genetic sequencing is, hence, referred to as *Sanger* sequencing, and spurred the initiation of the *Human Genome Project* in the 1990[385]. The aim of the project was to decipher the human genetic code and it was completed after 15 years in 2006[385]. The second generation of methods, referred to as *next generation* sequencing, was introduced during the 1990s, and, in comparison to the first generation, enabled high throughput analyses with sequencing of the whole genome all at once, utilizing *sequencing by synthesis* (SBS). These developments have had an extraordinary impact on biological research across different fields, not least in oncology[386]. Up until today, sequencing costs and time consumption have decreased remarkably, and a human genome can now be sequenced within 24 hours[386].

Sequencing of DNA is often divided into three categories dependent on the extent of genome capture (**Figure 21A**), i.e. whole genome sequencing (WGS), whole exome sequencing (WES, including only gene coding regions) and TDS, focusing on a lower number of genetic targets. In **paper I**, **III** and **IV**, different variants of TDS have been utilized together with the *Illumina NovaSeq 6000 platform*.

The basic steps of SBS are shown in **Figure 21B**.The first step is called *library preparation* and refers to the fragmentation of DNA (if not already fragmented such as in the case of cfDNA) followed by ligation of adaptors (oligonucleotides). To enable *multiplex sequencing*, i.e. analysing several samples at once, distinct adaptors are added to DNA from each specific sample prior to pooling of the samples. If using a WES or TDS approach, an additional step is included during library preparation which includes *target capture* and *enrichment*. In this step, molecular capture probes, matching the nucleotide sequences of interest, are added to extract specific DNA fragments.

The next step is the *cluster generation* and involves the attachment of the (selected) DNA fragments to the flow cell surface in the sequencer, via the adapters. This is then followed by amplification of the DNA fragments by *bridge PCR*, to enhance the fluorescent signal during the subsequent sequencing step. During *library sequencing*, primers first attach to each strand. followed by addition of fluorescently tagged nucleotides, one colour for each of the four bases. As the name indicates, DNA synthesis then takes place, where the fluorescent signal reveals the specific nucleotide that has been added. In this way, the sequencer can keep track of the nucleotide order of each fragment cluster, i.e. the DNA sequence. The last step involving sequence *alignment* and *data analysis* will be further detailed in the next section.

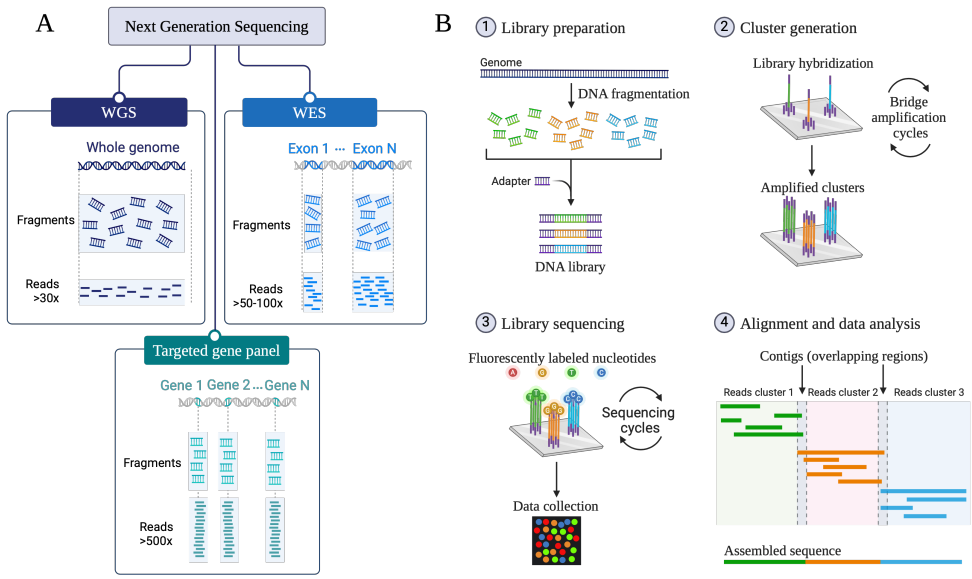


Figure 21. Next generation sequencing. A) Different types of next generation sequencing (NGS) approaches depending on genome coverage. **B)** Illustration of the four basic steps in sequencing by synthesis (NGS). Abbreviations: WGS; whole genome sequencing, WES; whole exome sequencing.

Principles of bioinformatics in targeted sequencing

The field of *bioinformatics* covers methods and computational tools for managing and understanding biological data, especially *big data* which refers to extremely large and complex datasets such as that from high throughput genomic sequencing. Distinct bioinformatic pipelines were utilized subsequent to targeted NGS in **paper I**, **III** and **IV**, and specific tools used in each study can be found in each respective original paper. However, although the pipelines differed from each other depending on the type of material (blood or tumour tissue) and the targeted gene panel used, the basic analytical steps remain the same and are emphasized in the section below.

The raw data analysis begins with the production of *fastq* files, which are large files containing information of each read from the sequencer, including a quality score for each individual nucleotide. This is followed by a quality check, where reads or bases with low quality are removed from subsequent analyses. The next step involves *mapping,* or *alignment,* of all reads to a *reference genome*, to ensure that the reads are ordered correctly. Of note, short reads increase the risk of "multi-mapping", referring to reads mapping to more than one genomic location, and hence, represents one of the challenges with fragmented DNA from FFPE specimens[377]. Yet, by utilizing *paired end sequencing*, i.e. sequencing of each DNA fragment from both ends, the chance of correct mapping is increased, and was thus used throughout the studies of this thesis. Information on individual reads and their mapping attributes is further stored in large Sequence Alignment Map (*SAM*) files, which are then compressed into Binary Alignment Map (*BAM*) files, enabling a more effective downstream process. This is followed by removal of duplicates (as described further in the next section) prior to *variant calling*. In addition, for each genomic position the *read depth* may be quantified by counting the number of unique reads covering a specific nucleotide (**Figure 21A**). For instance, a genomic locus covered by 20 unique reads has a read depth of 20x.

The next step, variant calling, refers to the detection of genetic variants which differ from that of the human reference genome. These include SNVs and InDels and can be found by various tools known as *variant callers*. Depending on the approach, different callers are more or less suitable, which should be taken into account in each specific case[387]. Detected variants are further annotated according to their potential significance for tumour development and progression[388], which enables filtering out variants which have a low probability of clinical relevance. This includes removal of variants commonly known to occur in the human population, such as SNPs, and variants unlikely to have an impact on the resulting gene product. The proportion of reads that support a specific genetic variant in a sample is often referred to as the *variant allele frequency* (VAF). This is calculated by dividing the number of reads harbouring the variant with the total number of reads for that exact genomic position. This is a crucial metric in genomic analyses and clinical decisions. It may also aid in estimating the sample cell fraction having the variant and, thus, provide insight into tumour heterogeneity[389].

SNP microarray

In **paper I** and paper **IV**, the *single nucleotide polymorphism microarray* (SNP array) *OncoScan CNV assay* was used to analyse large-scale CNA and LOH events, as this method is specifically designed for FFPE specimens[390]. The OncoScan assay utilizes *molecular inversion probe* (MIP) technology, which was originally intended for SNP genotyping, by targeting various SNP loci across the genome[391]. But over time, the application was extended to the detection of CNAs and later even somatic variants[392].

The basic principle behind the first part of the MIP technology is shown in **Figure 22**[392]. MIPs are added to a solution containing DNA fragments, where homology regions (20 bases long) matching the DNA segments before and after a SNP position will hybridise to the complementing DNA strand, whenever they are close enough. This will create a ring-like structure with a single nucleotide gap at the SNP position. Of note, in the OncoScan assay, 335 000 different SNPs positions are targeted. The mixture of DNA and probes is then divided into two separate vials, one with nucleotides A and T (A/T) and one with C and G (C/G). Depending on the SNP, the gap will be filled with one of the A/T or C/G nucleotides, and thereby form a complete circle in one of the vials exclusively. In the other tube, containing non-fitting nucleotides, the ring will remain incomplete.[391]

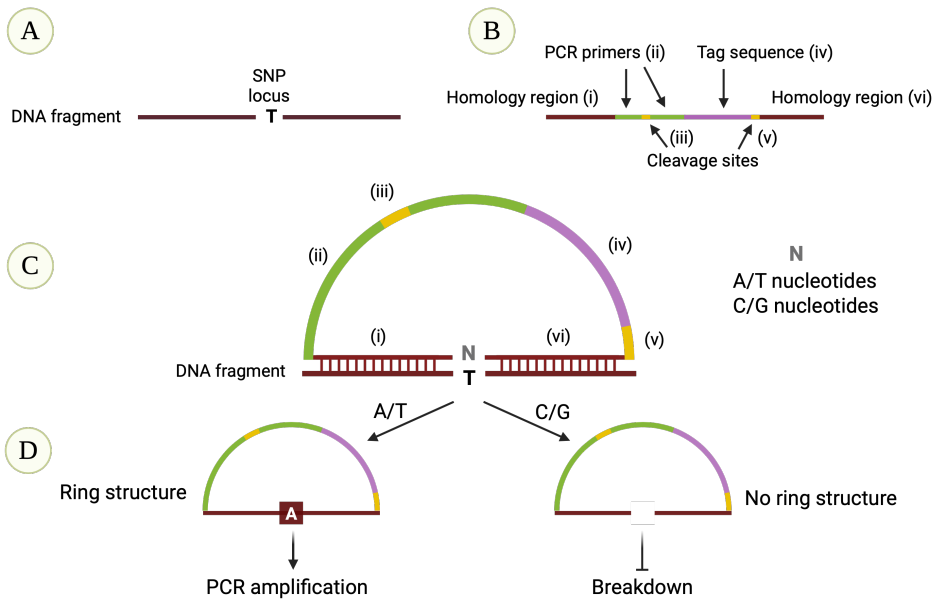


Figure 22. MIP technology. Illustration of the principle behind the first part of the molecular inversion probe (MIP) technology according to Jung et al[392]. **A**) DNA fragment with SNP locus (thymine, T). **B**) MIP construction. **C**) Hybridisation of probe to matching DNA fragment via the homology regions. The SNP position (N) will either be matched with A/T or C/G nucleotides. **D**) Gap filling with either A/T or C/G nucleotides in two separate vials. Ring structures get cleaved and processed further with PCR amplification. Incomplete circles are broken down.

The next step is the addition of exonucleases to each vial which will digest linear DNA such as the incomplete ring structure, unbound probes and gDNA. The ring structure is, however, resistant to exonuclease digestion and will thus be conserved and enriched for in downstream analyses. Following steps include cleavage of the ring into a linear form which can be amplified by PCR. The distinct tag sequence associated with each SNP is then used as a molecular barcode, representing the specific gDNA segment, which is specialized for hybridisation to the microarray. For the OncoScan assay, two different microarrays are used, one for each nucleotide pool. While on the microarray, the tag sequence hybridises to a specific spot, and emits a fluorescent signal which can be detected by the scanner.[391,392]

Two main metrics for each SNP loci obtained from the OncoScan assay are used in downstream analyses, the *log2-ratio* ($\log_2$R) and *B-allele frequency* (BAF)[393,394]. The $\log_2$R is calculated by comparing the combined intensity values for each SNP from the two arrays to the reference sample (equation *i*). A $\log_2$R value of 0 indicates the same copy number as the reference (often diploid, 1+1), whereas positive and negative values refer to copy number gains and losses, respectively. On the contrary, the BAF represents the proportion of the non-reference allele, B-allele, in a specific SNP position and is calculated by dividing the intensity of the B-allele to the total intensity values from all alleles (equation *ii*). Basically, this measure describes the relative number of one allele compared to the other. Heterozygous SNPs within a diploid segment (A+B) thus have a BAF value of 0.5, whereas homozygous SNPs can either have a BAF value of 0 (B+B) or 1 (A+A). In addition, the BAF values will also vary depending on the presence of CNAs which, thus, enables other combinations of allelic compositions.

(i) $$log_2R = log_2\left(\frac{A/T\ signal + G/C\ signal}{Reference\ signal}\right)$$

(ii) $$BAF = \frac{B\ allele\ intensity}{A\ allele\ intensity + B\ allele\ intensity}$$

When analysing bulk DNA from a tumour mass, the $\log_2$R and BAF values are also most often affected by the presence of normal cells. Of note, the deviation of the $\log_2$R and BAF values from the expected values, which assumes 100% tumour cell content, can further be utilized to calculate the *tumour cell fraction* (TCF) of each sample[394]. The $\log_2$R and BAF values will also vary depending on the presence of tumour heterogeneity.

Potential and hurdles with tumour tissue analyses

As previously stated, the tumour tissue analysed within the studies of this thesis were all collected from FFPE specimens, both newly processed and archival. The FFPE sample types included resected primary tumours and surrounding lymph node metastases (**paper I, III** and **IV**), diagnostic or excision biopsies (**paper III** and **IV**) and postmortem collected tissue (**paper III** and **IV**). As discussed, the FFPE process of tissue has a negative impact on the quality of DNA, and this effect is further enhanced over time[395]. Thus, for the archival FFPE tissue from the retrospective cohort used in **paper I**, some of the extracted DNA samples were excluded from downstream analyses due to their insufficient quality for adequate library construction prior to NGS.

Tumour purity

The high stromal content of PDAC poses a particular challenge for acquiring samples with a sufficient proportion of tumour cells, i.e. a high *tumour purity* or TCF. This is important for adequate genetic analyses, and especially for heterogeneity studies, as DNA from normal cells dilute the signal from tumour cells. This increases the risk for *type II errors*, i.e. false negative results. To overcome this, one should utilize methods which aim to enrich for areas with a high tumour purity. For the studies in this thesis, histology-guided manual acquisition of FFPE tissue cores in particularly tumour rich areas were performed after examination by a senior pathologist. Other available dissection methods include for instance laser capture protocols, which have shown promise in obtaining high purity samples from PDAC all the way down to the single cell level[396].

Postmortem effects

In **paper III** and **IV**, tumour specimens obtained at autopsy, by comprehensive multisite tissue collection, were used to enable downstream heterogeneity analyses of the terminal disease burden. The general notion about post-mortem effects on tissue quality is that an increased time between time of death and tissue collection, the *postmortem interval* (PMI), has a negative impact. However, although true in general, this has only been sparsely studied and, in fact, studies show that even with a PMI of 48h, tissue integrity may still be sufficient for nucleic acid studies, especially in regard to DNA[397]. This period has also been referred to as the "*twilight of death*", referring to the fact that different tissues and cells may still function, to various extents, during this time[398]. Of note, the fact that cardiopulmonary arrest does not directly trigger death of all cells in the body have laid the groundwork for the possibility of organ donation and the development of xenograft models.

It should, however, be noted that in the specific case of pancreatic tissue, autolytical processes leading to tissue degradation have been shown to be particularly rapid due to the release of digestive enzymes[399,400]. Thus, within the CHAMP study, the aim is to

perform all autopsies within a PMI of 48h, and the majority are carried out within 24h. Of note, and importance, tumour tissue has been shown to be more resistant to autolysis compared to matched normal tissue in the same patient, further spurring the performance of research autopsies for studying cancer[401]. In addition, it has been proposed that an increased proportion of stroma may have a preservative effect[402].

Exploring the spatial genetic heterogeneity

In an ideal situation, with high TCF and DNA quality, WGS may provide an adequate complete profile of both small and large-scale genetic alterations in the tumour. However, fragmented and low-quality DNA in combination with low purity poses various challenges for WGS. For instance, degraded DNA constitutes a risk of multi-mapping and uneven genome coverage, i.e. where some regions get sequenced with higher coverage than others. Nevertheless, recent research has shown a strong correlation between the number of SNVs detected by WGS from matched FF and FFPE tissue, with an overall concordance of 71%[403]. Less consistency was however observed for copy number profiles, as fluctuations in coverage for the FFPE samples complicated the analysis[403]. Moreover, to detect low frequency variants, as in cases with limited tumour purity, an increased sequencing depth is often required, which can lead to exceedingly high costs when sequencing the entire genome.

With the above-mentioned hurdles in mind, the below sections will briefly go through the different choices made for small-scale and large-scale DNA mutations, respectively.

Somatic small-scale variants and downstream analyses

To evaluate the presence of SNVs and InDels, TDS represents a common choice for FFPE tumour tissue, by enabling deeper sequencing at a fair cost[404]. This, together with the known limited mutation burden of PDAC[59], spurred the use of TDS for analyses of small-scale alterations throughout this thesis. Both commercial and in-house methods were utilized, with similar target profiles including coding regions of more than 300 genes. Overall coverage for the tumour tissue analyses in the studies were ~800x, facilitating the detection of variants down to VAFs of approximately 1%.

No matter the sequencing analysis, a tail of low frequency variants with uncertain significance almost always poses difficulties for downstream interpretation. Some of them might be true subclonal genetic alterations in the tumour, while others may just be artifacts, such as technical mistakes created during sequencing. Hence, it can be time-consuming to distinguish the true variants from false positives, as it is complicated to find the perfect balance between sensitive detection of tumour heterogeneity and removal of false positive variants. These technical artifacts may, however, be detected and filtered away by manual curation using the software *Integrative Genomics Viewer* (IGV)[405]. Another important aspect is to always include a matched normal sample from

each patient to filter out germline variants. In **paper I**, adjacent normal pancreatic tissue was used as normal controls, and in **paper III** and **IV**, DNA from circulating white blood cells was primarily used.

Lastly, in heterogeneity analyses, when a detected variant is not shared between all samples from a patient, it is of great importance to ensure that the position of the variant has proper sequencing coverage in *all* tumour samples to prevent the introduction of false heterogeneity. Along the same line, samples with too low tumour purity are not suited for heterogeneity studies as it is impossible to deduce if the absence of a subclonal variant is due to true heterogeneity or simply an effect of the low TCF. Thus, in **paper I** and **IV**, all sequenced variants were manually curated across all samples to validate their presence or absence, and tumour samples with insufficient tumour purity were removed from downstream phylogenetic analyses.

Copy number alterations and tumour heterogeneity

Due to the challenges of detecting high quality genome-wide copy number profiles from FFPE material by sequencing approaches such as WGS or WES, CNAs in **paper I** and **IV** were analysed using the OncoScan SNP array assay. The MIP technology in the OncoScan assay has been proven to be particularly suitable for degraded FFPE DNA, as it does not amplify the DNA fragments themselves, but rather the tag sequence[392].This was followed by careful evaluation of CNA breakpoints, as the presence or absence of a specific CNA laid the foundation for the ancestral tumour relationships, as described in the section below. It is, however, possible to analyse CNAs across the genome with targeted panels as well, as performed in **paper III**, although with an inferior resolution.

To evaluate the CNAs following the SNP array analysis, three main software were used: the *Chromosome Analysis Suite* (ChAS), for raw data analysis, and the *Nexus Copy Number* software together with the *Tumor Aberration Prediction Suite* (TAPS)[406] for assessment of allelic composition, clonality and ploidy level. Nexus and TAPS both utilize combined information from the $\log_2$R and BAF values to enable visualization and evaluation of all CNAs. Thereafter, the presence and allelic composition of each CNA were manually explored across all samples, based on thoroughly defined break points. By this method, it was possible to detect both similarities and heterogeneity between individual tumour regions.

Inferring evolutionary routes of cancer

Clonal deconvolution – detangling the subclonal landscape

Deducing genetically distinct *subclones* from *bulk* DNA sequencing poses several challenges. First, bulk sequencing results in a combined signal from thousands of cells

in one single sample. In addition, each individual tumour sample may contain various distinct subclones, detected at specific proportions, and the total tumour fraction most often varies as well. It is therefore common practice to perform a *clonal deconvolution* prior to phylogenetic analysis. Clonal deconvolution refers to the procedure where data, in this case specific genetic alterations and their frequencies, from individual tumour samples is exploited to infer which subclones that are present in each tumour region. This information can then be used to infer ancestral relationships between the detected subclones by phylogenetic reconstruction (**Figure 23**).

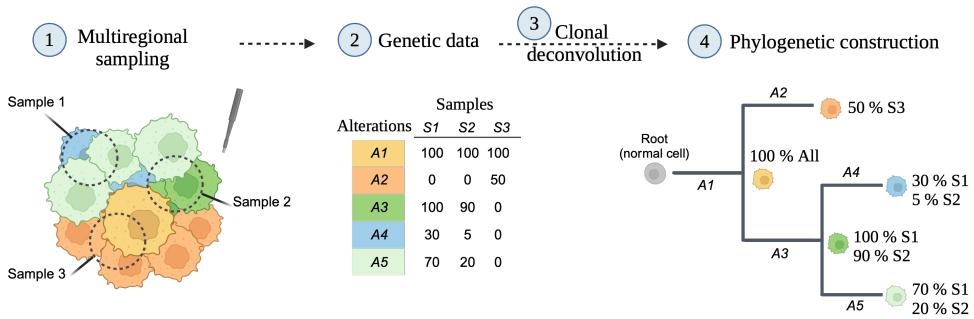


Figure 23. Schematic overview from tumour sampling to phylogenetic reconstruction. 1) Multiregional sampling of tumour tissue before bulk DNA sequencing according to Andersson et al[67]. 2) Formation of a matrix containing all samples and the percentages of cells having a specific alteration within each sample. 3) Clonal deconvolution and 4) phylogenetic reconstruction of detected subclones.

In **paper I** and **IV**, the subclonal landscape and subsequent phylogenies were inferred from tumour samples collected across both space and time. To facilitate clonal deconvolution, a crucial part of these studies was to estimate the tumour purity of each sample and then the proportion of tumour cells harbouring a specific genetic alteration, i.e. the *clone size*. For CNAs, this was done by utilizing either the $\log_2$R or BAF values, depending on the CNA type, and for TDS variants, VAF values were employed together with the allelic composition (copy number state) of that particular genomic locus.

Clonal deconvolution was then performed using the algorithm *DEVOLUTION*[67]. This is one of several mathematical tools that can infer evolutionary relationships between subclones, although many do not include both deconvolution and phylogenetic analysis in the same software[407], which is one of the strengths with DEVOLUTION. Another advantage, especially for tumours such as PDAC which are known to harbour complex chromosomal aberrations[285], is that it is not limited to only considering small-scale sequencing data.

The DEVOLUTION workflow starts with clonal deconvolution and the formation of an *event matrix*, containing the identified subclones along with the genetic alterations defining them. This is performed by clustering genetic information from the input *segmentfile*, containing the clone size, genomic position, and type, for the detected alterations in each sample. In **paper I**, due to overall low TCFs, and hence decreased resolution, the proportion of each genetic alteration in the tumour samples was dichotomized and annotated as either "clonal" or "subclonal" to minimize the introduction of false heterogeneity.

Phylogenetic reconstruction

In **paper I**, DEVOLUTION was also used for phylogenetic tree construction. By taking the created event matrix as input, it utilizes both the *maximum parsimony* (MP) and the *maximum likelihood* (ML) method to construct two phylogenies based on the detected subclones. The MP method tries to find the *simplest* phylogenetic solution, i.e. the tree with the lowest number of changes that can explain the input data, whereas the ML method tries to identify the most likely evolutionary tree[408]. The ML method was chosen for phylogenetic construction in **paper I**. It should, however, be mentioned that the MP and ML solutions often demonstrated a high degree of concordance throughout the study.

The evolutionary trees in **paper IV**, on the other hand, were created with a third algorithm, the *modified maximum parsimony* (MMP) method[409]. The MMP method, like MP, aims to find the simplest tree solution but, in addition, also focuses on limiting the introduction of back mutations and may take in information about specific alterations that are more likely to occur in parallel. This was particularly suitable for phylogenetic construction of subclones detected at the different tissue regions post-mortem, as they showed a highly complex chromosomal landscape which was challenging to detangle with the MP or ML method.

Evolutionary complexity assessment

To describe phylogenetic biodiversity, different measures can be used such as phylogenetic richness, divergence and regularity[409,410], or more elementary metrics such as stem length or the number of subclones. Nevertheless, to describe the visual phylogenetic complexity observed between the phylogenies produced in **paper I**, we developed a new combined complexity score, built on previous methods for decision trees[411]. This scoring system was specifically designed to distinguish the trees based on their network architecture, as exemplified in **Figure 24**, and is described in detail in the original paper. Taken together, the newly developed complexity score may be viewed as a multiplicative and additively combined summary of the above-mentioned three established biodiversity metrics.

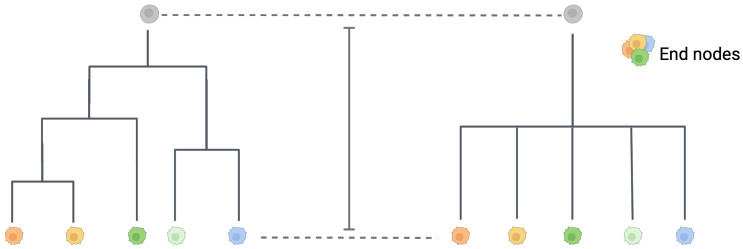


Figure 24. Phylogenetic network attributes. Two phylogenies of the same size and number of subclones (end nodes), although with different diversity characteristics, i.e. branching structures.

Evaluation of the immune microenvironment

Owing to their known influence on tumour progression, infiltrating IC populations were evaluated and correlated to the spatial genetic heterogeneity in the various tumour areas in **paper I**. This was performed by utilizing the TMA technique to create *single patient tissue chips* (SPTCs), i.e. individualized tissue libraries for each patient. To enable comparisons between the TME and genetic profiles, three adjacent FFPE tissue cores from each tumour cell enriched region were obtained, where two were added to the SPTC block, and one was utilized for DNA extraction. Of note, additional tumour cores were also collected from tumour adjacent stroma. Infiltrating ICs were then investigated by staining for specific cell markers, such as *cell surface* proteins, using *immunohistochemistry* (IHC) as described below.

Immunohistochemistry

Tissue analysis using IHC is one of the most commonly used analytical methods for investigating the presence of specific antigens, and it is an important complement to clinical pathology assessment and biomarker studies[412]. Antigens may for instance be protein cell surface markers or molecules inside the cell, such as in the cytoplasm or nucleus. It is thus of great importance to have prior knowledge of how the specific protein of interest is distributed and expressed in the cell prior to analysis to ensure a correct interpretation. The antigens are detected by the binding of specific *antibodies*, which may either be *monoclonal* or *polyclonal*, i.e. binding to either just one or several epitopes of the antigen, respectively[413]. However, no matter the type, it is crucial to validate antibody specificity and selectivity prior to usage to ensure correct and consistent results[414].

The most widely used material for IHC analyses are FFPE specimens. However, prior to IHC staining, the tissue slides must be pre-treated in a stepwise process. First the paraffin is removed, followed by tissue rehydration, and then, crosslinks created during formalin fixation are resolved by heat induction to enable access to the antigens, also

called *antigen retrieval*. After this, the tissue specimen is often counterstained with haematoxylin, to visualise the cell nucleus, before applying a solution containing the *primary* antibody, which binds to the specific antigen (**Figure 25**). For visualisation, a second antibody, tagged with an enzyme called *horseradish peroxidase* (HRP) is then added, which binds to the first antibody. Finally, a chromogenic substrate is added to the slide, such as 3,3'diaminobenzidine (DAB), which reacts with the HRP enzyme and produces a brown precipitate at the specific antigen site which can be detected by microscopic evaluation.

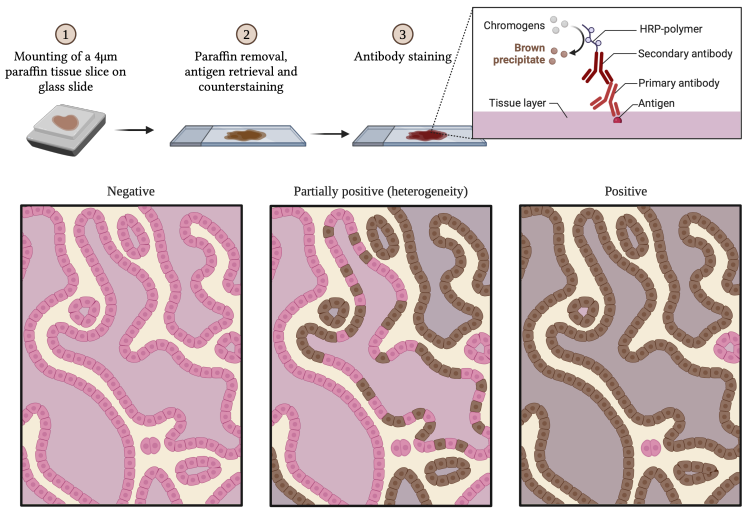


Figure 25. Immunohistochemistry. Overview of the general workflow for immunohistochemistry staining of FFPE tissue. **1**) A thin paraffin slice is obtained from the FFPE tissue block and mounted on a glass slide. **2**) The slide is then processed to remove the paraffin and rehydrate the tissue, followed by haematoxylin counterstaining. **3**) Primary and secondary antbodies are added in a stepwise process. Chromogens are then added which react with the horseradish peroxidase (HRP) and forms a brown precipitate at the antigen location. The three visual illustrations in the lower panel show negative, partial and positive staining, from left to right.

Investigating molecular heterogeneity

Utilizing TMAs for studies of heterogeneity comes with both benefits and drawbacks. The rapid high-throughput histology assessment, together with effective usage of reagents and tumour tissue, account for some of its advantages. In addition, staining all tissue areas at once, and on the same slide, minimizes the introduction of false heterogeneity due to batch effects. However, due to the relatively small area of each tumour core (1 mm for studies in this thesis), the technique has been criticised for not providing a representative image of the distribution of a particular protein expression in the tumour as a whole. This has, however, been extensively evaluated, and the degree of molecular heterogeneity detected by utilizing TMAs has shown a high overall

concordance with whole tissue sections[379,415]. Yet, to exercise extra caution, two cores were obtained from each tumour region in **paper I**, further addressing the raised concerns.

There are also other important aspects to consider during the manual evaluation of each IHC staining. For example, the observer should be blinded from outcome data such as patient survival, to avoid confounding the results. In addition, in **paper I**, each staining was reviewed by two observers, including one senior pathologist, to minimize the risk of "diagnostic drift", i.e. the gradual change in interpretation over time[416]. Another issue is that several different cell types may express the same protein, an example being PD-L1 which can be expressed on the cell surface of both different IC populations and tumour cells. Thus, prior knowledge of cell morphology and cell expression patterns is crucial for proper interpretation and heterogeneity assessment. This may, however, partially be aided by staining for several markers at once. Along that line, two markers were always evaluated together throughout the first study, referred to as *double* IHC staining, to enable IC subtyping of for instance different T cell and NK cell populations.

Moreover, quantitative assessment of a positive staining can be done by various approaches such as the exact cell count, percentage or intensity. For example, when evaluating cells with an overall low tissue abundancy, it could be suitable to count the exact number of cells. On the other hand, for the presence of a specific subpopulation, like PD-L1 positive leukocytes, it may be more appropriate to report this as a percentage of the total number of detected leukocytes. The different markers and ICs investigated by IHC analysis in **paper I** may be found in the original paper along with the individual scoring systems, adapted according to the overall abundance of the specific IC subtype. In addition, each IC population was further assessed in the context of its proximity to the tumour nests.

Blood-based monitoring of the systemic tumour burden

Clinical blood sampling and isolation of analytes

Collection and pre-analytical processing of whole blood for the analyses in **paper III** and **IV** was performed by research nurses in conjunction with patent treatment visits at the hospital (**Figure 26**). The blood was collected in both serum tubes, without any additives, and tubes coated with *ethylenediaminetetraacetic acid* (EDTA), acting as an anticoagulant. The choice of collection tube is important, especially for downstream cfDNA analyses, as some additives may be detrimental for subsequent sequencing or PCR analyses, e.g. *heparin*[86,417]. One of the main concerns involves leakage of gDNA from leukocytes into the plasma, which may dilute the proportion of cfDNA in the following analyses. Serum may also be used for isolation of cfDNA, although it has

been shown to yield less sensitive and robust results compared to plasma-derived cfDNA[417]. Thus, in the CHAMP stuy, plasma is used for subsequent isolation of cfDNA, and serum has so far mainly been used for protein analyses.

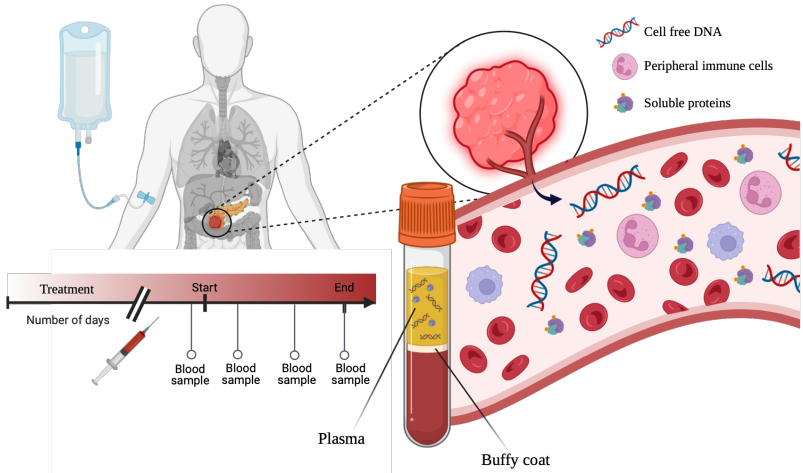


Figure 26. Blood sampling overview. An illustration of on-treatment blood sampling in the CHAMP study and blood analytes such as cell free DNA, immune cells and proteins.

Following centrifugation of the EDTA tubes, plasma and buffy coat were separated (**Figure 26**), and a second centrifugation was performed on the plasma fraction to remove possible cell debris and consequently minimize contamination of gDNA. In the beginning of the CHAMP study, however, the second centrifugation was not included in the laboratory procedure prior to storage. Hence, for some of the initial samples of the study, this was instead performed immediately after sample thawing. Of importance, size distribution analyses of these samples still showed comparably low percentages of gDNA and thus adequate quality for subsequent analyses, as also shown by others[418]. Size distribution analyses were carried out using automated electrophoresis which enables both a total quantification of the cfDNA and provides the proportion of gDNA present in the sample.

To also enable downstream analyses of viable circulating immune cells, the buffy coat was diluted in a freezing solution (ratio 1:10) containing cryoprotective dimethyl sulfoxide (DMSO), which reduces the formation of ice crystals that otherwise may damage, or in worst case, kill the cells. Thus, to ensure cell viability, the 1:10 ratio is crucial. Buffy coat may also serve as a source for normal control DNA, which was utilized in **paper III** and **IV** for comparisons between germline and somatic variants and for detection of variants associated with clonal haematopoiesis.

Parameters impacting quantity and quality of cfDNA

Besides the choice of collection tube and isolation protocol, several other parameters should also be considered when performing experiments on cfDNA. For instance, processing delays, such as that between blood draw and cfDNA isolation, or incorrect storage, have both been shown to increase contamination of gDNA and, thus, impact sample quality[419,420]. Another important aspect is the available plasma volume. The low levels of cfDNA in the blood, together with an often sparse ctDNA fraction, as in PDAC[118], demands a certain volume of starting material to maximize the chances of detecting low frequency variants. Thus, to reach a sufficient yield of cfDNA for downstream NGS approaches in cancer patients, it is preferable to use at least 5 ml of plasma for cfDNA isolation[421].

Lastly, shedding of cfDNA into the blood stream also varies over time. The total cfDNA plasma concentration has for instance been observed to decline during the day, with the lowest levels in the evening, and exercise has been shown to increase the cfDNA yield[422,423]. However, although this should be kept in mind from a ctDNA perspective, finding the optimal time of day for a blood draw would most likely pose a practical challenge, as this is most often performed in conjunction with hospital visits such as treatment administration at specific times.

Development of the CHAMP ctDNA panel

The choice of method for ctDNA detection in plasma is often based on balancing breadth with depth. At one end, ddPCR or ultra-deep sequencing approaches, focusing on only a few distinct genetic targets, provide remarkably sensitive assays for ctDNA detection[86,160-162]. At the other end, often with limited sensitivity due to rising costs, broad targeting sequencing and WES have shown clinical utility by enabling identification of genetic variants which might qualify patients for precision medicine[146,147,293]. These aspects set the stage for the ctDNA sequencing approach that was developed within the frames of the CHAMP study, and this thesis.

Hence, the *CHAMP panel*, used for detecting ctDNA before and during treatment in **paper III** and **IV**, was designed as a middle way between the two above-mentioned extremes. It is a cost-effective focused panel, including relevant genes for precision medicine, but at the same time aiming to provide a sensitivity down to 0.1 % VAFs by ultra-deep sequencing. The rationale behind the choice of genome coverage was guided by focusing on the vast majority of patients who are diagnosed with metastatic PDAC, for whom ctDNA levels have been shown to be increased compared to patients with operable disease[361]. Thus, although ctDNA fractions are still mainly found to be below 1%, these data suggest a window of opportunity for detection of selected clinically relevant targets with a sensitive-*enough* assay. Additionally, but of great importance, such a method could also be of particular value in advanced cases whenever core needle biopsies are not feasible.

The CHAMP panel includes two separate probe pools (**Figure 27**), where the first includes the complete coding regions of 22 selected genes (106 kb), along with common *KRAS* hotspots (e.g. G12, G13 and Q61). The genes were chosen based on either their high prevalence in PDAC[272], implications for precision medicine, or their status as known tumour-agnostic driver genes. The other pool (140 kb) includes probes targeting 1130 SNP positions across six chromosomes, namely chromosome 3, 7, 8, 9, 17 and 18. The aim of the SNP backbone is to enable the detection of CNAs and allelic imbalances as previous research, along with the results from **paper I**, have highlighted the importance and high prevalence of CNAs in PDAC development[271,285,290].

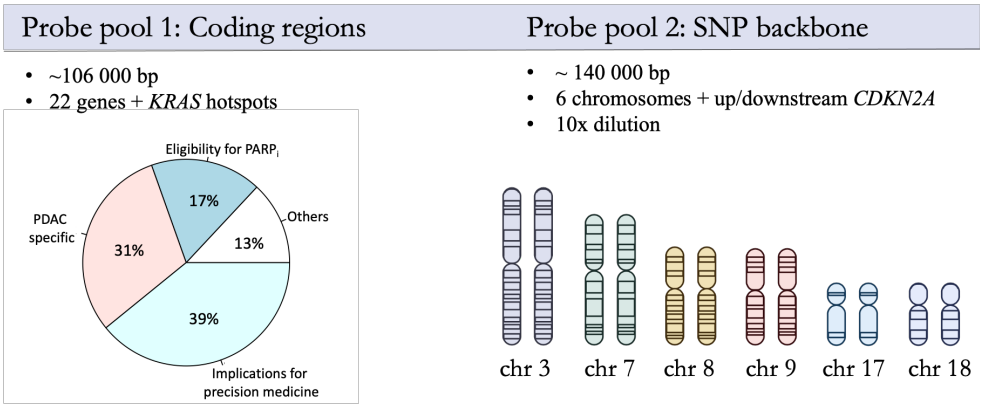


Figure 27. The CHAMP panel. An overview of the CHAMP panel design, divided into two different probe pools.

Acquiring copy number data from ctDNA using targeted sequencing has previously been shown to be feasible by others, although the most common approach includes WES or WGS[122,424,425]. In addition, probe pool two also includes probes targeting regions up and downstream of the *CDKN2A* gene to enhance detection of the frequently observed homozygous deletions in this area. However, to keep the cost down, probe panel two was diluted 10-fold compared to probe pool one. The CHAMP panel also included molecular *error suppression* to further enhance the sensitivity, which is described in detail below.

Suppression of sequencing errors using UMIs

During genetic sequencing, different biases are often introduced, for instance during the PCR amplification step and subsequent sequencing[426]. These biases include PCR *duplications*, which may result in overrepresentation of distinct DNA fragments in the final library and, thus, skew downstream analyses. To confidently adjust for the varying representation of specific DNA molecules, different methods, such as incorporation of *unique molecular identifiers* (UMIs), are often used. UMIs are short oligonucleotides

that are added to DNA fragments prior to the PCR amplification step, and may, hence, serve as molecular barcodes during sequencing to keep track of fragments originating from the same DNA molecule[427].

Moreover, mistakes during the PCR amplification and sequencing steps are common, resulting in the introduction of false positive variants in the final sequencing read. Of importance, the error rate is elevated by additional PCR cycles and increased sequencing depth, such as in the case of cfDNA sequencing, as more duplicates are produced. Thus, distinguishing these technical false positives from true genetic variants is crucial to increase variant detection sensitivity, and for this, information from UMIs may provide enhanced error suppression (**Figure 28**)[428].

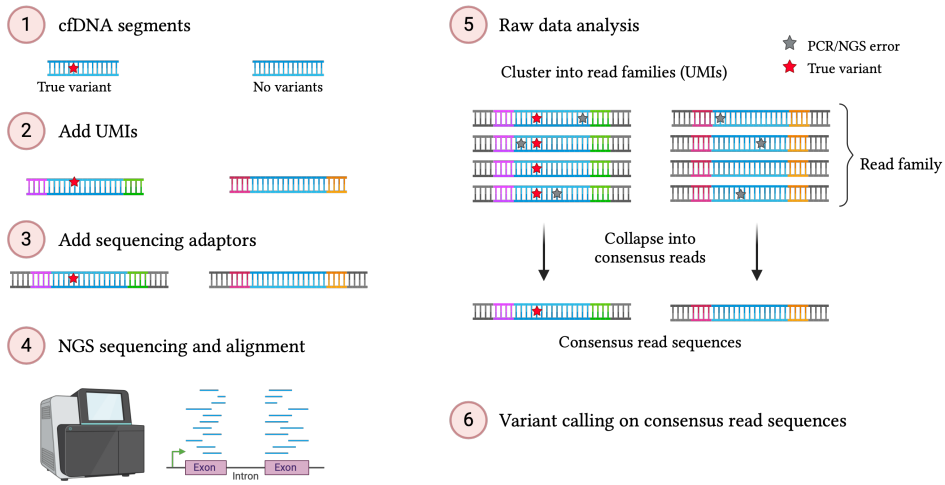


Figure 28. Utilization of UMIs during sequencing. Schematic overview of sequencing using incorporation of unique molecular identifiers (UMIs) and downstream data analysis.

During bioinformatic processing of the raw sequencing data, reads with matching UMIs, i.e. duplicates, are clustered together into *read families*[428]. These are then collapsed into a final *consensus read sequence*, containing only variants which were detected in all individual reads, and, thus, technical errors are removed[428].

Exploring the host response during chemotherapy

Although immune players in the TME have been widely explored, less is known about the systemic immune response in PDAC. In an attempt to address this, analyses of host immune response dynamics, before and during chemotherapy, were performed in **paper III**.

Investigating circulating immune cells by flow cytometry

Different single cell methods are available for the evaluation of circulating immune cells, such as *flow cytometry*, *mass cytometry* (CyTOF) and *single cell RNA sequencing* (scRNAseq). They each come with different advantages and disadvantages, especially in terms of throughput, multiparametric capability and cost. The flow cytometry method, although comparably limited by spectral overlaps (described below), enables high throughput and multiparametric analyses at a reasonable cost, and was, thus, considered an adequate assay for the overarching focus of **paper III**.

The flow cytometry method enables the study of different molecular cell parameters in a suspension containing a heterogenous cell population[429]. By utilizing a specialized fluidics system, cells are passed through one or several laser beams, one by one, and the scattered light is measured and translated into metrics such as cell size, shape and degrees of granularity (**Figure 29**). Like the IHC procedure, different antibodies may also be used to stain for specific antigens present on the cell surface or within the cells, which are coupled with various fluorescent dyes. Hence, by utilizing multicolour panels, several cell types may be detected and quantified by their distinct emission wavelengths, in one and the same cell suspension, as they pass individually by the lasers[429,430].

As described in detail in **paper III**, two antibody panels were developed to allow for a broad overview of both various circulating IC populations (*general* panel) and different T cell subtypes (*T cell* panel). As for the IHC assessment, subsequent data analysis differed depending on the specific cell population. IC subtypes found by the general panel were reported as the percentage of the live total non-granulocytic cells, and the frequency of T cell subtypes were either presented as a percentage of the total live T cell population or the respective parent population. The analysis also included detection of checkpoint molecules such as PD-1 and PD-L1.

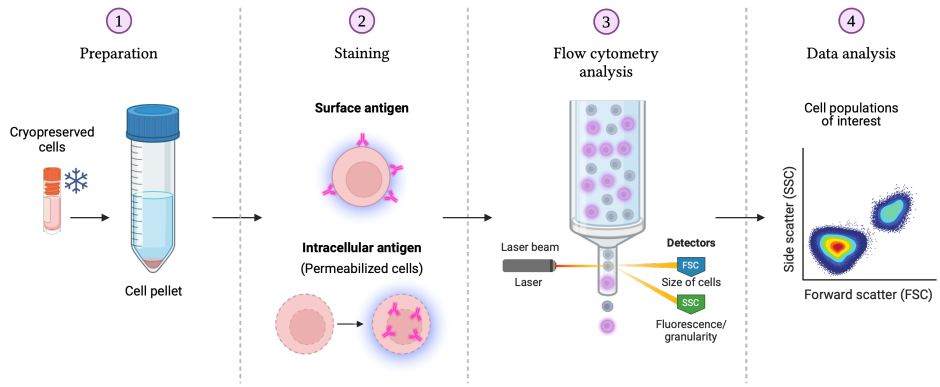


Figure 29. Flow cytometry. Schematic overview of the workflow for flow cytometry analysis, according to Maecker et al.[431], from **1**) cell suspension preparation, **2**) staining of antigens using antibodies, **3**) flow cytometry analysis and **4**) data analysis.

To successfully discern different cell populations by flow cytometry, it is important to carefully select appropriate antigen targets, but also to match antigen abundance with a suitable brightness of the antibody-coupled fluorophore[432]. In addition, each fluorophore comes with a specific emission range, which often poses a challenge when utilizing multicolour panels, as emission from one may spill into the detection of another. This phenomenon is also known as "*spillover*" and a *compensation* process is often required to correct for these overlaps[432]. The compensation process is highly important to adequately interpret the different cell signals and to conduct proper downstream data analyses.

The search for new circulating protein biomarkers

As a complement to the cell based immune analyses, circulating proteins known to be involved in tumour immunity, inflammation and tissue remodelling, amongst others, were also investigated in **paper III**. This was performed by utilizing a commercial panel targeting 92 soluble proteins, i.e. the *Olink Target 96 Immuno-Oncology assay*. The panel is based on a specific and sensitive high-throughput, multiplex, technology, i.e. the *proximity extension assay* (PEA)[433]. PEA utilizes antibody pairs which are tagged with unique complementing oligonucleotides. When the antibody pair binds to the specific protein antigen, it enables hybridization of the now adjacent oligonucleotides which forms a unique DNA tag sequence. This antigen specific DNA tagged is further amplified by qPCR and quantified[433].

Statistical considerations

Statistics are widely used for describing and comparing different types of data and for drawing conclusions about a population based on a sample. In order to ensure reliable and robust results, it is crucial to understand the nature of the specific data at hand and to choose an appropriate statistical method. Various tests and methodologies have been utilized throughout the studies of this thesis, and the specific tests can be found in each original paper (**I** and **III**).

In general, statistical tests are often applied to evaluate whether there is a true difference between two or more groups, for instance comparing OS between two patient groups which have been given two distinct treatment options. The actual difference in OS between the groups are in this case referred to as the *effect*, and a *p-value* (ranging from 0 to 1) is often calculated to describe the probability of the results to be caused solely by chance. In the field of medicine, a threshold of 0.05 ($p < 0.05$) is often used, stating that the likelihood for the observed difference to be a *false positive* (type I error) is less than 5%. The opposite, a *false negative* (type II error) refers to the presence of a true difference which is missed, for instance due to small sample sizes affecting the statistical power. It should, however, be noted that a *significant* difference, i.e. a difference found

with a p-value below the set threshold, should not be mistaken for a *clinically relevant* difference, for which the specific effect must be taken into account as well.

Moreover, whenever comparing descriptive measures of different groups, as in the example above, there are two main choices of methods to use. If your data follows a normal distribution, which is often the case with sufficiently large sample sizes, a *parametric* test is the most suitable choice. However, for smaller sample sizes, as in the case of all patient cohorts used throughout the studies in this thesis, *non-parametric* tests are preferred. These tests are in comparison not as powerful as parametric tests but are on the other hand less sensitive to outliers.

A common method used for examining how an outcome of interest is influenced by the change in one or several variables, is *regression* analysis. Various types of regression models, both univariable and multivariable, were used in **paper I** and **III** to evaluate relationships between different variables. In **paper III**, a regression analysis called the *Cox proportional hazards regression model* was used for survival analyses, which specifically utilizes time-to-event data under a certain time period. For instance, this method specializes in evaluating the effect of several predictor variables on the time until an event occurs, such as death or disease recurrence, and the output is the hazard ratio of each variable. Another survival analysis used in **paper III**, representing perhaps the most common in medical research, is the *Kaplan Meier* method. This analysis also utilizes time-to-event data and is particularly suitable whenever survival probabilities are to be compared between different groups.

Furthermore, a recurrent problem in medical research is the risk that comes whenever a large number of comparisons, or tests, are performed within the same data set. This is also referred to as *multiple testing,* and states that for each test performed, the likelihood of detecting a false positive increases. For instance, if twenty tests are performed, a significance threshold of 0.05 means that one of these results will be significant just due to random chance. This should, hence, always be taken into account and be adjusted for, as performed by different approaches in **paper I** and **III**.

In conclusion, statistical models are useful and important tools in the field of medicine, as well as in the studies of this thesis, as long as one knows *how* and *when* to utilize them.

Results in brief

A summary of the main results generated in each of the respective **papers I, III and IV** is provided below, together with a status update on the CHAMP study outlined in **paper II**. Further details are provided in the original papers.

Paper I

Branching copy number evolution and parallel immune profiles across the regional tumour space of resected pancreatic cancer

Study outline

The scarcity of biomarkers able to predict the risk of recurrence for patients with resectable PDAC, following surgery and adjuvant chemotherapy, prompted the investigation in **paper I** (**Figure 30**). Multiregional sampling and detailed genetic analyses of the primary tumour (35 samples) and adjacent lymph node metastases (five samples) were performed for nine patients with operable disease. In addition, parallel spatial analyses on ICs present in the surrounding TME were explored.

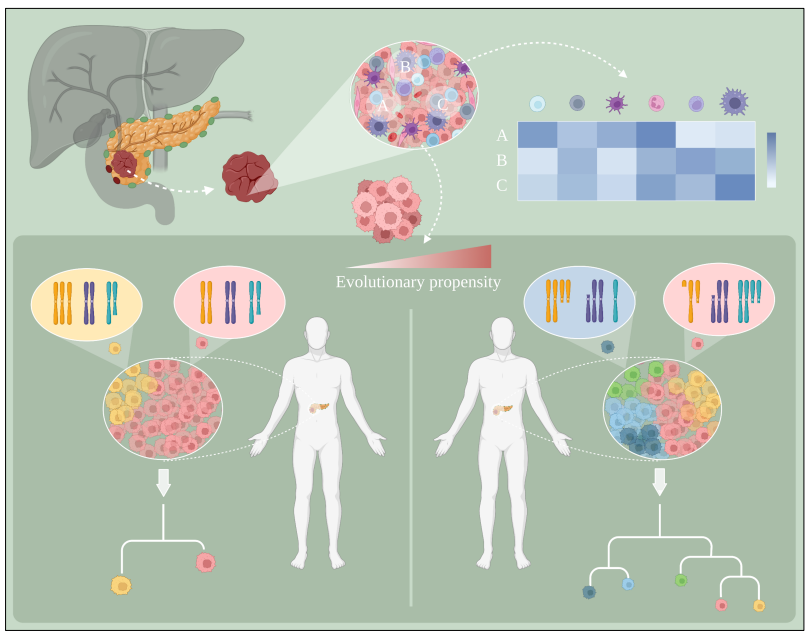


Figure 30. Graphical abstract of paper I. Visual overview of the main findings in paper I, adapted from Petersson et al.[434] which is licensed under CC BY-NC-ND 4.0 (https://creativecommons.org/licenses/by-nc-nd/4.0/).

The overall CNA burden and detected driver events

A wide discrepancy in CNA burden was detected between the nine regional tumour areas. However, common features such as a heterozygous loss of genomic regions covering *TP53* on 17p and *SMAD4* on 18q were detected across all patients, and homozygous deletions of *CDKN2A* were found in three patients. Genes frequently altered by SNVs and InDels included *KRAS* (9/9 patients), *TP53* (8/9 patients), *SMAD4* (5/9 patients) and *CDKN2A* (2/9 patients), also known to be early events in PDAC evolution. Additional potential driver events included variants in the *RNF43*, *GNAS* and *dihydropyrimidine dehydrogenase* (*DPYD*) genes, and amplifications covering the *MYC* oncogene. Several of the tumours also showed signs of early WGD events, resulting in a tetraploid genomic state.

Phylogenetic analysis depicting notable heterogeneity of CNAs

Following clonal deconvolution and phylogenetic analyses of the genetic subclones, inferred by all detected alterations, heterogeneity was seen in all tumours. Moreover, while only a sparse heterogeneity was detected for variants in the above-mentioned driver genes, CNA diversity was found to constitute the branching framework of the five phylogenies. By an in-depth evaluation of each phylogenetic structure, both early and late branching events from the trunk were observed, and all trees except one demonstrated different degrees of both linear and branching evolution. Metastatic dissemination to regional lymph nodes, analysed whenever applicable, was detected as an early evolutionary event for most tumours (3/4 cases). No signs of positive selection of specific CNA were found, although loss of 8p was exclusively found in patients with shorter OS (< 18 months).

The prognostic value of phylogenetic complexity

To systematically evaluate differences in the overarching branching frameworks, a networked based formula was derived and used to calculate a complexity score for each phylogeny. Subsequent comparisons between branching complexity and OS showed a significant association with higher evolutionary complexity, indicating a shorter OS. This was also true in multivariable analysis together with a combined score for vascular invasion.

Spatial analyses of the surrounding immune TME

To explore whether an increased genetic heterogeneity was mirrored by a greater diversity in infiltrating ICs in the TME, ten distinct IC populations together with the presence of B cell aggregates and the expression of PD-L1 on both ICs and tumour cells were analysed. Although no significant association was found between genetic heterogeneity and spatial IC diversity, evident interpatient discrepancies were detected. However, an interesting and hypothesis-generating finding was the association between a high overall CNA burden and increased PD-L1 expression on tumour cells.

Paper II

Chemotherapy, Host response and Molecular Dynamics in Periampullary cancer: the CHAMP Study

From start to present

Since the start of the CHAMP study in 2018, a total of 180 patients has been enrolled (30th of June 2024) as displayed in **Figure 31**. The study early expanded to not only include patients treated at Skåne University Hospital in Malmö, but also in Lund. Two ethical amendments have also been approved along the way, including the autopsy study, approval for increased blood sampling volumes and the continuation of the study beyond 150 participants. Since 2021, autopsies have been performed on 27 study participants. Moreover, pancreatic tumour origin has been confirmed by histopathology, or estimated by radiology, in all but seven patients, three with distal cholangiocarcinomas and four with ampullary carcinomas.

As further demonstrated in **Figure 31**, 126 patients within the study have been, or are being, treated with palliative intent and 49 have undergone surgery followed by adjuvant chemotherapy. At this moment, five patients included in the study are also undergoing neoadjuvant chemotherapy, for whom the treatment intention remains to be decided. In addition, 10 patients who were included in the CHAMP study when receiving adjuvant chemotherapy, have been included again upon receiving first-line palliative treatment for recurrent disease. The median age at diagnosis is 70 years, ranging from 38 to 83. Additionally, pathological germline variants have hitherto been found in eleven patients: two in mismatch repair genes *MutL protein homolog 1* (*MLH1*) and *MutS homolog 2* (*MSH2*), hence confirming Lynch syndrome, four in *BRCA2*, one in *BRCA1*, three in *CDKN2A,* and one in *PALB2*. Of these, two cases with *BRCA2* mutations and one with a mutation in *CDKN2A*, were detected by the analyses performed within the CHAMP study (**paper III**), the others were known prior to diagnosis, or detected as part of the clinical workup.

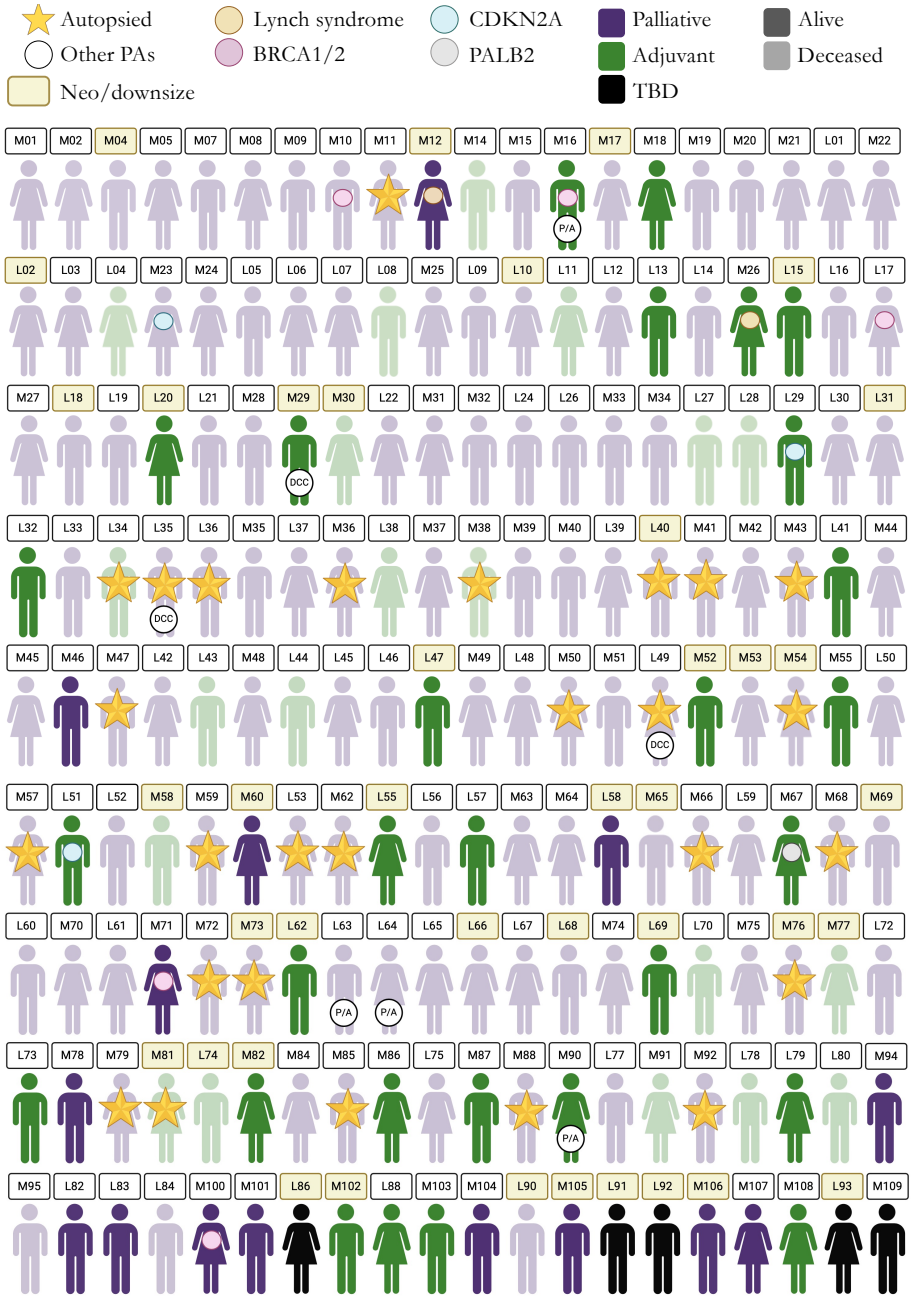


Figure 31. Patients of the CHAMP study. All patients enrolled in the CHAMP study until June 30, 2024. Patients are noted according to inclusion in the autopsy study, tumour origin, treatment intention (palliative, adjuvant, neoadjuvant/downsizing), inherited syndromes, germline variants in PDAC predisposition genes, sex and vital status. Courtesy of Karin Jirström. Abbreviations: PA; periampullary, P/A; Ampulla of Vater, DCC; distal cholangiocarcinoma, TBD; to be decided.

Paper III

Temporal dynamics of circulating tumour DNA and the systemic host response during chemotherapy in patients with newly diagnosed pancreatic cancer

Study outline

The constrained accessibility of tumour tissue from patients diagnosed with PDAC in general, and the inherent challenge of performing repeat biopsies during chemotherapy to evaluate tumour evolution in particular, spurred the investigation in **paper III**. In a total number of 201 blood samples from 60 CHAMP patients, collected before and during treatment, we explored ctDNA dynamics, 92 soluble proteins relevant for immune-oncology (196/201 samples), and circulating immune cell populations (160/201 samples). For 34 patients, genetic tumour tissue analyses were also included for variant comparison.

The multifaced nature of PDAC

A detailed review of patient demographics showed considerable disparity within the cohort considering *KRAS* status, detected pathological germline variants, metastatic burden, and OS, reflecting the complex nature of the disease. Moreover, initial analyses showed that palliative patients displayed an increased ctDNA burden before start of treatment at baseline (BL), compared to adjuvant patients. Significant differences between the groups were also seen for levels of CA19-9, *carcinoembryonic antigen* (CEA), and *C reactive protein* (CRP), which were all observed to be higher in palliative patients.

Prognostic value of the absolute tumour burden in palliative patients

In subsequent ctDNA evaluations, primarily focusing on palliative patients, we compared the dichotomized prognostic value of ctDNA detection (positive versus negative) in plasma at BL, as this had been shown to be of value in previous studies[359,367,435]. This was, however, not associated with OS in our cohort. Instead, we set out to investigate the prognostic relevance of the total number of tumour molecules per ml plasma, defined as *mutated genome equivalents* (mGEs). Comparisons of the estimated number of ctDNA molecules, at BL, to OS revealed a significant negative association. This was also true in multivariable analysis, including established prognostic factors such as metastatic stage at diagnosis (M0/M1). An optimal prognostic cutoff was further identified at BL (350 mGEs/ml plasma) and used for stratification of palliative patients into either a $ctDNA^{high}$ (n=15) or $ctDNA^{low}$ (n=28) group. Thereafter, the Kaplan-Meier analysis showed that patients in the $ctDNA^{low}$ group had a significantly increased OS compared to patients in the $ctDNA^{high}$ group (median OS 11.9 versus 3.7 months, $p < 0.0001$). Clinically relevant tumour characteristics were also compared between the two ctDNA groups at BL, revealing

higher total cfDNA levels, a higher frequency of metastatic disease, decreased levels of albumin, and increased concentrations of CA19-9, CEA and CRP, and a lower age median, in the ctDNA^{high} group. However, in multivariable analysis, including metastatic stage, age, regimen and performance status, the dichotomized ctDNA based stratification was the sole independent prognostic biomarker.

ctDNA monitoring in the ctDNA^{high} and ctDNA^{low} group

We further explored the potential clinical utility of ctDNA dynamics in disease monitoring within each ctDNA group. In general, detected variants in different samples from each patient showed limited temporal heterogeneity between the various sampling time points. In addition, an overall close coherence to the clinical disease course was observed, although this was primarily seen for patients in the ctDNA^{low} group. Evaluations comparing each change in ctDNA levels to the associated disease status further validated the prognostic cutoff, as ctDNA concentrations persisting or rising above it were associated with progression or death within three months for all cases.

ctDNA-derived variants and CNA profiles align with matched tumour tissue and add further value to the temporal genetic evolution

To further evaluate spatial and temporal heterogeneity, all genetic variants detected in plasma were further evaluated and compared to matched tumour tissue whenever possible. SNVs and Indels detected in ctDNA displayed a high overall concordance to the paired tumour tissue profile, although a few variants were exclusively detected in either plasma or tumour tissue in seven cases, thus reflecting genetic heterogeneity. Variants implying opportunities for precision medicine were also noted, such as a *KRAS^{G12C}* variant in one patient, for which there is a targeted treatment option available, i.e. sotorasib (KRAS-G12C inhibitor).

Whenever possible in terms of sufficient ctDNA fractions, assessment of genome-wide CNA profiles was also carried out by utilization of a novel bioinformatic tool[436]. Notably, although the sequencing panel used for ctDNA analyses only included SNP coverage for six chromosomes, CNAs could be detected throughout the whole genome and demonstrated a striking resemblance to matched tumour profiles. CNAs relevant for PDAC tumorigenesis were, hence, frequently detected even in cfDNA, such as amplifications covering *KRAS* and *MYC*, and deletions covering *CDKN2A*, *TP53* and *SMAD4*. In addition, for one patient, an amplification covering *KRAS* was exclusively detected in plasma after treatment, and not in the matched diagnostic biopsy or BL plasma sample, indicative of genetic evolution.

The prognostic value of MUC-16, CD20$^+$ B cells and HLA-DR$^+$CD8$^+$ T_c cells in palliative patients

Complementary analyses of the systemic immune response during treatment were further performed for all patients. Immune markers with significant changes over time were only detected in the ctDNA^{low} patient group and included *interferon gamma* (IFN-γ) and *CXC motif chemokine ligand 12* (CXCL12), amongst others. Biomarkers detected to be significantly associated with OS in palliative patients encompassed three serum proteins; *mucin 16* (MUC-16), *tumour necrosis factor related apoptosis inducing ligand* (TRAIL) and *C-X3-C motif chemokine ligand 1* (CX3CL1), and two IC populations; CD20$^+$ B cells and HLA-DR$^+$CD8$^+$ T_c cells, by multivariable time-varying regression analysis performed for proteins and ICs separately. However, when combined all together, only MUC-16, CD20$^+$ B cells and HLA-DR$^+$CD8$^+$ T_c cells remained statistically significant. This was also true when adjusting the analyses for ctDNA groups, age, performance status, treatment, and metastatic stage, indicating their independent, and complementary, prognostic values.

Patterns of covariation between systemic immune biomarkers, in comparison to cfDNA and ctDNA, and in different regimen contexts

Comparisons of dynamic on-treatment patterns between circulating immune biomarkers to cfDNA and ctDNA levels, and in different treatment regimens, were performed by utilization of *dynamic time warping*. Proteins and IC populations showing the closest fluctuation patterns to total levels of cfDNA and ctDNA were *hepatocyte growth factor* (HGF) and Vδ1$^+$ γδ T cells, and *nitric oxide synthase 3* (NOS3) and intermediate monocytes, respectively. Evaluations of general immune processes in different patient groups and according to treatment regimens were further performed by network analysis of the 50 top pairwise inter-protein covariations in each group. A common theme throughout the results was the central role of PD-L1 in all networks, except in the nab-paclitaxel treatment group, although in different immune contexts. For instance, PD-L1 closely correlated to immune-stimulatory proteins in adjuvant patients, such as CXC3CL1, and to other immunosuppressive proteins in ctDNA^{high} patients, such as PD-L1. Additionally, a close connection between *epidermal growth factor* (EGF) and *angiopoietin 1* (ANGPT1) was observed in all treatment-based networks.

Paper IV

The genetic landscape of terminal pancreatic cancer and preceding temporal events: An initial CHAMP autopsy study

Study outline

The findings in **paper I**, where the degree of spatial heterogeneity in the regional tumour space was indicative of OS following adjuvant treatment, encouraged the study design of **paper IV**. Thus, we now wanted to further evaluate genetic heterogeneity, and especially that of CNAs, in terminal disease by analysing tumour tissue collected postmortem, from both the primary tumour, local recurrence, and all metastatic lesions of sufficient size (42 samples, five CHAMP patients). In addition, this was related to antemortem tumour tissue whenever available (eight samples, four patients), such as diagnostic biopsies and one resected tumour, and ctDNA in plasma samples (21 samples, five patients) obtained before and during chemotherapy treatment.

Disease diversity and genetic profiles of terminal PDAC

Analyses of the five patients, four treated with palliative and one with curative intent, further manifested the notably varying survival times of patients with PDAC, despite the overall dismal prognosis. Two of the palliative patients reflected the observed extremes; one with a $KRAS^{WT}$ tumour who survived for 36.2 months, and another, displaying a high metastatic burden already at diagnosis, who only lived for 2.3 months. Moreover, metastatic dissemination was most commonly seen to the liver, although metastases were also detected in other sites, such as the peritoneum, lungs and pericardial fat. Subsequent genetic analyses revealed only modest diversity between patient-specific tumour lesions in terms of deleterious SNVs and InDels, such as known drivers of PDAC. In addition, one patient harboured a variant in the *tuberous sclerosis complex 2* (*TSC2*) gene, which was found in all tumour samples.

On the other hand, varying degrees of CNA heterogeneity were observed for individual tumour sites in each patient, with a median CNA count per sample ranging from 27 in one patient to 109 in another. Genomic regions known to be predilected for CNAs in PDAC were also seen to be altered within this cohort, i.e. deletions including 9p, 17p and 18q, with low intrapatient diversity. Amplifications covering the *MYC* gene were further detected in two patients, a homozygous deletion of *CDKN2A* in three patients and a homozygous deletion of *SMAD4* in one patient.

Phylogenetic reconstruction of terminal PDAC

Similar to **paper I**, phylogenetic reconstruction based on genetically defined subclones, comprised by SNVs, InDels and CNAs, was performed to further evaluate evolutionary traits and genetic heterogeneity of terminal PDAC. The results showed that spatial discrepancies in CNA profiles constituted the main part and architectural structure of

each tree, with great interpatient variation in both size and branching complexity. As an example, one tree encompassed 85% of all genetic events in the trunk and a total of eleven subclones, compared to another where only 17% of the events were truncal and where 36 distinct subclones were identified.

Detailed analyses of each ancestry further demonstrated a combination of linear and branching evolution for the majority of cases, even though two of the patients had distinguishably more homogenous phylogenies. Inclusion of antemortem samples also revealed that CNA diversity was evident to different extents in all cases already before treatment administration. Additionally, considering patterns of metastatic seeding, a mixture between both monophyletic and polyphyletic spread was proposed for most cases. The analyses also suggested that the majority of metastatic clones had disseminated already at an early stage, as exemplified by one patient where subclones detected at autopsy were identified as descendants from an ancestral clone to the ones detected in the resected tumour specimen.

On-treatment ctDNA profiles align with truncal genetic events and captures intrapatient CNA heterogeneity

Lastly, ctDNA was further investigated by ultra-deep sequencing for detection of SNVs, InDels and CNAs, in serial on-treatment plasma samples, and compared to the terminal tumour landscape. The findings showed that two patients had a high plasma-derived tumour burden at diagnosis, with ctDNA fractions above 20%, whereas the rest had relatively low levels, all detectable but with ctDNA fractions below 5%. A high interpatient discrepancy was also acknowledged in terms of total cfDNA levels during treatment. Considering detected SNVs and Indels, a high concordance was observed between plasma and truncal variants in matched tumour tissue. In addition, the potential utilization of ctDNA dynamics in PDAC monitoring was further validated by the finding that fluctuations of ctDNA levels aligned closely with the clinical disease course.

Moreover, CNA profiles were compared between plasma and matched tumour tissue for the two patients with high concentrations of ctDNA, at either one or several time points. The results showed a strong agreement between plasma and tumour lesions in regard to truncal CNAs in each respective phylogeny. Interestingly, however, while the ctDNA copy number profile at BL in one of the patients, with a relatively long OS (14.5 months), closely resembled that of the diagnostic pretreatment biopsy, the ctDNA profile in the other patient, with a particularly aggressive disease and short OS (2.3 months), revealed a combination of CNAs from all tumour lesions found at autopsy. Moreover, in the latter, discrepancies in the ctDNA CNA profile were detected between plasma samples collected before treatment and after one month, indicative of temporal genetic evolution.

Discussion

The work of this thesis underlines future opportunities for improved clinical management across various stages of PDAC, ranging from better prognostication to non-invasive disease monitoring and individualized treatment strategies. Patient focus differed between the studies, concentrating on adjuvant treated patients with resectable disease in **paper I**, while emphasizing palliative treated patients in **paper III** and **IV**, although not excluding patients with resected tumours. Consistent throughout all the studies were, however, the pronounced disparities within the different cohorts considering disease progression, chemoresistance and OS, reflecting the stage-independent intricate nature of PDAC. This was also mirrored in the diversity of metastatic sites of dissemination, as revealed by autopsies, confirming previous autopsy reports[437]. In addition, the performed autopsies further enabled comprehensive mapping of the terminal genetic landscape, which provides invaluable information, especially for patients with insufficient tumour tissue availability.

The following sections will discuss the main findings of **studies I**, **III** and **IV,** and how these new insights may spur further investigations of PDAC and impact future clinical management.

The role of copy number alterations across all PDAC stages

Copy number heterogeneity for tracking clonal relationships

Previous studies on other types of cancer, such as lung, renal and paediatric tumours, have demonstrated that CNA heterogeneity is important for OS[56,69,438]. Additionally, although quantitative CNA metrics and specific CNA events have shown prognostic value in PDAC[271,290,439], the impact of spatial heterogeneity remains less explored. Thus, in **paper I** and **IV**, the main focus was to map the diversity of CNAs across the tumour landscape in resected and terminal metastatic disease, respectively. Of note, specimens from one resected tumour and several diagnostic biopsies were also included in **paper IV**, enabling CNA analyses of treatment naïve tumour tissue as well. In general, although the overall burden differed, a varying extent of CNA heterogeneity was apparent in both studies before administration of treatment, and even more so in the terminal landscape.

To depicture this spatial diversity, phylogenetic trees were constructed by inferring ancestral relationships between all detected tumour subclones. These subclones were primarily based on thoroughly defined chromosomal breakpoints of each CNA, although other potential driver events such as SNVs and InDels were also included. This approach has been used in previous research for other types of malignancies[69,409], but to our knowledge, **paper I** and **IV** represent the first studies utilizing this

methodology for PDAC. Earlier studies of PDAC have, on the contrary, primarily utilized SNVs for phylogenetic reconstruction[70,305,440]. For instance, a study by Makohon-Moore et al. in 2017 revealed a remarkable homogenous genetic landscape in terms of known driver events between treatment naïve metastatic sites collected at autopsy, where the detected heterogeneity was foremost denoted to passenger mutations[305]. In another study, recurrent disease following adjuvant platinum-based chemotherapy showed a positive selection of subclones with drivers stimulating the proliferative MAPK/ERK and PI3K/AKT signalling pathways[70].

The sparse overall heterogeneity of genetic driver events in **paper I** and **paper IV**, although with some variations between different metastatic sites posttreatment, are in line with the studies above[70,305]. Contrastingly, the phylogenetic architectures were instead foremost outlined by spatial discrepancies in CNA profiles. Thus, in an attempt to benchmark SNV phylogenies to the ones founded by CNAs, a second phylogenetic construction was performed in **paper I** by utilization of all detected SNVs, regardless of their driver potential. The results revealed similar frameworks of the trees, although with lower resolution. It is however important to acknowledge that the analyses of small-scale mutations throughout this thesis were performed on FFPE tumour tissue using targeted sequencing approaches which, hence, limits the detection range. Nonetheless, these results promote the relevance of incorporating defined CNAs in the reconstruction of tumour phylogenies to achieve an enhanced resolution of the genetic landscape, as small- and large-scale mutations appear to be complementary, previously also discussed for other malignancies[69].

Copy number based phylogenetic diversity and its clinical implications

In both **paper I** and **IV**, the phylogenetic trees varied both by size and branching pattern between patients. Some trees had long trunks, with late emergence of the MRCA and limited branching, whereas others showed early branching events, promptly dividing the trees into different phylogenetic clades. Hence, signs of both *linear* and *branching* evolution were apparent for the majority of the phylogenies. Of note, in **paper IV**, where comparisons between several antemortem tumour areas to postmortem tumour sites could be performed for two patients, outlining contours of the terminal genetic landscape were observed already before treatment initiation.

Moreover, in **paper I**, although limited by the small cohort size (nine patients), the prognostic value of several tree attributes was evaluated. The overall CNA burden has previously been shown to be clinically relevant in resected PDAC[271,290], and an increased number of alterations has been correlated to worse prognosis in patients treated with adjuvant gemcitabine based regimens[290]. Although a similar trend was found in **paper I**, this finding could not be statistically confirmed, most likely due to the size of the cohort. On the contrary, one of the palliative treated patients in **paper IV** who displayed a particularly high CNA burden, already before treatment, had a relatively long OS.

Thus, whether the burden of CNAs has clinical implications in the metastatic setting remains to be seen. Of note, PDAC patients with deficient HRR and thus increased CIN, often but not always due to germline variants in genes such as *BRCA1/2*, have shown increased sensitivity to both PARP-inhibitors[229,230,329] and oxaliplatin-based regimens[271,330-332]. Hence, future studies are also needed to further evaluate the prognostic value of CNA burden in the context of CIN and treatments exploiting homologous recombination *deficient* tumours, as performed for resected tumours by Waddell et al.[271].

A visual review of the trees in **paper I** instead spurred numerical assessment of the branching structure, as also recently performed by utilizing established biodiversity measurements in a paediatric cancer study[409,410]. These measurements were further combined into a novel summarizing metric, considering both clonal architecture and the number of detected subclones, which was applied to each of the nine phylogenies. Interestingly, the following comparisons of branching complexity with OS demonstrated a significant negative association. Thus, yet acknowledging the small sample size, this observation promoted the hypothesis that increased evolutionary complexity before treatment might provide a heightened risk for chemoresistance and early relapse, in line with data from other malignancies[56,69,438].

Importantly, possible confounders were also evaluated, such as the total number of included samples, distinct FFPE blocks utilized and whether samples from regional lymph node metastases were included, but did not account for any substantial contribution to the phylogenetic architecture. On further note, as the complexity formula does not take branch *lengths* into account, perhaps the evolutionary framework and CNA burden could provide complementary clinically relevant information. In **paper IV**, the evolutionary complexity of the primary tumour prior to treatment was not assessable. Only two pre-treatment samples were adequate for phylogenetic analysis in the adjuvant treated patient and the others only had a diagnostic biopsy, usually from a metastatic lesion. Hence, for palliative patients, other approaches are required to evaluate whether some tumours are more *evolutionary prone* and how this affects treatment response and survival, as discussed in forthcoming sections. Nevertheless, one of the patients in **paper IV**, who survived less than three months from diagnosis, had a particularly complex terminal phylogenetic tree.

PDAC evolution

Specific genetic events during PDAC development

Genetic events known to play a pivotal role in PDAC tumorigenesis were also frequently detected in study **I**, **III** and **IV** of this thesis. These included SNVs activating *KRAS*, various small-scale loss-of-function variants affecting *TP53*, *CDKN2A* and

SMAD4[258,271,272,280], and specific CNAs such as focal amplification of *MYC*, homozygous deletion of *CDKN2A* and loss of heterozygosity covering regions 9p (*CDKN2A*), 17p (*TP53*) and 18q (*SMAD4*)[271,273,290]. Thus, in line with the plethora of previous data on frequent genetic events in PDAC, and with the two-hit hypothesis proposed by Knudson in mind[15], these results further underscore the importance of these events in PDAC.

From the evolutionary analyses performed in **paper I** and **IV**, these above-mentioned events were also validated as occurring evolutionary early during PDAC carcinogenesis, as they were predominantly detected in the trunk of the phylogenetic trees, in line with previous reports[70,258,271,272,280]. The main exception was the occasionally subclonal, and in a few cases also evolutionary parallel, emergence of *SMAD4* mutations. Thus, despite the 18q LOH being almost exclusively truncal, small-scale variants causing loss-of-function in the secondary *SMAD4* allele were found in the tree branches from a few patients in both studies. That variants in *SMAD4* often emerge late during PDAC evolution has been established previously and has been associated with the moment of invasion[277,286,289], thus validating these results.

Moreover, the extensive, although varying, presence of CNAs in tumour regions across all PDAC stages (**paper I**, **III** and **IV**), stresses their role in the development and progression of invasive PDAC. This notion has also been pointed out by the work of several others, for example suggesting that invasive growth may be initiated by one, or a few, catastrophic chromosomal rearrangement events, and by demonstrating that a more prominent CNA burden is observed in high-grade compared to low-grade dysplasias[280,285,441].

Signs of natural selection for specific CNAs, and their effect on OS, were also evaluated in the genetic landscape of resected PDAC in **paper I**, as previously performed in a large pan-cancer study[442]. This was carried out by examining commonly detected CNAs[275] and their point of emergence in each respective phylogeny, classified as being either truncal, subclonal or absent. Although likely affected by the small sample size, no specific alterations were found to be significantly associated with early or late emergence, which promoted the assumption that the branching trajectories were a result of neutral evolution. Yet, an interesting finding was that chromosomal deletion of 8p was only detected in patients with a shorter OS. This finding is in line with the association of 8p loss to tumour progression in breast cancer[443] and to increased metastatic potential and decreased OS in liver cancer, amongst others[444]. Consequently, these insights motivate future analyses on larger PDAC cohorts, including posttreatment samples from metastases, to investigate positive or negative selection of specific chromosomal events and their evolutionary significance.

Metastatic subclones and their phylogenetic origin

The evolutionary point of metastatic dissemination was evaluated in both **paper I** and **IV**, for local (regional lymph nodes) and distant metastases, respectively. Overall, in the reconstructed phylogenetic trees, subclones detected in metastases were often found to have emerged evolutionary early, even before surgery, confirming results from previous studies[70,295]. In particular, early dissemination to the liver and lymph node metastases was denoted. To provide an example, for one patient in each study at least one of the metastatic subclonal populations shared only truncal alterations with those found in the matched primary tumour region. Of importance, the term *early* in this context does not refer to the actual time frame, but rather to a consecutive order of evolutionary events.

In **paper IV**, multiregional postmortem sampling was performed on all available tumour lesions found in the thoracic and abdominal cavities. Metastatic subclones often showed discrepancies in ancestral origin, i.e. signs of *polyphyletic* dissemination, in all patients but one. *Polyclonal* seeding of the liver was also frequently detected, exemplified by the sole operated patient where subclones in the resected pancreatic tumour and lymph node metastasis were observed to have seeded individual metastases to the liver, detected postmortem. Metastases were also observed to seed each other, i.e. providing evidence of *intermetastatic* seeding, such as in one patient where a subclones in the diaphragm and rectouterine pouch were proposed to be descendants from subclones in the liver metastasis. These results are in line with the metastatic origins described in an earlier investigation of posttreatment dissemination patterns of PDAC by Sakamoto et al.[70], further emphasizing the utility of CNA based phylogenetic analyses from FFPE specimens. However, although the acquisition of multiple *pre*treatment samples provided a glint of the following final genetic landscape in two of the patients, unsurprisingly, single biopsies did not. It must, although, be pointed out that these biopsies were obtained from liver metastases, possibly representing a more homogenous genetic landscape due to clonal selection in the metastatic process. Either way, it highlights the inevitable problem of single biopsies and their limited ability to provide a holistic view of the tumour landscape[55,58].

Building on the findings of **paper IV**, one could also speculate on potential signs of natural selection of subclones due to the systemic pressure from administrated therapeutic drugs. In one patient, several pre-treatment samples from a resected metastasis to the ileum were available, from which a branched phylogenetic pattern was detected, dividing the subclones into two main tree clades. Interestingly, at autopsy, only one of these clades were detected, including linear descendants, indicating signs of negative selection of the other clade. Similarly, in a second case, no linear descendants were found postmortem from subclones detected in the pre-treatment liver biopsy. Of note, this specific tumour also displayed the highest overall CNA burden and a relatively positive response to chemotherapy. It should however be pointed out that

although multiregional sampling enables a good read-out for identification of heterogeneity, *all* tumour areas are not sampled, and hence, additional subclones may have been missed.

Blood based on-treatment tumour monitoring

Given the limitations with single tissue biopsies and that biopsies, in general, can cause significant discomfort for patients, a plethora of research has been conducted to assess the utility of liquid biopsies as either a replacement or complement[77,84-86,354]. Building on that, the combination of an overall sparse accessibility to tumour tissue, a high stromal tumour component and limited knowledge about the temporal heterogeneity of PDAC, encouraged the evaluation of longitudinal ctDNA dynamics in **paper III** and **IV**. A focused sequencing panel was developed, primarily designed for monitoring of palliative patients during chemotherapy, in which an increased ctDNA burden has been observed compared to patients with operable disease[361].

Validation of the CHAMP panel and its clinical utility

Throughout **paper III** and **IV**, the sensitivity of the CHAMP sequencing assay enabled overall detection of VAFs below 0.5%, and even lower whenever prior knowledge of variants in matched tumour tissue was available. These numbers are comparable to other similar assays in the field of precision oncology, although methodologies such as ddPCR have demonstrated even higher sensitivities[157]. The panel also facilitated detection of chromosomal changes across the genome, and allelic imbalances for six chromosomes frequently altered in PDAC, although at a lower sensitivity.

Nevertheless, as touched upon before, all methods come with both benefits and drawbacks. Methods such as ddPCR, focusing on only a few gene targets, are limited by the presence of the specific genetic variants and the number of ctDNA fragments[157]. The high frequency of *KRAS* mutations in PDAC does, however, make it a suitable cost-effective method for the purpose of monitoring MRD, although the minor proportion, approximately 10%, of patients with $KRAS^{WT}$ tumours will be missed. Another popular way to monitor ctDNA is by using a tumour-informed approach, where knowledge about specific tumour variants is leveraged for increased sensitivity[445]. However, this method obviously comes with the evident drawback of being dependent on available tumour tissue. None of these methods may, however, detect *de novo* variants, i.e. mutations in genes outside the target span of the panel. In contrast, ultra-sensitive unbiased approaches for determining the ctDNA burden, such as sWGS are less restricted by the presence of specific ctDNA molecules, and may detect numerous different mutations associated with the tumour[164,165], although the majority will most likely be passenger variants. Taken together, the specific research question at hand

should guide the particular choice of method, utilizing its respective strengths and weaknesses.

On that account, the customized CHAMP panel utilized in **paper III** and **IV** allows for detection of the most common genetic PDAC variants[272], together with a number of genes known to provide clinically valuable information when mutated. The panel is, thus, independent of available tumour tissue and increases the chances of detecting *KRAS*^{WT} tumours, which is of importance for prognostication[446]. Whilst one could argue that the intermediate panel design in this thesis is neither the most sensitive, nor the widest in terms of gene targets, findings of **paper III** and **IV** demonstrated the adequacy for its purpose – cost efficient sensitive monitoring of the ctDNA burden in the palliative patient group while still enabling identification of targets for precision medicine. In **paper III**, ctDNA was detected in plasma from the majority of palliative patients, and several of the adjuvant patients, and in **paper IV**, ctDNA was detected in plasma from all patients. Nevertheless, for the purpose of monitoring MRD in operated patients, the results from **paper III** suggest that an alternative, more sensitive method would be more suitable.

The close coherence between mutations detected in plasma and corresponding tumour tissue in **paper III** and **IV** also validated the utility of the design. This was true both for small-scale variants, but also for genome-wide copy number profiles. Notably, although probes targeting SNP positions were only incorporated for six chromosomes, CNAs generating a change in $\log_2$R were detectable across the entire genome in plasma samples with high ctDNA fractions, as also recently shown by Lapin et al. using a similar approach[292]. Of importance, even at low levels, information from specific CNAs enhanced the accuracy of ctDNA fraction estimations by enabling normalization to the specific allelic background. This is highly relevant, as the ctDNA fraction is often used for monitoring purposes, further elaborated on in forthcoming sections. However, for an increased sensitivity of CNA detection specifically, one could proposedly also consider sWGS, as performed by others[447].

Liquid versus tumour biopsies

Studies comparing the extent of identified genetic heterogeneity by utilizing either ctDNA or tissue biopsies have shown at least comparable, if not superior, reflections of the genetic tumour landscape from ctDNA analyses[152]. Thus, one of the aims of **paper III** and **IV** was also to perform similar evaluations whenever matched tumour tissue was available. Findings from **paper IV** did not provide any signs of heterogeneity for small-scale driver mutations between longitudinal plasma samples and tumour tissue, other than those attributed to varying ctDNA fractions. Instead, identified ctDNA variants cohered completely and exclusively to the truncal variants in the reconstructed phylogenies. It should, however, be mentioned that the vast majority of subclonal driver mutations detected in the tumour tissue were found in genes not included in the

CHAMP panel. On the contrary, in **paper III**, inconsistencies of small-scale driver variants were detected between plasma and matched tumour tissue in a few cases, as also seen in previous studies[361,368], but no temporal disparities were found. These findings support ctDNA evaluations as a complement in PDAC characterization, especially whenever access to tumour tissue is sparse or non-available.

Contrastingly, although only assessable in a few cases, signs of temporal CNA heterogeneity were identified in both **paper III** and **IV**. In **paper III**, a *KRAS* amplification, which was undetectable in the diagnostic tumour biopsy and ctDNA sample before treatment initiation, was found in ctDNA in association with disease progression. Increased KRAS burden due to multiple DNA copies has been noted in other studies[292,448], and in distinct association to treatment resistance[449]. In one patient included in **paper IV**, the ctDNA copy number profile of the sample obtained before treatment differed from the sample obtained after two months by several alterations, and substantially from CNAs detected in the diagnostic tumour biopsy. Similar results were also recently published by Huebner et al.[291], emphasizing the value of longitudinal CNA assessment using cfDNA for monitoring of disease progression.

Opportunities for precision medicine

The focused sequencing approach utilized in **paper III** and **IV** facilitated identification of a few clinically relevant alterations derived from ctDNA, as also demonstrated by others using both cfDNA and tissue analyses[355,450,451]. These variants included a $KRAS^{G12C}$ mutation, targetable with novel KRAS inhibitors[337], and variants in *BRCA2*, motivating therapy with PARP-inhibitors[229]. Moreover, utilizing broader gene panels, additional actionable targets were detected in tumour tissue, such as a clonal *TSC2* variant in one patient in **paper IV**. Loss-of-function mutations in *TSC2* have been shown to enhance the effect of systemic treatment with immunotherapy or *mammalian target of Rapamycin* (mTOR) inhibitors[452,453], which could have been fundamental in the above-mentioned case considering the observed rapid disease evolvement and chemo-resistance. Building on these results, utilizing more comprehensive panels should also be considered whenever permitted by sufficient ctDNA fractions, to enable a wider identification of less frequent actionable targets[146]. In addition, this would simultaneously provide information regarding *germline* variants, which is of equal importance for clinical management[329,332,334].

Collectively, these findings further motivate clinical implementation of genetic testing in PDAC[451,454], and the complementary value of ctDNA analyses to tumour biopsies. Of note, before considering targeted treatment strategies, one should always consider the clonality of the genetic variant, as alterations only detected in a fraction of the tumour cells would likely not cause a sufficient tumour response, but rather spur the emergence of resistant clones[455].

Clinical management of PDAC by ctDNA-based stratification

A range of studies have evaluated the clinical value of detectable, i.e. positive versus negative, ctDNA levels in plasma of PDAC patients, both in the adjuvant and palliative settings, and concluded an adverse relationship with prognosis[357,359,367,435]. Hence, equivalent comparisons were also carried out in **paper III**, and although similar trends were found for palliative patients, these results could not be statistically validated in either patient group. For adjuvant patients, the small cohort size (fifteen patients) and, as previously discussed, a likely suboptimal limit of detection for the lower tumour burden, probably contributed to the discrepant results. For the palliative patient group, apart from the sample size (45 patients), other factors potentially contributing to the discrepancy include variations in preprocessing of cfDNA, detection limits of the utilized assays and the extent of genome targets. For instance, utilizing the CHAMP panel, it was possible to detect ctDNA even in patients with a $KRAS^{WT}$ tumour, whereas other studies have focused purely on $KRAS^{mut}$ tumours[367]. It should also be noted that in terms of detection limit, a slightly higher proportion of PDAC patients was found to be ctDNA positive prior to chemotherapy administration in our cohort compared to others[367,435].

A possible confounding factor for sensitive sequencing analyses of plasma is also the presence of mutated cfDNA from normal cells, especially in elderly patients[158,159,456]. This problem is particularly pronounced for early detection but cannot be ruled out even in advanced disease. However, this can be partly mitigated by utilizing parallel sequencing of matched white blood cells, the most prominent source of cfDNA mutations[158,159], as performed throughout this thesis. Thus, the challenges with potential false positive mutations shed from normal solid tissues, and the high proportion of palliative patients with detectable ctDNA, further motivated the exploration of a more quantitative prognostic metric.

The majority of analyses in **paper III** were instead performed by utilizing the absolute number of mGEs in each cfDNA sample. This measurement is calculated by normalizing the fraction of ctDNA to the total concentration of cfDNA per ml plasma[118], leading to a decreased vulnerability to fluctuations in normal cfDNA concentration, as further discussed in the forthcoming section. The following survival analyses showed a significant prognostic value for the number of mGEs per ml plasma before treatment initiation, and a prognostic *cutoff* was calculated. Building on those results, the cohort was divided into a $ctDNA^{high}$ and $ctDNA^{low}$ patient group, by classifying them according to their ctDNA burden, which revealed a significant difference in OS, 3.7 vs 11.9 months, respectively. Thus, although numerous studies have evaluated the dichotomized value of positive versus negative ctDNA detection, this is, to our knowledge, the first to utilize a quantitative cutoff value to stratify the patients.

Comparisons of clinical parameters between the two groups further revealed that ctDNA^{high} patients displayed an increased frequency of distant metastases, particularly dissemination to the liver. Thus, of importance, multivariable analyses were carried out to ensure that the prognostic value did not only serve as a proxy for patients with or without metastatic disease. The analysis was performed together with several clinical parameters, including metastatic stage, and revealed that the ctDNA burden was the sole independent factor. The association between increased ctDNA levels and metastatic spread to the liver has also been reported by others[362,435]. Nevertheless, future studies are needed to confirm whether liver metastases in general shed a higher amount of cfDNA to the circulation, or if the metastases simply obstruct the overall cfDNA clearance, as the liver remains one of the most important organs for this purpose. Proposedly, one could for instance utilize *methylation profiling* of cfDNA, which has been shown to be a sensitive method for tracing the cfDNA tissue of origin[457,458].

Monitoring the systemic disease burden

Similar concentration measures to the one used throughout **paper III** have been utilized previously for ctDNA quantification[110,118], and its utility for clinical purposes has been discussed in comparison to the more frequently reported ctDNA *fraction*[86,459]. The two metrics both come with benefits and drawbacks. The ctDNA fraction is, as previously mentioned, dependent on the total cfDNA concentration, whereas the number of copies is more sensitive to modifications in overall clearance[86]. As an example, in **paper IV**, two of the patients had exceptionally high (> 20 %) ctDNA fractions before treatment, but only slightly elevated total cfDNA levels. On the contrary, after one month, the total concentration of cfDNA had expanded significantly. Of importance, although this increase was accompanied by a magnified ctDNA concentration for one of the patients, ctDNA could no longer be detectable in the other, highlighting the dynamics between plasma-derived cfDNA and ctDNA levels. Thus, the respective concentrations of ctDNA and total cfDNA, together with the ctDNA fraction, are seemingly complementary, and all three metrics should be considered for evaluations of tumour dynamics[86]. Yet, as the absolute ctDNA concentration takes the ctDNA fraction into an account, this was chosen for monitoring purposes in **paper III** and **IV**.

In **paper III**, fluctuations in ctDNA levels were found to be of particular clinical value in the ctDNA^{low} patient group, and it was especially useful for predicting disease progression whenever the levels reached above the prognostic cutoff. The same could not be seen in the ctDNA^{high} group, as a dramatic decrease below the cutoff after treatment initiation was not associated with neither progression nor stable disease/regression. Even so, all cases showing persistently high levels of ctDNA were associated with rapid progression or death within a three-month time frame, in line with a recent study[292]. Collectively, these findings spur clinical implementation of PDAC-monitoring utilizing ctDNA. However, the longitudinal analyses were mainly based on ctDNA level follow-up at one month and three months, and considering the

short half-life of cfDNA[114,115], future experiments should also aim for temporal analyses with shorter time intervals to allow for increased resolution.

Can we detect an evolutionary prone tumour from a blood sample?

To change the outcome for patients diagnosed with PDAC, it is essential to individualize the treatment strategy according to the molecular characteristics of the specific cancer at hand. Today, patients are treated with similar systemic regimens, modified mainly by their performance status, with a wide range of outcomes but no adequate prediction models to foresee who will benefit and who will not. Thus, one of the fundamental questions throughout this thesis have been

"Could genetic heterogeneity serve as a marker to distinguish tumours prone for rapid evolution and chemoresistance from those having a more indolent disease course and being more sensitive to treatment, already at diagnosis?"

This hypothesis was encouraged by the findings in **paper I**, proposing that the extent of clonal complexity, i.e. a broad range of substrates for natural selection to act upon, may be indicative of an increased evolutionary propensity and resistance to treatment. Speculatively, such differences might also explain the lack of consensus for ctDNA fluctuations and the clinical disease course in the ctDNA^{high} group in **paper III**. However, performing phylogenetic analyses from multiregional sampling data is a time-consuming process, but above all, it requires sufficient access to tumour tissue. Hence, palliative patients would seldom be eligible for such an evaluation, and particularly not within a reasonable timeframe. Consequently, this motivated the pursuit of blood-based markers capable of discerning particularly evolutionary prone tumours which require an alternative treatment strategy.

With that in mind, a study by Pereira et al.[460] recently demonstrated that the mutation profile of cfDNA sufficiently reflected the terminal genetic landscape of several metastatic cancers at autopsy. In addition, they showed that cfDNA mutation clonality varied depending on the number of metastases harbouring the specific variant. These results are in line with the findings in **paper IV**, proposing that the extent of genetic heterogeneity observed in ctDNA before treatment, i.e. the subclonal proportion of CNAs, may be suggestive of the overall tumour diversity and potential treatment response. These deductions were based on two patients with comparably high pretreatment ctDNA levels, but profound differences in treatment response and OS. The two patients displayed notable disparities in CNA clonality derived from ctDNA before treatment initiation, where an increased clonal complexity was observed in the patient with a more aggressive and chemo-resistant tumour, similar to the reasoning from the results in **paper I**. Furthermore, in this patient, the ctDNA profile demonstrated a vast mixture of alterations detected across all postmortem sampled metastatic tumour sites. Contrastingly for the other patient, with a higher proportion

of clonal CNAs, the genetic profile was instead closely related to that of the pretreatment tumour biopsy.

It is, however, important to mention that a higher proportion of clonal alterations in the ctDNA profile does not necessarily equal a homogenous tumour, but rather a dominating clone causative of the majority of DNA shedding into the circulation. Either way, for the patient with a more uniform ctDNA profile included in **paper IV**, the dominating clone appeared to be sensitive to chemotherapy, further strengthening our reasoning. This was supported by the dramatic drop in ctDNA levels following one month of treatment, the sustained response to therapy and the lack of linear descendants from subclones detected in the diagnostic biopsy after treatment.

Evidently, it is essential to also acknowledge that these conclusions were drawn from only two patients, and therefore require exploration and validation in larger cohorts. In addition, the findings from both **paper III** and **IV** demonstrate biological limitations for CNA analyses using targeted sequencing approaches in ctDNA low samples[291], despite utilizing novel bioinformatic approaches[436]. Low ctDNA fractions should, thus, *always* be taken into account whenever exploring the extent of genetic heterogeneity and its clinical implications. Yet, it is logical from a biological and evolutionary point of view to speculate on whether a homogenous ctDNA profile could in fact be indicative of patients who are more likely to benefit from chemotherapy treatment[54,56,460]. For cases where tumour tissue is available, one could also proposedly assess the genetic diversity with increased resolution by comparing ctDNA and tumour profiles. In addition, future incorporation of sensitive cfDNA methylation profiling might shed further light on the metastatic burden and ctDNA heterogeneity by tracing the tissue origin of the circulating fragments[457,458].

The complex multifaced host response in PDAC

Local and systemic immune response

Like any naturally occurring ecosystem, the TME imposes various constraints for tumour progression, not least in PDAC with its hypoxic, desmoplastic and immunosuppressive characteristics[372,461]. In this case, the harsh environment has further been shown to promote an aggressive and invasive phenotype and to hinder the penetration of therapeutic agents[372,462]. Hence, in **paper I** and **III** of this thesis, the local and systemic immune responses were evaluated, respectively, in combination with the genetic tumour profiles for various patient groups. In both studies, one should, however, bear in mind that the relatively small sample sizes most likely influenced the observed results. The hypothesis for **paper I** was built upon previous research showing that the clonal evolution of tumours is in constant interplay with the surrounding TME, and that the IC subtypes and their topographical distribution are important for

prognosis[463-466]. Thus, the research question motivating the analyses in **paper I** was whether an increased genetic heterogeneity of the tumour was also reflected by a diverse spatial immune landscape. Multiregional analyses of each resected tumour showed both great intrapatient and interpatient heterogeneity of various IC populations. Yet, no significant associations were observed between the overall diversity of IC subsets in the TME and the genetic evolutionary complexity of the tumour. Hence, although the utilization of double IHC analyses enabled spatial investigations of ten different IC populations, other multiplex methods, capable of mapping subpopulations in further detail, are most probably required[467].

Moreover, for an effective adaptive immune response, specific lymphocytes such as T_c cells must be able to detect the cancer cells by, for example, recognizing MHC class I bound neoantigens on their cell surface. Recent research has looked deeper into this in PDAC long-term survivors and found that specific characteristics of tumour neoantigens are important for proper T cell activation and OS[333,463,468]. Thus, looking further into the link between neoantigen repertoire, T cell infiltration and genetic heterogeneity could be a logical approach in future investigations.

Likewise, in **paper III**, changes in levels of different immune related proteins and ICs were analysed both in adjuvant patients and in palliative patients divided into the $ctDNA^{high}$ and $ctDNA^{low}$ group. Immune markers with significant fold changes over time were only found in the $ctDNA^{low}$ group, most likely influenced by the small sample size of the other groups, and included a significant increase of IFN-γ and CXCL12. The impact of each specific treatment regimens, however, remains a potential bias in all groups and needs further investigation in larger cohorts. For instance, therapeutic agents targeting proliferating cells have been shown to affect B cell maturation and impair cytokine production in T cells, leading to impaired communication and a weakened adaptive response[469].

Signs of immune evasion

The most interesting finding from the IC analyses in **paper I** was the positive association between CNA burden and PD-L1 molecules on the tumour cells. In line with these results, an elevated PD-L1 expression has previously been associated with an increased number of neoantigens[470]. In addition, correlations between tumour aneuploidy and immunosuppression have been shown for other cancers, together with a decreased response to immunotherapies[188]. Thus, speculatively, the observed association in **paper I** promotes the hypothesis that a higher CNA burden results in an increased tumour immunogenicity, and, hence, the need for upregulating immune check point molecules to dampen the surrounding anti-tumour activity[189]. The true relationship between CNA burden, neoantigen load, immunoediting strategies and response to immunotherapies, however, remains to be unveiled[189,455].

Similarly, in **paper III**, PD-L1 was shown to be a centrality when investigating covarying proteins in serum between different the patient groups by dynamic time warping. This method is of particular use when comparing patterns between two time series, as it allows for a certain degree of flexibility. Importantly, however, it must be noted that this analysis was built upon the assumption that proteins covarying closely together represent ongoing active immune processes, which needs further validation. Interestingly, though, in the two most opposing patient groups, adjuvant treated patients and palliative patients stratified into the ctDNA^{high} group, soluble PD-L1 was not observed to fluctuate together with the same proteins. In the adjuvant group, PD-L1 covaried with immunostimulatory proteins, and in the ctDNA^{high} group, it instead covaried with for instance other immune checkpoint molecules. The same analysis was also performed by grouping the patients according to which treatment regimen they received. Again, PD-L1 was observed to be a central node both for patients treated with oxaliplatin and gemcitabine, with similar disparities in covarying proteins, i.e. a close covariation with immune activating molecules in patients treated with oxaliplatin and with immunosuppressive molecules in patients treated with gemcitabine.

Theoretically, the two distinct PD-L1 networks observed in **paper III** could propose ongoing immune exhaustion and stimulation processes in different patient groups and treatment regimens. However, to which extent these findings are affected by the given treatment and the tumour burden remains to be explored. Taken together, these hypothesis-generating results promote further evaluations of the systemic immune response in PDAC and its potential for providing important information regarding ongoing immunoediting processes.

Circulating nucleic acids and their immunological effects

In addition to analysing signs of ongoing immune processes, dynamic time warping in **paper III** was also utilized to explore protein and immune cell markers covarying with plasma concentrations of circulating nucleic acids. Of importance for these discussions is that cfDNA may act as a DAMP, which can trigger activation of innate ICs through pattern recognition[471,472]. In addition, the total cfDNA concentration has been negatively associated with prognosis in various malignancies, including PDAC[112,113,473].

Temporal patterns of the protein marker NOS3 and intermediate monocytes were found to have the closest similarities to variations in ctDNA concentration specifically. In line with these results, NOS3 has been shown to contribute to oxidative stress and, in turn, tumour aggressiveness in PDAC[474]. The role of intermediate monocytes, representing a transition state between classical and non-classical populations in the circulation[170], is however less understood, and has been shown to contribute to both pro-inflammatory and anti-inflammatory processes[475]. It should however be noted that fluctuations of plasma-derived ctDNA are inevitably affected by overall ctDNA levels

and thereby the detection limit of the methodology, which might impact the resolution of the analysis.

Moving forward with temporal variations of total cfDNA concentrations, the closest covarying patterns were seen for Vδ1$^+$ γδ T cells and HGF. Vδ1$^+$ γδ T cells are sparsely found in the circulation but have been shown to be capable of exerting cytotoxic activity in tumours, including PDAC, after activation[476,477]. Of particular interest, and in line with its association to cfDNA levels in this study, previous research has demonstrated that they can be activated by innate signalling from DAMPs[477,478]. HGF on the other hand has been associated with tumour metastasis and progression, and previous studies have demonstrated that it may be produced by pancreatic stellate cells in the TME[479]. Of note, these cells are further known for their contribution to the hypoxic fibrotic environment in PDAC[480].

Conclusively, these results highlight the intricate dynamics between circulating nucleic acids, both tumour specific and in general, and the complex host response. In particular for PDAC, the findings further proposes that the desmoplastic TME not only impacts the local, but also the systemic immune response.

Prognostic immune biomarkers for monitoring treatment response

The lack of reliable routine biomarkers in PDAC, both predictive and prognostic, also motivated the search for novel biomarker candidates in **paper III**. Of importance, the longitudinal blood sampling further enabled detection of immune markers capable of providing *temporal* clinical guidance. Both circulating immune cells and serum proteins were explored, which in the end demonstrated three markers with significant prognostic value over time, and superior performance compared to routine biomarkers. These included the protein MUC-16, also known as *cancer antigen 125* (CA125), HLA-DR$^+$ T$_c$ cells and CD20$^+$ B cells.

It is, however, important to mention that peripheral immune cell data was only included for patients with samples available from at least the following three time points: start of treatment, one month and three months. Thus, these analyses should be interpreted with caution in terms of survival as the analysis may be slightly affected by an overall longer OS of the included patients. Nevertheless, the prognostic value of the immune cell populations HLA-DR$^+$ T$_c$ cells and CD20$^+$ B cells has, to the best of our knowledge, not been shown for PDAC previously. The negative association between CD20$^+$ B cells and OS could, proposedly, be due to a specific subset of CD20$^+$ B cells called *B-1 B cells*, which have shown capable of exerting both pro-tumour and anti-tumour activity[481]. Specific B-1 B cells have, for example, demonstrated regulatory abilities, dampening the overall immune reaction and facilitating tumour progression, and they can be activated by DAMPs in an innate manner[481,482]. Thus, in line with the discussions in the aforementioned section, associations between specific B cell subsets and circulating nucleic acids merit further study, especially in the context of systemic

cytotoxic treatments. For this purpose, in-depth analyses of specific B cells could be facilitated by methodologies such as scRNAseq. The role of HLA-DR$^+$ T_c cells in cancer has been less studied, although previous research has proposed regulatory activities similarly to other defined T_{regs}[483,484]. Speculatively, their adverse prognostic relationship with OS **in paper III** is in line with this data, although their particular impact in PDAC remains largely unexplored.

The sole protein biomarker associated with OS was MUC-16, which is a commonly used marker in clinical practice for ovarian and endometrial cancers. Previous research has, however, highlighted its tumour-*agnostic* utility, as increased expression has been shown to be associated with disease progression in various cancers, including PDAC[485,486]. The functional role of MUC-16 in PDAC has been attributed to invasive traits, such as increased cell migration[487], and in ovarian cancer, studies have shown that it may protect tumour cells from destruction by NK cells[488]. Thus, although its association with prognosis in **paper III** did not come as a surprise, it further stresses its potential utility in future PDAC management.

If further validated, these markers could proposedly be utilized in tandem with the suggested ctDNA stratification, to refine prognostication and aid clinical monitoring and management of PDAC.

Concluding thoughts

The work of this thesis addresses and explores several aspects of PDAC across all disease stages, including genetic diversity, clonal evolution and immunological traits, both local and systemic. It explicitly underlines the clinical implications of tumour heterogeneity and stresses the inadequacy of utilizing single tumour biopsies for capturing a comprehensive view of the PDAC landscape. It also suggests modern strategies for PDAC monitoring and prognostication, utilizing liquid biopsies, and a novel patient stratification based on the systemic tumour burden. In addition, it highlights the possibilities of utilizing archival FFPE material for evolutionary analyses, promoting usage of already established repositories world-wide.

In conclusion, although PDAC evolution is impacted by a plethora of factors, this thesis has provided new insights which may hopefully contribute to an improved patient outcome and quality of life.

Future management of PDAC

"Cure sometimes, treat often, comfort always."

by Hippocrates, "Father of Medicine" (460-370 BC)

From an *engineer's point of view*, acknowledging the fact that all oncological considerations might not be fully covered, several approaches for future PDAC management may be proposed from the results of **paper I**, **III** and **IV** of this thesis. It is, for instance, apparent that for many PDAC cases, a combined assessment of clinicopathological data, together with information from genetic analyses and various immune biomarkers, may result in a more accurate prognostication. The forthcoming section will, hence, summarize proposed analyses and future approaches that could be used in different PDAC stages, utilizing already clinically approved therapeutic drugs.

Starting with patients eligible for operation, complementary liquid biopsy analyses should be carried out after surgical removal of the tumour to evaluate the presence of ctDNA in plasma and various biomarkers such as CA19-9 and CA125. The ctDNA analysis should proposedly be carried out by utilizing ultra-sensitive methods such as ddPCR or sWGS, as the ctDNA fraction will most probably be sparse (**paper III**). In addition, after surgery, genome-wide sequencing of different tumour areas should be performed to evaluate genetic tumour heterogeneity and to explore targets enabling future alternatives with precision medicine. Of note, targets may include both germline alterations and somatic variants detected in *all* tumour areas. In addition, one should also evaluate potential signs of "*BRCAness*", i.e. ongoing CIN, even in the absence of variants in HRR associated genes.

Patients with a homogenous tumour profile, negative resection margins and negative ctDNA status in plasma, could suggestively be offered a similar linear adjuvant protocol as currently used in clinical practice, as these tumours will, speculatively, according to the findings of **paper I**, be less prone to adaptation. However, for patients with detectable ctDNA in plasma after surgery and/or a heterogenous genetic tumour profile, microscopic disease is likely still present, and the increased clonal diversity might indicate a heightened risk of evolutionary propensity and rapid relapse (**paper I**). Thus, for these patients, sequential administration of drugs with different antagonistic mechanisms, exploiting the vulnerable state that comes with small populations, should be considered as an alternative adjuvant therapy approach[64]. In the best of worlds, this strategy would lead to an *extinction vortex*, as the tumour cells will probably not be resistant to all agents, which will eventually eliminate the remaining tumour cells. Nevertheless, for both patient groups, sensitive

monitoring should be carried out by analysing cfDNA and other biomarkers, in order to adjust the strategy in the event of tumour recurrence and spare patients from ineffective harsh treatments.

For palliative patients, the findings in **paper III** propose an initial stratification into either a ctDNA^{high} or ctDNA^{low} group following focused targeted sequencing of cfDNA. In cases where ctDNA fractions are deemed sufficient, when tumour tissue is available, or else utilizing circulating leukocytes, broad targeted sequencing should also be carried out to enable opportunities for precision medicine purposes, as described above. In the ctDNA^{low} group, results generated in **paper III** further showed an increased chance of survival benefits from current clinical treatment protocols, which should again be offered. This should, however, be combined with close surveillance by liquid biopsies to detect imminent tumour progression and treatment resistance, as ctDNA monitoring was found to be particularly useful in this patient group. However, novel arising strategies, such as adaptive treatment protocols exploiting ecoevolutionary dynamics to either control or eliminate the disease[372], will hopefully provide an even more markedly improved outcome for these patients in the future. For this purpose, the close coherence between ctDNA fluctuations and the clinical disease course could prove to be particularly useful.

For patients stratified into the ctDNA^{high} group, findings from **paper III** and **IV** indicate a high risk of treatment resistance. Thus, in this group, upfront broad targeted sequencing, or WGS, of cfDNA would be particularly important to evaluate alternative therapeutic agents. In cases where no targeted treatments are declared suitable, these patients should instead be offered best supportive care, which is of equal essence to prevent toxic side effects without any survival benefit from chemotherapy. However, and of importance if validated, the findings in **paper IV** propose that assessment of genetic heterogeneity in ctDNA, and compared to tumour tissue when available, might provide further evidence of evolutionary propensity and predictions of chemotherapy effectiveness. As in the case of one ctDNA^{high} patient in **paper IV**, where only a limited CNA heterogeneity was detected in the ctDNA profile before treatment, a durable treatment response was observed and ctDNA levels dropped below the limit of detection after treatment initiation.

Taken together, from the results of this thesis, and all insights that have come from being a part of the CHAMP study, we foresee various potential approaches towards individualised treatment strategies for patients with PDAC. In an initial step, we intend to follow up on these essential takeaways with a treatment trial specifically designed for the palliative patient group, where the treatment protocol will be adapted based on combined molecular and clinical factors.

Acknowledgements

This thesis would not have been possible without the support and help from all the *fantastic* people around me. I would particularly want to thank:

Karin Jirström, main supervisor and life of every party. I will forever be grateful that you invited me to join your amazing research team, for your never-ending support and for your continuous belief in me. Thank you for always being available, for letting me explore things in my own way, and for mentoring me in both research endeavours and in life. You're a *real world* queen!

My co-supervisor **David Gisselsson**. Thank you for all your guidance throughout the years, for helping me to bridge engineering with medicine, already as an undergraduate student, and for all your invaluable support during my thesis projects. Your passion for research is a true inspiration!

Karin Leandersson, co-supervisor and immunology guru. Thank you for always being available for expert input and advice, and for your enthusiasm and curiosity in every discussion – it´s highly contagious!

Co-supervisor **Markus Heidenblad**. Thank you for all your encouragement, sequencing expertise, career advice and fun times!

Jakob Eberhard, **Sofie Olsson Hau** and **Christina Siesing**, my oncology anchors. Thank you for all the valuable discussions, for providing this engineer with the patient perspective, and for all the fun!

My best lab partners and life coaches, **Björn Nodin** and **Noori Zendehrokh**. Thank you for all your invaluable help in the lab, for the never-ending pranks and for all the laughs.

Sara Wahlin, **Maja Svensson** and **Hedda Jacobsen**, my dynamic trio of excellence. How lucky am I to have had you by my side during this journey? Thank you for your support, throughout all the ups and downs, I could *never* have done this without you!

Carina Eriksson, **Marie Sjögren** and **Mattias Björnesäter**, my Kamprad cornerstones and role models. Thank you for always providing a space for comfort and for all the daily laughs. You're the best!

Former, current and affiliated members of the Jirström group not previously mentioned: **Gustav Andersson**, **Jonna Berntsson**, **Karolina Boman**, **David Borg**, **Jacob Elebro**, **Margareta Heby**, **Charlotta Hedner**, **Philip Falk**, **Emelie Karnevi**, **Danyil Kuznyecov**, **Anna Larsson**, **Sebastian Lundgren**, **Bahar Rastegar** and **Maria Svensson**. Thank you for all your help, fruitful discussions and great times on the dance floor!

All the collaborators I've had the privilege to work with:

Jenny Karlsson, thank you for all your constant support, kindness and help, regardless of whether it was a math problem or just the usual mental overload. I truly admire your ability of making the best of everything you do.

Subhayan Chattopadhyay, thank you for being my solid support for all questions regarding statistics, R and math, no matter how basic they were. Furthermore, thank you for every interesting discussion and for all the fun during our travels. I am so excited about our future research endeavours!

Natalie Andersson, queen of trees and clonal deconvolution! Thank you so much for a great collaboration and for always being available to help me understand and use your cool software. In addition, thank you for all the fun in Tampa and Banff!

Present and former members of the **Gisselsson group** not previously mentioned: **Geoffroy Durand**, **Linda Holmquist Mengelbier**, **Caroline Jansson**, **Kaname Uno**, **Anders Valind** and **Hiroaki Yasui**. Thank you for always making feel so welcome and for letting me join both your group meetings and discussions, but also BBQ evenings. It's been a blast!

Kristina Lundberg and **Viktor Sincic** at the Department of Immunotechnology, thank you for a great collaboration during Majas master thesis and for all the enjoyable discussions thereafter. It's always a lot of fun spending time with you!

Collaborators at KI and SciLifelab: **Johan Lindberg**, **Rebecka Bergström** and **Markus Mayrhofer**. Thank you for all your help with sequencing analyses and for your guidance in the development of the ctDNA panel. I have learned so much and really enjoyed all our discussions.

Björn Hallström, for all your invaluable help with the bioinformatic processing of sequencing data and for your patience in addressing all my *numerous* questions.

Göran Jönsson, thank you for great input and discussions during the development of paper III!

Collaborators at the **Center for Translational Genomics** and **SciLifeLab**. Especially a large thank you to **Tina Catela Ivkovic**, **Annica Björnbäck** and **Liesl Joubert** for all your work within the various sequencing development projects and for guiding me through all DNA preprocessing steps. Your help has been invaluable to the genetic parts of this thesis.

I also want to express my warmest gratitude to all people within the CHAMP study:

The amazing research nurses: **Märta Adauktusson**, **Mattias Björnesäter, Carina Eriksson**, **Lise-Lott Johansson**, **Solveig Karlsson**, **Rosita Nordkvist Marie Sjögren**, **Charlotta Wollheim** and **Lina Zander**. The CHAMP study would be nothing without you!

Clinicians at the Oncology Department and at ASIH and Palliative Care, for making the CHAMP study possible.

Magnus Andersson, **Jonas Duckert**, **Åsa Larsson** and **Pernilla Strandqvist** for all your assistance and support during the CHAMP research autopsies.

All patients, and relatives to patients in the CHAMP autopsy program.

Other people contributing to the engaging research environment:

Former and current Heads of the Department of Clinical Sciences Lund, **Bo Baldetorp** and **Mikael Bodelsson**. In addition, thank you **Bosse** for always supporting me in all my work, one engineer to another!

Susanne André and **Anette Saltin** for all your valuable administrative assistance throughout the years.

Magnus Zätterström, for all your financial assistance, every great table tennis game and for always providing a fun weather report in every email.

To former and current members of all the Kamprad building research groups: **Adrian**, **Belting**, **Darabi**, **Jernström**, **Rosendahl** and **Wittrup**, for all the fun events, great discussions and help throughout these years. To **Hanke** and **Johanna**, thank you for all the exciting table tennis tournaments, laughs and moral support!

I also want to express my immense gratitude to all the *funders* for every grant making the work of this thesis possible: the **Sjöberg Foundation**, **BioCARE**, the **Governmental Funding of Clinical Research within the National Health Service** (**ALF**), the **Faculty of Medicine at Lund University**, the **Ingrid and Sverker Persson Foundation**, the **Lennart Glans Foundation**, the **Mrs Berta Kamprad Foundation**, the **Royal Physiographic Society of Lund**, the **Swedish Cancer Society**, **Skåne University Hospital Funds and Donations** and the **Swedish Research Council**. I would also particularly want to thank the **John and Augusta Persson's Foundation** whose generous support have enabled me to attend various external conferences and courses.

Lastly, but certainly not least, I would like to thank all my friends and family who have supported me along the way, particularly:

My awesome high school friends **Clausen**, **Elin**, **Emma**, **Thea**, **Alexander**, **Filip** and **Ludvig**. Thank you for all the great memories, even the awkward ones, for showing me the fun in science and for helping me find my path forward.

My Lunda-family, especially **Johan**, **Lisa**, **Hans**, **Kaka**, **Johanna**, **Klara** and **Magda**. Thank you for making Skåne my second home, for supporting me throughout these years and for the best of times!

Zackarias, my fellow study partner and best after-work buddy. Thank you for every all-to-nerdy discussion at Inferno bar, for teaching me about everything between 3D printing to how to make the best GT, and for the never ceasing questions.

My soul sisters, **Jessica** and **Louice**. You have no idea how lucky I feel to have had you two by my side all my life. Thank you for your endless comfort and for always being in my corner.

My amazing parents in law, **Anne** and **Rolf**. Thank you for all your support and encouragement, and for being such fantastic grandparents to Freja. An additional thank you for helping me in every way during the past year so that I could focus on finishing my thesis.

To **mum** and **dad**, for your endless encouragement and faith in me throughout all my life and for being such awesome grandparents. **Mum**, for all your warmth and immense support no matter what challenge I have faced. **Dad**, for all your tireless help and curiosity. You are my absolute best *bollplank*! I love you both.

Freja, my sunshine. It's such a privilege being your mum. Thank you for all the joy you bring and for the perspective of life you have given me. I love you.

To **Simon**, best friend and love of my life. Thank you for always steering me in the right (and often more fun!) direction, for knowing me better that I know myself and for endlessly supporting me in whatever challenge I pursue. My life would not be half as happy, half as healthy (except for all the ice cream), nor half as filled with laughs, without you. I love you, *always*.

References

1. Aktipis, C.A.*, et al.* Cancer across the tree of life: cooperation and cheating in multicellularity. *Philos Trans R Soc Lond B Biol Sci* **370**(2015).
2. Vincze, O.*, et al.* Cancer risk across mammals. *Nature* **601**, 263-267 (2022).
3. Caulin, A.F. & Maley, C.C. Peto's Paradox: evolution's prescription for cancer prevention. *Trends Ecol Evol* **26**, 175-182 (2011).
4. Peto, R., Roe, F.J., Lee, P.N., Levy, L. & Clack, J. Cancer and ageing in mice and men. *Br J Cancer* **32**, 411-426 (1975).
5. Hajdu, S.I. A note from history: landmarks in history of cancer, part 1. *Cancer* **117**, 1097-1102 (2011).
6. Mazzarello, P. A unifying concept: the history of cell theory. *Nat Cell Biol* **1**, E13-15 (1999).
7. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
8. Abegglen, L.M.*, et al.* Potential Mechanisms for Cancer Resistance in Elephants and Comparative Cellular Response to DNA Damage in Humans. *JAMA* **314**, 1850-1860 (2015).
9. Watson, J.D. & Crick, F.H. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**, 737-738 (1953).
10. Boveri, T. Concerning the origin of malignant tumours by Theodor Boveri. Translated and annotated by Henry Harris. *J Cell Sci* **121 Suppl 1**, 1-84 (2008).
11. Soto, A.M. & Sonnenschein, C. The tissue organization field theory of cancer: a testable replacement for the somatic mutation theory. *Bioessays* **33**, 332-340 (2011).
12. Jassim, A., Rahrmann, E.P., Simons, B.D. & Gilbertson, R.J. Cancers make their own luck: theories of cancer origins. *Nat Rev Cancer* **23**, 710-724 (2023).
13. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov* **12**, 31-46 (2022).
14. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
15. Knudson, A.G., Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* **68**, 820-823 (1971).
16. Sherr, C.J. Principles of tumor suppression. *Cell* **116**, 235-246 (2004).
17. Martincorena, I. & Campbell, P.J. Somatic mutation in cancer and normal cells. *Science* **349**, 1483-1489 (2015).
18. Stratton, M.R., Campbell, P.J. & Futreal, P.A. The cancer genome. *Nature* **458**, 719-724 (2009).
19. Tokheim, C.J., Papadopoulos, N., Kinzler, K.W., Vogelstein, B. & Karchin, R. Evaluating the evaluation of cancer driver genes. *Proc Natl Acad Sci U S A* **113**, 14330-14335 (2016).
20. Garraway, L.A. & Lander, E.S. Lessons from the cancer genome. *Cell* **153**, 17-37 (2013).
21. McLaren, W.*, et al.* The Ensembl Variant Effect Predictor. *Genome Biol* **17**, 122 (2016).
22. Vogelstein, B.*, et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).

23. Kurosaki, T., Popp, M.W. & Maquat, L.E. Quality and quantity control of gene expression by nonsense-mediated mRNA decay. *Nat Rev Mol Cell Biol* **20**, 406-420 (2019).
24. Cherry, S. & Lynch, K.W. Alternative splicing and cancer: insights, opportunities, and challenges from an expanding view of the transcriptome. *Genes Dev* **34**, 1005-1016 (2020).
25. Frazer, K.A., Murray, S.S., Schork, N.J. & Topol, E.J. Human genetic variation and its contribution to complex traits. *Nat Rev Genet* **10**, 241-251 (2009).
26. Deng, N., Zhou, H., Fan, H. & Yuan, Y. Single nucleotide polymorphisms and cancer susceptibility. *Oncotarget* **8**, 110635-110649 (2017).
27. Albertson, D.G., Collins, C., McCormick, F. & Gray, J.W. Chromosome aberrations in solid tumors. *Nat Genet* **34**, 369-376 (2003).
28. Hansemann, D. Ueber asymmetrische Zelltheilung in Epithelkrebsen und deren biologische Bedeutung. *Archiv für pathologische Anatomie und Physiologie und für klinische Medicin* **119**, 299-326 (1890).
29. Ben-David, U. & Amon, A. Context is everything: aneuploidy in cancer. *Nat Rev Genet* **21**, 44-62 (2020).
30. Turajlic, S.*, et al.* Tracking Cancer Evolution Reveals Constrained Routes to Metastases: TRACERx Renal. *Cell* **173**, 581-594 e512 (2018).
31. Thompson, S.L., Bakhoum, S.F. & Compton, D.A. Mechanisms of chromosomal instability. *Curr Biol* **20**, R285-295 (2010).
32. Gisselsson, D. Classification of chromosome segregation errors in cancer. *Chromosoma* **117**, 511-519 (2008).
33. Gisselsson, D.*, et al.* Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors. *Proc Natl Acad Sci U S A* **98**, 12683-12688 (2001).
34. Thompson, L.H. & Schild, D. Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutat Res* **477**, 131-153 (2001).
35. Hanel, W. & Moll, U.M. Links between mutant p53 and genomic instability. *J Cell Biochem* **113**, 433-439 (2012).
36. Drews, R.M.*, et al.* A pan-cancer compendium of chromosomal instability. *Nature* **606**, 976-983 (2022).
37. Bielski, C.M.*, et al.* Genome doubling shapes the evolution and prognosis of advanced cancers. *Nat Genet* **50**, 1189-1195 (2018).
38. Zack, T.I.*, et al.* Pan-cancer patterns of somatic copy number alteration. *Nat Genet* **45**, 1134-1140 (2013).
39. Dewhurst, S.M.*, et al.* Tolerance of whole-genome doubling propagates chromosomal instability and accelerates cancer genome evolution. *Cancer Discov* **4**, 175-185 (2014).
40. Kuznetsova, A.Y.*, et al.* Chromosomal instability, tolerance of mitotic errors and multidrug resistance are promoted by tetraploidization in human cells. *Cell Cycle* **14**, 2810-2820 (2015).
41. Pienta, K.J., Hammarlund, E.U., Brown, J.S., Amend, S.R. & Axelrod, R.M. Cancer recurrence and lethality are enabled by enhanced survival and reversible cell cycle arrest of polyaneuploid cells. *Proc Natl Acad Sci U S A* **118**(2021).
42. Zhang, S.*, et al.* Generation of cancer stem-like cells through the formation of polyploid giant cancer cells. *Oncogene* **33**, 116-128 (2014).

43. Lin, K.C., *et al.* The role of heterogeneous environment and docetaxel gradient in the emergence of polyploid, mesenchymal and resistant prostate cancer cells. *Clin Exp Metastasis* **36**, 97-108 (2019).
44. Fei, F., *et al.* The number of polyploid giant cancer cells and epithelial-mesenchymal transition-related proteins are associated with invasion and metastasis in human breast cancer. *J Exp Clin Cancer Res* **34**, 158 (2015).
45. Zhang, L., Wu, C. & Hoffman, R.M. Prostate Cancer Heterogeneous High-Metastatic Multi-Organ-Colonizing Chemo-Resistant Variants Selected by Serial Metastatic Passage in Nude Mice Are Highly Enriched for Multinucleate Giant Cells. *PLoS One* **10**, e0140721 (2015).
46. Mittal, K., *et al.* Multinucleated polyploidy drives resistance to Docetaxel chemotherapy in prostate cancer. *Br J Cancer* **116**, 1186-1194 (2017).
47. Cortes-Ciriano, I., *et al.* Comprehensive analysis of chromothripsis in 2,658 human cancers using whole-genome sequencing. *Nat Genet* **52**, 331-341 (2020).
48. Stephens, P.J., *et al.* Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell* **144**, 27-40 (2011).
49. Darwin, C. *On the origin of species by means of natural selection, or preservation of favoured races in the struggle for life*, (London : John Murray, 1859, 1859).
50. Gregory, T.R. Understanding Natural Selection: Essential Concepts and Common Misconceptions. *Evolution: Education and Outreach* **2**, 156-175 (2009).
51. Greaves, M. & Maley, C.C. Clonal evolution in cancer. *Nature* **481**, 306-313 (2012).
52. Nowell, P.C. The clonal evolution of tumor cell populations. *Science* **194**, 23-28 (1976).
53. Birkbak, N.J. & McGranahan, N. Cancer Genome Evolutionary Trajectories in Metastasis. *Cancer Cell* **37**, 8-19 (2020).
54. Gerlinger, M., *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* **366**, 883-892 (2012).
55. Iacobuzio-Donahue, C.A., Litchfield, K. & Swanton, C. Intratumor heterogeneity reflects clinical disease course. *Nature Cancer* **1**, 3-6 (2020).
56. Jamal-Hanjani, M., *et al.* Tracking the Evolution of Non-Small-Cell Lung Cancer. *N Engl J Med* **376**, 2109-2121 (2017).
57. Mengelbier, L.H., *et al.* Intratumoral genome diversity parallels progression and predicts outcome in pediatric cancer. *Nat Commun* **6**, 6125 (2015).
58. Swanton, C. Intratumor heterogeneity: evolution through space and time. *Cancer Res* **72**, 4875-4882 (2012).
59. Alexandrov, L.B., *et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415-421 (2013).
60. Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S. & Baradaran, B. The Different Mechanisms of Cancer Drug Resistance: A Brief Review. *Adv Pharm Bull* **7**, 339-348 (2017).
61. Vasan, N., Baselga, J. & Hyman, D.M. A view on drug resistance in cancer. *Nature* **575**, 299-309 (2019).
62. Gatenby, R.A., Brown, J. & Vincent, T. Lessons from applied ecology: cancer control using an evolutionary double bind. *Cancer Res* **69**, 7499-7502 (2009).
63. Gatenby, R.A., Silva, A.S., Gillies, R.J. & Frieden, B.R. Adaptive therapy. *Cancer Res* **69**, 4894-4903 (2009).
64. Gatenby, R.A., Zhang, J. & Brown, J.S. First Strike-Second Strike Strategies in Metastatic Cancer: Lessons from the Evolutionary Dynamics of Extinction. *Cancer Res* **79**, 3174-3177 (2019).

65. Zhang, J., Cunningham, J.J., Brown, J.S. & Gatenby, R.A. Integrating evolutionary dynamics into treatment of metastatic castrate-resistant prostate cancer. *Nat Commun* **8**, 1816 (2017).

66. Schwartz, R. & Schaffer, A.A. The evolution of tumour phylogenetics: principles and practice. *Nat Rev Genet* **18**, 213-229 (2017).

67. Andersson, N., Chattopadhyay, S., Valind, A., Karlsson, J. & Gisselsson, D. DEVOLUTION-A method for phylogenetic reconstruction of aneuploid cancers based on multiregional genotyping data. *Commun Biol* **4**, 1103 (2021).

68. Alves, J.M., Prieto, T. & Posada, D. Multiregional Tumor Trees Are Not Phylogenies. *Trends Cancer* **3**, 546-550 (2017).

69. Andersson, N.*, et al.* Extensive Clonal Branching Shapes the Evolutionary History of High-Risk Pediatric Cancers. *Cancer Res* **80**, 1512-1523 (2020).

70. Sakamoto, H.*, et al.* The Evolutionary Origins of Recurrent Pancreatic Cancer. *Cancer Discov* **10**, 792-805 (2020).

71. Zhang, M.*, et al.* Clonal architecture in mesothelioma is prognostic and shapes the tumour microenvironment. *Nat Commun* **12**, 1751 (2021).

72. de Queiroz, K. Nodes, branches, and phylogenetic definitions. *Syst Biol* **62**, 625-632 (2013).

73. Alexandrov, L.B.*, et al.* Clock-like mutational processes in human somatic cells. *Nat Genet* **47**, 1402-1407 (2015).

74. Rodon, J.*, et al.* Genomic and transcriptomic profiling expands precision cancer medicine: the WINTHER trial. *Nat Med* **25**, 751-758 (2019).

75. Long, G.V.*, et al.* Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N Engl J Med* **371**, 1877-1888 (2014).

76. Goodman, A.M.*, et al.* Tumor Mutational Burden as an Independent Predictor of Response to Immunotherapy in Diverse Cancers. *Mol Cancer Ther* **16**, 2598-2608 (2017).

77. Lone, S.N.*, et al.* Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments. *Mol Cancer* **21**, 79 (2022).

78. Mattox, A.K.*, et al.* The Origin of Highly Elevated Cell-Free DNA in Healthy Individuals and Patients with Pancreatic, Colorectal, Lung, or Ovarian Cancer. *Cancer Discov* **13**, 2166-2179 (2023).

79. Ulz, P.*, et al.* Whole-genome plasma sequencing reveals focal amplifications as a driving force in metastatic prostate cancer. *Nat Commun* 7, 12008 (2016).

80. Manier, S.*, et al.* Prognostic role of circulating exosomal miRNAs in multiple myeloma. *Blood* **129**, 2429-2436 (2017).

81. Siravegna, G.*, et al.* Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat Med* **21**, 827 (2015).

82. Liu, Q.*, et al.* Circulating exosomal microRNAs as prognostic biomarkers for non-small-cell lung cancer. *Oncotarget* **8**, 13048-13058 (2017).

83. Connal, S.*, et al.* Liquid biopsies: the future of cancer early detection. *J Transl Med* **21**, 118 (2023).

84. Crowley, E., Di Nicolantonio, F., Loupakis, F. & Bardelli, A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol* **10**, 472-484 (2013).

85. Batool, S.M.*, et al.* The Liquid Biopsy Consortium: Challenges and opportunities for early cancer detection and monitoring. *Cell Rep Med* **4**, 101198 (2023).

86. Wan, J.C.M.*, et al.* Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* **17**, 223-238 (2017).

87. Aceto, N.*, et al.* Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell* **158**, 1110-1122 (2014).

88. Lin, D.*, et al.* Circulating tumor cells: biology and clinical significance. *Signal Transduct Target Ther* **6**, 404 (2021).

89. Magbanua, M.J.M.*, et al.* Synchronous Detection of Circulating Tumor Cells in Blood and Disseminated Tumor Cells in Bone Marrow Predicts Adverse Outcome in Early Breast Cancer. *Clin Cancer Res* **25**, 5388-5397 (2019).

90. Basso, U.*, et al.* Prognostic Role of Circulating Tumor Cells in Metastatic Renal Cell Carcinoma: A Large, Multicenter, Prospective Trial. *Oncologist* **26**, 740-750 (2021).

91. Cieslikowski, W.A.*, et al.* Circulating Tumor Cells as a Marker of Disseminated Disease in Patients with Newly Diagnosed High-Risk Prostate Cancer. *Cancers (Basel)* **12**(2020).

92. Lozano, R.*, et al.* Value of Early Circulating Tumor Cells Dynamics to Estimate Docetaxel Benefit in Metastatic Castration-Resistant Prostate Cancer (mCRPC) Patients. *Cancers (Basel)* **13**(2021).

93. Matsushita, D.*, et al.* Clinical significance of circulating tumor cells in the response to trastuzumab for HER2-negative metastatic gastric cancer. *Cancer Chemother Pharmacol* **87**, 789-797 (2021).

94. Thery, L.*, et al.* Circulating Tumor Cells in Early Breast Cancer. *JNCI Cancer Spectr* **3**, pkz026 (2019).

95. Mouliere, F., El Messaoudi, S., Pang, D., Dritschilo, A. & Thierry, A.R. Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer. *Mol Oncol* **8**, 927-941 (2014).

96. Mouliere, F.*, et al.* High fragmentation characterizes tumour-derived circulating DNA. *PLoS One* **6**, e23418 (2011).

97. Cohen, J.D.*, et al.* Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **359**, 926-930 (2018).

98. Heitzer, E., Auinger, L. & Speicher, M.R. Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living. *Trends Mol Med* **26**, 519-528 (2020).

99. Lui, Y.Y.*, et al.* Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. *Clin Chem* **48**, 421-427 (2002).

100. Gil, M.M., Quezada, M.S., Revello, R., Akolekar, R. & Nicolaides, K.H. Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: updated meta-analysis. *Ultrasound Obstet Gynecol* **45**, 249-266 (2015).

101. Norwitz, E.R. & Levy, B. Noninvasive prenatal testing: the future is now. *Rev Obstet Gynecol* **6**, 48-62 (2013).

102. Mandel, P. & Metais, P. [Nuclear Acids In Human Blood Plasma]. *C R Seances Soc Biol Fil* **142**, 241-243 (1948).

103. Jahr, S.*, et al.* DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* **61**, 1659-1665 (2001).

104. Schwarzenbach, H., Hoon, D.S. & Pantel, K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* **11**, 426-437 (2011).

105. Anker, P., Stroun, M. & Maurice, P.A. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. *Cancer Res* **35**, 2375-2382 (1975).

106. Grabuschnig, S.*, et al.* Putative Origins of Cell-Free DNA in Humans: A Review of Active and Passive Nucleic Acid Release Mechanisms. *Int J Mol Sci* **21**(2020).

107. Ramachandran, S. & Henikoff, S. Replicating Nucleosomes. *Sci Adv* **1**(2015).

108. Diehl, F., *et al.* Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci U S A* **102**, 16368-16373 (2005).

109. Diaz, L.A., Jr. & Bardelli, A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol* **32**, 579-586 (2014).

110. Parkinson, C.A., *et al.* Exploratory Analysis of TP53 Mutations in Circulating Tumour DNA as Biomarkers of Treatment Response for Patients with Relapsed High-Grade Serous Ovarian Carcinoma: A Retrospective Study. *PLoS Med* **13**, e1002198 (2016).

111. Abbosh, C., *et al.* Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature* **545**, 446-451 (2017).

112. Viller Tuxen, I., *et al.* Plasma total cell-free DNA is a prognostic biomarker of overall survival in metastatic solid tumour patients. *Br J Cancer* **121**, 125-130 (2019).

113. Mamis, K. & Bozic, I. Early-stage cancer results in a multiplicative increase in cell-free DNA originating from healthy tissue. *bioRxiv*, 2024.2001.2026.577500 (2024).

114. Yu, S.C., *et al.* High-resolution profiling of fetal DNA clearance from maternal plasma by massively parallel sequencing. *Clin Chem* **59**, 1228-1237 (2013).

115. Lo, Y.M., *et al.* Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* **64**, 218-224 (1999).

116. Stroun, M., *et al.* Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* **46**, 318-322 (1989).

117. Sorenson, G.D., *et al.* Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer Epidemiol Biomarkers Prev* **3**, 67-71 (1994).

118. Bettegowda, C., *et al.* Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* **6**, 224ra224 (2014).

119. Creemers, A., *et al.* Clinical value of ctDNA in upper-GI cancers: A systematic review and meta-analysis. *Biochim Biophys Acta Rev Cancer* **1868**, 394-403 (2017).

120. Forshew, T., *et al.* Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med* **4**, 136ra168 (2012).

121. Murtaza, M., *et al.* Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* **497**, 108-112 (2013).

122. Leary, R.J., *et al.* Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. *Sci Transl Med* **4**, 162ra154 (2012).

123. Kamat, A.A., *et al.* Circulating cell-free DNA: a novel biomarker for response to therapy in ovarian carcinoma. *Cancer Biol Ther* **5**, 1369-1374 (2006).

124. Lecomte, T., *et al.* Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. *Int J Cancer* **100**, 542-548 (2002).

125. Gautschi, O., *et al.* Origin and prognostic value of circulating KRAS mutations in lung cancer patients. *Cancer Lett* **254**, 265-273 (2007).

126. Nygaard, A.D., Garm Spindler, K.L., Pallisgaard, N., Andersen, R.F. & Jakobsen, A. The prognostic value of KRAS mutated plasma DNA in advanced non-small cell lung cancer. *Lung Cancer* **79**, 312-317 (2013).

127. Wang, S., *et al.* Potential clinical significance of a plasma-based KRAS mutation analysis in patients with advanced non-small cell lung cancer. *Clin Cancer Res* **16**, 1324-1330 (2010).

128. Scherer, F., *et al.* Distinct biological subtypes and patterns of genome evolution in lymphoma revealed by circulating tumor DNA. *Sci Transl Med* **8**, 364ra155 (2016).

129. Udomruk, S., Orrapin, S., Pruksakorn, D. & Chaiyawat, P. Size distribution of cell-free DNA in oncology. *Crit Rev Oncol Hematol* **166**, 103455 (2021).

130. Underhill, H.R.*, et al.* Fragment Length of Circulating Tumor DNA. *PLoS Genet* **12**, e1006162 (2016).

131. Lapin, M.*, et al.* Fragment size and level of cell-free DNA provide prognostic information in patients with advanced pancreatic cancer. *J Transl Med* **16**, 300 (2018).

132. Yamamoto, Y.*, et al.* Increased level and fragmentation of plasma circulating cell-free DNA are diagnostic and prognostic markers for renal cell carcinoma. *Oncotarget* **9**, 20467-20475 (2018).

133. Dawson, S.J.*, et al.* Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* **368**, 1199-1209 (2013).

134. Diehl, F.*, et al.* Circulating mutant DNA to assess tumor dynamics. *Nat Med* **14**, 985-990 (2008).

135. Gray, E.S.*, et al.* Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. *Oncotarget* **6**, 42008-42018 (2015).

136. Newman, A.M.*, et al.* An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med* **20**, 548-554 (2014).

137. Pantel, K. & Alix-Panabieres, C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. *Nat Rev Clin Oncol* **16**, 409-424 (2019).

138. Garcia-Murillas, I.*, et al.* Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. *Sci Transl Med* **7**, 302ra133 (2015).

139. Olsson, E.*, et al.* Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. *EMBO Mol Med* **7**, 1034-1047 (2015).

140. Reinert, T.*, et al.* Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. *Gut* **65**, 625-634 (2016).

141. Tie, J.*, et al.* Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci Transl Med* **8**, 346ra392 (2016).

142. Reinert, T.*, et al.* Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. *JAMA Oncol* **5**, 1124-1131 (2019).

143. Gormally, E.*, et al.* TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: a prospective study. *Cancer Res* **66**, 6871-6876 (2006).

144. Amant, F.*, et al.* Presymptomatic Identification of Cancers in Pregnant Women During Noninvasive Prenatal Testing. *JAMA Oncol* **1**, 814-819 (2015).

145. Normanno, N., Denis, M.G., Thress, K.S., Ratcliffe, M. & Reck, M. Guide to detecting epidermal growth factor receptor (EGFR) mutations in ctDNA of patients with advanced non-small-cell lung cancer. *Oncotarget* **8**, 12501-12516 (2017).

146. Pishvaian, M.J.*, et al.* Molecular Profiling of Patients with Pancreatic Cancer: Initial Results from the Know Your Tumor Initiative. *Clin Cancer Res* **24**, 5018-5027 (2018).

147. Schwaederle, M.*, et al.* Use of Liquid Biopsies in Clinical Oncology: Pilot Experience in 168 Patients. *Clin Cancer Res* **22**, 5497-5505 (2016).

148. De Mattos-Arruda, L.*, et al.* Establishing the origin of metastatic deposits in the setting of multiple primary malignancies: the role of massively parallel sequencing. *Mol Oncol* **8**, 150-158 (2014).

149. De Mattos-Arruda, L.*, et al.* Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. *Ann Oncol* **25**, 1729-1735 (2014).

150. Jamal-Hanjani, M., *et al.* Detection of ubiquitous and heterogeneous mutations in cell-free DNA from patients with early-stage non-small-cell lung cancer. *Ann Oncol* **27**, 862-867 (2016).
151. Murtaza, M., *et al.* Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nat Commun* **6**, 8760 (2015).
152. Parikh, A.R., *et al.* Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. *Nat Med* **25**, 1415-1421 (2019).
153. Abbosh, C., *et al.* Tracking early lung cancer metastatic dissemination in TRACERx using ctDNA. *Nature* **616**, 553-562 (2023).
154. Romanel, A., *et al.* Plasma AR and abiraterone-resistant prostate cancer. *Sci Transl Med* **7**, 312re310 (2015).
155. Ruas, J.S., *et al.* Somatic Copy Number Alteration in Circulating Tumor DNA for Monitoring of Pediatric Patients with Cancer. *Biomedicines* **11**(2023).
156. Nguyen, M.T.N., *et al.* Circulating tumor DNA-based copy-number profiles enable monitoring treatment effects during therapy in high-grade serous carcinoma. *Biomed Pharmacother* **168**, 115630 (2023).
157. Deveson, I.W., *et al.* Evaluating the analytical validity of circulating tumor DNA sequencing assays for precision oncology. *Nat Biotechnol* **39**, 1115-1128 (2021).
158. Abbosh, C., Swanton, C. & Birkbak, N.J. Clonal haematopoiesis: a source of biological noise in cell-free DNA analyses. *Ann Oncol* **30**, 358-359 (2019).
159. Hu, Y., *et al.* False-Positive Plasma Genotyping Due to Clonal Hematopoiesis. *Clin Cancer Res* **24**, 4437-4443 (2018).
160. Phallen, J., *et al.* Direct detection of early-stage cancers using circulating tumor DNA. *Sci Transl Med* **9**(2017).
161. Kennedy, S.R., *et al.* Detecting ultralow-frequency mutations by Duplex Sequencing. *Nature Protocols* **9**, 2586-2606 (2014).
162. Newman, A.M., *et al.* An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nature Medicine* **20**, 548-554 (2014).
163. Newman, A.M., *et al.* Integrated digital error suppression for improved detection of circulating tumor DNA. *Nature Biotechnology* **34**, 547-555 (2016).
164. Zviran, A., *et al.* Genome-wide cell-free DNA mutational integration enables ultra-sensitive cancer monitoring. *Nature Medicine* **26**, 1114-1124 (2020).
165. Widman, A.J., *et al.* Ultrasensitive plasma-based monitoring of tumor burden using machine-learning-guided signal enrichment. *Nat Med* (2024).
166. Anderson, N.M. & Simon, M.C. The tumor microenvironment. *Current Biology* **30**, R921-R925 (2020).
167. Dvorak, H.F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**, 1650-1659 (1986).
168. Swann, J.B. & Smyth, M.J. Immune surveillance of tumors. *J Clin Invest* **117**, 1137-1146 (2007).
169. Vivier, E., *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44-49 (2011).
170. Olingy, C.E., Dinh, H.Q. & Hedrick, C.C. Monocyte heterogeneity and functions in cancer. *J Leukoc Biol* **106**, 309-322 (2019).
171. Blériot, C., Chakarov, S. & Ginhoux, F. Determinants of Resident Tissue Macrophage Identity and Function. *Immunity* **52**, 957-970 (2020).

172. Wynn, T.A., Chawla, A. & Pollard, J.W. Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445-455 (2013).

173. Mantovani, A., Allavena, P., Marchesi, F. & Garlanda, C. Macrophages as tools and targets in cancer therapy. *Nature Reviews Drug Discovery* **21**, 799-820 (2022).

174. Mosser, D.M. & Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **8**, 958-969 (2008).

175. Wculek, S.K.*, et al.* Dendritic cells in cancer immunology and immunotherapy. *Nature Reviews Immunology* **20**, 7-24 (2020).

176. Gardner, A. & Ruffell, B. Dendritic Cells and Cancer Immunity. *Trends Immunol* **37**, 855-865 (2016).

177. Dudek, A.M., Martin, S., Garg, A.D. & Agostinis, P. Immature, Semi-Mature, and Fully Mature Dendritic Cells: Toward a DC-Cancer Cells Interface That Augments Anticancer Immunity. *Front Immunol* **4**, 438 (2013).

178. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat Immunol* **9**, 503-510 (2008).

179. Luckheeram, R.V., Zhou, R., Verma, A.D. & Xia, B. $CD4^+$T cells: differentiation and functions. *Clin Dev Immunol* **2012**, 925135 (2012).

180. Vignali, D.A.A., Collison, L.W. & Workman, C.J. How regulatory T cells work. *Nature Reviews Immunology* **8**, 523-532 (2008).

181. Togashi, Y., Shitara, K. & Nishikawa, H. Regulatory T cells in cancer immunosuppression — implications for anticancer therapy. *Nature Reviews Clinical Oncology* **16**, 356-371 (2019).

182. LeBien, T.W. & Tedder, T.F. B lymphocytes: how they develop and function. *Blood* **112**, 1570-1580 (2008).

183. Nutt, S.L., Hodgkin, P.D., Tarlinton, D.M. & Corcoran, L.M. The generation of antibody-secreting plasma cells. *Nature Reviews Immunology* **15**, 160-171 (2015).

184. Largeot, A., Pagano, G., Gonder, S., Moussay, E. & Paggetti, J. The B-side of Cancer Immunity: The Underrated Tune. *Cells* **8**(2019).

185. Pardoll, D.M. The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews Cancer* **12**, 252-264 (2012).

186. Buchbinder, E.I. & Desai, A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol* **39**, 98-106 (2016).

187. Wherry, E.J. T cell exhaustion. *Nature Immunology* **12**, 492-499 (2011).

188. Davoli, T., Uno, H., Wooten, E.C. & Elledge, S.J. Tumor aneuploidy correlates with markers of immune evasion and with reduced response to immunotherapy. *Science* **355**(2017).

189. Bakhoum, S.F. & Cantley, L.C. The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment. *Cell* **174**, 1347-1360 (2018).

190. Laughney, A.M., Elizalde, S., Genovese, G. & Bakhoum, S.F. Dynamics of Tumor Heterogeneity Derived from Clonal Karyotypic Evolution. *Cell Rep* **12**, 809-820 (2015).

191. Santaguida, S.*, et al.* Chromosome Mis-segregation Generates Cell-Cycle-Arrested Cells with Complex Karyotypes that Are Eliminated by the Immune System. *Dev Cell* **41**, 638-651.e635 (2017).

192. Mackenzie, K.J.*, et al.* cGAS surveillance of micronuclei links genome instability to innate immunity. *Nature* **548**, 461-465 (2017).

193. McGranahan, N.*, et al.* Allele-Specific HLA Loss and Immune Escape in Lung Cancer Evolution. *Cell* **171**, 1259-1271.e1211 (2017).

194. Zhang, A.W., *et al.* Interfaces of Malignant and Immunologic Clonal Dynamics in Ovarian Cancer. *Cell* **173**, 1755-1769.e1722 (2018).

195. Hester, C.A., *et al.* Incidence and comparative outcomes of periampullary cancer: A population-based analysis demonstrating improved outcomes and increased use of adjuvant therapy from 2004 to 2012. *J Surg Oncol* **119**, 303-317 (2019).

196. McGuigan, A., *et al.* Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol* **24**, 4846-4861 (2018).

197. Siegel, R.L., Miller, K.D., Wagle, N.S. & Jemal, A. Cancer statistics, 2023. *CA Cancer J Clin* **73**, 17-48 (2023).

198. Park, W., Chawla, A. & O'Reilly, E.M. Pancreatic Cancer: A Review. *JAMA* **326**, 851-862 (2021).

199. Bray, F., *et al.* Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **74**, 229-263 (2024).

200. Rawla, P., Sunkara, T. & Gaduputi, V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J Oncol* **10**, 10-27 (2019).

201. Lynch, S.M., *et al.* Cigarette smoking and pancreatic cancer: a pooled analysis from the pancreatic cancer cohort consortium. *Am J Epidemiol* **170**, 403-413 (2009).

202. Molina-Montes, E., *et al.* Pancreatic Cancer Risk in Relation to Lifetime Smoking Patterns, Tobacco Type, and Dose-Response Relationships. *Cancer Epidemiol Biomarkers Prev* **29**, 1009-1018 (2020).

203. Arslan, A.A., *et al.* Anthropometric measures, body mass index, and pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan). *Arch Intern Med* **170**, 791-802 (2010).

204. Michaud, D.S., *et al.* Physical activity, obesity, height, and the risk of pancreatic cancer. *JAMA* **286**, 921-929 (2001).

205. Naudin, S., *et al.* Lifetime and baseline alcohol intakes and risk of pancreatic cancer in the European Prospective Investigation into Cancer and Nutrition study. *Int J Cancer* **143**, 801-812 (2018).

206. Lucenteforte, E., *et al.* Alcohol consumption and pancreatic cancer: a pooled analysis in the International Pancreatic Cancer Case-Control Consortium (PanC4). *Ann Oncol* **23**, 374-382 (2012).

207. Duell, E.J., *et al.* Pancreatitis and pancreatic cancer risk: a pooled analysis in the International Pancreatic Cancer Case-Control Consortium (PanC4). *Ann Oncol* **23**, 2964-2970 (2012).

208. Kirkegard, J., Mortensen, M.R., Johannsen, I.R., Mortensen, F.V. & Cronin-Fenton, D. Positive predictive value of acute and chronic pancreatitis diagnoses in the Danish National Patient Registry: A validation study. *Scand J Public Health* **48**, 14-19 (2020).

209. Fan, X., *et al.* Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study. *Gut* **67**, 120-127 (2018).

210. Michaud, D.S., *et al.* Plasma antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study. *Gut* **62**, 1764-1770 (2013).

211. Chari, S.T., *et al.* Probability of pancreatic cancer following diabetes: a population-based study. *Gastroenterology* **129**, 504-511 (2005).

212. Gupta, S., *et al.* New-onset diabetes and pancreatic cancer. *Clin Gastroenterol Hepatol* **4**, 1366-1372; quiz 1301 (2006).

213. Munigala, S., Singh, A., Gelrud, A. & Agarwal, B. Predictors for Pancreatic Cancer Diagnosis Following New-Onset Diabetes Mellitus. *Clin Transl Gastroenterol* **6**, e118 (2015).

214. Cotterchio, M., Lowcock, E., Hudson, T.J., Greenwood, C. & Gallinger, S. Association between allergies and risk of pancreatic cancer. *Cancer Epidemiol Biomarkers Prev* **23**, 469-480 (2014).

215. Gandini, S., Lowenfels, A.B., Jaffee, E.M., Armstrong, T.D. & Maisonneuve, P. Allergies and the risk of pancreatic cancer: a meta-analysis with review of epidemiology and biological mechanisms. *Cancer Epidemiol Biomarkers Prev* **14**, 1908-1916 (2005).

216. Klein, A.P. Pancreatic cancer epidemiology: understanding the role of lifestyle and inherited risk factors. *Nat Rev Gastroenterol Hepatol* **18**, 493-502 (2021).

217. Singhi, A.D.*, et al.* A histomorphologic comparison of familial and sporadic pancreatic cancers. *Pancreatology* **15**, 387-391 (2015).

218. Norris, A.L.*, et al.* Familial and sporadic pancreatic cancer share the same molecular pathogenesis. *Fam Cancer* **14**, 95-103 (2015).

219. Shi, C.*, et al.* Increased Prevalence of Precursor Lesions in Familial Pancreatic Cancer Patients. *Clin Cancer Res* **15**, 7737-7743 (2009).

220. Hu, C.*, et al.* Association Between Inherited Germline Mutations in Cancer Predisposition Genes and Risk of Pancreatic Cancer. *JAMA* **319**, 2401-2409 (2018).

221. Hu, C.*, et al.* Multigene Hereditary Cancer Panels Reveal High-Risk Pancreatic Cancer Susceptibility Genes. *JCO Precis Oncol* **2**(2018).

222. Golan, T.*, et al.* Geographic and Ethnic Heterogeneity of Germline BRCA1 or BRCA2 Mutation Prevalence Among Patients With Metastatic Pancreatic Cancer Screened for Entry Into the POLO Trial. *J Clin Oncol* **38**, 1442-1454 (2020).

223. Shindo, K.*, et al.* Deleterious Germline Mutations in Patients With Apparently Sporadic Pancreatic Adenocarcinoma. *J Clin Oncol* **35**, 3382-3390 (2017).

224. Giardiello, F.M.*, et al.* Increased risk of cancer in the Peutz-Jeghers syndrome. *N Engl J Med* **316**, 1511-1514 (1987).

225. van Lier, M.G.*, et al.* High cancer risk in Peutz-Jeghers syndrome: a systematic review and surveillance recommendations. *Am J Gastroenterol* **105**, 1258-1264; author reply 1265 (2010).

226. Goldstein, A.M., Struewing, J.P., Fraser, M.C., Smith, M.W. & Tucker, M.A. Prospective risk of cancer in CDKN2A germline mutation carriers. *J Med Genet* **41**, 421-424 (2004).

227. Vasen, H.F.*, et al.* Risk of developing pancreatic cancer in families with familial atypical multiple mole melanoma associated with a specific 19 deletion of p16 (p16-Leiden). *Int J Cancer* **87**, 809-811 (2000).

228. Kastrinos, F.*, et al.* Risk of pancreatic cancer in families with Lynch syndrome. *JAMA* **302**, 1790-1795 (2009).

229. Bryant, H.E.*, et al.* Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913-917 (2005).

230. Villarroel, M.C.*, et al.* Personalizing cancer treatment in the age of global genomic analyses: PALB2 gene mutations and the response to DNA damaging agents in pancreatic cancer. *Mol Cancer Ther* **10**, 3-8 (2011).

231. Le, D.T.*, et al.* Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* **357**, 409-413 (2017).

232. Hahn, S.A.*, et al.* BRCA2 germline mutations in familial pancreatic carcinoma. *J Natl Cancer Inst* **95**, 214-221 (2003).

233. Zhen, D.B., *et al.* BRCA1, BRCA2, PALB2, and CDKN2A mutations in familial pancreatic cancer: a PACGENE study. *Genet Med* **17**, 569-577 (2015).

234. Slater, E.P., *et al.* PALB2 mutations in European familial pancreatic cancer families. *Clin Genet* **78**, 490-494 (2010).

235. Jones, S., *et al.* Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science* **324**, 217 (2009).

236. Grant, R.C., *et al.* Prevalence of germline mutations in cancer predisposition genes in patients with pancreatic cancer. *Gastroenterology* **148**, 556-564 (2015).

237. Whitcomb, D.C., *et al.* Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* **14**, 141-145 (1996).

238. Whitcomb, D.C., *et al.* A gene for hereditary pancreatitis maps to chromosome 7q35. *Gastroenterology* **110**, 1975-1980 (1996).

239. Rebours, V., *et al.* Risk of pancreatic adenocarcinoma in patients with hereditary pancreatitis: a national exhaustive series. *Am J Gastroenterol* **103**, 111-119 (2008).

240. Artinyan, A., *et al.* The anatomic location of pancreatic cancer is a prognostic factor for survival. *HPB (Oxford)* **10**, 371-376 (2008).

241. Walter, F.M., *et al.* Symptoms and patient factors associated with diagnostic intervals for pancreatic cancer (SYMPTOM pancreatic study): a prospective cohort study. *Lancet Gastroenterol Hepatol* **1**, 298-306 (2016).

242. Strasberg, S.M., *et al.* Jaundice: an important, poorly recognized risk factor for diminished survival in patients with adenocarcinoma of the head of the pancreas. *HPB (Oxford)* **16**, 150-156 (2014).

243. Aslanian, H.R., Lee, J.H. & Canto, M.I. AGA Clinical Practice Update on Pancreas Cancer Screening in High-Risk Individuals: Expert Review. *Gastroenterology* **159**, 358-362 (2020).

244. Tseng, D.S.J., *et al.* The Role of CT in Assessment of Extraregional Lymph Node Involvement in Pancreatic and Periampullary Cancer: A Diagnostic Accuracy Study. *Radiol Imaging Cancer* **3**, e200014 (2021).

245. Park, H.S., *et al.* Preoperative evaluation of pancreatic cancer: comparison of gadolinium-enhanced dynamic MRI with MR cholangiopancreatography versus MDCT. *J Magn Reson Imaging* **30**, 586-595 (2009).

246. Kleeff, J., *et al.* Pancreatic cancer. *Nat Rev Dis Primers* **2**, 16022 (2016).

247. Amin, M.B., *et al.* The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin* **67**, 93-99 (2017).

248. Allen, P.J., *et al.* Multi-institutional Validation Study of the American Joint Commission on Cancer (8th Edition) Changes for T and N Staging in Patients With Pancreatic Adenocarcinoma. *Ann Surg* **265**, 185-191 (2017).

249. Lee, T., Teng, T.Z.J. & Shelat, V.G. Carbohydrate antigen 19-9 - tumor marker: Past, present, and future. *World J Gastrointest Surg* **12**, 468-490 (2020).

250. Scara, S., Bottoni, P. & Scatena, R. CA 19-9: Biochemical and Clinical Aspects. *Adv Exp Med Biol* **867**, 247-260 (2015).

251. Wood, L.D., Canto, M.I., Jaffee, E.M. & Simeone, D.M. Pancreatic Cancer: Pathogenesis, Screening, Diagnosis, and Treatment. *Gastroenterology* **163**, 386-402 e381 (2022).

252. Matsuda, Y., *et al.* The Prevalence and Clinicopathological Characteristics of High-Grade Pancreatic Intraepithelial Neoplasia: Autopsy Study Evaluating the Entire Pancreatic Parenchyma. *Pancreas* **46**, 658-664 (2017).

253. Basturk, O., *et al.* A Revised Classification System and Recommendations From the Baltimore Consensus Meeting for Neoplastic Precursor Lesions in the Pancreas. *Am J Surg Pathol* **39**, 1730-1741 (2015).

254. Laffan, T.A., *et al.* Prevalence of unsuspected pancreatic cysts on MDCT. *AJR Am J Roentgenol* **191**, 802-807 (2008).

255. Noe, M., *et al.* Genomic characterization of malignant progression in neoplastic pancreatic cysts. *Nat Commun* **11**, 4085 (2020).

256. Taherian, M., Wang, H. & Wang, H. Pancreatic Ductal Adenocarcinoma: Molecular Pathology and Predictive Biomarkers. *Cells* **11**(2022).

257. Luchini, C., Capelli, P. & Scarpa, A. Pancreatic Ductal Adenocarcinoma and Its Variants. *Surg Pathol Clin* **9**, 547-560 (2016).

258. Bailey, P., *et al.* Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* **531**, 47-52 (2016).

259. Collisson, E.A., *et al.* Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med* **17**, 500-503 (2011).

260. Moffitt, R.A., *et al.* Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. *Nat Genet* **47**, 1168-1178 (2015).

261. Chan-Seng-Yue, M., *et al.* Transcription phenotypes of pancreatic cancer are driven by genomic events during tumor evolution. *Nat Genet* **52**, 231-240 (2020).

262. Maurer, C., *et al.* Experimental microdissection enables functional harmonisation of pancreatic cancer subtypes. *Gut* **68**, 1034-1043 (2019).

263. Puleo, F., *et al.* Stratification of Pancreatic Ductal Adenocarcinomas Based on Tumor and Microenvironment Features. *Gastroenterology* **155**, 1999-2013 e1993 (2018).

264. Halbrook, C.J., Lyssiotis, C.A., Pasca di Magliano, M. & Maitra, A. Pancreatic cancer: Advances and challenges. *Cell* **186**, 1729-1754 (2023).

265. Aung, K.L., *et al.* Genomics-Driven Precision Medicine for Advanced Pancreatic Cancer: Early Results from the COMPASS Trial. *Clin Cancer Res* **24**, 1344-1354 (2018).

266. Almoguera, C., *et al.* Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell* **53**, 549-554 (1988).

267. Caldas, C., *et al.* Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet* **8**, 27-32 (1994).

268. Scarpa, A., *et al.* Pancreatic adenocarcinomas frequently show p53 gene mutations. *Am J Pathol* **142**, 1534-1543 (1993).

269. Hahn, S.A., *et al.* DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**, 350-353 (1996).

270. Jones, S., *et al.* Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* **321**, 1801-1806 (2008).

271. Waddell, N., *et al.* Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* **518**, 495-501 (2015).

272. Cancer Genome Atlas Research Network. Electronic address, a.a.d.h.e. & Cancer Genome Atlas Research, N. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* **32**, 185-203 e113 (2017).

273. Witkiewicz, A.K., *et al.* Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. *Nat Commun* **6**, 6744 (2015).

274. Yousef, A.*, et al.* Impact of KRAS mutations and co-mutations on clinical outcomes in pancreatic ductal adenocarcinoma. *NPJ Precis Oncol* **8**, 27 (2024).

275. Sandhu, V.*, et al.* The Genomic Landscape of Pancreatic and Periampullary Adenocarcinoma. *Cancer Res* **76**, 5092-5102 (2016).

276. Hayashi, A., Hong, J. & Iacobuzio-Donahue, C.A. The pancreatic cancer genome revisited. *Nat Rev Gastroenterol Hepatol* **18**, 469-481 (2021).

277. Makohon-Moore, A.P.*, et al.* Precancerous neoplastic cells can move through the pancreatic ductal system. *Nature* **561**, 201-205 (2018).

278. Wu, J.*, et al.* Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. *Proc Natl Acad Sci U S A* **108**, 21188-21193 (2011).

279. Kuboki, Y.*, et al.* Single-cell sequencing defines genetic heterogeneity in pancreatic cancer precursor lesions. *J Pathol* **247**, 347-356 (2019).

280. Braxton, A.M.*, et al.* 3D genomic mapping reveals multifocality of human pancreatic precancers. *Nature* **629**, 679-687 (2024).

281. Kim, H.J., Lee, H.N., Jeong, M.S. & Jang, S.B. Oncogenic KRAS: Signaling and Drug Resistance. *Cancers (Basel)* **13**(2021).

282. Huang, L., Guo, Z., Wang, F. & Fu, L. KRAS mutation: from undruggable to druggable in cancer. *Signal Transduct Target Ther* **6**, 386 (2021).

283. Huang, L., Guo, Z., Wang, F. & Fu, L. KRAS mutation: from undruggable to druggable in cancer. *Signal Transduction and Targeted Therapy* **6**, 386 (2021).

284. Vogelstein, B., Lane, D. & Levine, A.J. Surfing the p53 network. *Nature* **408**, 307-310 (2000).

285. Notta, F.*, et al.* A renewed model of pancreatic cancer evolution based on genomic rearrangement patterns. *Nature* **538**, 378-382 (2016).

286. Hosoda, W.*, et al.* Genetic analyses of isolated high-grade pancreatic intraepithelial neoplasia (HG-PanIN) reveal paucity of alterations in TP53 and SMAD4. *J Pathol* **242**, 16-23 (2017).

287. Ruas, M. & Peters, G. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1378**, F115-F177 (1998).

288. Massague, J. TGFbeta in Cancer. *Cell* **134**, 215-230 (2008).

289. Huang, W.*, et al.* Pattern of Invasion in Human Pancreatic Cancer Organoids Is Associated with Loss of SMAD4 and Clinical Outcome. *Cancer Res* **80**, 2804-2817 (2020).

290. Detlefsen, S.*, et al.* High overall copy number variation burden by genome-wide methylation profiling holds negative prognostic value in surgically treated pancreatic ductal adenocarcinoma. *Hum Pathol* **142**, 68-80 (2023).

291. Huebner, A.*, et al.* ACT-Discover: identifying karyotype heterogeneity in pancreatic cancer evolution using ctDNA. *Genome Med* **15**, 27 (2023).

292. Lapin, M.*, et al.* Comprehensive ctDNA Measurements Improve Prediction of Clinical Outcomes and Enable Dynamic Tracking of Disease Progression in Advanced Pancreatic Cancer. *Clin Cancer Res* **29**, 1267-1278 (2023).

293. Sivapalan, L.*, et al.* Longitudinal profiling of circulating tumour DNA for tracking tumour dynamics in pancreatic cancer. *BMC Cancer* **22**, 369 (2022).

294. Hata, T.*, et al.* Genome-Wide Somatic Copy Number Alterations and Mutations in High-Grade Pancreatic Intraepithelial Neoplasia. *Am J Pathol* **188**, 1723-1733 (2018).

295. Haeno, H.*, et al.* Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. *Cell* **148**, 362-375 (2012).

296. Roder, J.D., Thorban, S., Pantel, K. & Siewert, J.R. Micrometastases in bone marrow: prognostic indicators for pancreatic cancer. *World J Surg* **23**, 888-891 (1999).

297. Vogel, I.*, et al.* Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor. *Clin Cancer Res* **5**, 593-599 (1999).

298. Yachida, S.*, et al.* Implications of peritoneal washing cytology in patients with potentially resectable pancreatic cancer. *Br J Surg* **89**, 573-578 (2002).

299. Kamisawa, T., Isawa, T., Koike, M., Tsuruta, K. & Okamoto, A. Hematogenous metastases of pancreatic ductal carcinoma. *Pancreas* **11**, 345-349 (1995).

300. Disibio, G. & French, S.W. Metastatic patterns of cancers: results from a large autopsy study. *Arch Pathol Lab Med* **132**, 931-939 (2008).

301. Mao, C., Domenico, D.R., Kim, K., Hanson, D.J. & Howard, J.M. Observations on the developmental patterns and the consequences of pancreatic exocrine adenocarcinoma. Findings of 154 autopsies. *Arch Surg* **130**, 125-134 (1995).

302. Pour, P.M., Bell, R.H. & Batra, S.K. Neural invasion in the staging of pancreatic cancer. *Pancreas* **26**, 322-325 (2003).

303. Takahashi, H.*, et al.* Transcriptomic Profile of Lymphovascular Invasion, a Known Risk Factor of Pancreatic Ductal Adenocarcinoma Metastasis. *Cancers (Basel)* **12**(2020).

304. Kim, J. Cell Dissemination in Pancreatic Cancer. *Cells* **11**(2022).

305. Makohon-Moore, A.P.*, et al.* Limited heterogeneity of known driver gene mutations among the metastases of individual patients with pancreatic cancer. *Nat Genet* **49**, 358-366 (2017).

306. Da Costa, J.M. *On the morbid anatomy and symptoms of cancer of the pancreas*, (JB Lippincott & Company, 1858).

307. Halsted, W.S. Contributions to the surgery of the bile passages, especially of the common bile-duct. *The Boston Medical and Surgical Journal* **141**, 645-654 (1899).

308. Whipple, A.O., Parsons, W.B. & Mullins, C.R. TEEATMENT of carcinoma of the ampulla of VATER. *Annals of surgery* **102**, 763-779 (1935).

309. Huttner, F.J.*, et al.* Pylorus-preserving pancreaticoduodenectomy (pp Whipple) versus pancreaticoduodenectomy (classic Whipple) for surgical treatment of periampullary and pancreatic carcinoma. *Cochrane Database Syst Rev* **2**, CD006053 (2016).

310. Scholten, L.*, et al.* Systematic review of functional outcome and quality of life after total pancreatectomy. *Br J Surg* **106**, 1735-1746 (2019).

311. Passeri, M.J.*, et al.* Total compared with partial pancreatectomy for pancreatic adenocarcinoma: assessment of resection margin, readmission rate, and survival from the U.S. National Cancer Database. *Curr Oncol* **26**, e346-e356 (2019).

312. Conroy, T.*, et al.* FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* **364**, 1817-1825 (2011).

313. Burris, H.A., 3rd*, et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* **15**, 2403-2413 (1997).

314. Alcindor, T. & Beauger, N. Oxaliplatin: a review in the era of molecularly targeted therapy. *Curr Oncol* **18**, 18-25 (2011).

315. Longley, D.B., Harkin, D.P. & Johnston, P.G. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* **3**, 330-338 (2003).

316. Mini, E., Nobili, S., Caciagli, B., Landini, I. & Mazzei, T. Cellular pharmacology of gemcitabine. *Ann Oncol* **17 Suppl 5**, v7-12 (2006).

317. Yardley, D.A. nab-Paclitaxel mechanisms of action and delivery. *J Control Release* **170**, 365-372 (2013).

318. Kciuk, M., Marciniak, B. & Kontek, R. Irinotecan-Still an Important Player in Cancer Chemotherapy: A Comprehensive Overview. *Int J Mol Sci* **21**(2020).

319. Neoptolemos, J.P.*, et al.* Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. *Lancet* **358**, 1576-1585 (2001).

320. Neoptolemos, J.P.*, et al.* A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* **350**, 1200-1210 (2004).

321. Oettle, H.*, et al.* Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial. *JAMA* **310**, 1473-1481 (2013).

322. Conroy, T.*, et al.* Five-Year Outcomes of FOLFIRINOX vs Gemcitabine as Adjuvant Therapy for Pancreatic Cancer: A Randomized Clinical Trial. *JAMA Oncol* **8**, 1571-1578 (2022).

323. Walko, C.M. & Lindley, C. Capecitabine: a review. *Clin Ther* **27**, 23-44 (2005).

324. Versteijne, E.*, et al.* Meta-analysis comparing upfront surgery with neoadjuvant treatment in patients with resectable or borderline resectable pancreatic cancer. *Br J Surg* **105**, 946-958 (2018).

325. Janssen, Q.P., O'Reilly, E.M., van Eijck, C.H.J. & Groot Koerkamp, B. Neoadjuvant Treatment in Patients With Resectable and Borderline Resectable Pancreatic Cancer. *Front Oncol* **10**, 41 (2020).

326. Sultana, A.*, et al.* Meta-analyses of chemotherapy for locally advanced and metastatic pancreatic cancer. *J Clin Oncol* **25**, 2607-2615 (2007).

327. Cunningham, D.*, et al.* Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. *J Clin Oncol* **27**, 5513-5518 (2009).

328. Von Hoff, D.D.*, et al.* Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* **369**, 1691-1703 (2013).

329. Golan, T.*, et al.* Maintenance Olaparib for Germline BRCA-Mutated Metastatic Pancreatic Cancer. *N Engl J Med* **381**, 317-327 (2019).

330. Wattenberg, M.M.*, et al.* Platinum response characteristics of patients with pancreatic ductal adenocarcinoma and a germline BRCA1, BRCA2 or PALB2 mutation. *Br J Cancer* **122**, 333-339 (2020).

331. Reiss, K.A.*, et al.* Retrospective Survival Analysis of Patients With Advanced Pancreatic Ductal Adenocarcinoma and Germline BRCA or PALB2 Mutations. *JCO Precis Oncol* **2**, 1-9 (2018).

332. Golan, T.*, et al.* Overall survival and clinical characteristics of pancreatic cancer in BRCA mutation carriers. *Br J Cancer* **111**, 1132-1138 (2014).

333. Balachandran, V.P.*, et al.* Identification of unique neoantigen qualities in long-term survivors of pancreatic cancer. *Nature* **551**, 512-516 (2017).

334. Humphris, J.L.*, et al.* Hypermutation In Pancreatic Cancer. *Gastroenterology* **152**, 68-74 e62 (2017).

335. Canon, J.*, et al.* The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity. *Nature* **575**, 217-223 (2019).

336. Hofmann, M.H.*, et al.* BI-3406, a Potent and Selective SOS1-KRAS Interaction Inhibitor, Is Effective in KRAS-Driven Cancers through Combined MEK Inhibition. *Cancer Discov* **11**, 142-157 (2021).

337. Krishnan, T., Roberts-Thomson, R., Broadbridge, V. & Price, T. Targeting Mutated KRAS Genes to Treat Solid Tumours. *Mol Diagn Ther* **26**, 39-49 (2022).

338. Wang, X., *et al.* Identification of MRTX1133, a Noncovalent, Potent, and Selective KRAS(G12D) Inhibitor. *J Med Chem* **65**, 3123-3133 (2022).

339. Leidner, R., *et al.* Neoantigen T-Cell Receptor Gene Therapy in Pancreatic Cancer. *N Engl J Med* **386**, 2112-2119 (2022).

340. O'Reilly, E.M. & Hechtman, J.F. Tumour response to TRK inhibition in a patient with pancreatic adenocarcinoma harbouring an NTRK gene fusion. *Ann Oncol* **30**, viii36-viii40 (2019).

341. Heining, C., *et al.* NRG1 Fusions in KRAS Wild-Type Pancreatic Cancer. *Cancer Discov* **8**, 1087-1095 (2018).

342. Quinonero, F., *et al.* The challenge of drug resistance in pancreatic ductal adenocarcinoma: a current overview. *Cancer Biol Med* **16**, 688-699 (2019).

343. Seyedi, S., *et al.* Resistance Management for Cancer: Lessons from Farmers. (2023).

344. Tabashnik, B.E. Managing resistance with multiple pesticide tactics: theory, evidence, and recommendations. *J Econ Entomol* **82**, 1263-1269 (1989).

345. Basanta, D. & Anderson, A.R. Exploiting ecological principles to better understand cancer progression and treatment. *Interface Focus* **3**, 20130020 (2013).

346. Acar, A., *et al.* Exploiting evolutionary steering to induce collateral drug sensitivity in cancer. *Nat Commun* **11**, 1923 (2020).

347. Archetti, M. & Pienta, K.J. Cooperation among cancer cells: applying game theory to cancer. *Nat Rev Cancer* **19**, 110-117 (2019).

348. Greaves, M. Darwinian medicine: a case for cancer. *Nat Rev Cancer* **7**, 213-221 (2007).

349. West, J., *et al.* A survey of open questions in adaptive therapy: Bridging mathematics and clinical translation. *Elife* **12**(2023).

350. Frei, E., 3rd, Elias, A., Wheeler, C., Richardson, P. & Hryniuk, W. The relationship between high-dose treatment and combination chemotherapy: the concept of summation dose intensity. *Clin Cancer Res* **4**, 2027-2037 (1998).

351. Zhang, J., Cunningham, J., Brown, J. & Gatenby, R. Evolution-based mathematical models significantly prolong response to abiraterone in metastatic castrate-resistant prostate cancer and identify strategies to further improve outcomes. *Elife* **11**(2022).

352. Hockings, H., *et al.* Adaptive therapy achieves long-term control of chemotherapy resistance in high grade ovarian cancer. *bioRxiv* (2023).

353. Mouliere, F., *et al.* Enhanced detection of circulating tumor DNA by fragment size analysis. *Sci Transl Med* **10**(2018).

354. Heitzer, E., Haque, I.S., Roberts, C.E.S. & Speicher, M.R. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet* **20**, 71-88 (2019).

355. Takai, E., *et al.* Clinical Utility of Circulating Tumor DNA for Molecular Assessment and Precision Medicine in Pancreatic Cancer. *Adv Exp Med Biol* **924**, 13-17 (2016).

356. Pietrasz, D., *et al.* Plasma Circulating Tumor DNA in Pancreatic Cancer Patients Is a Prognostic Marker. *Clin Cancer Res* **23**, 116-123 (2017).

357. Hadano, N., *et al.* Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer. *Br J Cancer* **115**, 59-65 (2016).

358. Cohen, J.D., *et al.* Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. *Proc Natl Acad Sci U S A* **114**, 10202-10207 (2017).

359. Kruger, S., *et al.* Repeated mutKRAS ctDNA measurements represent a novel and promising tool for early response prediction and therapy monitoring in advanced pancreatic cancer. *Ann Oncol* **29**, 2348-2355 (2018).

360. Watanabe, F., *et al.* Longitudinal monitoring of KRAS-mutated circulating tumor DNA enables the prediction of prognosis and therapeutic responses in patients with pancreatic cancer. *PLoS One* **14**, e0227366 (2019).

361. Patel, H., *et al.* Clinical correlates of blood-derived circulating tumor DNA in pancreatic cancer. *J Hematol Oncol* **12**, 130 (2019).

362. Uesato, Y., *et al.* Evaluation of circulating tumor DNA as a biomarker in pancreatic cancer with liver metastasis. *PLoS One* **15**, e0235623 (2020).

363. Bernard, V., *et al.* Circulating Nucleic Acids Are Associated With Outcomes of Patients With Pancreatic Cancer. *Gastroenterology* **156**, 108-118 e104 (2019).

364. Umemoto, K., *et al.* Clinical significance of circulating-tumour DNA analysis by metastatic sites in pancreatic cancer. *Br J Cancer* **128**, 1603-1608 (2023).

365. Del Re, M., *et al.* Early changes in plasma DNA levels of mutant KRAS as a sensitive marker of response to chemotherapy in pancreatic cancer. *Sci Rep* **7**, 7931 (2017).

366. Dayimu, A., *et al.* Clinical and biological markers predictive of treatment response associated with metastatic pancreatic adenocarcinoma. *Br J Cancer* **128**, 1672-1680 (2023).

367. Edland, K.H., *et al.* Monitoring of circulating tumour DNA in advanced pancreatic ductal adenocarcinoma predicts clinical outcome and reveals disease progression earlier than radiological imaging. *Mol Oncol* **17**, 1857-1870 (2023).

368. Zill, O.A., *et al.* Cell-Free DNA Next-Generation Sequencing in Pancreatobiliary Carcinomas. *Cancer Discov* **5**, 1040-1048 (2015).

369. Guan, S., *et al.* Evaluation of circulating tumor DNA as a prognostic biomarker for metastatic pancreatic adenocarcinoma. *Front Oncol* **12**, 926260 (2022).

370. Wei, T., *et al.* Monitoring Tumor Burden in Response to FOLFIRINOX Chemotherapy Via Profiling Circulating Cell-Free DNA in Pancreatic Cancer. *Mol Cancer Ther* **18**, 196-203 (2019).

371. Karamitopoulou, E. Tumour microenvironment of pancreatic cancer: immune landscape is dictated by molecular and histopathological features. *British Journal of Cancer* **121**, 5-14 (2019).

372. Raja Arul, G.L., Toruner, M.D., Gatenby, R.A. & Carr, R.M. Ecoevolutionary biology of pancreatic ductal adenocarcinoma. *Pancreatology* **22**, 730-740 (2022).

373. Haqq, J., *et al.* Pancreatic stellate cells and pancreas cancer: current perspectives and future strategies. *Eur J Cancer* **50**, 2570-2582 (2014).

374. Ando, R., *et al.* Good and Bad Stroma in Pancreatic Cancer: Relevance of Functional States of Cancer-Associated Fibroblasts. *Cancers (Basel)* **14**(2022).

375. Hau, S.O., *et al.* Chemotherapy, host response and molecular dynamics in periampullary cancer: the CHAMP study. *BMC Cancer* **20**, 308 (2020).

376. Fox, C.H., Johnson, F.B., Whiting, J. & Roller, P.P. Formaldehyde fixation. *J Histochem Cytochem* **33**, 845-853 (1985).

377. Mathieson, W. & Thomas, G.A. Why Formalin-fixed, Paraffin-embedded Biospecimens Must Be Used in Genomic Medicine: An Evidence-based Review and Conclusion. *J Histochem Cytochem* **68**, 543-552 (2020).

378. Hedegaard, J., *et al.* Next-generation sequencing of RNA and DNA isolated from paired fresh-frozen and formalin-fixed paraffin-embedded samples of human cancer and normal tissue. *PLoS One* **9**, e98187 (2014).

379. Kononen, J., *et al.* Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* **4**, 844-847 (1998).

380. Voduc, D., Kenney, C. & Nielsen, T.O. Tissue microarrays in clinical oncology. *Semin Radiat Oncol* **18**, 89-97 (2008).

381. Vogelstein, B. & Gillespie, D. Preparative and analytical purification of DNA from agarose. *Proc Natl Acad Sci U S A* **76**, 615-619 (1979).

382. Boom, R.*, et al.* Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* **28**, 495-503 (1990).

383. Diefenbach, R.J., Lee, J.H., Kefford, R.F. & Rizos, H. Evaluation of commercial kits for purification of circulating free DNA. *Cancer Genet* **228-229**, 21-27 (2018).

384. Sanger, F., Nicklen, S. & Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* **74**, 5463-5467 (1977).

385. Collins, F.S. & Fink, L. The Human Genome Project. *Alcohol Health Res World* **19**, 190-195 (1995).

386. Goodwin, S., McPherson, J.D. & McCombie, W.R. Coming of age: ten years of next-generation sequencing technologies. *Nature Reviews Genetics* **17**, 333-351 (2016).

387. Koboldt, D.C. Best practices for variant calling in clinical sequencing. *Genome Medicine* **12**, 91 (2020).

388. Li, M.M.*, et al.* Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists. *J Mol Diagn* **19**, 4-23 (2017).

389. Boscolo Bielo, L.*, et al.* Variant allele frequency: a decision-making tool in precision oncology? *Trends Cancer* **9**, 1058-1068 (2023).

390. Foster, J.M.*, et al.* Cross-laboratory validation of the OncoScan® FFPE Assay, a multiplex tool for whole genome tumour profiling. *BMC Med Genomics* **8**, 5 (2015).

391. Wang, Y., Cottman, M. & Schiffman, J.D. Molecular inversion probes: a novel microarray technology and its application in cancer research. *Cancer Genet* **205**, 341-355 (2012).

392. Jung, H.-S., Lefferts, J.A. & Tsongalis, G.J. Utilization of the oncoscan microarray assay in cancer diagnostics. *Applied Cancer Research* **37**, 1 (2017).

393. Peiffer, D.A.*, et al.* High-resolution genomic profiling of chromosomal aberrations using Infinium whole-genome genotyping. *Genome Res* **16**, 1136-1148 (2006).

394. Staaf, J.*, et al.* Segmentation-based detection of allelic imbalance and loss-of-heterozygosity in cancer cells using whole genome SNP arrays. *Genome Biol* **9**, R136 (2008).

395. Watanabe, M.*, et al.* Estimation of age-related DNA degradation from formalin-fixed and paraffin-embedded tissue according to the extraction methods. *Exp Ther Med* **14**, 2683-2688 (2017).

396. Funel, N.*, et al.* Laser microdissection and primary cell cultures improve pharmacogenetic analysis in pancreatic adenocarcinoma. *Laboratory Investigation* **88**, 773-784 (2008).

397. Iacobuzio-Donahue, C.A.*, et al.* Cancer biology as revealed by the research autopsy. *Nat Rev Cancer* **19**, 686-697 (2019).

398. Pozhitkov, A.E. & Noble, P.A. Gene expression in the twilight of death: The increase of thousands of transcripts has implications to transplantation, cancer, and forensic research. *Bioessays* **39**(2017).

399. Gill, J.R. Pancreatitis: A Forensic Perspective. *Acad Forensic Pathol* **6**, 237-248 (2016).

400. Cocariu, E.*, et al.* Correlations Between the Autolytic Changes and Postmortem Interval in Refrigerated Cadavers. *Romanian Journal Of Internal Medicine* **54**(2016).

401. Fan, J.*, et al.* Quantification of nucleic acid quality in postmortem tissues from a cancer research autopsy program. *Oncotarget* 7, 66906-66921 (2016).

402. Alwelaie, Y.*, et al.* Acinar cell induced autolysis is a frequent occurrence in CytoLyt-fixed pancreatic fine needle aspiration specimens: An analysis of 157 cytology samples. *Cancer Cytopathol* **129**, 283-290 (2021).

403. Robbe, P.*, et al.* Clinical whole-genome sequencing from routine formalin-fixed, paraffin-embedded specimens: pilot study for the 100,000 Genomes Project. *Genetics in Medicine* **20**, 1196-1205 (2018).

404. Spencer, D.H.*, et al.* Comparison of clinical targeted next-generation sequence data from formalin-fixed and fresh-frozen tissue specimens. *J Mol Diagn* **15**, 623-633 (2013).

405. Robinson, J.T.*, et al.* Integrative genomics viewer. *Nat Biotechnol* **29**, 24-26 (2011).

406. Rasmussen, M.*, et al.* Allele-specific copy number analysis of tumor samples with aneuploidy and tumor heterogeneity. *Genome Biol* **12**, R108 (2011).

407. Lu, B. Cancer phylogenetic inference using copy number alterations detected from DNA sequencing data. *Cancer Pathogenesis and Therapy* (2024).

408. Steel, M. & Penny, D. Parsimony, Likelihood, and the Role of Models in Molecular Phylogenetics. *Molecular Biology and Evolution* **17**, 839-850 (2000).

409. Rastegar, B.*, et al.* Resolving the Pathogenesis of Anaplastic Wilms Tumors through Spatial Mapping of Cancer Cell Evolution. *Clin Cancer Res* **29**, 2668-2677 (2023).

410. Tucker, C.M.*, et al.* A guide to phylogenetic metrics for conservation, community ecology and macroecology. *Biological Reviews* **92**, 698-715 (2017).

411. Martin, J.K., & Hirschberg, D. . On the complexity of learning decision trees. in *International Symposium on Artificial Intelligence and Mathematics* 112-115 (1996).

412. Matos, L.L., Trufelli, D.C., de Matos, M.G. & da Silva Pinhal, M.A. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights* **5**, 9-20 (2010).

413. Coons, A.H. The development of immunohistochemistry. *Ann N Y Acad Sci* **177**, 5-9 (1971).

414. Bordeaux, J.*, et al.* Antibody validation. *Biotechniques* **48**, 197-209 (2010).

415. Jourdan, F.*, et al.* Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression. *Virchows Arch* **443**, 115-121 (2003).

416. Meyerholz, D.K. & Beck, A.P. Principles and approaches for reproducible scoring of tissue stains in research. *Lab Invest* **98**, 844-855 (2018).

417. Gerber, T.*, et al.* Assessment of Pre-Analytical Sample Handling Conditions for Comprehensive Liquid Biopsy Analysis. *The Journal of Molecular Diagnostics* **22**, 1070-1086 (2020).

418. Swinkels, D.W., Wiegerinck, E., Steegers, E.A. & de Kok, J.B. Effects of blood-processing protocols on cell-free DNA quantification in plasma. *Clinical Chemistry* **49**, 525-526 (2003).

419. Jung, M., Klotzek, S., Lewandowski, M., Fleischhacker, M. & Jung, K. Changes in concentration of DNA in serum and plasma during storage of blood samples. *Clin Chem* **49**, 1028-1029 (2003).

420. Chan, K.A., Yeung, S.-W., Lui, W.-B., Rainer, T.H. & Lo, Y.D. Effects of Preanalytical Factors on the Molecular Size of Cell-Free DNA in Blood. *Clinical Chemistry* **51**, 781-784 (2005).

421. Devonshire, A.S.*, et al.* Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. *Anal Bioanal Chem* **406**, 6499-6512 (2014).

422. Madsen, A.T., Hojbjerg, J.A., Sorensen, B.S. & Winther-Larsen, A. Day-to-day and within-day biological variation of cell-free DNA. *EBioMedicine* **49**, 284-290 (2019).

423. Pokrywka, A.*, et al.* The influence of hypoxic physical activity on cfDNA as a new marker of vascular inflammation. *Archives of Medical Science* **11**, 1156-1163 (2015).

424. Heitzer, E.*, et al.* Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. *Genome Med* **5**, 30 (2013).

425. Paweletz, C.P.*, et al.* Bias-Corrected Targeted Next-Generation Sequencing for Rapid, Multiplexed Detection of Actionable Alterations in Cell-Free DNA from Advanced Lung Cancer Patients. *Clin Cancer Res* **22**, 915-922 (2016).

426. Aird, D.*, et al.* Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. *Genome Biology* **12**, R18 (2011).

427. Smith, T., Heger, A. & Sudbery, I. UMI-tools: modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy. *Genome Res* **27**, 491-499 (2017).

428. Mansukhani, S.*, et al.* Ultra-Sensitive Mutation Detection and Genome-Wide DNA Copy Number Reconstruction by Error-Corrected Circulating Tumor DNA Sequencing. *Clinical Chemistry* **64**, 1626-1635 (2018).

429. Picot, J., Guerin, C.L., Le Van Kim, C. & Boulanger, C.M. Flow cytometry: retrospective, fundamentals and recent instrumentation. *Cytotechnology* **64**, 109-130 (2012).

430. Perfetto, S.P., Chattopadhyay, P.K. & Roederer, M. Seventeen-colour flow cytometry: unravelling the immune system. *Nature Reviews Immunology* **4**, 648-655 (2004).

431. Maecker, H.T., McCoy, J.P. & Nussenblatt, R. Standardizing immunophenotyping for the Human Immunology Project. *Nature Reviews Immunology* **12**, 191-200 (2012).

432. Maecker, H.T., Frey, T., Nomura, L.E. & Trotter, J. Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry A* **62**, 169-173 (2004).

433. Assarsson, E.*, et al.* Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS One* **9**, e95192 (2014).

434. Petersson, A.*, et al.* Branching copy number evolution and parallel immune profiles across the regional tumor space of resected pancreatic cancer. *Mol Cancer Res* (2022).

435. Pietrasz, D.*, et al.* Prognostic value of circulating tumour DNA in metastatic pancreatic cancer patients: post-hoc analyses of two clinical trials. *Br J Cancer* **126**, 440-448 (2022).

436. Mayrhofer, M.*, et al.* Sensitive detection of copy number alterations in samples with low circulating tumor DNA fraction. *medRxiv*, 2024.2005.2004.24306860 (2024).

437. Yachida, S. & Iacobuzio-Donahue, C.A. The pathology and genetics of metastatic pancreatic cancer. *Arch Pathol Lab Med* **133**, 413-422 (2009).

438. Turajlic, S.*, et al.* Deterministic Evolutionary Trajectories Influence Primary Tumor Growth: TRACERx Renal. *Cell* **173**, 595-610 e511 (2018).

439. Asting, A.G.*, et al.* Alterations in Tumor DNA Are Related to Short Postoperative Survival in Patients Resected for Pancreatic Carcinoma Aimed at Cure. *Pancreas* **45**, 900-907 (2016).

440. Yachida, S.*, et al.* Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* **467**, 1114-1117 (2010).

441. Murphy, S.J.*, et al.* Genetic alterations associated with progression from pancreatic intraepithelial neoplasia to invasive pancreatic tumor. *Gastroenterology* **145**, 1098-1109 e1091 (2013).

442. Watkins, T.B.K.*, et al.* Pervasive chromosomal instability and karyotype order in tumour evolution. *Nature* **587**, 126-132 (2020).

443. Cai, Y.*, et al.* Loss of Chromosome 8p Governs Tumor Progression and Drug Response by Altering Lipid Metabolism. *Cancer Cell* **29**, 751-766 (2016).

444. Huth, T.*, et al.* Chromosome 8p engineering reveals increased metastatic potential targetable by patient-specific synthetic lethality in liver cancer. *Sci Adv* **9**, eadh1442 (2023).

445. Watanabe, K.*, et al.* Tumor-Informed Approach Improved ctDNA Detection Rate in Resected Pancreatic Cancer. *Int J Mol Sci* **23**(2022).

446. Ben-Ammar, I.*, et al.* Precision medicine for KRAS wild-type pancreatic adenocarcinomas. *Eur J Cancer* **197**, 113497 (2024).

447. Wei, T.*, et al.* Genome-wide profiling of circulating tumor DNA depicts landscape of copy number alterations in pancreatic cancer with liver metastasis. *Mol Oncol* **14**, 1966-1977 (2020).

448. Mohan, S.*, et al.* Analysis of circulating cell-free DNA identifies KRAS copy number gain and mutation as a novel prognostic marker in Pancreatic cancer. *Sci Rep* **9**, 11610 (2019).

449. Pittella-Silva, F., Kimura, Y., Low, S.K., Nakamura, Y. & Motoya, M. Amplification of mutant KRAS(G12D) in a patient with advanced metastatic pancreatic adenocarcinoma detected by liquid biopsy: A case report. *Mol Clin Oncol* **15**, 172 (2021).

450. Reissig, T.M.*, et al.* Smaller panel, similar results: genomic profiling and molecularly informed therapy in pancreatic cancer. *ESMO Open* **8**, 101539 (2023).

451. Pishvaian, M.J.*, et al.* Overall survival in patients with pancreatic cancer receiving matched therapies following molecular profiling: a retrospective analysis of the Know Your Tumor registry trial. *Lancet Oncol* **21**, 508-518 (2020).

452. Ye, Y. & Zheng, S. Successful Immunotherapy for Pancreatic Cancer in a Patient With TSC2 and SMAD4 Mutations: A Case Report. *Front Immunol* **12**, 785400 (2021).

453. Kwiatkowski, D.J. & Wagle, N. mTOR Inhibitors in Cancer: What Can We Learn from Exceptional Responses? *EBioMedicine* **2**, 2-4 (2015).

454. Bhutani, M.S., Cazacu, I.M., Roy-Chowdhuri, S., Maitra, A. & Pishvaian, M.J. Upfront molecular profiling of pancreatic cancer patients - An idea whose time has come. *Pancreatology* **20**, 391-393 (2020).

455. McGranahan, N. & Swanton, C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell* **168**, 613-628 (2017).

456. Acha-Sagredo, A., Ganguli, P. & Ciccarelli, F.D. Somatic variation in normal tissues: friend or foe of cancer early detection? *Ann Oncol* **33**, 1239-1249 (2022).

457. Lehmann-Werman, R.*, et al.* Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc Natl Acad Sci U S A* **113**, E1826-1834 (2016).

458. Sun, K.*, et al.* Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci U S A* **112**, E5503-5512 (2015).

459. Bos, M.K.*, et al.* Comparison of variant allele frequency and number of mutant molecules as units of measurement for circulating tumor DNA. *Mol Oncol* **15**, 57-66 (2021).

460. Pereira, B.*, et al.* Cell-free DNA captures tumor heterogeneity and driver alterations in rapid autopsies with pre-treated metastatic cancer. *Nat Commun* **12**, 3199 (2021).

461. Knudsen, E.S.*, et al.* Stratification of Pancreatic Ductal Adenocarcinoma: Combinatorial Genetic, Stromal, and Immunologic Markers. *Clin Cancer Res* **23**, 4429-4440 (2017).

462. Taube, J.M.*, et al.* Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin Cancer Res* **20**, 5064-5074 (2014).

463. Rosenthal, R.*, et al.* Neoantigen-directed immune escape in lung cancer evolution. *Nature* **567**, 479-485 (2019).

464. Lundgren, S., *et al.* Quantitative, qualitative and spatial analysis of lymphocyte infiltration in periampullary and pancreatic adenocarcinoma. *Int J Cancer* **146**, 3461-3473 (2020).

465. Lundgren, S., *et al.* Topographical Distribution and Spatial Interactions of Innate and Semi-Innate Immune Cells in Pancreatic and Other Periampullary Adenocarcinoma. *Front Immunol* **11**, 558169 (2020).

466. Wartenberg, M., *et al.* Integrated Genomic and Immunophenotypic Classification of Pancreatic Cancer Reveals Three Distinct Subtypes with Prognostic/Predictive Significance. *Clin Cancer Res* **24**, 4444-4454 (2018).

467. Tan, W.C.C., *et al.* Overview of multiplex immunohistochemistry/immunofluorescence techniques in the era of cancer immunotherapy. *Cancer Commun (Lond)* **40**, 135-153 (2020).

468. Łuksza, M., *et al.* Neoantigen quality predicts immunoediting in survivors of pancreatic cancer. *Nature* **606**, 389-395 (2022).

469. Sharma, A., Jasrotia, S. & Kumar, A. Effects of Chemotherapy on the Immune System: Implications for Cancer Treatment and Patient Outcomes. *Naunyn-Schmiedeberg's Archives of Pharmacology* **397**, 2551-2566 (2024).

470. Teng, M.W., Ngiow, S.F., Ribas, A. & Smyth, M.J. Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Res* **75**, 2139-2145 (2015).

471. Schlee, M. & Hartmann, G. Discriminating self from non-self in nucleic acid sensing. *Nat Rev Immunol* **16**, 566-580 (2016).

472. Motwani, M., Pesiridis, S. & Fitzgerald, K.A. DNA sensing by the cGAS-STING pathway in health and disease. *Nat Rev Genet* **20**, 657-674 (2019).

473. Singh, N., Gupta, S., Pandey, R.M., Chauhan, S.S. & Saraya, A. High levels of cell-free circulating nucleic acids in pancreatic cancer are associated with vascular encasement, metastasis and poor survival. *Cancer Invest* **33**, 78-85 (2015).

474. Ortega, M.A., *et al.* Oxidative Stress Markers Are Associated with a Poor Prognosis in Patients with Pancreatic Cancer. *Antioxidants (Basel)* **11**(2022).

475. Kapellos, T.S., *et al.* Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. *Front Immunol* **10**, 2035 (2019).

476. Maeurer, M.J., *et al.* Human intestinal Vdelta1+ lymphocytes recognize tumor cells of epithelial origin. *J Exp Med* **183**, 1681-1696 (1996).

477. Siegers, G.M. & Lamb, L.S., Jr. Cytotoxic and regulatory properties of circulating Vdelta1+ gammadelta T cells: a new player on the cell therapy field? *Mol Ther* **22**, 1416-1422 (2014).

478. Schwacha, M.G., *et al.* Dermal γδ T-Cells Can Be Activated by Mitochondrial Damage-Associated Molecular Patterns. *PLoS One* **11**, e0158993 (2016).

479. Pothula, S.P., *et al.* Hepatocyte growth factor inhibition: a novel therapeutic approach in pancreatic cancer. *Br J Cancer* **114**, 269-280 (2016).

480. Apte, M.V., *et al.* Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells. *Pancreas* **29**, 179-187 (2004).

481. Rodriguez-Zhurbenko, N. & Hernandez, A.M. The role of B-1 cells in cancer progression and anti-tumor immunity. *Front Immunol* **15**, 1363176 (2024).

482. Jang, G.Y., *et al.* Interactions between tumor-derived proteins and Toll-like receptors. *Exp Mol Med* **52**, 1926-1935 (2020).

483. Machicote, A., Belén, S., Baz, P., Billordo, L.A. & Fainboim, L. Human CD8+HLA-DR+ Regulatory T Cells, Similarly to Classical CD4+Foxp3+ Cells, Suppress Immune Responses via PD-1/PD-L1 Axis. *Frontiers in Immunology* **9**(2018).

484. Arruvito, L.*, et al.* Identification and Clinical Relevance of Naturally Occurring Human CD8+HLA-DR+ Regulatory T Cells. *The Journal of Immunology* **193**, 4469-4476 (2014).

485. Luo, X.*, et al.* The CA125 level postoperative change rule and its prognostic significance in patients with resectable pancreatic cancer. *BMC Cancer* **23**, 832 (2023).

486. Napoli, N.*, et al.* Ca 125 is an independent prognostic marker in resected pancreatic cancer of the head of the pancreas. *Updates Surg* **75**, 1481-1496 (2023).

487. Muniyan, S.*, et al.* MUC16 contributes to the metastasis of pancreatic ductal adenocarcinoma through focal adhesion mediated signaling mechanism. *Genes Cancer* **7**, 110-124 (2016).

488. Gubbels, J.A.*, et al.* MUC16 provides immune protection by inhibiting synapse formation between NK and ovarian tumor cells. *Mol Cancer* **9**, 11 (2010).

"Mischief Managed"

by J.K. Rowling

About the thesis

This thesis represents a true interdisciplinary team effort, blending the knowledge from both clinicians and engineers, like myself, in the pursuit of improving the dismal outcome for patients with pancreatic cancer. If this research brings benefit to just one person, I will consider it a significant achievement.

Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2024:97
ISBN 978-91-8021-593-0
ISSN 1652-8220