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INTERVENTION AND TECHNOLOGY
Karolinska Institutet, Stockholm, Sweden

DIAGNOSTIC AND PREDICTIVE FACTORS IN PANCREATIC CANCER: CLINICAL AND TRANSLATIONAL STUDIES

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Stockholm 2022

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2022

ISBN 978-91-8016-766-6

Diagnostic and Predictive Factors in Pancreatic Cancer: Clinical and Translational Studies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at lecture hall C1.87, Karolinska University Hospital Huddinge

Friday October 21, 2022 at 09:00

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To my family

POPULAR SCIENCE SUMMARY OF THE THESIS

Every day, about 150 people in Sweden get a cancer diagnosis. Three to four of them have pancreatic cancer. They, together with their families and loved ones, face a vicious disease. Many patients with pancreatic cancer live shorter than one year from the diagnosis. Pancreatic cancer is hard to treat and medical progress with this cancer has been painstakingly difficult to make.

The pancreas is a gland in the upper part of the belly behind the stomach and close to the liver and gallbladder. It has two functions. It produces enzymes that go into the gut and are important for digestion and it produces hormones, like insulin, that go into the blood and regulate e.g., the blood sugar level. The common form of pancreatic cancer develops when cells in the tissues that produce and transport the enzymes grow out of control and form a mass. If this mass is small enough, and if there are no signs that the cancer has spread to other parts of the body, a surgeon will try to remove it. However, the cancer will come back in most cases although patients get chemotherapy after surgery to reduce this risk. Patients who have a relapse or tumors that are too big for surgery or spread at diagnosis can get chemotherapy to push back the cancer and slow down the course of the disease, but this treatment cannot cure them.

In **Study I** of my thesis, I wanted to understand the results of different types of chemotherapy to slow down advanced pancreatic cancer in the normal hospital setting in Stockholm. Together with my co-authors, we looked at almost 600 patients who had been treated in 2013–2017. We found that most results were similar to what had been published in the scientific literature. However, one finding that we had not expected was that a treatment called FOLFIRINOX was not better than two other, gemcitabine/capecitabine and gemcitabine/nab-paclitaxel. We used statistical methods to remove the influence of other factors, e.g., younger or healthier patients in one group or another, but still got the same results. When we looked for clues in the amount of each chemotherapy that had been given to patients, side-effects, and what other treatment patients might have received later in the course of their disease, we found that gemcitabine/capecitabine and gemcitabine/nab-paclitaxel had been given with less deviation from their treatment schedules.

Cancer cells behave differently from healthy cells because they have acquired changes in their DNA – the manual of all processes in the body. Usually, we have the same DNA in all our cells, but different instructions, called genes, may be used by cells depending on which tissue and organ they are in and how we live. In cancer, genes involved in controlling cell growth and letting cells survive outside their normal environment are damaged and permanently overactive or inactive. In pancreatic cancer this is usually caused by random DNA damage as we grow older. Which gene damages a tumor has can be different from patient to patient. One promising strategy to make the treatment of many cancers more efficient has been to find such specific damages to certain genes because they can in some

cases forecast if a patient benefits from specific drug. In **Study II**, we tried to characterize a set of more than 600 genes in the tumor tissue of 39 patients who had already been treated for advanced pancreatic cancer with DNA sequencing. We then used a commercial software, MH Guide, that can identify gene changes and automatically finds matches to specific drugs. In our study, this software suggested drugs that otherwise wouldn't have been considered in 27/31 cases. We had a group of experts evaluate these suggestions and they found them potentially meaningful in 75% of cases.

Changes to the genes of cancer cells can not only be found by directly testing cells from the tumor tissue. Tumor cells release tiny amounts of free DNA but also various kinds of small particles, called extracellular vesicles, that contain DNA and other molecules from their inside into the blood stream. Cancer cell DNA that is circulating in the blood is difficult to detect because there is also plenty of DNA from healthy cells, a needle-in-the-haystack situation. Strategies to pinpoint the DNA that comes from cancer cells are stepstones towards blood tests to find, analyze and monitor cancers. In **Studies III–IV**, I collected blood from patients with pancreatic cancer. Together with other scientists, I could separate different components of one sample of whole blood: Red and white blood cells, responsible for oxygen transport and the immune defense, platelets, cell fragments responsible for wound healing, apoptotic bodies, the remnants of cells undergoing controlled cell death, large and small extracellular vesicles, and large soluble molecules. In **Study III**, we systematically tested which of these components contained most DNA from cancer cells. In samples taken at late stages of pancreatic cancer, we found high levels of soluble free cancer DNA, something we had expected. The new finding was that cancer DNA at early stages was much more concentrated in small extracellular vesicles. In **Study IV**, we tested which role the size of circulating DNA pieces might play. We found that pancreatic cancer patients have higher levels of soluble small DNA pieces as well as larger chunks of DNA in apoptotic bodies compared to healthy people. When we separated small and large DNA fragments to separately test the amount of cancer DNA in both types, it turned out that small soluble DNA had the highest share compared to all other blood components and larger DNA pieces.

Together, the results of the four studies in my thesis tackle pancreatic cancer in different areas. Hands-on, they might help to optimize how we use chemotherapy to treat pancreatic cancer patients in everyday care and highlight that it is feasible and worthwhile to look for individualized treatment options. From a wider perspective, they may be important for the development of next generation cancer blood tests.

ABSTRACT

Aims. The aim of **Study I** was to assess the effectiveness of chemotherapy for patients with advanced pancreatic cancer in a real-world setting. **Study II** aimed to evaluate the utility of a clinical decision support system to identify precision oncology opportunities for pancreatic cancer. The aim of **Study III** was to identify the blood component most enriched with pancreatic cancer-derived circulating DNA; that of **Study IV** was to characterize long and short tumor-derived circulating DNA fragments.

Methods. Study I is a single-institution retrospective cohort study of prospectively generated clinical data. We included a total of 595 patients and used univariate and multivariate models, including flexible parametric models to analyze overall survival and time to treatment failure according to different first-line chemotherapy regimens. Exploratory analyses included the adherence to different protocols, adverse events, and second-line chemotherapy. **Study II** is a prospective observational study of 39 patients with pancreatic cancer who were enrolled in the PePaCaKa-01 study. Archival tumor tissue was sequenced, and data was processed with the proprietary clinical decision support system MH Guide and results were evaluated by a study-specific molecular tumor board. Endpoints of the study were the frequency of successful generation of support system reports, the frequency of actionable molecular targets, and their evaluation by the tumor board. We performed a post-hoc analysis to determine the proportion of patients who received molecular informed therapies. **Studies III–IV** analyzed blood samples from a prospective cohort of patients with advanced pancreatic cancer. We systematically separated whole blood into red and white blood cells, platelets, apoptotic bodies, large and small extracellular vesicles, and soluble protein using differential centrifugation. We confirmed efficient separation with protein assays (Western blotting and multiplex bead-based extracellular vesicle flow cytometry), nanoparticle tracking analysis and transmission electron microscopy, and extracted DNA from all components. We used digital PCR to quantify the abundance of *KRAS*^{mut} DNA, a hallmark of pancreatic cancer-derived DNA. In **Study IV** we additionally used automated electrophoresis to quantify the lengths of circulating DNA fragments and ligation-based sequencing library preparation and tagmentation to selectively target short and long DNA fragments, respectively.

Results. In **Study I,** we observed similar overall survival for gemcitabine/capecitabine, gemcitabine/nab-paclitaxel, and FOLFIRINOX (including modified regimens) compared to gemcitabine. Combinations of 5-fluorouracil/oxaliplatin and best supportive care were associated with poorer outcomes. Models adjusting for other demographic and clinical variables showed a survival benefit for gemcitabine-combinations and FOLFIRINOX. Exploratory analyses revealed differences in protocol adherence across different treatments, a relatively low frequency of AEs, and a difference between different sequences of first- and second-line therapy. In **Study II**, a CDSS report was generated for 31/39 patients, 28/31 reports were evaluated at the study-specific molecular tumor board The clinical decision support system made 80 individual recommendations to use molecularly informed therapies

based on 61 genomic variants. In 21/28 cases, the tumor board classified at least one molecularly informed therapy as a potential clinical option. The post-hoc analysis revealed that six patients received molecularly informed treatment in routine care. In **Study III**, *KRAS*^{mut} DNA had the highest concentrations in the soluble protein and small vesicles blood fractions at late stages of PDAC. At early stages, it was highest in large and small extracellular vesicles. Small extracellular vesicles also contained the highest ratio of the concentrations of mutant : wild type *KRAS* DNA at this stage. In **Study IV**, blood from PDAC patients had significantly higher concentrations of short cell-free DNA in the soluble protein fraction than that of healthy individuals. The mutant allele frequency of *KRAS*^{mut} was highest in this blood component. Long genomic DNA fragments were most reliably measured in association with apoptotic bodies but *KRAS*^{mut} genomic DNA occurred in all assessed blood fractions.

Conclusions. Chemotherapy in clinical routine use can result in outcomes that reflect relevant randomized controlled trials and gemcitabine-based regimens are highly effective this setting. Differences between different treatments might be related to how they are applied (**Study I**). The clinical decision support system MH Guide could identify clinically relevant opportunities for molecularly informed treatments of advanced pancreatic cancer (**Study II**). At early stages of pancreatic cancer, tumor-derived DNA is mostly associated with small extracellular vesicles; in more advanced disease it is mainly a feature of the soluble protein fraction (**Study III**). Short DNA fragments in this fraction are a more robust source of tumor-derived DNA than longer genomic DNA fragments (**Study IV**).

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CONTENTS

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AE	Adverse event
AJCC	American Joint Committee on Cancer
APC	Allophycocyanin
bp	Base pair
CDSS	Clinical decision support system
cfDNA	Cell-free DNA
CE	Conformité européenne [European conformity]
CI	Confidence interval
CTC	Circulating tumor cell
CTCAE	Common Terminology Criteria for Adverse Events
ctDNA	Circulating tumor DNA
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
DOR	Duration of response
dPCR	Digital polymerase chain reaction
DSB	Double-strand break
ECOG	Eastern Cooperative Oncology Group
EU	European Union
EV	Extracellular vesicle
evDNA	Extracellular vesicle-associated DNA
FOLFIRINOX	Comibination of 5-FU, folinic acid, irinotecan and oxaliplatin
HR	Hazard ratio
HRD	homologous recombination double-strand break repair deficiency
HRR	homologous recombination double-strand break repair
Kb	Kilobase pair
kDa	Kilodalton
LAPC	Locally advanced pancreatic cancer
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinases
min.	Minute(s)
MMR	Mismatch repair

mo.	Month(s)
MSI	Microsatellite instability
MTB	Molecular tumor board
NR	Not reached
NTA	Nanoparticle tracking analysis
NSCLC	Non-small cell lung cancer
OR	Odds ratio
ORR	Overall response rate
OS	Overall Survival
PARP	poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	programmed cell death protein
PDAC	Pancreatic ductal adenocarcinoma
PFS	Progression-free survival
RBC	Red blood cell
RCT	Randomized controlled trial
RNA	Ribonucleic acid
SIR	Standardized incidence ratio
TCR	T-cell receptor
TEM	Transmission electron microscopy
TTF	Time to treatment failure
UMI	Unique molecular identifier
WBC	White blood cell
WES	Whole exome sequencing
WGS	Whole genome sequencing
WT	Wild type

GENE AND PROTEIN NOMENCLATURE

Gene names and symbols follow the system of the Human Genome Organization (HUGO) Gene Nomenclature Committee.
Available at: https://www.genenames.org/

Protein nomenclature follows the guidelines of the European Bioinformatics Institute (EMBL-EBI), the National Center for Biotechnology Information (NCBI), the Protein Information Resource (PIR) and the Swiss Institute for Bioinformatics (SIB).
Available at: ncbi.nlm.nih.gov/genome/doc/internatprot_nomenguide/

1 INTRODUCTION

In 2020, pancreatic cancer was the seventh most common cancer in Europe with an estimated 140,100 new cases, or 3.5% of all newly diagnosed cancers. Slightly more men (70,200; 3.3%) than women (69,900; 3,7%) were affected although the age-standardized incidence rate was clearly higher in men than women, 22.2 versus 16 per 100,000 person years (1). In the same year, there were 132,100 estimated deaths caused by pancreatic cancer making it the fourth most common cause of cancer-related death in Europe with a mortality rate that is almost the same as the incidence rate (1). Because the risk to develop pancreatic cancer is associated with older age, the demographic aging of the European population is expected to increase the number of cases by almost one-third by 2040 (2). In addition to the expected additional disease burden and mortality caused by this development, PDAC is also the only major malignant neoplasm with an age-standardized incidence rate that did not decrease between 2014 and 2019 (3).

In Sweden, the median overall survival across all stages of pancreatic cancer did not exceed six months in the decade between 2005 and 2015 (4). Long-term survival occurs almost exclusively among patients who undergo surgery followed by adjuvant chemotherapy, with a 5-year relative survival rate of approximately 20% (5,6). The 5-year relative survival of patients with advanced disease is much poorer, typically below 5%, and it is 5–10% across all stages (7). Compared to other common cancer types, advances in early detection and treatment of pancreatic cancer have been slow although there has been a minor trend towards improved patient outcomes over the past decades (8–11). Together, pancreatic cancer is the area of a significant and increasing personal and societal unmet medical need (12).

Pancreatic ductal adenocarcinoma (PDAC) is the most common histologic subtype of malignant neoplasms of the pancreas, accounting for 80–85% of all cases (13). Ductal adenocarcinomas are highly malignant tumors that originate from the exocrine part of the pancreas. Generally, epithelial cells in the pancreatic duct have been considered to be the precursor cells of PDAC based on its morphological and phenotypical features (14). In good accordance with this presumed origin, a distinct PDAC cancer stem cell population with epithelial features has been identified (15). There is, however, convincing evidence that PDAC also can originate from other precursors like acinar cells that undergo metaplasia into duct-like cells (16) or a sub-set of cells directed towards endocrine differentiation (17). But although there might be different paths to PDAC tumorigenesis, common clinical and biological features characterize the vast majority of manifest PDAC cases.

2 BACKGROUND

2.1 CLINICAL PRESENTATION OF PANCREATIC CANCER

Patients with PDAC have often few or unspecific early symptoms. Although abdominal pain, back pain, bloating, dyspepsia, constipation or nausea, as well as unintentional weight loss or new onset diabetes are common prior to a diagnosis of PDAC, the positive predictive value of each symptom is well below one percent (18). The only high-risk symptom of PDAC is jaundice which can be caused by obstruction of the common biliary duct by a tumor in the pancreatic head. The often-diffuse symptoms of PDAC are reflected in frequent delays in the diagnostic work-up. As a consequence, about four in five patients are diagnosed with advanced disease (19,20).

Irrespective of the stage at diagnosis and ensuing therapeutic options, PDAC often progresses rapidly. Although this can result in a broad range of symptoms at later stages of the disease, a particularly complex clinical challenge affecting a large proportion of patients with PDAC is cachexia (21). Cachectic patients experience skeletal muscle wasting often together with a loss of adipose tissue which cannot be sufficiently mitigated by nutritional support and which leads to increasing impairment of the entire organism (22).

2.2 MEDICAL PANCREATIC CANCER TREATMENT

2.2.1 Adjuvant treatment of pancreatic cancer

Clinical trials performed in the 1980s and 1990s provided the first evidence that patients who underwent radical surgery of resectable, non-metastatic tumors, the only potentially curative treatment of PDAC, benefit from adjuvant chemotherapy or chemoradiotherapy (23–25). Later, two randomized controlled trials (RCT), ESPAC-1 and CONKO-001, demonstrated a survival benefit from adjuvant treatment with gemcitabine compared to observation alone (26,27). In the ESPAC-1 trial, the median overall survival (OS) in the gemcitabine group was 19.7 months compared to 14 months in the observation group (Hazard ratio [HR], 0.66 [95% CI, 0.52–0.83]). The CONKO-001 trial reported a median OS of 22.8 months among patients treated with gemcitabine compared to 20.2 months in the control group (HR, 0.76 [95% CI, 0.61-0.95]) together with a 5-year OS of 20.7% (95% CI, 14.7%–26.6%) compared to 10.4% (95% CI, 5.9%–15.0%) (27,28). The efficacy of gemcitabine was confirmed in the follow-up trial, ESPAC-3, that randomized patients after surgery between six months of gemcitabine or 5-fluorouracil (5-FU) and folinic acid (29). The median overall survival (OS) among patients treated with gemcitabine was 23.6 months (95% CI, 21.4–26.4) and 23.0 months (95% CI, 21.1–25.0) in the 5-FU and folinic acid group with no differences in survival rates at 12 and 24 months. Treatment with gemcitabine monotherapy has thereafter been used in the control arms of trials with combination chemotherapy after surgery for PDAC. The combination of gemcitabine with capecitabine was associated with a modest but statistically significant difference in median OS (HR, 0.82 [95% CI, 0.68–0.98]) and led to a more pronounced difference in the 5-years survival rate, 18.6% compared to 11.9% (30). In a more rigorously

selected study population, the modified quadruple combination of 5-FU, folinic acid, irinotecan and oxaliplatin (FOLFIRINOX) was associated with a median OS of 54.4 months (95% CI, 41.8–NR) compared to 35.0 months (95% CI, 28.7–43.9) in the gemcitabine arm and with impressive 3-year survival rates of 63.4% and 48.7, respectively (31). A phase III RCT of the combination of gemcitabine with nab-paclitaxel, an established treatment for advanced PDAC, did not reach its primary endpoint of independently reviewed improved disease-free survival (DFS) but resulted in improved median OS and 5-year survival compared to gemcitabine alone (32). The combination of orally administered tegafur/gimeracil/oteracil (S1) proved more effective than adjuvant gemcitabine in a large Japanese phase III trial but is not widely used in Western populations (33).

2.2.2 Pre- and perioperative treatment of pancreatic cancer

Neoadjuvant treatment of upfront resectable or borderline resectable PDAC is a developing strategy, and no standard has been defined, yet. Based on experiences with chemoradiotherapy with concomitant 5-FU, it has been hypothesized that delivering oncological treatment before surgery is more feasible than delivering adjuvant treatment and that better exposure to chemotherapy might improve outcomes (34). Only few smaller trials in the pre-FOLFIRINOX era investigated a formal neoadjuvant approach for patients with resectable tumors demonstrating improved R0-resection rates and indicators of improved oncological outcomes (35–37). In contrast, several trials investigated combined chemoradiotherapy and sequential systemic oncological treatment often including both resectable and borderline resectable cases of PDAC. Borderline resectable PDAC is defined by a set of anatomical criteria based on the extent of involvement of the celiac axis, superior mesenteric artery, the portal vein and the superior mesenteric vein together with biological factor and the patient's performance status (38). While inclusion of borderline tumors eliminates the important distinction between formal neoadjuvant treatment of resectable disease and downstaging of locally advanced tumors that cannot be directly resected, the conclusion of a large meta-analysis of 38 trials with almost 3,500 patients was that pre-operative treatment improves OS and R0-resection rates despite a lower overall resection rate (39). However, this approach failed to improve OS of patients with resectable or borderline resectable tumors in the largest, randomized phase III RCT in this setting to date that compared 2.4 Gy × 15 pre-operative radiotherapy and adjuvant gemcitabine with direct surgery and adjuvant gemcitabine alone (40). In this trial, secondary analyses suggested a potential benefit of neoadjuvant chemoradiotherapy, but the focus of current trials is nonetheless rather the escalation of chemotherapy than further investigating the role of local irradiation. In this way, incorporation of modified FOLFIRINOX in the pre-operative treatment has shown promising results (41,42). Two randomized phase II trials of peri-operative versus adjuvant nab-paclitaxel/gemcitabine or peri-operative versus adjuvant modified FOLFIRINOX have been conducted to study complete neoadjuvant treatment with modern chemotherapy regimens for resectable tumors (43,44). Unfortunately, results from the NEONAX trial investigating nab-paclitaxel/gemcitabine were largely inconclusive and results from the Scandinavian NorPACT-1 trial of modified FOLFIRINOX are still pending.

2.2.1 Chemotherapy against advanced pancreatic cancer

The vast majority of PDAC patients presents with either locally advanced non-resectable tumors or metastatic disease. Advanced disease is defined by (i) encasement of the superior mesenteric vein greater than 180 degrees or involvement of the coeliac axis, (ii) portal vein or superior mesenteric vein involvement without the technical possibility of reconstruction, (iii) involvement of the aorta, (iv) non-regional lymph node involvement, or (v) the presence of metastatic lesions (45). In addition to multimodal supportive and palliative care, patients in an adequate performance status are offered systemic chemotherapy to alleviate symptoms and extend survival. Since the introduction of gemcitabine (46), different combination therapies have been tested with a successive improvement of overall survival (Table 1), generally at the price of increased risk for toxicity. Evidence from RCTs supports the use of the combination therapies FOLFIRINOX and gemcitabine/nab-paclitaxel for patients with metastatic PDAC in good performance status, while gemcitabine monotherapy is the preferred option for patients in a reduced performance status or with other contraindications against combination treatment (47,48). Frail patients should not receive systemic therapies. It is, however, important to note that the definition of advanced PDAC has not been consistent across trials. Earlier trials tended to include patients with locally advanced and metastatic disease, but more recent trials only included patients with metastatic PDAC. The overall efficacy of chemotherapy for advanced PDAC was also assessed in a recent Cochrane systematic review. The review confirmed the efficacy of FOLFIRINOX and gemcitabine/nab-paclitaxel but highlighted that even other therapies can be efficacious and that better markers are needed to guide treatment selection for individual patients (49).

Table 1. Pivotal RCTs of systemic first-line chemotherapy for advanced PDAC

Authors	Patients (*n*)	Treatment	OS, mo. (95% CI)	HR (95% CI)	Stage	Ref.
Burris et al. (1997)	63	Gemcitabine	5.7 (–)	–	Locally advanced and metastatic	(46)
	63	5-FU	4.4 (–)			
Louvet et al. (2005)	156	Gemcitabine	7.1 (–)	1.2 (0.95–1.54)	Locally advanced and metastatic	(54)
	157	Gemcitabine/oxaliplatin	9.0 (–)			
Heineman et al. (2006)	97	Gemcitabine	6.0 (–)	0.8 (–)	Locally advanced and metastatic	(55)]
	98	Gemcitabine/cisplatin	7.5 (–)			

Table 1 continues on the next page

Table 1. Pivotal RCTs of systemic first-line chemotherapy for advanced PDAC – continued from previous page

Moore et al. (2007)	284	Gemcitabine/placebo	5.9 (–)	0.82 (0.69–0.99)	Locally advanced and metastatic	(56)
	285	Gemcitabine/erlotinib	6.2 (–)			
Cunningham et al (2009)	266	Gemcitabine	6.2 (5.5–7.2)	0.86 (0.72–1.02)	Locally advanced and metastatic	(57)
	267	Gemcitabine/capecitabine	7.1 (6.2–7.8)			
Von Hoff et al. (2013)	430	Gemcitabine	6.7 (6–7.2)	0.72 (0.62–0.83)	Metastatic	(58)
	431	Gemcitabine/nab-paclitaxel	8.5 (7.9–9.5)			
Conroy et al. (2011)	171	Gemcitabine	6.8 (5.5–7.8)	0.57 (0.45–0.73)	Metastatic	(59)
	171	FOLFIRINOX	11.1 (9–13.1)			

For patients with locally advanced non-metastatic PDAC, neither FOLFIRINOX nor gemcitabine/nab-paclitaxel were studied in a phase III RCT. A phase III trial that assessed the effect of consolidation with chemoradiotherapy after induction treatment with gemcitabine did not demonstrate any additional benefit compared to chemotherapy maintenance (50). Thus, the evidence from randomized trials so far only supports the use of gemcitabine in this setting (47). However, a patient-level meta-analysis of 315 patients across eleven studies demonstrated a high efficacy of FOLFIRINOX in this patient population (51). In clinical practice, FOLFIRINOX is therefore commonly used in the setting of locally advanced PDAC, not least because of the prospect to proceed to surgery in the case of a good oncological response (52). A prospective nonrandomized phase II trial of six months of treatment with gemcitabine/nab-paclitaxel demonstrated a high disease-control rate (DCR; 77.6% [90% CI 70.3%–83.5%) and overall response rate (ORR; 33.6% [90% CI 26-6%–41.5%]) which supports the use also of this regimen (53).

Patients in a good performance status after first-line treatment can be offered second-line systemic treatment. There is conflicting evidence from two randomized trials if the combination of 5-FU/folinic acid and oxaliplatin is associated with a survival benefit compared to best supportive care for patients who had previously been treated with gemcitabine-based chemotherapy (60,61). The combination of 5-FU/folinic acid and nanoliposomal irinotecan demonstrated a survival benefit over second-line treatment with either agent alone (62). An earlier direct comparison of FOLFOX and FOLFIRI containing non-nanoliposomal irinotecan in a randomized phase II trial demonstrated similar efficacy of both treatments (63). Evidence from a phase III trial also supported the use of capecitabine after gemcitabine in combination with erlotinib but the strategy has now been superseded by other combinations (64). Additionally, combinations of gemcitabine with other platinum

compounds or taxanes, and various single agent chemotherapies have been tested after failure of gemcitabine without any conclusive results (65). No data from randomized trials is available for the second-line treatment of patients who previously received FOLFIRINOX or other 5-FU-based treatment for advanced PDAC. A prospective series of 57 consecutive patients treated with gemcitabine/nab-paclitaxel after failure of FOLFIRINOX showed a promising 58% DCR and 17.5% ORR (66).

2.2.2 Advanced pancreatic cancer in clinical practice

Patients with advanced PDAC in the RCTs underlying the current treatment recommendations are selected by inclusion and exclusion criteria that might introduce a substantial difference between the trial populations and the patient population in clinical practice. Data from the real-world clinical practice of treating PDAC patients is therefore valuable to assess if the efficacy of treatments in RCTs translates into benefits for patients in routine care (67). In fact, a retrospective analysis of patients treated with first-line chemotherapy over an 11-year period in the Canadian province of British Columbia demonstrated that only 25% and 45% of them would have fulfilled the inclusion criteria for the phase III trials of FOLFIRINOX or gemcitabine/nab-paclitaxel, respectively (68).

Two recent systematic reviews and meta-analyses summarized direct comparisons of the real-world effectiveness of FOFLIRINOX and gemcitabine-nab-paclitaxel. The pooled analysis of sixteen studies with a mixed population of patients with locally advance and metastatic disease found no difference between regimens and calculated a HR for death of 0.99 (95% CI, 0.84–1.16; random effect model). The pooled HR for disease progression was 0.88 (95% CI, 0.71–1.1) and favored gemcitabine/nab-paclitaxel without reaching statistical significance. In the same meta-analysis, FOLFIRINOX was associated with a numerically longer median OS by 1.15 months (95% CI, 0.08–2.22) (69). The second recent meta-analysis compared the median OS associated with both treatments across 31 studies with more than 5,200 patients (70). In this analysis, 30 studies reported a direct comparison of both treatment regimens. Of these, 18 reported a numerically longer median OS with FOLFIRINOX and nine reported a longer median OS with gemcitabine/nab-paclitaxel. Three studies reported no numerical difference. Among the fourteen statistical comparisons included in the meta-analysis, however, twelve did not find any significant differences in median OS, one comparison favored FOLFIRINOX and one favored gemcitabine/nab-paclitaxel. Not included in these meta-analyses was the largest single analysis of real-world data on combination chemotherapy for advanced PDAC to date (71). In this analysis of 1,130 patients, FOLFIRINOX was clearly associated with a longer median OS of 9.6 months compared to 6.1 months for gemcitabine/nab-paclitaxel with an improved propensity-score weighted HR 0.77 (95% CI, 0.70–0.85) for FOLFIRINOX and a similarly improved HR in a multivariate adjusted COX model. Notably, this advantage was observed in all sub-group analyses. In summary, reported OS data in real-world cohorts differ significantly. Consequently, the extent to which survival benefits documented in RCTs can be translated into a routine clinical setting varies so much that FOLFIRINOX does not appear be a better

choice than other treatments in all cases. Few factors contributing to on this discrepancy between real-world data and RCTs have been investigated.

2.3 MOLECULAR CHARACTERISTICS OF PANCREATIC CANCER

2.3.1 The pancreatic cancer genome

The mutational landscape that drives PDAC biology is dominated by a limited number of frequently mutated genes and a broad range of lower-frequency variants in a plethora of other genes with various roles in tumorigenesis (72). Somatic missense single nucleotide variants (SNV) of *KRAS* are the most common alteration in the PDAC cancer genome and found in 85–90% of tumors (73–76). Typically, *KRAS* variants affect codon 12 of exon 2, resulting in a handful of prominent activating oncogenic driver mutations in the KRAS protein with the $KRAS^{G12D}$, $KRAS^{G12V}$, and $KRAS^{G12R}$ variants accounting for 33%, 26%, and 14% of cases, respectively (Figure 1A).

In addition to *KRAS*, the only genes in which somatic mutations occur in the tumors of larger groups of patients are *TP53*, *SMAD4*, and *CDKN2A* (Figure 1A). In most cases, combinations of variants in these genes can be found and they represent rather different aspects of pancreatic cancer pathophysiology than mutually exclusive pathways (74,78,79). A second tier of genes, altered in 2–10% of PDAC cases, comprises *ARID1A*, *RNF43*, *LRB1B*, *KMT2C*, *TGFBR2*, *GNAS*, *RBM10*, *FAT4*, *ATM*, *ELP2*, *FANCF*, *KDM6A*, *AXIN1*, *RELN*, *MAP2K4*, *KAT6A*, *PCLO*, *PTPRT*, *ACVR1B*, *DNMT3A*, *RNF213*, *PIK3CA*, *TGFBR1* *SMARCA4*, and *FAT1* (Figure 1B). The prevalence of mutations of other individual genes is markedly lower, typically in the range of 1–0.5% (Figure 1C).

Whole genome sequencing has revealed that the PDAC genome is not only defined by aberrations of individual genes but also by different degrees of genomic structural variation (74). Tumors with the highest degree of genomic instability are associated with flawed DNA maintenance, specifically loss of *BRCA1*-, *BRCA2*-, or *PALB2*-function, leading to homologous recombination double-strand break repair deficiency (HRD) (74). The ensuing structural variants in these tumors form a characteristic pattern of multiple deletions up to 50 base pairs (bp) and alignment of microhomology adjacent to breakpoints as cells compensate HRD by relying more on microhomology-mediated end joining (MMEJ), a form of error-prone alternative non-homologous end joining, to repair double strand breaks (80,81). In the dataset in which this unstable genomic subtype was initially identified, it accounted for 14% of cases (74). Still, some PDAC with this specific mutational signature appear to have no pathogenic variant in any HRD gene (74,82).

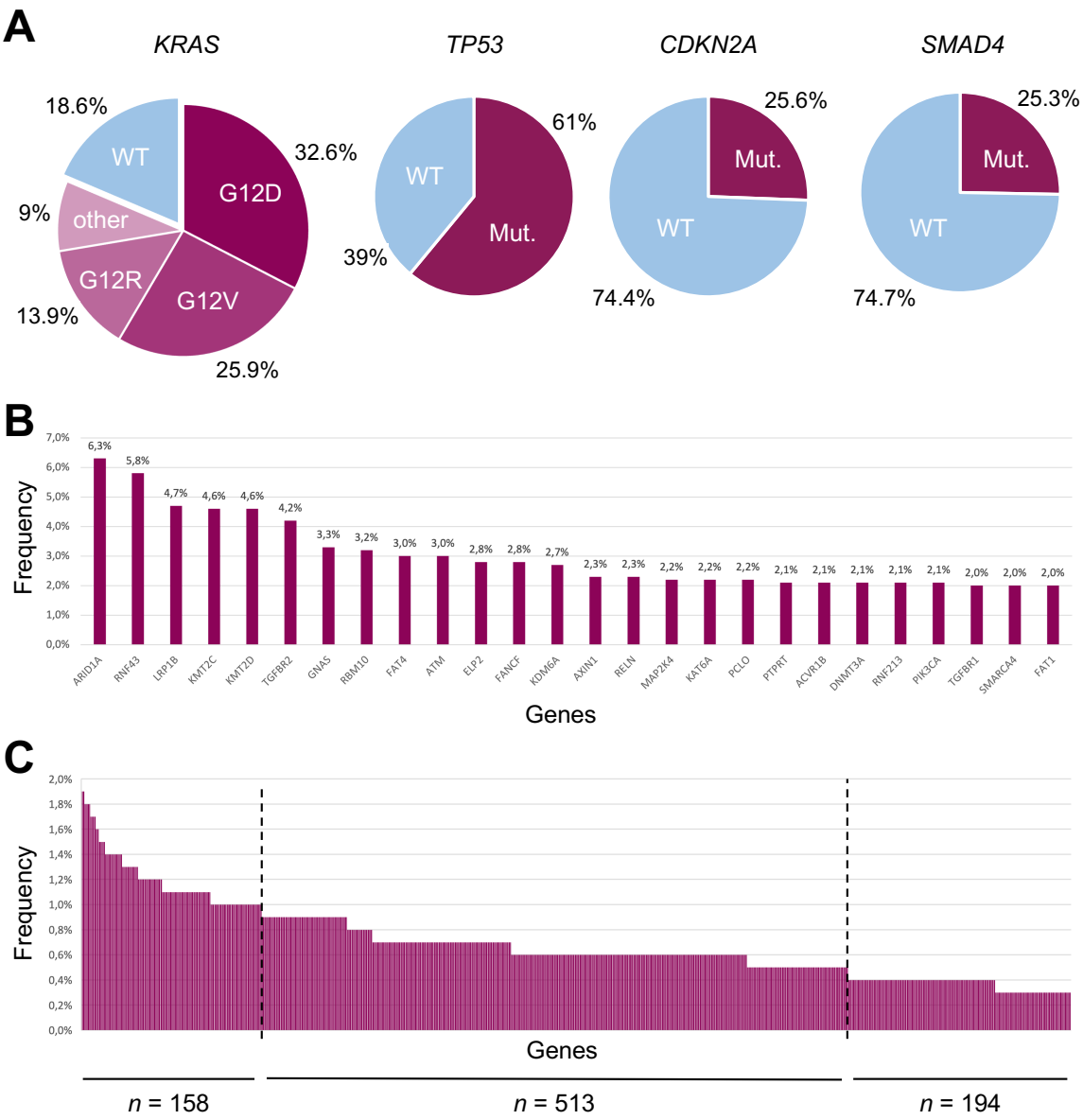

Figure 1. The genomic landscape of pancreatic adenocarcinoma. Cumulative data from five independent datasets with 988 tumor samples (ICGC, WES, *n* = 99 (75); QCMG, WGS/WES, *n* =456 (73); TGCA, WES, *n* = 184(73); UTSW, WES, *n* = 109 (76); CPTAC, WGS/WES, *n* = 140 (77)). (**A**) Fraction of mutant (Mut) and wild-type (WT) alleles in the four most frequently mutated genes *KRAS* (with common *KRAS* codon 12 variants), *TP53*, *SMAD4*, and *CDKN2A*. (**B**) Genes altered in >2% to <10% of tumors (*n* = 26). (**C**) Summary of genes altered in <2% to >1%, <1% to >0.5%, and <0.5% of tumors.

Raw data accessed through cBioPortal (https://www.cbioportal.org; August 20, 2022).

The genome of another subset of PDAC is characterized by copy number variations (CNVs) and structural rearrangements limited to certain regions. In most of these cases, tumors acquire copy number gains of oncogenic drivers, e.g. copy number gains of mutated *KRAS* (74,83), or amplifications of other genes with implications for tumor biology, e.g., *GATA6* which is associated with concordant expression changes of this gene and a more slow-growing phenotype (84,85). This group also includes CNVs and rearrangements of various

targets of molecularly informed therapies, albeit with a low or very low individual prevalence (86).

2.3.2 Hereditary pancreatic cancer and pathogenic germline variants

In populations with European ancestry, between one fifth and one third of PDAC cases has been attributed to hereditary factors (87,88). While genome-wide association studies suggest that genetic risk factors accounting for a substantial proportion of PDAC heritability are still incompletely understood (87,89), pathogenic germline variants (PGVs) in several high-risk susceptibility genes have be linked to PDAC (90). Their impact is particularly obvious in families with at least one pair of first-degree relatives with PDAC, the criterion which defines the familial pancreatic cancer syndrome (91). Individuals from these families have at least a more than six-fold increased relative risk compared to the general population to become affected by PDAC (92,93).

Although the genetic basis of familial pancreatic cancer appears to be heterogeneous, established susceptibility genes typically cluster around distinct pathophysiological mechanisms (94). Among these, heterozygous mutations of the homologous recombination double-strand break repair (HRR) genes, *BRCA1*, *BRCA2*, and *PALB2,* and pathogenic variants of genes involved in the activation of the G_1/S checkpoint of the cell cycle, *ATM*, *CHEK2*, *CDKN2A*, and *TP53,* are most prevalent (Table 2). Other PGVs associated with increased PDAC risk affect the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*, or the hereditary pancreatitis genes *PRSS1*, *SPINK1*, and *CFTR*. The highest genetic risk of PDAC is associated with Peutz–Jeghers syndrome caused by PGVs of the *STK11* gene that encodes the serine/threonine kinase LKB1 which acts as a tumor suppressor by maintaining cell polarity and controlling the mTOR pathway (95). Studies that investigated various sets of these genes, sometimes together with other germline genetic alterations, detected PGVs in 8–14.5% of patients from FPC families (94,96–98). In the context of an unselected population-based cohort of Western patients with pancreatic cancer, the prevalence of PGVs in susceptibility genes was 3.8% (95% CI, 2.1%−5.6%) (99).

Table 2. Heterozygous deleterious germline variants in susceptibility genes and increased risk of PDAC

Gene	Associated syndrome	Relative PDAC risk (95% CI)	Cumulative lifetime PDAC risk (95% CI)	Population frequency	Ref.
General population	–	Reference	1.7%	–	(100)
ATM	Ataxia-telangiectasia	RR 6.5 (4.5-9.5) OR, 3.44, (2.58–4.6) to 5.71 (4.38–7.33)	9.5% (5%–14%)*	2.8%†	(101–104)

Table 2 continues on the next page

Table 2. Germline mutations associated with increased risk of PDAC – continued from previous page

BRCA1	Hereditary breast–ovarian cancer syndromes	OR 2.58 (1.54-4.05) SIR 2.55 (1.03–5.31)	2.9% (1.9%–4.5%; men), 2.3% (1.5%–3.6%; women)*	0.15–0.65%	(103,105–107)
BRCA2	Hereditary breast–ovarian cancer syndromes	OR 6.20 (4.62–8.17) SIR 2.13 (0.36–7.03)	3.0% (1.7%–5.4%; men), 2.3% (1.3%–4.2%; women)*	0.26–0.45%	(103,105–107)
CDKN2A	Familial atypical multiple mole melanoma	OR 8.69, (4.69–16.12) to 12.33 (5.43–25.61)	17% ‡ –20.7%§	rare	(103,104,108,109)
CFTR	Cystic fibrosis (homozygous)§	OR 1.40 (1.04–1.89) to 3.13 (1.0–9.55)	Not reported	1.2–3.7%	(110–112)
CHEK2	Li–Fraumeni-like syndrome	1.31 (0.91–183	Not reported	0.4%	(97,103,113,114)
MLH1	Lynch syndrome	HR 7.5 (2.4-23.0) OR 6.66 (1.94-17.53)	6.2%‡	<0.4%	(103,104,115–117)
MSH2/EPCAM	Lynch syndrome	HR 10.9 (5.5–21.9) OR 3.17 (2.70–5.91) to 7.10 (1.04–37.16)	0.5%–1.6%‡	<0.4%	(104,115–118)
MSH6	Lynch syndrome	OR 7.79 (3.85–15.16)	1.4%–1.6%‡	<0.5%	(116–118)
PALB2	Fanconi anemia	RR 2.37 (1.24–4.50) OR 3.09, (2.02–4.74)	3% (2%–5%; men), 2% (1%–4%; women)*	<1%	(104,119,120)
PMS2	Lynch syndrome	OR 0.7 (0.12–2.22)	≤1%–1.6%	<0.4%	(103,116,117)
PRSS1	Hereditary pancreatitis	SIR 87 (42–113)	33.3%*‖ – 55%¶	rare	(121,122)
SPINK1	Idiopathic pancreatitis	OR 1.52 (0.67–3.45)	51.8%*	1.7%	(123,124)

Table 2 continues on the next page

Table 2. Germline mutations associated with increased risk of PDAC – continued from previous page

STK11	Peutz-Jeghers syndrome	HR 76 (36–160)	26% (4%–47%)§	0.002–0.0005%	(125,126)
TP53	Li-Fraumeni syndrome	OR 6.7 (2.52–14.95) to 7.25 (2.78–18.13)	Not reported**	0.07%	(103,118)

*by age 80 years. †range estimate 0.68–7.7%. ‡by age 75 years. §by age 70 years; §heterozygous carrier status of *CFTR* variants is associated with various exocrine pancreas disorders (112). ‖*PRSS1*variants were present in 79% of PDAC cases. ¶by age 75 years. Risk is attributable to hereditary pancreatitis irrespective of *PRSS1* mutational status. **Carriers of TP53 PGV have a 50% cumulative risk for any cancer by age 30 years (127).

Most PDAC susceptibility genes are classical tumor suppressors. The carcinogenic potential of PGVs in these genes depends on a second somatic event resulting in biallelic loss-of-function (128). This process is exemplified by *BRCA1/2*-mutations, currently the clinically most relevant PGVs for PDAC patients because of their prevalence and potential therapeutic implications (129). Studies in breast and ovarian cancer have shown that 90% of *BRCA1*^{mut} and 64% of *BRCA2*^{mut} breast cancer and 93% of *BRCA1*^{mut} and 84% of *BRCA2*^{mut} ovarian cancer have locus-specific loss of heterozygosity (LOH) with deletion of the normal allele (130). Other mechanisms of inactivation of the second allele appear to be rare. Two studies with a total of 30 tumor samples from individuals with *BRCA1/2* PGVs but tumors with biallelic balance described, e.g., hypermethylation of the *BRCA1* and *BRCA*2 promoters only in two cases (131,132). One of these studies also assessed deleterious somatic mutations of the second allele but found none (132). In PDAC, data on the frequency of LOH in *BRCA1/2*^{mut} is scarce. In a small series, locus-specific LOH was observed in 4/8 patients (133). However, even haploinsufficiency might be therapeutically addressable in specific cases of *BRCA1/2*-associated cancers (134).

Based on the frequency of PGVs in patients with PDAC, implications for kindreds, and potential clinical consequences, the American National Comprehensive Cancer Network (NCCN) guidelines recommended germline testing of all PDAC patients in their 2021 updated guidelines (48). European Society for Medical Oncology (ESMO) guidelines currently recommend multigene panel testing of families fulfilling FPC criteria (135). The recently updated Swedish “Nationellt vårdprogram bukspottkörtelcancer “ [National care program for pancreatic cancer] recommends genetic counseling with the possibility to perform genetic testing for kindreds with more than two PDAC cases or one case of PDAC and two cases of melanoma (136). These discrepancies between recommendations reflect different appreciations of the absolute lifetime risks of PDAC associated with PGVs and the therapeutic options linked to them.

2.3.3 Transcriptomics and other molecularly informed classifications

Insights into the genomic events that underpin PDAC tumorigenesis have been complemented by several studies that defined molecular subtypes based on gene expression

data (137). Moffit et al., Collisson et al., and Bailey et al. have developed three different classification systems that identified two, three, and four different PDAC subtypes, respectively; all of which also correspond to distinct histological tumor features (73,138,139). These three tumor classifiers have substantial overlap with each other and were generally confirmed in external datasets (140–143). The classifiers by Moffit et al. and Bailey et al. have also externally validated prognostic value for PDAC survival. The classifier by Collisson et al. distinguishes a subtype associated with poor prognosis, termed quasi-mesenchymal, but its prognostic value could not be externally validated (140). Later studies have promoted the integration of stromal features into PDAC classification systems and raised questions about contamination with acinar cells as the origin of a previously described tumor subtype similar to normal exocrine pancreatic tissue, termed exocrine-like or aberrantly differentiated endocrine–exocrine (144). Although this highlights that no consensus on transcriptomics-based molecular subtypes exists, these studies have already provided important insights into the heterogeneity of PDAC biology and further clinical research needs to clarify whether they have clinical utility beyond the stratification of prognostic groups.

Several other classification systems using technology-driven comprehensive profiling of PDAC patients have been suggested (145). Among others, genome-wide methylation patterns, metabolomic signatures, or the composition of the gut microbiota could be associated with patient clinical outcomes but lack currently clinical applications (146–148).

2.4 PRECISION ONCOLOGY FOR PANCREATIC CANCER

2.4.1 Targeting the RAS-RAF-MEK-ERK pathway

As the most pertinent driver of PDAC, $KRAS^{mut}$ and its downstream signaling through mitogen-activated protein kinase (MAPK) signaling pathways are priority therapeutic targets. However, the KRAS protein structure lacks hydrophobic pockets for binding of inhibitory compounds making it notoriously resistant to direct inhibition (149). To date, this can only be overcome by targeting the specific chemical properties of the cysteine residue in the $KRAS^{G12C}$ variant (150). This has led to the development of exclusive $KRAS^{G12C}$ inhibitors, sotorasib and adagrasib, with remarkable efficacy in phase II trials in patients with $KRAS^{G12C}$ non-small cell lung cancer (NSCLC) (151,152). Unfortunately, this is a rare *KRAS* variant in PDAC and occurs only in about 1–2% of all cases (73–77). Data from the phase I trial of sotorasib showed nonetheless that this small molecularly defined subgroup of patients may benefit from $KRAS^{G12C}$-directed treatment (153). Preliminary phase I/II data reported an ORR of 21.1% (95% CI, 9.6%–37.3%), DCR of 84.2% (95% CI, 68.8%–94.0%), and a median OS of 6.9 months (95% CI, 5.0–9.1) among 38 patients with refractory PDAC treated with sotorasib (154). The ORR was 50%, the DCR was 100% and median PFS was 6.6 months (95% CI 1.0–9.7) among heavily pretreated patients who received adagrasib (155).

Other emerging strategies to directly target aberrant *KRAS* in PDAC involves the use of genetically engineered allogeneic T-cell receptor (TCR)-based adoptive cell therapy. This

approach has been pioneered in two patients with refractory *KRAS*^{G12D} PDAC using slightly different techniques resulting in one prolonged partial response (156).

Several clinical trials have also studied targeting the downstream oncogenic signaling of *KRAS*^{mut} although with insufficient signs of efficacy. The canonical signaling pathway of KRAS is the RAF/MEK/ERK pathway but mouse models of PDAC have shown that KRAS also activates other signaling systems including the PI3K/AKT/mTOR pathway to promote cell survival and proliferation (157). This has been the rationale for phase I trials of compounds that target PI3K/AKT/mTOR signaling in combination with MEK inhibitors. A trial of the mTOR inhibitor everolimus, in combination with trametinib, enrolled 21 patients with PDAC, one of whom achieved a partial response. The investigators were, however, not able to define a safe recommended phase II dose and schedule of everolimus and trametinib due to excess toxicity (158). Similarly, combinations of trametinib with the AKT inhibitor afuresertib or the dual PI3K/mTOR inhibitor GSK2126458 were poorly tolerated and showed minimal clinical benefit in the patients with PDAC included in these trials (159,160).

Preclinical *in vitro* and *ex vivo* data suggests an impaired interaction of the *KRAS*^{G12R} gene product with PI3K although tumor cells might compensate this deficit by upregulation of *PI3K* transcription (161). This observation has led to the hypothesis that *KRAS*^{G12R} PDAC might be more responsive to MEK inhibition which was subsequently tested in a minor trial. Among eight patients treated with the MEK1/2 inhibitor selumetinib, none had an objective response but three had SD >6 months; the median PFS for all patients was 3.0 months (95% CI, 0.8–8.2) (162).

Pathogenic or likely pathogenic variants in other genes encoding the RAF/MEK/ERK pathway are rare. Such variants in *BRAF* occur in 1.4% of PDAC cases, one third of which is the highly actionable *BRAF*^{V600E} variant (73–77). Several case reports exist that patients with *BRAF*^{V600E} PDAC respond exceptionally well to BRAF and MEK inhibitors (163–165). Oncogenic *BRAF* in-frame deletions are rare (78). One reported patient with such a deletion responded initially well to treatment with trametinib and again to subsequent treatment with the ERK1/2 inhibitor ulixertinib after emergence of a MEK2 resistance mutation (78).

2.4.2 Targeting DDR and MMR deficiencies in pancreatic cancer

Tumors with deficient DNA repair pathways have emerged as the major molecularly defined subtype of PDAC that can be addressed with molecularly informed treatment strategies. Two particularly important drug classes are platinum-based antineoplastics and poly(ADP-ribose) polymerase (PARP) inhibitors, compounds that either directly or indirectly induce increased double-strand breaks (DSB), leading to the selective death of tumors cells with HRD because of excess unrepaired DNA damage (166,167). While platinum-based agents directly cross-link DNA strands and induce DSBs during DNA synthesis, PARP inhibitors block single-strand break repair resulting in subsequent DSBs, a concept termed synthetic lethality (168,169). Additionally, PARP is also involved in MMEJ and several other DNA maintenance pathways which represent other potential mechanism of for PARP inhibitors.

However, the individual contributions of these mechanisms to synthetic lethality are not entirely understood (80,169). The most prominent population for HRD-directed therapies are patients with *BRAC1/2* or *PALB2* PGVs but PDAC patients with somatic mutations in HRR-associated genes might also be candidates for such therapies. In a large systematic review and meta-analysis of HRD in 21,842 PDAC patients, its cumulative prevalence ranged from 14.5%–44% with different techniques and definitions used across studies. In a consensus set of HRR genes, the pooled prevalence estimates of germline and somatic mutations were: *BRCA2*, 3.5%; *ATM*, 2.2%; *BRCA1,* 0.9%; *FANC*-family, 0.5%; *CHEK2*, 0.3%; *PALB2*, 0.2%; *ATR*, 0.1%, and *RAD51*-family: 0.0% (170).

In agreement with the biological function of HRR, retrospective studies indicated that patients with *BRCA1/2*^{mut} tumors had a significantly longer OS with platinum-based chemotherapy than with other systemic treatment (171). Likewise, 22% of pretreated patients with advanced disease enrolled in an unrandomized phase II trial of the PARP inhibitor olaparib (germline *BRCA1/2*^{mut} PDAC, n = 23) had an objective response (including four patients with a complete response) and 47% had stable disease ≥8 weeks (172). These observations provided a good rationale for the phase III POLO trial that tested maintenance therapy with olaparib versus placebo for germline *BRCA1/2*^{mut} PDAC patients who had not progressed after ≥16.weeks of platinum-based chemotherapy with FOLFIRINOX. In the initial analysis, maintenance was associated with a significantly prolonged PFS of 7.4 compared to 3.8 months (HR 0.53 [95% CI, 0.35–0.82]) but the final OS analysis did not confirm a survival benefit of olaparib (HR 0.83 [95% CI, 0.56–1.22]). Still, a subgroup of patients might benefit from olaparib as suggested by a 3-year survival rate of 33.9% compared to 17.8% for placebo (173,174). Another PARP inhibitor, rucaparib, similarly showed antitumoral efficacy in an unrandomized phase II trial (PDAC, n = 19; including three patients with somatic *BRCA2* mutations). The ORR was 16% and the DCR was 32% (175). Rucaparib was also tested as maintenance therapy for PDAC patients with pathogenic germline or somatic *BRCA1/2* or *PALB2* variants without progression after ≥16.weeks of any platinum-based therapy with an ORR of 41.7% (3 complete responses; 12 partial responses; 95% CI, 25.5–59.2) and high PFS rates at 6 and 12 months (176). Concomitant use of platinum-based chemotherapy and PARP inhibitors is, in contrast, discouraged by a randomized phase II trial of gemcitabine and cisplatin with or without veliparib. In this trial, the triple combination did not confer an additional survival benefit (median OS 15.5 months [95% CI, 12.2–24.3] versus 16.4 months [95% CI, 11.7–23.4]) to previously untreated patients with PDAC and *BRCA1/2* or *PALB2* PGVs (177).

The DNA mismatch repair (MMR) mechanism responds to certain types of DNA damage, but its main function is the correction of replication errors during DNA synthesis (178). A defective MMR (dMMR) machinery results in the accumulation of mutations in microsatellites, areas of repetitive DNA motifs, that are more prone to replication errors than other regions of the genome – a phenomenon called microsatellite instability (MSI) (179). Tumors with MSI also have an clearly increased rate of coding somatic mutations compared to MMR-proficient tumors (180–182). These mutations can represent neoepitopes which

increase the T cell reactivity against hypermutated tumors and promote the activity of immune checkpoint inhibitors, particularly programmed cell death protein (PD)-1 inhibitors (183). The prevalence of dMMR/MSI among unselected patients with PDAC is approximately 2% (184). Besides PGVs in the MMR genes *MLH1*, *MSH2, MSH6*, *PMS2*, and *EPCAM*, somatic homozygous deletion of *MLH1* or hypermethylation of its promoter occur in sporadic cases of PDAC and might promote a dMMR/MSI phenotype (185,186).

In the absence of dMMR/MSI, PDAC responds poorly to immune checkpoint inhibitors and has one of the lowest levels of tumor mutational burden of all solid tumors (182). Against this background, a total of six patients with dMMR/MSI PDAC were included in five multicenter, nonrandomized clinical trials of pembrolizumab, five of whom had an objective response including response durations beyond nine months (187). A later nonrandomized phase II basket trial of pembrolizumab for dMMR/MSI tumors included 22 patients with PDAC who had an ORR of 18.2% (95 CI, 5.2%–40.3%) with a median duration of response (DOR) of 13.4 months (95 CI, 8.1–NR). Across all PDAC patients, the median PFS was 2.1 months (95% CI, 1.9–3.4) and median OS was 4.0 (95 CI, 2.1–9.8) highlighting a substantial difference between responders and non-responders (188).

2.4.3 Other molecularly informed treatment strategies

Somatic variants of *CDKN2A* are among the most common alterations in PDAC (Figure 1A). *CDKN2A* codes for two proteins, p16 and p14(ARF), that act as tumor suppressors through control of the G_1/S checkpoint of the cell cycle (189). Because p16 acts through inhibition of the cyklin-dependent kinase CDK4/6 in healthy cells, tumors with *CDKN2A* aberrations might respond to treatment with a selective CDK4/6 inhibitor. Despite this biological rationale, palbociclib did not have any clinical activity in twelve PDAC patients with *CDKN2A*^{mut} or *CDKN2A* loss (190).

Table 3. Low-frequency actionable genomic variants in PDAC*

Genomic marker		Patients (*n*)	Study type	Treatment	Outcome	ESCAT tier	Ref.
ALK	*EML4–ALK*	5	Case reports	Alectinib; ceritinib crizotinib; lorlatinib	1 PR, alectinib (PPFIBP1–ALK) 3 SD, alectinib; ceritinib; crizotinib	III-A	(194)
	Other *ALK* fusion	3			1 PD, crizotinib → alectinib		

Table 3 continues on the next page

Table 3. Low-frequency actionable genomic variants in PDAC – continued from previous page

ERBB2	*ERBB2*^{V842I}	1	Case reports	Trastuzumab	Unknown	III-A	(191, 195)
		1		T-DM1	PR		
	ERBB2 amp	17†	Phase II	Trastuzumab/ capecitabine	$PFS_{6\ mo.}$ 11.8% (95% CI, 0%–27.1%)	III-A	
FGFR1–3	FGFR2-USP33	1	Phase I/II	Pemigatinib	PR	III-A	(196)
	FGF/FGFR amp.	3			3 PD	III-B	
IDH1	*IDH1*^{R132H}	1	Case report	Ivosedinib	PD	III-A	(197)
NRG1	*NRG1* fusions	3	Case report	Afatinib; erlotinib/ pertuzumab; erlotinib/ trastuzumab/ chemotherapy	2 PR < 6 months, afatinib, erlotinib/ pertuzumab 1 SD, erlotinib/ trastuzumab/ chemotherapy	II-B	(198)
		10	Phase I/II	Zenocotuzumab	ORR 40% (90% CI, 15–70),		(199)
NTRK	*NTRK fusions*	1	Phase I–II	Larotrectinib	PR	II-B	(200)
		2	Phase I	Entrectinib	2 PR		(201)
ROS1	*ROS1 fusions*	1	Phase I	Entrectinib	PR	II-B	(201)
STK11	*STK11*^{D194E} and LOH	1	Case Report	Everolimus	PR	IV-A	(202)

*not including *KRAS*^{G12C}, *KRAS*^{G12R}, *BRAF*^{V600E}, *BRAF* in-frame deletions, and HRD- or dMMR/MSI-related variants. †including patients with immunohistochemistry +3 HER2 expression without *ERBB2* amp. ‡meeting abstract. Abbreviations: amp, amplification; mo., months; PD, progressive disease; $PFS_{6\ mo}$, progression-free survival rate at 6 months; PR, partial response; SD, stable disease; T-DM1, trastuzumab emtansine

Several studies have used comprehensive genomic profiling of tumor samples in order to identify predictive molecular markers including variants with a low frequency in PDAC. Four prospective clinical trials that characterized patients with advanced PDAC at referral centers reported results from 62, 336, 71, and 3,594 patients and identified actionable targets in 30%, 10.1%, 48%, and 17%, respectively (78,191–193). These included pathogenic variants in genes that encode proteins in MAPK pathways, missense mutations, gene fusions, and CNVs. Generally, limited and fragmented evidence for the clinical efficacy of PDAC treatment informed by low-frequency molecular targets exists (Table 3).

2.4.4 Knowledge management and clinical decision support

In addition to molecularly defined therapeutic targets of PDAC with evidence of actionability from clinical trials or at least case reports, a large number of hypothetical matched marker–drug pairs exist that have not been clinically tested (86). Practical reasons for this might be that patients with such variants are difficult to identify in a timely manner, which is crucial given the often-aggressive course of disease, or that no relevant clinical trials or a framework for off-label drug repurposing exist. Publication bias against negative outcomes is also likely to contribute to underreporting of cases without clinical benefit of a molecularly targeted treatment (203). It is therefore often not obvious if an observed molecular alteration is biologically relevant or if it can be considered an actionable target. This can result in inconsistent evaluations of the variable degrees of evidence that underpin the assessment if a certain genomic variant is predictive of response to a targeted drug. In the context of real-time precision oncology trials, this is likely to be a major reason for the erratic proportions of PDAC cases with actionable target that have been reported (78,191–193).

Many medical centers have established multidisciplinary molecular tumor boards (MTBs) to advance consistent assessment of individual tumor molecular profiles. However, the same clinical context and molecular marker can result in different recommendations from different institutions (204,205). In response to the need for a standardized evaluation across the entire oncology community, different classification systems have been developed. Examples of these are the ESMO Scale of Clinical Actionability for molecular Targets (ESCAT) and the evidence system of the OncoKB knowledgebase, developed by researchers at Memorial Sloan Kettering Cancer Center with certain content of this system recognized by the United States Food and Drug Administration (206–208).

Knowledgebases are resources that aggregate information on the biological and clinical significance of genomic alterations in cancer. They are a systematic approach to collecting and organizing available evidence by a group of dedicated experts or the broader oncology community and provide an interface to later query the data (209). In addition, standards for the reporting and interpretation of variants have been published which is important to harmonize parallel curation initiatives (210,211). Regardless, an analysis of the source publications for specific interpretations in different knowledgebases has shown an overall concordance of less than 25% (212). This highlights the difficulties to entirely capture all available evidence in a setting of a rapidly expanding biomedical literature and has been met with the increasing integration of different knowledgebases (209).

On an individual patient level, the annotation of genomic data, evaluation of evidence supporting actionability, and identification of potential treatment options can be integrated using clinical decision support systems (CDSS) (213,214). These systems can eliminate the risk of error or bias introduced by a manual review of the literature in any of these steps, engage various sources of biomedical knowledge, and improve the turnaround time to review sequencing results. Outputs from CDSS can be either used directly or reviewed by an expert panel. Several commercial and academic CDSS are available but relatively little is known

about how they impact on the identification of molecularly informed treatment options for PDAC patients (215,216).

2.5 BLOOD TESTS FOR PANCREATIC CANCER

2.5.1 Blood assays for detection and monitoring of pancreatic cancer

The histological verification of PDAC with a tissue biopsy of the primary tumor or a metastatic lesion is mandatory in the initial work-up of newly diagnosed patients who are not candidates for immediate surgery (47,48). Tissue samples allow for a morphological assessment and grading of the tumor as well as various routine or investigational molecular analyses. Blood samples, in contrast, do not require invasive procedures and can be easily repeated along the care path of a patient. Because tumors and metastatic lesions release disease-related molecules, particles, and cells, these can be detected and analyzed in human biofluids, particularly peripheral blood. The approach to analyze these samples for tumor-specific molecular patterns has been termed liquid biopsy (217). The rapid technical improvements in this field have sparked considerable research into blood assays to detect PDAC, stratify the prognosis of patients, predict the response to cancer drugs, and monitor disease response or progression (145).

Despite ongoing efforts to develop novel blood assays, the only established blood marker of PDAC to date is the carbohydrate antigen (CA) 19-9, the sialylated form of the Lewis blood group antigen, that was initially described in 1981 (218). Individuals with the Le(a-b-) phenotype cannot express CA 19-9 limiting its clinical utility (219). The prevalence of Le(a-b-) is 6% in European populations and individuals with European ancestry in the Unites States, 7.2% in South Asian and 8% in Chinese populations but 22% among individuals with African-American ancestry and 35% in West African populations (219–222). This highlights a specific need for better blood markers for these groups. In individuals who can express CA 19-9, various other malignant and non-malignant diseases might increase its serum levels, which further limit its diagnostic value (223). Overall, CA 19-9 performs weakly as a diagnostic assay with a sensitivity of 78.2% (95% CI, 76.1%–80.2%) and a specificity of 82.8% (95% CI, 79.9%–85.3%) in a large meta-analysis (219). CA 19-9 has the greatest clinical value in the context of other clinical and radiological information to support the management of patients who have already been diagnosed with PDAC. As a prognostic marker, longer survival of patients with advanced PDAC is correlated to low CA 19-9 levels and vice versa, which can factor into the decision how aggressively the disease should be treated (224). Even more importantly, serial measurements of CA 19-9 under ongoing treatment showed that a decline of its serum levels correlate with treatment response and survival (225,226). In the post-hoc analysis of the MPACT trial of nab-paclitaxel and gemcitabine versus gemcitabine for metastatic PDAC, this meant that patients with any CA 19-9 decline after 8 weeks had an approximately 3 months longer median OS (HR 0.53 [95% CI, 0.36–0.78]), irrespective of the treatment arm they had been randomized to (227). Patients with a larger decrease of CA 19-9 levels appeared also to survive longer than patients with a small or moderate decline although subgroups were small in this exploratory analysis.

2.5.2 Cell-free DNA

Cell-free DNA (cfDNA) is the best-studied type of tumor-derived material that can be detected in blood from cancer patients. Several oncology assays based on cfDNA technology have been introduced or are close to introduction as clinical tools, e.g., for identification of actionable target in NSCLC, measurement of minimal residual disease of hematological malignancies, or tumor agnostic early detection (228). The term cfDNA comprises DNA from various sources in the organism. A synonymous term is circulating free DNA, also abbreviated to cfDNA, and the two terms are used interchangeably throughout the literature. Circulating tumor DNA (ctDNA) is a more narrowly defined type of cfDNA originating from malignant cells in cancer patients.

The presence of free DNA in human blood plasma was first described in 1948 (229) and more than 40 years ago the minute amounts of cfDNA could be quantified for the first time (230–232). Increased levels of cfDNA were soon recognized in the blood plasma of patients with gastrointestinal cancers, including PDAC, compared to cfDNA levels in healthy individuals (233). In healthy individuals, the main origin of cfDNA are apoptotic cells, mainly in the hematopoietic system, and cfDNA is typically described as double-stranded fragments approximately 176 bp in length, although there is a significant variation of fragment length (234,235). Because tumor-derived ctDNA appears to be slightly shorter, selection of fragments in the 90–150 bp range has been suggested to enrich samples with ctDNA that otherwise only is a small fraction of all circulating DNA (236–238). Various somatic alterations of the tumor genome can be detected in ctDNA, including SNVs, CNVs, MSI, and genome-wide LOH (239,240). Structural variants can be more difficult to discover, especially in the context of a low ctDNA fraction, but their detection is possible with modified technical or bioinformatic approaches (241,242).

One of the first SNVs in any solid tumor that could be detected in ctDNA was $KRAS^{mut}$ in the blood of three patients with PDAC. Already in this paper, the authors speculated that $KRAS^{mut}$ ctDNA could be used to detect PDAC, determine the prognosis of patients and monitor treatment response (243). However, $KRAS^{mut}$ ctDNA could only be detected in 18.8% (n = 82/437) to 30% (n = 66/221) of patients with localized PDAC in two cohort studies. In both studies, $KRAS^{mut}$ ctDNA could also be detected in the control groups, i.e., 0.5–3.7% of healthy individuals and 4.3% of patients with chronic pancreatitis, albeit at substantially lower mutant allele frequencies (MAFs) than in in the plasma of PDAC patients (244,245). Thus, the sensitivity of $KRAS^{mut}$ ctDNA appears to be insufficient to reliably detect early PDAC, while the clinical significance of small amounts of mutated DNA in the blood of individuals without cancer is unknown. In one of the studies, the sensitivity to detect PDAC could be improved to 64% (95% CI, 57%–70%) by combination of $KRAS^{mut}$ ctDNA with four protein markers, CA 19-9, carcinoembryonic antigen (CEA), hepatocyte growth factor (HGF), and osteopontin (OPN) (245). Using an extended protein panel in a pan-cancer study (PDAC, n = 93), the sensitivity could be further improved to approximately 75% at >99% specificity (246). However, none of these tests is currently widely accepted for PDAC diagnostic testing.

Many studies have investigated the relationship between levels of ctDNA and PDAC survival (Table 4). Most of these studies used PCR assays to measure *KRAS*$^{G12D/G12V}$ as a surrogate for the amount of ctDNA, but other techniques were used in some cases (83,247–250). In summary, the presence and/or higher levels of ctDNA were a negative prognostic factor associated with shorter survival and an increased risk of death, although the exact strength of this correlation is difficult to gauge from a heterogeneous set of studies.

Table 4. Plasma ctDNA and prognosis of patients with PDAC

Authors	Study type	Stage	Patients (n)	Marker	Readout	Outcome	Ref.
Del Re et al. 2017	Prospective cohort	LAPC, metastatic	27	*KRAS*^{mut} MAF dynamics under chemotherapy	*KRAS*^{mut} MAF stability/decrease vs increase at C1D15†	mOS 6.5 vs 11.5 mo. ($p = 0.009$)	(252)
Earl et al. 2015	Prospective cohort	Resectable, LAPC, metastatic	31	*KRAS*^{mut} ctDNA	Presence/absence *KRAS*^{mut} ctDNA	HR death 12.2 (95% CI, 3.3-45.1)*	(253)
Hadano et al. 2016	Prospective cohort	Resectable PDAC	105	*KRAS*^{mut} ctDNA	Presence/absence *KRAS*^{mut} ctDNA	HR death 3.2 (95% CI,1.8–5.4)*	(254)
Kim et al. 2018	Prospective cohort	Resectable, LAPC, metastatic	106	*KRAS*^{mut} ctDNA conc.	*KRAS*^{mut} ctDNA conc. ≥ vs < median‡	HR death 2.0 (95% CI, 1.1–3.7)*	(255)
Kruger at al. 2018	Prospective cohort	LAPC, metastatic	54	*KRAS*^{mut} ctDNA conc.	Presence/absence *KRAS*^{mut} ctDNA *KRAS*^{mut} ctDNA conc.	Levels of *KRAS*^{mut} ctDNA conc. are inversely correlated with OS	(256)
Lapin et al. 2018	Prospective cohort	LAPC, metastatic	61	cfDNA conc.	cfDNA ≤167 bp conc. ≥ vs < median§	HR death 2.2 (95% CI, 1.1–4.6)*	(257)
Mohan et al. 2019	Prospective cohort	LAPC, metastatic	55	*KRAS*^{mut} ctDNA	any CNV	HR death 7.1 (95% CI, 3.2-15.8)	(83)
					KRAS^{mut} amp.	HR death 10.9 (95% CI, 3.8-31.1	
					Presence/absence *KRAS*^{mut} ctDNA	HR death 3.5 (95% CI, 1.8-6.8)	
Nakano et al. 2018	Retrospective cohort	Resectable	45	*KRAS*^{mut} ctDNA	Post-operative presence/absence *KRAS*^{mut} ctDNA	HR death 2.9 (95% CI,1.1–5.6)	(258)

Table 4 continues on the next page

Table 4. Plasma ctDNA and prognosis of patients with PDAC – continued from previous page

Perets et al. 2018	Prospective cohort	Metastatic	17	*KRAS*^{mut} ctDNA	Presence/absence *KRAS*^{mut} ctDNA	mOS 8.0 v. 37.5 mo. ($p = 0.004$)	(250)
Piettrasz et al. 2017	Prospective cohort	LAPC, metastatic	104	*KRAS*^{mut} MAF	Presence/absence *KRAS*^{mut} ctDNA	HR death 2.0 (95% CI, 1.1–3.5)*	(259)
					KRAS^{mut} MAF tertiles	mOS 18.9 vs 7.8 vs 4.9 mo. (p <0.001)	
Singh et al. 2015	Retrospective cohort	Resectable, LAPC, metastatic	127	*KRAS*^{mut} ctDNA	cfDNA conc > vs ≤ 62 ng/mL.	HR death 2.8 (95% CI, 1.8–4.6)*	(260)
					Presence/absence *KRAS*^{mut} ctDNA	HR 0.8 death (95% CI, 0.5-1.3)‖	
Strijker et al. 2019	Prospective cohort	Metastatic	58	NGS	Presence/absence *KRAS*^{mut} ctDNA	HR death 2.2 (95% CI, 1.2–3.9)‖	(248)
					KRAS^{mut} MAF (per 1% increase)	1.1 (1.0–1.1)*	
Tjensvoll et al. 2016	Prospective cohort	LAPC, metastatic	14	*KRAS*^{mut} ctDNA	Presence/absence *KRAS*^{mut} ctDNA	HR death 5.9 ($p = 0.099$)*	(261)
Uesato et. al. 2020	Prospective cohort	Metastatic PDAC	109	NGS	Presence/absence *KRAS*^{mut} ctDNA	HR death 3.1 (95% CI, 1.9–5.0)*	(247)
Watanabe et al. (2019	Prospective cohort	Resectable, LAPC, metastatic	78	*KRAS*^{mut} ctDNA	Presence/absence *KRAS*^{mut} ctDNA	mOS 33.7 v. 15.8 mo. (p = 0.07)	(262)
Wei et al. 2019	Prospective cohort	Advanced PDAC	38	NGS; *KRAS*^{mut} MAF	*KRAS*^{mut} MAF ≥5% vs <5%	HR death 2.1 (95% CI, 0.8–5.1)	(249)

*multivariate analysis. †fifteen days after treatment initiation. ‡median 0.165 *KRAS*^{mut} copies/µl; §locally advanced, median 3.26 ng/ml; metastatic, median 6.58 ng/ml plasma. ‖univariate analysis. Abbreviations: amp., amplification; LAPC, locally advanced pancreatic cancer; mOS, median overall survival

One strategy to improve the detection of ctDNA in the blood of PDAC patients is selection of short or ultrashort fragments of cfDNA. This is supported by the observation that the median DNA fragment length of cfDNA with *KRAS*^{mut} is significantly shorter than that of cfDNA with the wild-type allele, 135 bp compared to 164 bp (p <0.0001). Interestingly, the *KRAS*^{mut} MAF is also highest in fragments in the 60–100 bp range and drops significantly in fragments longer than 150 bp (263). This finding was corroborated in another study in which overlapping different-size amplicons spanning *KRAS* codon 12 were used for library

preparation from cfDNA for sequencing. While long amplicons excluded short DNA fragments as PCR templates, short amplicons did not. The *KRAS*^{mut} MAF increased 4.6-fold (95% CI, 2.6–8.1) when 57 bp amplicons were compared to 218 bp amplicons. Compared to 167 bp amplicons, the range of commonly used cfDNA library preparation protocols, the increase was still 4.1-fold (95% CI, 2.3–7.3). A consequence was that almost twice as many patients had detectable *KRAS*^{mut} ctDNA using the 57 bp amplicon compared to 218 bp amplicon assay (94.2% vs 59.6%). This suggests that short DNA fragments should be targeted to improve PDAC ctDNA assays (264). This conclusion is supported by circumstantial evidence that a short median cfDNA fragment size ≤ 167 bp is associated with both shorter PFS (HR 0.41 [95% CI, 0.23–0.74], univariable Cox regression) and OS (HR 0.39 [95% CI, 0.22–0.71]) (257).

In addition to characterization of somatic tumor mutations in ctDNA, PDAC specific epigenetic methylation patterns can be discerned (265). Several studies have investigated the methylation patterns of different sets of genes in ctDNA from PDAC patients, usually in comparison to healthy individuals or patients with benign pancreatic condition (266–273). In summary, three of these studies reported an area under the receiver operating characteristics (AUROC) between 0.86 and 0.99 for the detection of PDAC, but neither assay has been externally validated (268,269,273). Similarly, a correlation between increased hypermethylation and poorer overall survival has been postulated but not been validated (269).

2.5.3 Extracellular vesicle-associated DNA

Extracellular vesicles (EV) are a heterogeneous group of lipid bilayer membrane nanoparticles that are actively secreted or shed from all types of cells, including cancer cells, and that can be detected in all body fluids (217). Depending on vesicle size and the biogenesis pathway, apoptotic bodies, microvesicles, and exosomes can be distinguished (Table 5). However, there is no consensus on markers that can link EVs to a specific subcellular origin. Thus, size-based classifications, e.g., the terms “small vesicles” and “large vesicles”, are currently recommended in the field (274). Purification of different EV populations from complex biofluids is technically challenging and neither separation by size nor based on molecular characteristics yields pure biologically defined vesicles (275,276).

The physiological function of extracellular vesicles was initially believed to be that of “garbage bags”, used by the cell to discard redundant molecules (281,282). Later, their role in the transfer of nucleic acids and proteins between cells was recognized (283,284). Together with the already known function of EVs in the regulation of adaptive immunity, this has opened a fast-growing field of research into an extensive range of mechanisms in human health and disease (285,286).

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Table 5. Characteristics of extracellular vesicles

Vesicle type	Size (nm)	Cellular origin	Postulated physiological functions	Ref.
Apoptotic bodies	500–4,000*	Disintegration of apoptotic cells	Clearance of apoptotic cell remnants by phagocytosis.	(277,278)
Microvesicles	50–2,000	Direct budding from the plasma membrane through phospholipid redistribution and cytoskeleton contraction	Cell-cell interaction, immunomodulation, coagulation	(278,279)
Exosomes	30–150	Formation in the endosomal network and release by fusion of the multivesicular endosome with the plasma membrane	Cell-cell interaction, protein and RNA transfer, induction of immune tolerance	(280)

*Release of smaller vesicles, 50–500 nm in size, occurs through membrane blebbing

In cancer, EVs have been implicated in promoting neoangiogenesis, immune evasion, and metastatic dissemination (287). In the pathophysiology of PDAC, EVs play a particular role by preparation of pre-metastatic niches in the liver through transfer of macrophage migration inhibitory factor (MIF) from tumor to Kupffer cells (288). Likewise, EVs are vehicles for the dissemination of different proteins that promote loss of muscle mass and lipolysis in PDAC associated cachexia (21). In fact, the proteome of EVs isolated from the blood of patients with cancer has such distinct patterns, that it could be used to distinguish various cancer types from each other as well as from healthy individuals with a remarkably high sensitivity and specificity in a landmark trial (289).

The dominant concept of ctDNA holds that it circulates freely in the blood plasma, bound to histone octamers in nucleosome configuration (290). In contrast to this model, some studies have suggested EVs as a major carrier of ctDNA in PDAC. The first study to describe ctDNA in small EVs in the 100 nm range found them to contain double-stranded genomic DNA fragments >10 kilobase pairs (kb) with tumor specific *KRAS* and *TP53* mutation as well as smaller ctDNA fragments (291). The presence of double stranded DNA in circulating small vesicles from PDAC patients was also confirmed in other studies (292,293). In contrast, ctDNA was not associated with small EVs in the context of prostate cancer where it was detected in large EVs in the 1–6 μm range instead. These vesicles were enriched with tumor-derived chromosomal DNA, including large DNA fragments up to 2 megabase pairs (294). *In vitro* studies of EVs from human medulloblastoma cell lines found DNA with *c-MYC* amplifications mostly associated with intermediately-sized vesicles, mainly consistent with

microvesicles (295). If these distinctions to PDAC represent cancer type-specific difference in tumor biology is currently unknown.

In a prospective study to assess the diagnostic utility of EV-associated ctDNA (evDNA), *KRAS*^{mut} was detectable in 66.7%, 80%, and 85% of patients with localized (n = 53), locally advanced (n = 15), or metastatic disease (n = 20), respectively. The corresponding numbers for conventional ctDNA extracted from plasma were 45.5%, 30.8%, and 57.9%. In healthy individuals (n = 54) evDNA and ctDNA were detected in 7.4% and 14.8%, respectively. In a validation cohort of 39 patients with localized disease and 82 healthy controls, the sensitivity and specificity of evDNA was, however, markedly lower (296). A subsequent analysis of the impact of evDNA on progression and survival from the same researchers showed that a evDNA *KRAS* MAF ≥5% was a predictor of shorter PFS (HR, 2.28 [95% CI, 1.18–4.40]) and OS (HR, 3.46; [95% CI, 1.40–8.50]) for patient with metastatic PDAC.

2.5.1 RNA and protein markers in extracellular vesicles

There is compelling evidence that EVs contain different types of RNA (297). These include messenger RNA (mRNA), microRNAs (miRNA), ribosomal RNA (rRNA) and various other small RNA types (298). Some *in vitro* studies suggest that apoptotic bodies contain rRNA and that large EVs hardly contain any RNA, whereas small EVs are enriched with small RNA types (299). There is contending evidence though that both, large and small EVs, contain small non-coding RNAs and mRNA (300). In PDAC, tumor cells secret EVs with RNAs that increase invasiveness and stimulate the induction of cancer-associated fibroblasts (301,302). The PDAC microenvironment also interacts with tumor cells through EVs with miRNAs that promote metabolic changes, induction of epithelial-mesenchymal transition and cancer stemness (303,304). Regarding the mRNA content of EVs, a pilot study of three patients with PDAC or periampullary carcinoma demonstrated that the EV transcriptome is largely consistent with results from matched tissue analyses (293).

A large number of diagnostic assays for PDAC based on EV-associated tumor RNA have been tested. A recent systematic review summarized seventeen case-control studies that reported various EV-associated non-coding RNAs and a few gene transcripts that were used to distinguish PDAC from healthy individuals or patients with benign pancreatic conditions (305). The AUROC of these studies varied between 0.65 and 1. Generally, the assays lacked external validation and have not been adopted. EV-associated RNAs were also tested as part of various panels of multiple miRNAs, long non-coding RNAs, mRNAs or in combination with additional proteins. In total, panels from five of these studies, have been externally validated but further translational development is currently unclear.

In addition to RNAs, there are far more than 100 reports on the performance of individual EV-associated proteins as diagnostic markers of PDAC across a large number of studies (305). Glypican-1 is arguably the most eminent EV-associated protein marker and could be used to detected patients with PDAC with absolute sensitivity and specificity in the initial report. EV-associated glypican-1 expression was also correlated with disease burden, and

predicted survival after pancreas surgery in this study (306). Scaling the approach for clinical applications has, however, been difficult not least because enzyme linked immunosorbent assays (ELISA) for glypican-1 perform poorly (306–309). A later study could confirm the specific overexpression of glypican-1 on EVs in the blood of PDAC patients. Using manual EV isolation and bead-based flow-cytometry, the authors reported 98.3% sensitivity and 86.2% specificity with an AUROC of 0.96 (309). Glypican-1 is also one of five protein markers in a highly accurate multiplexed plasmonic assay that has been tested and validated for PDAC. Using a nanoplasmonic sensors it allows for high throughput and overcomes practical limitations of multiplexed EV protein assays but it is currently not available for clinical use (310).

2.5.2 Other liquid biopsy strategies

Tumor-educated platelets have been suggested as another source of circulating tumor-derived mRNA that is transferred from cancer cells and alters the platelet transcriptome (311). Based on these findings, a tumor-agnostic RNA sequencing assay was developed and tested in a pan-cancer cohort. In a subset of 35 patients with PDAC, this assay achieved a detection rate of 98% (312). In a proof-of-concept study, it was also possible to identify distinct protein patterns in platelets from patients with early PDAC using mass spectrometry-based proteomics (313).

In addition to circulating tumor-derived molecules, extracellular vesicles, and tumor-educated platelets, entire circulating tumor cells (CTC) can be found in the blood stream of PDAC patients (314). The frequency with which CTCs can be detected varies considerably in the literature, between 11% and 92% (315). The fact that CTC detection is largely technology-driven is probably one important factor for this variation but fewer patients with early and localized disease appear to have detectable CTCs irrespective of the method that was used. This is reflected in a limited sensitivity of 64% but a good specificity of 99.5% for a combination of CTC detection and four blood protein markers in the largest effort to use CTC for early PDAC detection to date (245). This is consistent with the meta-analysis of 44 studies that estimated that the pooled fraction of CTC-positive patients was 65% (95% CI, 55%–75%). In the pooled analysis of survival data, detection of CTCs was associated with poorer outcomes (HR for OS 1.82 [95% CI, 1,61–2,05]).

3 RESEARCH AIMS

3.1 OBJECTIVES

The overall objectives of the thesis are to evaluate established treatment strategies for advanced disease, to explore avenues for the implementation of precision oncology, and to optimize liquid biopsy strategies for PDAC.

3.2 SPECIFIC AIMS

Study I

- Assessment of the effeciveness of chemotherapy for patients with advanced PDAC in a real-world setting.

Study II

- Evaluation of the utility of the CDSS MH Guide to prospectively suggest molecularly informed treatment options for PDAC patients.

Study III

- Identification of the blood component most enriched with PDAC-derived circulating DNA.

Study IV

- Characterization of long and short tumor-derived circulating DNA fragments and their association with extracellular vesicles and other blood components.

4 MATERIALS AND METHODS

4.1 STUDY DESIGN

Different designs were used for the four studies that constitute this thesis. Study I is a retrospective cohort study that captured prospectively generated clinical data on the real-world practice of treating advanced PDAC. The design of Study I considered the requirements of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines to ensure adequate data capture and analysis (316). Study II is a single-center prospective observational clinical trial of the CDSS MH Guide, a CE marked medical device, ClinicalTrials.gov Identifier: NCT02767700. The primary outcome of this study was the fraction of patients with a complete CDSS report of actionable gene variants in three domains: Molecularly informed therapies, markers of resistance to cancer drugs, and increased risk of toxicity. Secondary outcomes included the frequency of genomic variants, of actionable markers, and assessment of actionability by a study-specific MTB. No treatment interventions were specified by the study protocol, but we performed a post-hoc analysis with additional retrospective data to compare CDSS recommendations and what treatment patients received in clinical practice. Studies III and IV are translational studies that used blood samples from a prospective blood sample collection cohort of patients with newly diagnosed locally advanced, metastatic or recurrent PDAC that was established as part of this thesis.

4.2 PATIENTS AND HEALTHY VOLUNTEERS

4.2.1 Study I

Patients who initiated treatment for advanced PDAC at Karolinska University Hospital between January 1, 2013, and July 31, 2017, and at its satellite site located at Södersjukhuset between January 1, 2013, and September 30, 2016, were identified using ICD-10 codes C25.x and C24.1. The study was submitted to the former Regional Ethical Review Council in Stockholm, now part of the Swedish Ethical Review Authority, and performed under a waiver to obtain individual informed consent, registration number 2015/2185-31/4.

4.2.2 Study II

Patients with locally advanced, metastatic, or recurrent PDAC who progressed after one line of oncological treatment were eligible for inclusion in the observational PePaCaKa-01 trial. Inclusion and exclusion criteria were defined by the study protocol. Briefly, main criteria comprised patient age, performance status, comorbidity and prognosis as well as sufficient archival tissue for gene panel tumor sequencing (Next generation sequencing; NGS). Written informed consent to participate was obtained from all patients prior to inclusion in the trial. The trial was performed in accordance with the Declaration of Helsinki and ICH E6 (R2) guidelines for good clinical practice. The study was approved by the former Regional Ethical Review Council in Stockholm, now part of the Swedish Ethical Review Authority, registration number 2015/1732-31. The post-hoc analysis was performed under the framework a retrospective analysis, registration number 2015/2185-31/4.

4.2.3 Studies III and IV

Patients ≥18 years of age with newly diagnosed locally advanced, metastatic or recurrent PDAC without secondary malignancies were enrolled in a prospective biofluid collection cohort for comprehensive EV analysis. Except for three pilot patients, all participants were treatment naïve. Neither patients nor healthy cancer-free donors, recruited as controls, were reimbursed for their participation. All participants gave written informed consent to sample collection, molecular profiling and the use of relevant clinical data before inclusion in the project. The research plan was approved by the former Regional Ethical Review Council in Stockholm, now part of the Swedish Ethical Review Authority, registration number 2017/912-31.

4.3 METHODS

4.3.1 Study I

4.3.1.1 Patient and tumor characteristics

Baseline patient characteristics comprised sex, age at diagnosis, performance status per the Eastern Cooperative Oncology Group (ECOG) scale (317), length and weight. Additional patient variables were broadly stratified bona fide estimates of alcohol and tobacco use. Information on medical procedures included any surgery, endoscopic or percutaneous bile duct interventions, radiotherapy, and medical treatment against PDAC. Laboratory and pathology variables included levels of CA 19-9 from the closest available date to the first patient visit related to the treatment of advanced PDAC, tumor morphology and grading. The tumor stage was retrospectively assigned according to the American Joint Committee on Cancer (AJCC) Cancer Staging Atlas, 7th Edition (318).

4.3.1.2 Outcome measures

The main outcome measure of Study I was OS measured from the date of the patient visit related to the treatment of advanced PDAC, i.e., generally the initial visit to an oncologist at which the treatment decision was made, until the date of death recorded in the Swedish Population Register. Patients who were alive or lost to follow-up were censored at the date of record curation or the date of last contact, respectively. Time to treatment failure/discontinuation (TTF), was used as an ancillary endpoint, measured from the same starting date as OS until the visit at which discontinuation of treatment was decided. If patients receive subsequent treatment, this date served also as the starting date for measurement of OS and TTF related to second-line therapy in an exploratory analysis. Adverse events (AEs) were curated by bona fide appraisal of available clinical information and classified according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 (319).

4.3.1.3 Statistical analyses

Kaplan-Meier survival proportions and univariate Cox proportional hazards regression model HRs were used to report unadjusted estimates of OS and TTF. HRs from a Cox proportional hazards regression model adjusted for potential confounders were calculated but Schoenfeld residuals testing indicated that the proportional hazard assumption was violated in some cases. Thus, we calculated HRs from flexible parametric models that allow non-proportional effects of the covariates and visualization of the HR over time (320). We used standard statistic methods for testing differences between groups of patients (Pearson's chi-squared test for categorical variables; t test and related tests for continuous variables).

4.3.2 Study II

4.3.2.1 DNA preparation and NGS

DNA was extracted using commercially available DNA extraction kits and Swedish Board for Accreditation and Conformity Assessment (SWEDAC) certified workflows of the Department of Clinical Pathology and Cytology, Karolinska University Hospital. The tumor cellularity of formalin-fixed paraffin embedded samples was assessed on hematoxylin-eosin-stained sections and DNA was extracted from areas with high tumor content with column-based DNA extraction kits (Qiagen). After quantitation and quality control of the isolated DNA, libraries were prepared using hybrid capture-based target enrichment for a custom designed 620 gene panel with the SureSelect^{XT} Reagent Kit (Agilent). Samples were sequenced at the Clinical Genomics facility, SciLifeLab at Karolinska Institutet and the Royal Institute of Technology, on a HiSeq 2500 platform (Illumina). Casava 1.8.4 was used to demultiplex data and create FASTQ files for downstream analysis (Figure 2).

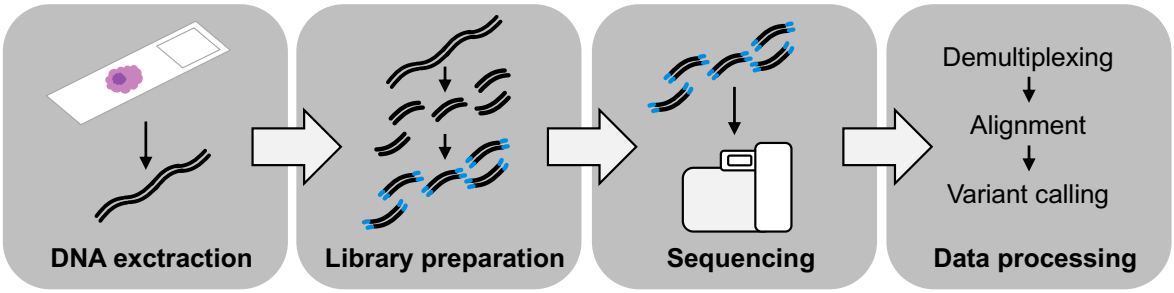

Figure 2. Workflow of tumor tissue next generation sequencing.

4.3.2.2 CDSS data processing

FASTQ files were transferred trough a virtual private network to Molecular Health for analysis with the CDSS MH Guide version 3.0. The CDSS (i) aligns data with a reference genome, (ii) calls variants, including, SNVs, indels and structural variants, and (iii) estimates their impact on protein sequence and functional from publicly available resources. In a next step, variants are evaluated by proprietary clinical annotation algorithms that integrate various sources of information on actionability, prioritize molecular targets and compile a report

which is available through the MH Guide web portal. MH Guide is regulated as a class I medical product in the EU.

4.3.2.3 Study-specific MTB

CDSS reports were discussed in a study-specific MTB that met at least once a month during the study period. Members of the MTB were a physician trained by Molecular Health to use the MH Guide interface, a clinical oncologist, a pathologist and a tumor biologist with expertise in pancreatic cancer. Other experts could join the MTB as required. Based on all available clinical and biological information, the MTB offered a consensus statement that either a molecularly informed treatment option can be considered, that it supports continued treatment with cytotoxic chemotherapy, or that it considers best supportive care the best option.

4.3.2.4 Post-hoc analysis

The PePaCaKa-01 study protocol did not regulate any treatment decisions which were entirely in the responsibility of the study participants' medical teams. Results from the CDSS and MTB discussion were, however, disclosed to the patient and his/her treating physician. We performed a post-hoc analysis to capture the frequencies with which subsequent treatment was consistent with CDSS recommendations and MTB statements.

4.3.3 Studies III and IV

4.3.3.1 Sample preparation and differential centrifugation

Whole-blood samples of 20 ml were drawn either from a chest port or by phlebotomy into four 4.5 ml 3.2% sodium citrate containing glass tubes. The initial 2 ml were discarded to avoid contamination, especially by tissue components and air if obtained by phlebotomy. Citrate containing tubes were used to reduce platelet degranulation which potentially can compromise downstream EVs analyses (321,322). Blood samples were processed within two hours. Red (RBC) and white blood cells (WBC) were sedimented by an initial centrifugation step at 120 × *g* to generate platelet-rich plasma. Aliquots of 1 ml of platelet-rich plasma were then subjected to four steps of differential centrifugation followed by ultrafiltration on a 10 kDa spin filter to separate fractions preferentially enriched with platelets, apoptotic bodies, large vesicles/microvesicles, small vesicles/exosomes, soluble protein, and flow-through (Figure 3).

4.3.3.2 Size exclusion chromatography (Study III)

Size exclusion chromatography is a method in which suspended particles are loaded onto columns of porous resin and fractions of size-separated particles are collected. Particles larger than the resin pores, such as EVs flow directly through the column into the first fractions collected, while the progress of small proteins is impeded so that these elute in the last fractions collected. We used the qEVoriginal 70 nm SEC columns (Izon) and collected five

sequential 1.5mL fractions to further separate the small vesicle and soluble protein fractions obtained by differential centrifugation.

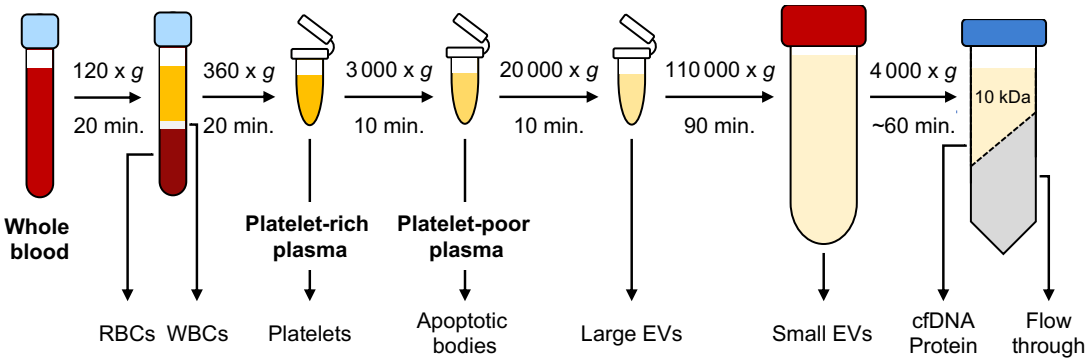

Figure 3. Differential centrifugation for separation of blood components. Whole blood was centrifuged at 120 × *g* for 20 min to pack RBCs and WBCs. WBC were obtained from the buffy coat and RBCs from the cell pack. Platelet-rich plasma was then aliquoted and transferred to a microcentrifuge tube and serially centrifuged at 360 × *g* for 20 min to separate platelets, at 3,000 × *g* for 10 min to separate ABs, and at 20,000 × *g* for 10 min to separate large EVs. At each step, the supernatant was transferred to a new microcentrifuge tube. After the 20,000 × *g* step, the supernatant from all aliquots of the same sample were pooled and diluted in 0.2 µm filtered PBS prior to ultracentrifugation at 100,000 × *g* for 90 min to pellet small EVs. The supernatant was centrifuged at 4000 × g on a 10 kDa filter (Amicon) to concentrate soluble factors to a residual volume of 100 µl for each aliquot from the initial sample. Flow-through was kept as negative controls. Abbreviations: AB, apoptotic bodies; EV, extracellular vesicle; min., minutes; RBC, red blood cell; WBC, white blood cell.

4.3.3.3 EV surface protein profiling (Study III)

Despite a lack of specific markers to differentiate EVs that originate from distinct cellular processes, multiplexed analysis of EV-associated proteins can reveal important information on the efficacy of differential centrifugation to separate different blood components, especially cells from EVs and the soluble fraction. We isolated proteins from fractionated samples with SpinTrap columns (GE Healthcare) and used a Western blotting assay with antibodies against Histone H3 (Santa Cruz FL-136, 1:250), a surrogate for nucleated WBCs, CD42a (Miltenyi 130-100-960, 1:150), a surface marker of platelets (323), cleaved-CASP9 (Cell Signalling 9505S, 1:1000) and BAX (Cell Signalling 2772S, 1:1000), markers of apoptosis (324), and the tetraspanins CD9 (Abcam ab92726, 1:2000) and CD81 (Santa Cruz sc-9158, 1:200), typically expressed on EVs (325), to characterize the different fractions resulting from differential centrifugation. Samples were run on a NuPAGE 4–12% Bis-Tris protein gel (Invitrogen) in 0.5 M dithiothreitol, 0.4 M sodium carbonate, 8% sodium dodecyl sulfate, and 10% glycerol and transferred to a nitrocellulose membrane with the iBlot system (Invitrogen). Signals were detected on an Odyssey Imager and captured with Image Studio 5.2 (LI-COR Biosciences).

Multiplex bead-based EV surface protein profiling employs the capture of EVs with up to 39 different antibody-coated bead subsets with antibodies against the protein of interest and detection with APC-conjugated anti-tetraspanin (anti-CD9, anti-CD63, and anti-CD8) antibodies (MACSPlex Exosome Kit, human, Miltenyi). W used the assay according to the manufacturer's instructions and previously published protocols (326). We used a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, Germany) for readout of the assay and FlowJo v10 software (FlowJo LLC).

4.3.3.4 Electron microscopy

Extracellular vesicles and apoptotic bodies can be visualized with transmission electron microscopy (TEM). Within their expected size-ranges, EVs are round to cup shaped particles (327), apoptotic bodies are round, oval or slightly irregular objects that often contain cell debris (328). We loaded diluted samples on formvar/carbon-coated nickel grids and stained them with 1% uranyl acetate before TEM with a Tecnai 12 Spirit BioTwin transmission electron microscope (FEI Company). Images were acquired at 5000x magnification with a 2kx2k Veleta CCD camera (Olympus Soft Imagining Solutions). Images were analyzed with ImageJ version 2.0 (National Institutes of Health).

4.3.3.5 Nanoparticle tracking analysis (Study III)

Nanoparticle tracking analysis (NTA) is a method that measures the size distribution of suspended particles in the 30–1000 nm range. The instrument captures the Brownian motion of nanoparticles which is only related to their size at a known temperature and viscosity of the medium. To this end it is equipped with a laser light scattering microscope and a charge-coupled device camera that can capture live images of moving particles. From this data their size can be calculated (329). We used a NanoSight NS500 instrument (Malvern) to characterize blood derived EV fractions. Samples with high EV concentrations were diluted with PBS before analysis.

4.3.3.6 Blood component DNA Extraction

DNA was extracted from samples using the phenol:chloroform:isoamyl alcohol and ethanol precipitation method. We used this method because of a higher yield and lack of bias for specific lengths of DNA compared to other methods (330).

4.3.3.7 Preparation of sequencing libraries (Study IV)

For library preparation from classical ctDNA in different blood components, we used the ligation-based ThruPLEX Tag-seq library preparation kit (Takara), optimized for short DNA fragments, without prior fragmentation of the sample, according to the manufacturer's instructions. This ligation-based method has three steps. Double stranded DNA fragments are end-repaired to generate blunt ends to which adapters with unique molecular identifiers (UMI) are ligated at the 5' end. Then, libraries are extended and dual molecular barcodes (i5 and i7) for demultiplexing of individual samples and adapters for attachment to the

sequencer's flow cell (P5 and P7) are added before dual-index labeled libraries, each containing two UIMs, are amplified by PCR (Figure 4A).

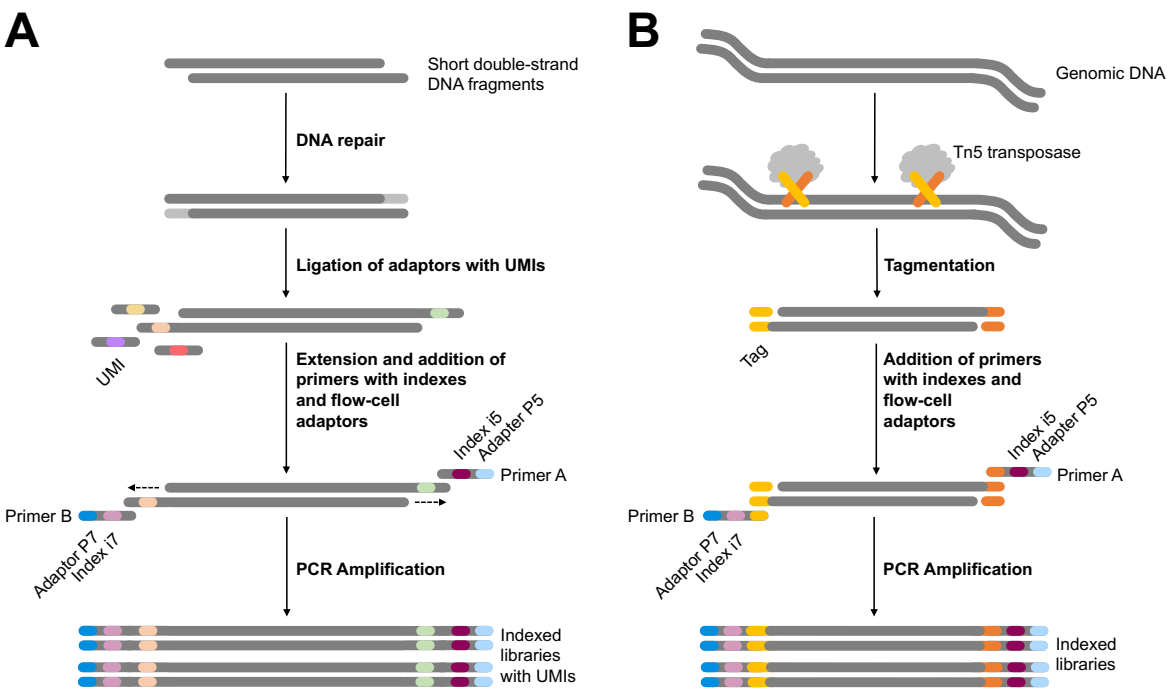

Figure 4. Flowchart of sequencing library preparation protocols. (**A**) Ligation-based ThruPLEX Tag-seq library preparation with UMIs (**B**) Tagmentation with Tn5 transposase. Abbreviations: UMI, unique molecular identifier.

To target long fragments of DNA, we used tagmentation for library construction. Tn5 transposase was prepared in-house according to a previously published protocol (331). This enzyme simultaneously cleaves DNA and ligates DNA tags to the fragments. Index primers including barcodes and flow-cell adapters P5/P7 can anneal to the tag and indexed library molecules are amplified by PCR (Figure 4B). Hyperparamegnetic Sera-Mag beads (Cytiva) were used for library cleanup.

4.3.3.8 DNA quantitation and fragment size analysis (Study IV)

The size and quantity of DNA fragment from separated blood fractions as well as from sequencing libraries was measured on a 4200 TapeStation system or a 2100 Bioanalyzer according to the manufacturer's instructions (Agilent). Both systems use automated electrophoresis.

4.3.3.9 Digital PCR

Digital PCR (dPCR) is based on the separation of individual DNA target molecules in a sample which then are individually amplified. The result is a homogeneous PCR product from each template that can be characterized, e.g, with a fluorescent-probe. This way, even few mutant gene copies can be detected in a sample and their frequency in relation to the

wild-type allele can be calculated (332). We used a chip-based assay on the QuantStudio 3D digital PCR system (Applied Biosystems) and commercially available Taqman dPCR (ThermoFisher) chemistry for the detection *KRAS*^{G12D} and *KRAS*^{G12V} mutant alleles.

4.3.3.10 Tumor tissue genotyping

DNA preparation from tumor tissue and gene panel sequencing were performed as the reference standard to determine the tumor's *KRAS* exon 2 codon 12 mutational status. We used similar workflows at the Department of Clinical Pathology and Cytology and the Clinical Genomics Facility at SciLifeLab as outlined under section *4.3.2.1 DNA preparation and sequencing*. In cases where insufficient archival material was available for sequencing, we attempted genotyping using *KRAS*^{G12D} and *KRAS*^{G12V} specific digital PCR on blood components.

4.4 ETHICAL CONSIDERATIONS

All studies in this thesis concerned human research subjects, either because it involved their data (Study I), their tissues and processing of related genomic and clinical information (Study II), or study-specific blood samples, generation of genomic data, and combination of this data with additional demographic and health information (Studies III–IV). The purpose of biomedical research with human subject is to generate knowledge that might benefit other, currently unknown, individuals in the future (333). Patients participate in research projects as a source of data from which such knowledge can be inferred. It is therefore ethically required that the potential future benefit can be reasonably expected to overweigh the risks and inconveniences for participating subjects.

This and other ethical principles for medical research involving humans are laid out in the Declaration of Helsinki of the World Medical Association that also stipulates that all human medical research must be subject to independent ethical review (334). These principles are enshrined in Swedish national law (335). In the application process to the Regional Ethical Review Council in Stockholm, we therefore outlined in detail how the knowledge that we wanted to generate with our research is in a fair balance with the relatively small individual risks for participants, especially with regards to minimal risks of harm.

The ethical considerations for PDAC patients in Studies II and III/IV were, however, more complex than a plain trade-off between individual risk and potential common benefit. As this research included genomic testing of tumor tissue, the generated data could potentially have direct implications for patients' individual prognosis or treatment, especially in the real-time setting of Study II. Such potential personal windfall from research can be a strong motivation for patients to participate in studies, but it can also cause unrealistic hopes and expectations. The uncertainty to obtained individually meaningful medical information was therefore particularly stressed in the informed consent process.

We followed Good Research Practice to limit additional indirect risks pertinent to all research with human subjects, including the integrity of personally identifiable information

5 RESULTS AND DISCUSSION

5.1 STUDY I

5.1.1 Results

The main finding of Study I is that first-line systemic oncological treatment of 595 patients with PDAC in the clinical real-world setting of Karolinska University Hospital in 2013–2017 largely reflected survival outcomes that had been reported in relevant RCTs. Univariate analysis showed a significantly longer OS associated with gemcitabine/capecitabine (n = 60; 10.6 months [95% CI, 7.8–13.3), gemcitabine/nab-paclitaxel (n = 66; 9.8 months [95% CI, 7.9–11.8]), as well as FOLFIRINOX and its derivatives (n = 31; 9.9 months [95% CI, 8.1–11.7]) compared to gemcitabine (n = 185; 6.6 months [95% CI, 5.5–7.7]) (Figure 5A). A surprising finding was the absence of a clear difference between these three regimens. We also found that patients with locally advanced pancreatic cancer had a trend towards nominally longer median OS compared to patients with metastatic disease if treated with gemcitabine/capecitabine or gemcitabine/nab-paclitaxel but not with FOLFIRINOX, although these subgroup analyses did not reach statistical significance. The survival benefit associated with the three combination therapies was even more pronounced when we adjusted for potential confounding by other demographic, clinical, and tumor parameters. When we modeled the adjusted HRs for death over time with flexible parametric models, it appeared, however, that this survival benefit disappeared after six to twelve months.

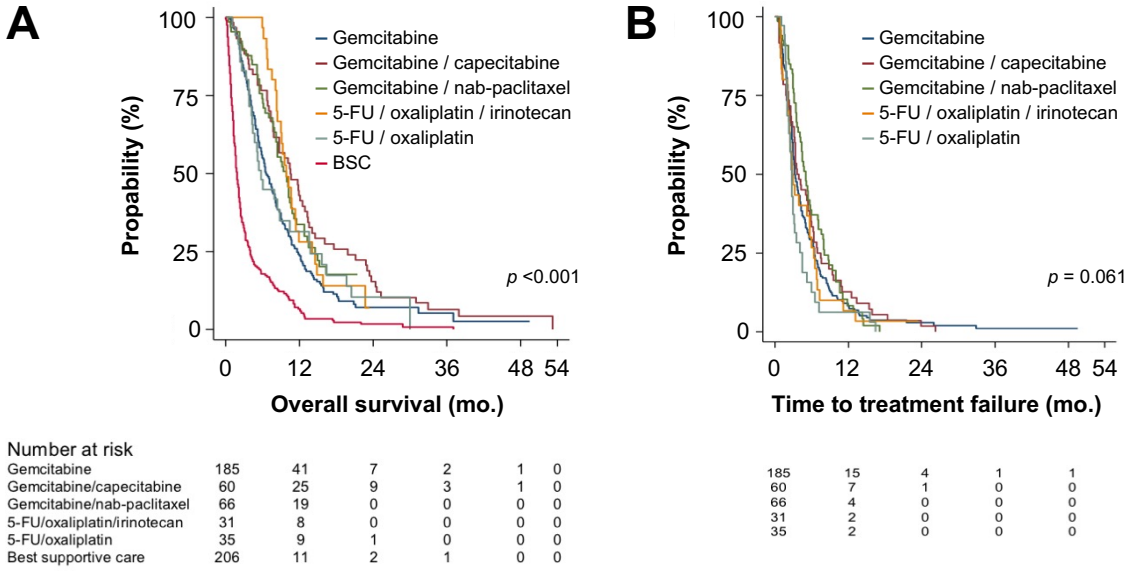

Figure 5. Kaplan-Meier estimates according to treatment group of patients with advanced PDAC. (**A**) Overall survival (**B**) Time to treatment failure. Abbreviations: 5-FU, 5-fluorouracil; BSC, best supportive care; mo., months.

The median TTF was 2.8–5.1 months without any statistically significant differences across groups except for gemcitabine/nab-paclitaxel compared to gemcitabine if adjusted for potentially confounding variables (Figure 5B). Only between 37% and 53%, of patients had disease progression at the time treatment was discontinued. Exploratory analyses revealed a marked difference in the protocol adherence of patients in the different groups with the best adherences to gemcitabine-based combinations. There was no significant difference between overall survival associated with second-line treatment with gemcitabine-base regimens compared to 5-FU-based regimens. Sequential treatment with gemcitabine-base treatment followed by 5-FU-based therapy was nonetheless associated with better outcomes than the inverse order, median OS 12.8 months (95% CI, 10.9–14.8) versus 9.9 months (95% CI, 10.9–14.8). No clear patterns of AEs emerged, and AE frequencies were generally low.

5.1.2 Discussion

Understanding how evidence from RCTs translates into the care of patients in a real-world setting is important to identify gaps in the optimal delivery of cancer care to patients (67). The results from Study I are important in this context, because they demonstrate that chemotherapy for advanced PDAC resulted in clinical outcomes that were comparable to the RCTs that had established them in clinical routine (46,57–59). The finding, that gemcitabine-based combination therapies were associated with OS similar to FOLFIRINOX and related combinations of 5-FU, folinic acid, oxaliplatin and irinotecan is a minor exception to this, but several other real-world studies have made similar observations (336–339). In contrast, a large European multi-center chart review study confirmed an increased survival associated with FOLFIRINOX compared to other regimens (340). Combinations of 5-FU, folinic acid, and oxaliplatin for advanced or recurrent PDAC lacks evidence from RCTs. Their use resulted in poor outcomes in this study and is discouraged if other treatment options are available. In our flexible parametric models, we observed rapid changes of HRs and no survival benefits with combination therapies compared to gemcitabine after a few months. Interestingly, the initially higher HRs for BSC also decreased at later timepoints. This phenomenon likely reflects the poor prognosis of patients with PDAC and that most patients were deceased by that time.

Gemcitabine/nab-paclitaxel was the only therapy associated with improved TTF. It was also one of the therapies with the highest proportions of patients who were treated until progression and who had a good protocol adherence. In contrast, the TTF for FOLFIRINOX was markedly shorter, a high proportion of patients was not evaluated for response, and the overall protocol adherence was poor. Given that patients who received FOFLFIRNOX were younger and in a better performance status at diagnosis, this points to a disparity in how the two most common combination treatments could be deployed. This is, however, not entirely reflected in the AE patterns that only shows minor differences between treatment groups and dramatically lower frequencies than what has been previously reported, especially for FOLFIRINOX (59,341). This might represent a methodological problem of retrospective AE curation or very careful patient management consistent with the low protocol adherence. The

exploratory analysis of second-line treatment confirms the efficacy of both regimens after previous treatment failure (342–347). The observed difference between different sequences is difficult to generalize and merely hypothesis-generating.

While real-world data is an important tool to assess the implementation of evidence in clinical practice, it cannot generate the same kind of robust evidence as a RCT (348). The main reason is that a retrospective design cannot entirely control confounding, despite best efforts to record confounding factors and adjust for them. The most obvious problem in the context of advanced PDAC is indication bias as healthier patients are more likely to receive more intensive regimens than older and frailer individuals. These problems do not only impact on the internal validity of the study but also affect the generalizability of our findings. Conclusions on the comparative effectiveness of treatments should therefore only be made with outmost care.

The strength of Study I is that it can guide the use of established therapies in a very hands-on way. It suggests, e.g., improvements to routines regarding FOLFIRINOX and highlights the efficacy of gemcitabine-based treatments in a heterogenous population that is difficult to study in RCTs.

5.2 STUDY II

5.2.1 Results

A total of 39 patients were enrolled in the PePaCaKa-01 study, a CDSS report could be generated for 31 of them, and 28 cases were discussed at the study-specific MTB (Figure 6A, 6C). The CDSS reported a median of 18 somatic variants with a MAF ≥10% across 32 samples (in one case sequencing was performed on tissue from the primary tumor and a metastatic lesion). The four most frequently mutated genes were *KRAS* (*n* = 15; 78%), *TP53* (*n* = 16; 50%), *TGFBR2* (*n* = 10; 31%), and *CDKN2A* (*n* = 9; 28%). Several mutations consistent with germline variants in genes with potential relevance in relation to hereditary PDAC were detected, but none of them was clinically relevant apart from an already known pathogenic *BRCA1* K434fs variant. The CDSS made a total of 80 recommendations for targeted drugs based on 61 detected actionable aberrations (Figure 6B). The most commonly recommended drugs were PARP inhibitors and inhibitors of the MAPK pathway. The overall strength of evidence assigned to these recommendations was weak. The CDSS raised alerts for potential inefficacy of cancer drugs or increased toxicity for 27/31 and 30/31 patients, respectively. The study-specific tumor board agreed with at least one CDSS-designated opportunity for molecularly informed treatment in 21/28 cases (Figure 6C).

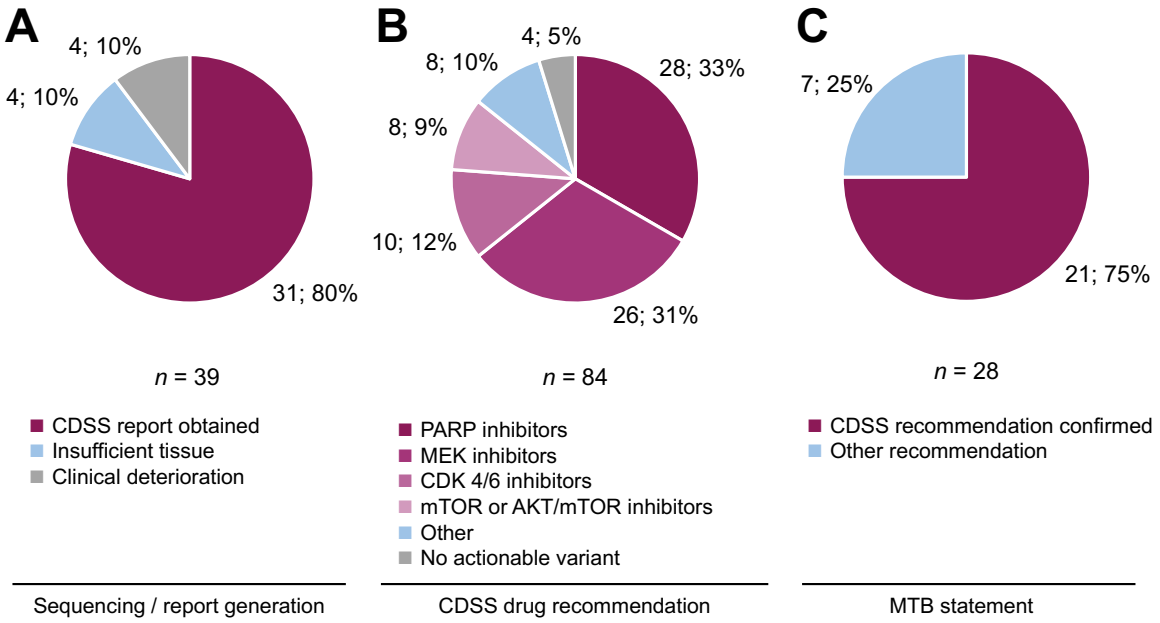

Figure 6. Outcomes of the PePaCaKa-01 study. (**A**) Frequency of successful DNA extraction, sequencing and generation of a CDSS report among all study participants (n = 39). (**B**) Frequency of various drug classes recommended by the CDSS based on potentially actionable alterations. (**C**) MTB evaluation of CDSS molecularly informed recommendations.

5.2.2 Discussion

The use of CDSS to annotate and interpret tumor sequencing results has been suggested as a way to implement precision oncology at scale (213). In Study II, we found that the CDSS MH Guide could be successfully deployed along the care path of PDAC patients with advanced disease. Using a prospective study design, we could generate reports with recommendations for 31/39 patients. Insufficient tissue and clinical deterioration were the main reasons for failure to produce a report; these problems have also been the main barriers to PDAC precision treatment in other studies (349). Actionable variants were reported in 87% of samples and there was a high consensus between the CDSS and the study-specific MTB on which therapies could be considered. Taking inefficacy or toxicity markers also into consideration, clinically relevant markers were reported in all cases.

High rates of clinically relevant variants, similar to that in Study II, were also detected by another proprietary CDSS in another study and in the previous retrospective analysis of MH Guide from our institution (215,216). In larger cohorts of patients, this rate is usually markedly lower, typically about one third of patients (191,192). A possible reason for this discrepancy is that the larger series relied on manual evaluation and interpretation of evidence and might have applied stricter criteria to classify aberrations as actionable. Still, the MTB in Study II found in 65% of all cases at least one marker-drug pair that could be considered. The CDSS might therefore have identified extra evidence that is not taken into account in a manual review or curated in knowledgebases. Such evidence is, however, usually weak and strong evidence is unlikely to be missed by other curation methods. This is consistent with the overall low level of evidence that the CDSS assigned to its recommendations.

A potential limitation of this study was the high proportion of primary tumor tissue that was resected before metastatic dissemination of the disease, but a comparison of primary and metastatic tissue has previously shown that genomic differences are small (350). Additionally, the MTB review of CDSS recommendations might have been vulnerable to acquiescence bias leading to an increased rate of approvals. The evidence scale used by the CDSS resembles established grading scales. Although it had been desirable to use a standardized system such as ESCAT, it is unclear if this had resulted in a more efficient separation of different recommendations.

Like several other clinical sequencing studies that explored molecular treatment opportunities we report only limited information on their clinical application through our post-hoc analysis. The proportion of patients who received treatment based on a CDSS-designated target was higher than in three comparable series (191,192,349).

5.3 STUDY III

5.3.1 Results

As a first step of a broad characterization of the association of different blood components with tumor-derived DNA, we assessed the efficacy of the differential centrifugation protocol to separate cells, EVs, and soluble factors. Using TME and NTA, we could confirm the presence of particles in the expected size ranges in the AB, large and small EV fractions. Western blotting confirmed the presence of histone H3 in the WBC fraction, CD42a in all cell fractions, and apoptosis markers in the platelet and AB fractions. The tetraspanins CD9 and CD81 occurred in all vesicle fractions and in platelets. These findings were corroborated by multiplex bead-based EV flow cytometry.

Because previous work had linked evDNA to early disease (296), we separated the samples (n = 23) based on patient survival after their acquisition, i.e., survival shorter or longer than 300 days, the median of the cohort. This resulted in 11 early and 12 late samples from patients with known *KRAS*$^{G12D/G12V}$ variants. As expected, when comparing the concentration of *KRAS*^{mut} ctDNA in unfractionated platelet-poor plasma, patients with a poorer prognosis had higher concentrations. Next, we measured the concentrations of *KRAS*^{mut} DNA in the individual blood components in late- and early-stage PDAC.

Late in the course of disease, the soluble protein fraction contained the highest levels of *KRAS*^{mut} DNA. Small and large EVs, ABs and platelets were associated with successively decreasing concentrations. In these late samples, the ratio of *KRAS*^{mut} to *KRAS*$^{wild\text{-}type}$ DNA was highest in the soluble protein and small EV fractions, approximately 1:10. At early stages of PDAC, we observed the highest concentrations of *KRAS*^{mut} DNA in large and small EVs but low levels in the soluble protein fraction. Although the overall *KRAS*^{mut} to *KRAS*$^{wild\text{-}type}$ ratio was, as expected, lower in early-stage samples, the difference between groups was much more distinct. Small EVs had an almost four times higher ratio than large EVs and an almost ten times higher ratio than soluble protein. Notably, the ratio was also significantly higher than in unfractionated platelet-poor plasma (Figure 7). Using size exclusion chromatography,

we could show that this association of *KRAS*^{mut} DNA with small EVs was not caused by contamination of the EV fraction with soluble ctDNA.

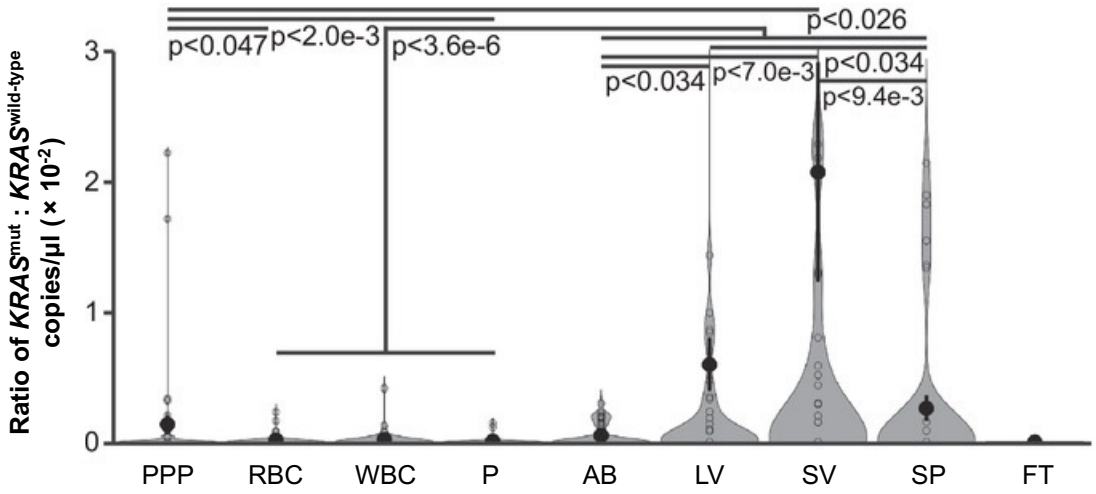

Figure 7. Ratio of *KRAS*^{mut} : *KRAS*$^{wild\text{-}type}$ DNA copies/µl for platelet-poor plasma and fractionated blood components from samples taken early in the course of PDAC. Abbreviations: AB, apoptotic bodies; FT, flow through; LV, large vesicles; P, platelets; PPP, platelet-poor plasma; RBC, red blood cell; SP, soluble protein; SV, small vesicle; WBC, white blood cell.

5.3.2 Discussion

Identification of the blood component most enriched with tumor-derived DNA is a strategy to improve liquid biopsies especially for patients early in the course of their disease when the levels of ctDNA are generally lower (351). Study III conducted the first systematic and comprehensive survey of the association of *KRAS*^{mut} DNA, a proxy for PDAC tumor DNA, with various blood components stratified by patient survival after sampling. The main finding was that *KRAS*^{mut} DNA at earlier stages of PDAC was most associated with small EVs whereas high levels of ctDNA were associated with the soluble protein fraction, the reservoir of classical cfDNA, in the blood of patients at late stages.

The groundwork of this work was the effective fractionation of blood components with differential centrifugation. This approach does not yield entirely pure blood fractions, but on the basis of the proteins associated with each fraction (352–354), we achieved a good separation of target components albeit with a certain overlap that was acceptable for our experimental design. One observation that can be pointed out in this context was the high concentration of tetraspanins CD9 and CD81 detected by Western blotting in the soluble

protein fraction. Using the multiplexed bead-based assay, we could show that most of these proteins are not on the surface of EVs. This was particularly important as the distribution of tumor-derived DNA between the soluble fraction and EVs required efficient separation of these fractions.

The increase of the *KRAS*^{mut} to *KRAS*$^{wild\text{-}type}$ ratio in the soluble fraction, and to some extent in the EV fractions, at later stages of PDAC is the driving factor of overall higher *KRAS*^{mut} DNA concentration of in plasma. This observation confirms previous studies that soluble ctDNA levels increase at later stages of various cancers (351). In contrast, the identification of EVs as the main source of *KRAS*^{mut} DNA at early stages of PDAC is a novel finding.

While the high frequency of *KRAS* codon 12 variants makes PDAC a very suitable model to investigate the distribution of tumor-derived DNA in blood, it is not sure our findings can be generalized to other cancer types. Previous studies in prostate cancer have, e.g., linked tumor derived DNA to large vesicles (294).

In the context of PDAC tumor biology, it is interesting that exosome formation, in contrast to ctDNA release that is driven by necrosis and apoptosis (355), is an energy-consuming process and as such subject to evolutionary pressure in tumor cells. This, at least indirectly, supports an oncogenic role of small EVs in early PDAC. Regardless of the biological function of small EVs, Study III identifies them as a promising target for liquid biopsies that aim to detect tumor signals with a low frequency.

5.4 STUDY IV

5.4.1 Results

After additional work to substantiate the efficacy of differential centrifugation by TEM and characterization of the size distribution of ABs, large and small EVs, the initial step of study IV was the measurement of DNA fragment lengths in fractionated blood components. All vesicle fractions and the soluble protein component contained measurable amounts of short DNA in the ~170 bp range. Such fragments could not be detected in red or white blood cells, platelets or in the flow through. The cell fractions, especially WBC, contained, as expected, large amounts of DNA ≥20 kb. Substantial amounts of long genomic DNA fragments were also found in the AB fraction with minor amounts associated with large and small vesicles. Long DNA fragments were not detectable in the soluble protein fraction. Compared to healthy individuals, PDAC patients had significantly higher concentrations of short cfDNA in the soluble fraction and of genomic DNA in the AB fraction. To be able to specifically target short and long DNA fragments for downstream analysis, we developed a separation strategy based on different protocols for sequencing library construction. Ligation based preparation resulted in libraries from AB, small and large EVs as well as the protein fraction but not from RBC, WBC, platelets or FT. After validation of the tagmentation approach to omit short DNA templates using a synthetic 177 bp fragment, we tested it on various blood components.

Tagmented libraries could be prepared from all blood components, including soluble protein, except for flow through.

Next, we turned to digital PCR to quantify the *KRAS* MAFs in both types of libraries to assess the distribution of tumor-derived circulating DNA across different fragment lengths stratified by ABs, large and small EVs and soluble protein. Irrespective of the preparation method, the mean MAF was highest in the soluble protein fraction, 15.1%, (95% CI, 6.7%–23.5%) in cfDNA libraries and 27.5% (95% CI, 15.2%–37.8%) in tagmented libraries. It was lowest in the AB fraction, 0.9% (95% CI, 0.4%–1.3%) and 6.5%, (95% CI, 4.4%–8.7%), respectively. EVs were associated with low $KRAS^{mut}$ MAFs in libraries from short DNA and intermediate to high MAFs in tagmented libraries albeit with a wide confidence interval. Questions about the reliability of the measurements in specific blood fractions were raised by weak $KRAS^{wild\text{-}type}$ signals in short-DNA libraries from ABs and in long-DNA libraries from EVs and the soluble protein fraction. Based on a limited set of samples, we could not discern an obvious correlation of $KRAS^{mut}$ MAFs in any type of DNA or blood component with clinical outcomes.

5.4.2 Discussion

Selection of DNA fragments of a specific length might be an avenue towards enrichment of tumor-derived signals in liquid biopsies. The main findings of Study IV were that the soluble blood fraction is the main source of short cfDNA and that larger genomic DNA fragments are mostly a feature of cells and ABs. These observations contradict claims that EVs are the main reservoirs of cfDNA in human blood (356), at least in the context of advanced cancer. As part of Study IV, we also developed a new, previously not described method to separate short and long DNA fragments from the same sample using ligation-based library preparation and tagmentation.

Measuring absolute levels of cfDNA in healthy individuals and cancer patients as well as the $KRAS^{mut}$ MAF in separated libraries from PDAC patients, the most robust associations were between $KRAS^{mut}$ ctDNA and between genomic $KRAS^{mut}$ DNA and ABs. Both observations are consistent with apoptosis as the main source of the respective DNA fragments. This supports previous *in vitro* findings that ABs are a richer source of gDNA than large and small vesicles from prostate cancer cell lines and that apoptosis is a main release mechanism for ctDNA (355). Unfortunately, we could not link the $KRAS^{mut}$ MAF in any type of DNA fragments or blood components to clinical features of PDAC.

The main limitation of Study IV is the relatively small number of samples, especially in the downstream analyses of libraries from defined-length templates. This is partially compensated for by the biological plausibility of our findings but discovery of specific associations in subgroups of patients or correlation with clinical features requires a larger study.

Study IV provides novel insights into the distribution of genomic DNA in the blood of PDAC patients and a powerful tool for future investigations of such DNA fragments in blood.

6 CONCLUSIONS

Treatment of PDAC patients in a real-world setting (Study I)

- Chemotherapy in clinical routine use can result in roughly similar or better survival outcomes as reported in RCTs.
- Gemcitabine-based regimens are highly effective in clinical routine use.
- Substantial differences in the ability to deploy different treatment regimens exist.

Precision oncology opportunities for PDAC patients using a CDSS (Study II):

- The prospective use of a CDSS to generate treatment recommendations for PDAC patients is feasible.
- Tumor tissue sequencing and CDSS processing yields clinically relevant information for a majority of PDAC patients.

Association of tumor-derived DNA with different blood components (Study III):

- Tumor-derived *KRAS*^{mut} DNA is primarily associated with EVs at early stages of PDAC.
- At late stages of PDAC, the amount of ctDNA, associated with the soluble blood fraction, increases and becomes the major source of *KRAS*^{mut} DNA.

Distribution of DNA fragments with different lengths in blood (Study IV)

- Long fragments of genomic DNA are primarily a feature of ABs.
- Short ctDNA fragments in the soluble fraction have the highest content of tumor derived *KRAS*^{mut} DNA.
- Short DNA is an overall more robust source of *KRAS*^{mut} DNA than circulating genomic DNA.

7 PERSPECTIVES

The four studies in my thesis cover a broad range within PDAC research. The common motivation behind them is to generate knowledge that eventually can benefit patients with this dreadful cancer or other malignant tumors. The scope of Study I was to benchmark the current practice of PDAC patient care. Understanding if we are "achieving the achievable" (67) will help clinicians to understand the current gaps and to identify opportunities for short-term improvements. Study II looked beyond the current practice and assessed the possibilities and challenges of using a precision oncology approach in a clinical setting. Studies III and IV might indirectly improve patient care through insights that can help to design assays to detect and monitor PDAC. The future perspectives in these different areas might be best summarized in the answers to three questions:

How can we assure to offer optimal treatment to each PDAC patient? Current evidence does only suggests that precision oncology will benefit subsets of PDAC patients in the near future (86). Similarly, there are significant barriers towards the use of immunotherapy. Therefore, cytotoxic chemotherapy will be a key element of the treatment of PDAC patients for years to come. I believe that results from **Study I**, despite limitations to the generalizability of all findings, will help to close some gaps to use available regimens as efficiently as possible. In the future, this effort might be supported by novel assays that can predict the efficacy of different cancer drugs. Steps into this direction have been taken, e.g., with the development of patient-derived organoid libraries that reproduce patient responses to current standard-of-care regimens (357). But even with better predictive tests, it will be necessary to systematically review the outcomes any treatment can deliver.

Will precision oncology finally arrive for PDAC patients? Tumor sequencing is finally available on a large scale at many centers. The challenge in treating PDAC patients is that *KRAS*-directed therapies are urgently needed. With the arrival of $KRAS^{G12C}$ inhibitors, steps into this direction have been taken (154,155) but $KRAS^{G12D/G12V}$ specific therapies will be needed to achieve a substantial impact for the majority of patients. In addition, there are some other encouraging molecularly informed strategies against PDAC. Their common challenge is that the frequency of most highly predictive molecular targets, that can be found with approaches like the one of **Study II,** is painfully low. As such, it is difficult, and in some cases unfeasible, to generate evidence for many marker–drug pairs from large RTCs. One response to this dilemma is the approval of tumor-type agnostic therapies with companion diagnostics, e.g., TRK inhibitors (358). Another approach is the repurposing of drugs without an indication for PDAC based on their mechanism of action (359). Shifting the focus towards exceptional responses in few molecularly defined cases instead of classical endpoints, like statistically significant OS benefits, both approaches challenge the current way efficacy is evaluated by European and national authorities. This will increase the need for a coherent societal definition of criteria for the health care system for providing often expensive therapies to small groups of patients.

What are the future technologies that can improve PDAC care? Within the current model of PDAC, knowledge is rapidly expanding. **Study III–IV** advanced the understanding of EVs as a promising avenue towards more efficient liquid biopsy methods. Such methods could result in better tests for early detection or allow the evaluation of ongoing treatment with serial samples. If feasible, characterization of gene expression information in EVs might in the future even allow for a completely new type of real-time disease monitoring. By the very nature of scientific progress, it is, however, impossible to know if the coming improvements in the care of PDAC patients will come from the step-wise organic growth of our current understanding of the disease or if a discovery that introduces a fundamental paradigm shift and breakthrough is around the corner (360).

8 ACKNOWLEDGEMENTS

Matthias Löhr, principal supervisor. Thank you for the freedom to pursue my scientific interests, the most generous support to thrive, and the fun it has been to be your student.

Maria Gustafsson Liljefors, co-supervisor, clinical colleague. Thank you for keeping me focused and for always having advice. You are worth more than gold.

Daniel Hagey, co-supervisor. I couldn't have found a better person to study EVs with. Thank you for your patience and endless support.

Rainer Heuchel, co-supervisor. Thank you for sharing your enormous knowledge about pancreatic cancer.

Samir EL Andaloussi, former co-supervisor. Thank you for believing in my project and your strategic input.

Jingru Yu, co-author. Thank you for excellent statistical skills and patient explanations.

Jeffrey Yachnin, mentor. Thank you for being a teacher in the art of being a doctor.

Pia Raja Pakse, **Lylie Mbuyi**, and **the staff at MBA Huddinge**, sample coordinators. Thank you for taking all these blood samples.

Maura Krook, research nurse. Thank you for keeping track of patients and things.

Carlos Fernandes Moro, pathologist. I could always count on you when I needed a pathologist. Thank you for that and for your massive knowledge of the pancreas.

Marja Engqvist and **Gittan Nässén**, research nurses. For running PePaCaKa-01.

Johanna Vernersson, enhetschef CKC. Thank you for supporting me with resources and logistics.

Cecilia Radkiewicz and **Serafeim Theodoroglou**. For building something together and for having my back.

Maria Helde Frankling, former ST-chef. For daring to hire me in the first place.

Clinical colleagues at the Phase 1-Unit and **the Dept. of Upper Abdominal Diseases**.

Linn, **Agnes**, and **Klara**. For endless love and support.

My mother Margot and Hinrich. For everything I got from you and the believe that anything can be possible – "Einfach mal machen".

I received financial support from the following funding bodies: Forskningsrådet KI-Region Stockholm (Forskar-ST), Robert Lundbergs minnesstiftelse, Gastrointestinal Onkologisk Förening, Radiumhemmets Forskningsfonder, Stockholms Läkarförenings understödsfond, and Tage Olssons stipendiefond.

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