Translational and clinical aspects of pancreatic cancer

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The painting shows a key moment in the dialog between cells of the immune system, when an antigen presenting cell (top) is displaying a small piece of a virus (red dot at center) with MHC, and using it to stimulate the action of immune T-cells (bottom) through T-cell receptors.

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“It always seems impossible until it’s done”

Nelson Mandela
1918-2013
Fighter for freedom and president of South Africa 1994-1999.
Nobel Prize for Peace in 1993.

To my father Rune and my brother Jerry with love

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ABSTRACT

Pancreatic cancer is a disease with dismal prognosis due to late detection and ineffective treatments. The majority of the patients already have disseminated disease at the time of diagnosis. The only potential curative treatment, surgery, is extensive with high morbidity and non-ignorable mortality. One of the most feared complications is postpancreatectomy hemorrhage. Circulating tumor cells (CTC) and extracellular vesicles (EVs), both analyzed in this thesis, are potential biomarkers. CTC are important in metastasis and might have subclones with higher disseminating capacity. Patient-derived xenografts (PDX) mouse models can be used to receive information of tumors and their response to treatments.

The overall aim of this thesis was to establish a translational research platform for pancreatic cancer to improve diagnostics and treatment. The specific aims of the studies were to detect CTC in blood and EVs in tissue to try to identify biomarkers for early detection, determine a fractional uptake of CTC in the lung-liver compartment perioperatively, evolve a PDX model of pancreatic cancer to evaluate the effect of immunotherapy and to define predictive factors of postpancreatectomy hemorrhage.

In paper I blood samples from the portal vein and peripheral artery were collected perioperatively during pancreaticoduodenectomy to detect CTC. The difference in the number of CTC in portal and arterial blood was calculated. In paper II pancreatic tumor tissue were removed from the specimen perioperatively and implanted into immune compromised NOG mice. Tumor infiltrating lymphocytes (TILs) from the same tumor tissues were expanded. Growing tumors were serial transplanted into NOG mice expressing human interleukin 2 (hIL2-NOG mice). When the tumors gained a volume of 80-100 mm3, TILs were injected in the mice to evaluate the effect of adoptive T-cell transfer (ACT) therapy. The pancreatic tumor specimens were also used to extract EVs in Paper III. Both tumor tissue and non-tumor pancreatic tissue were utilized. The protein profiles of the pancreatic tumor and non-tumor derived EVs were analyzed with mass spectrometry and compared. In paper IV potential pre-, peri- and postoperative predictive factors of postpancreatectomy hemorrhage after pancreaticoduodenectomy was evaluated.

A difference in the number of CTC in portal and peripheral blood was detected, indicating a possibility of a perioperative fractional uptake of CTC with a metastatic profile in liver and lung tissues. In the PDX study, three out of six established tumors in the hIL2-NOG mice were reduced in the size after the ACT, which might imply an effect of the immunotherapy. In the EV project, isolation of EVs was successful and potential biomarkers and interesting upregulated proteins and their connected pathways could be identified. The protein contents were significantly different in the tumor EVs compared to the non-tumor. In the last clinical project, high postoperative CRP was identified as a predictive factor for PPH C development.

A translational platform for pancreatic cancer research, that enable studies of tumor biology, prognostic biomarkers, new therapies and detection of postoperative complications was established.

Keywords: circulating tumor cells, extracellular vesicles, immunotherapy, PDX model, pancreatic cancer, pancreaticoduodenectomy, postoperative complications

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SAMMANFATTNING PÅ SVENSKA

Bukspottkörtelcancer har dyster prognos främst på grund av att den upptäcks sent, men även då det inte finns effektiva behandlingsalternativ. I en majoritet av fallen är sjukdomen redan spridd vid upptäckt. Av de som opereras, den enda potentiella möjligheten till bot, lever ungefär en fjärdedel efter fem år. Operationen är omfattande och har betydande komplikationer och ibland även dödlighet. Det finns ett stort behov av att upptäcka sjukdomen tidigare, identifiera mer effektiva terapier, samt att förebygga operations-komplikationer.

Syftet med den här avhandlingen var att skapa en plattform med blod- och vävnadsanalyser från bukspottkörtelcancerpatienter för att kunna studera potentiella tumörmarkörer för tidigupptäckt av bukspottkörtelcancer, utvärdera behandlingsalternativ, samt identifiera riskfaktorer vid operation.

I avhandlingens första studie analyserades cirkulerande tumörceller i blod. En skillnad mellan mängden cirkulerande tumörceller i portavenen och perifer artär identifierades, vilket kan tyda på att tumörceller med metastatisk potential fastnar i lung- och leverkretsloppet under operationen. Att identifiera cirkulerande tumörceller eller speciella grupper av dem med metastaserande egenskaper skulle kunna möjliggöra tidigare upptäckt av bukspottkörtelcancer, underlätta utvärdering av behandlingar och öka kunskapen om hur bukspottkörtelcancer sprids.

I den andra studien implanterades tumörvävnad från bukspottkörteln i underhuden på möss. Tumörer som tillväxte behandlades med immunterapi. En minskad tumörstorlek kunde då påvisas i hälften av fallen, vilket kan indikera att immunterapi under vissa förutsättningar kan ha en effekt på pankreascancer. Lyckas man definiera dessa förutsättningar kan man förhoppningsvis i framtiden utveckla mer personliga behandlingsalternativ för bukspottkörtelcancer, beroende på tumörernas egenskaper.

Extracellulära vesiklar är små avknoppade delar av celler som bär innehåll från ursprungscellen med sig. Bland annat cirkulerar extracellulära vesiklar i blodet, men de finns även i andra kroppsvätskor och i vävnader. I studie nummer tre kunde extracellulära vesiklar isoleras från bukspottkörteltumörvävnad och icke-tumörvävnad från bukspottkörteln och deras proteinmönster analyseras. Proteinmönstret i de extracellulära vesiklarna i tumörvävnaden skiljde sig från det i icke-tumörvävnad, vilket innebär att de går att särskilja. Det fanns också enskilda proteiner i de

extracellulära vesiklarna som potentiellt skulle kunna vara unika för bukspottkörtelcancer. Om samma proteinmönster eller enskilda proteiner kan bekräftas i mer omfattande studier i framtiden och även identifieras hos extracellulära vesiklar i blod, skulle detta kunna innebära att de kan användas som tumörmarkörer för att påvisa bukspottkörtelcancer.

I den sista studien gjordes en genomgång av patienter som fått livshotande sena blödningar efter bukspottkörtelkirurgi för att försöka hitta faktorer som kan identifiera patienter med förhöjd risk. Ett blodvärde som mäter inflammation, C-reaktivt protein (CRP) som rutinmässigt tas efter operation sågs vara förhöjt och vid en viss nivå korrelera med uppkomsten av sena blödningar. Dessa patienter kan man då röntga för att se om tecken till komplikationer finns.

Sammanfattningsvis har en forskningsmiljö som möjliggör studier av bukspottkörtelcancer skapats och fynden med potentiella tumörmarkörer och behandlingsalternativ, samt identifikation av operationskomplikationer lagt en grund för framtida forskning.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Fractional uptake of circulating tumor cells into liver-lung compartments during curative resection of periampullary cancer.

Vilhav C, Engström C, Naredi P, Novotny A, Fagman JB, Iresjö BM, Asting AG, Lundholm K. *Oncol Lett. 2018 Nov; 16(5): 6331–6338.*

II. Genetics and therapeutic responses to TIL therapy of pancreatic cancer PDX models.

Nilsson LM*, **Vilhav C***, Karlsson JW, Fagman JB, Naredi P, Engström C, Nilsson JA
*Shared authorship. 2021. *In manuscript.*

III. Proteomic profiling of extracellular vesicles in tumour tissue from pancreatic cancer patients.

Karimi N*, **Vilhav C*,** Fagman JB, Naredi P, Lötvall J[#], Lässer C[#]
*# Shared authorships. 2021.
In manuscript

IV. C-reactive protein identifies patients at risk of postpancreatectomy hemorrhage.

Vilhav C, Fagman JB, Holmberg E, Naredi P, Engström C**.**
2021. Submitted manuscript.

CONTENT

ABBREVIATIONS

ACT	Adoptive T cell transfer
APC	Antigen presenting cells
ASCP	Adenosquamous carcinoma of the pancreas
BCR	B cell receptor
CA19-9	Carbohydrate antigen 19-9
CAFs	Cancer-associated fibroblasts
CRP	C-reactive protein
CTC	Circulating tumor cells
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ERAS	Enhanced recovery after surgery
EVs	Extracellular vesicles
FACS	Fluorescence-activated single cell sorting
ICI	Immune checkpoint inhibitors
ISGPS	International study group of pancreatic surgery
MHC	Major Histocompatibility Complex
pMMR	Mismatch repair

MS	Mass spectrometry
MSI	Microsatellite instability
NK cell	Natural killer cell
PD	Pancreaticoduodenectomy
PDAC	Pancreatic ductal adenocarcinoma
PDX	Patient-derived xenograft
POPF	Postoperative pancreatic fistula
PPH C	Postpancreatectomy hemorrhage
PSCs	Pancreatic stellate cells
TAMs	Tumor-associated macrophages
TCR	T cell receptor
TDEs	Tumor-derived exosomes
TME	Tumor microenvironment
Tregs	Regulatory T cells

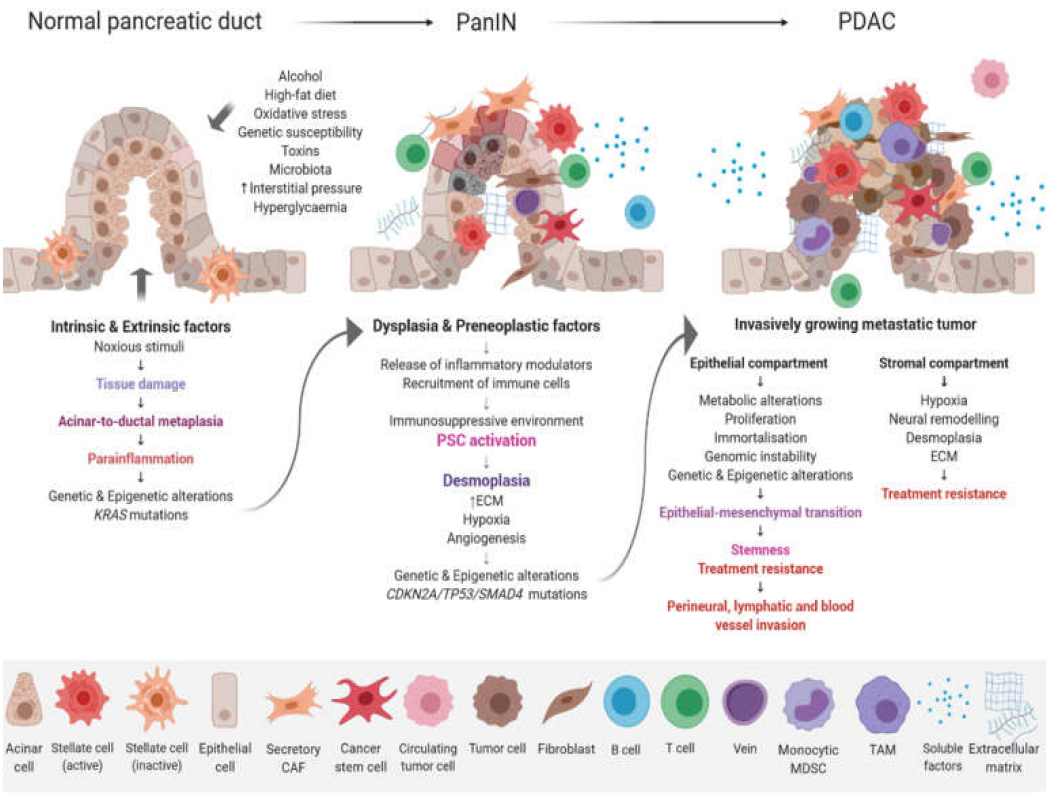
1 INTRODUCTION

Despite a relatively low incidence, the 14th most common cancer in the world, pancreatic cancer is the fourth leading cause of cancer-related deaths in most developed countries and is predicted to be the third in a close future [1-4]. The 5-years survival rate is only between 2 and 9 per cent in the population worldwide [4-6] and pancreatic adenocarcinoma is somewhat more common in men than women [1]. Pancreatic cancer is most common in the ages of 65 to 69 years in men and 75 to 79 in women [7]. In Sweden the incidence 2017 was 13-14 persons per 100 000 inhabitants, which means that around 1500 people are diagnosed every year. The incidence in Sweden has been increasing since 2008. The mortality rate has stayed almost the same since the 1990th. The main cause of the persistent high mortality is that up to 80 per cent of the patients has a non-curable disease at the time of diagnosis. Among the 20 per cent who get through treatment with curative intent, operation and chemotherapy, the 5 years mortality rate in Sweden has improved slightly from 20 to 25 per cent during the last five years [8].The only chance of cure is surgical resection. Adjuvant and palliative chemotherapies are used as a standard to prolong the survival. Neoadjuvant chemotherapy is mainly used in patients with borderline resectable or locally advanced pancreatic cancer as a bridge to surgery or in studies [9, 10].

The risk factors for pancreatic cancer can be grouped as non-modifiable and modifiable risk factors. The non-modifiable constitutes of advanced age, male gender, inherited genetic mutations, chronic pancreatitis and type 2 diabetes mellitus. The known modifiable risk factors are smoking, alcohol, obesity and dietary factors [7]. Strongest evidence as modifiable risk factor and considered to be most important is smoking [6, 11].

Exocrine epithelial cancers, where pancreatic ductal adenocarcinoma (PDAC) and its variants, among others adenosquamous carcinoma, colloid carcinoma and signet ring cell carcinoma, comprise up to 90 per cent of all pancreatic cancers. Other rare epithelial types are acinar cell carcinoma, pancreatoblastoma and solid pseudopapillary neoplasm with high-grade dysplasia. Endocrine tumors are uncommon, accounting for 2 per cent of all pancreatic neoplasms [12]. Benign epithelial tumors and precursors constitutes according to the WHO classification of serous cystadenoma, serous cystadenocarcinoma, pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), intraductal oncocytic papillary neoplasm (IOPN), intraductal tubulopapillary neoplasm (ITPN) and mucinous cystic neoplasm (MCN).

The uppermost common genetic defect of pancreatic adenocarcinoma is mutation in the kirsten rat sarcoma viral oncogene (KRAS) that is found in 90 per cent of the tumors [13]. Pancreatic adenocarcinoma has the highest frequency of KRAS mutations of all cancers [14]. KRAS mutations result in uncontrolled proliferation, increased invasion and cancer progression with worse prognosis and shorter survival [15, 16].



Progression model of PDAC. The stepwise accumulation of morphologic, histopathologic, genetic, and epigenetic changes are accompanied by immune cell infiltration and a desmoplastic stromal reaction. Abbreviations: CAF, cancer-associated fibroblast; ECM, extracellular matrix; MDSC, myeloid-derived suppressor cell; PSC, pancreatic stellate cell; TAM, tumor-associated macrophage.

Published in: Ciernikova, S.; Earl, J.; García Bermejo, M.L.; Stevurkova, V.; Carrato, A.; Smolkova, B. Epigenetic Landscape in Pancreatic Ductal Adenocarcinoma: On the Way to Overcoming Drug Resistance? *Int. J. Mol. Sci.* 2020, *21*, 4091. https://doi.org/10.3390/ijms21114091.

Inactivation of the tumor suppressor gene cyclin-dependent kinase inhibitor 2A (CDKN2A) is seen in 95% of the pancreatic tumors and lead to protein p16 reduction. P16 is fundamental for the regulation of the G1/S transition of the cell cycle and impaired control can result in cellular transformation [17, 18].

Tumor protein 53 (TP53) is a tumor suppressor gene inactivated in 50-70 per cent of the tumors, making it possible for damage cells to escape destruction control checkpoints and ignore apoptotic signals [17, 19]. In approximately 50 per cent of the pancreatic adenocarcinomas the tumor suppressor gene SMAD family member 4 (SMAD4) is inactivated. This inactivation causes cancer progression by defect signaling of the transforming growth factor β (TGF-β) cell-surface receptor [20].

There are many challenges left to improve the prognosis of pancreatic cancer. To find tumor markers for early detection and develop new improved treatments is of high priority to fight the cancer progression. This thesis main purpose is to create a platform for translational pancreatic cancer research to increase the knowledge of and the probability to cure one of the deadliest cancers in the world.

1.1 TUMOR MARKERS

Since the vast majority of the pancreatic tumors are spread at the time of detection, tumor markers suitable for early detection are urgently needed. At the moment there are no tumor markers that meet the requirements, but interesting research is in progress that hopefully in a near future will provide the desired results. In this thesis a selection of existing and promising tumor markers of pancreatic cancer will be presented.

1.1.1 SERUM MARKERS

Carbohydrate antigen 19-9

Carbohydrate antigen 19-9 (CA19-9) is the most used and evaluated biomarker in pancreatic cancer. Elevated Ca 19-9 has been described in many gastrointestinal malignancies, besides pancreatic adenocarcinoma, in colorectal cancer, cholangiocarcinoma and gastric cancers, but also breast, gynecological and lung cancers [21-24].

The sensitivity of Ca 19-9 in pancreatic cancer is between 70 and 90 per cent and the specificity between 68 and 91 per cent in studies [25]. One of the reasons of the low sensitivity is that around 7-22 per cent of humans do not express Lewis antigen on their red blood cells, why they cannot synthesize Ca 19-9 or only produce small amounts [26-28]. Ca 19-9 seems to correlate with the stage of the pancreatic cancer, meaning that often only low and more unspecific values are detected in early disease [29-31]. The problem with the specificity of Ca 19-9 is that it can be elevated in benign conditions in the pancreaticobiliary tract like pancreatitis, liver diseases, jaundice, but also in lung, gynecological and endocrine diseases [32, 33].

Extensive screening studies of Ca 19-9 have been performed. In asymptomatic patients the Ca 19-9 screenings were ineffective [34]. In Korea 70.940 asymptomatic patients were screened resulting in a positive predictive value to detect pancreatic cancer of only 0.9 per cent [35]. In symptomatic patients, foremost with jaundice, Ca 19-9 can be helpful in the diagnosis of pancreatic adenocarcinoma [34].

Ca 19-9 can be used as a complement in the diagnosis, but the main area of application is to monitor chemotherapy and relapses of pancreatic cancer. Preoperative Ca 19-9 levels have been seen to correlate with postoperative survival in resectable pancreatic cancer patients [36]. Even a decrease in Ca19-9 postoperatively is a predictor of survival, since patients with normalized or downtrending values live longer [29, 37].

The levels of Ca 19-9 also correlate with the efficacy of chemotherapy: neoadjuvant, adjuvant and palliative, and a decrease in Ca 19-9 predicts better survival [38-41]. Both the response to chemotherapy and postoperative recurrence of pancreatic adenocarcinoma can be detected earlier with Ca 19-9 monitoring than what is possible with imaging technics [38, 42].

Many serum tumor markers have been suggested to be useful for pancreatic cancer, among others carcinoembryonic antigen (CEA), Ca 242 and macrophage inhibitory cytokine 1 **(**Mic-1). No other marker has been proved to be more beneficial than CA 19-9 and there are no other markers consequently used in routine clinical practice [30].

1.1.2 TISSUE-BASED TUMOR MARKERS

Circulating tumor cells

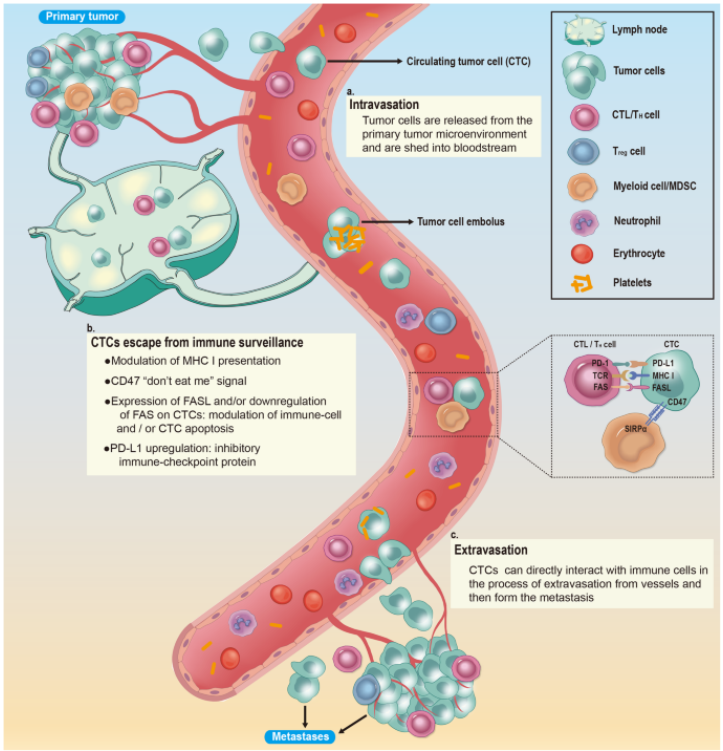
Circulating tumor cells (CTC) are tumor cells emitted from the primary tumor tissue or metastases into the blood stream. The CTC can passively detach from the tumors or be released after tumor invasion of other tissue and their blood supply [43, 44].

CTC are well documented to be connected to survival and to be adequate in monitoring response to therapy in many malignancies, for example breast-, prostate- and colorectal cancer [45-49]. No CTC isolation methods are yet sensitive enough to be used for early detection [50, 51]. There are many challenges in CTC identification. One essential problem is that there are extremely few CTC in the peripheral blood compared to the amount of blood cells, about one CTC per billion normal blood cells [52, 53]. The numbers of CTC are fewer in non-metastatic disease and seems to correlates with the tumor stage, why CTC are even more difficult to detect in early cancer stages [54-56]. In many cancer forms, including PDAC, epithelial-mesenchymal transition (EMT) frequently occurs. During EMT the tumor cells lose their epithelial features and develop a mesenchymal phenotype [57]. The tumor cells are through EMT thought to go from a more stable to an invasive form. EMT is connected with worse prognosis in PDAC due to increased invasiveness, chemoresistance and metastasis [58-60]. Many systems to recognize CTC are based upon epithelial markers catching CTC and they are consequently not completely reliably when EMT appears.

Several different methods of CTC detection have been developed. Roughly, they can be divided into two main ways of identification, by physical or biological attributes. In the first one, physical-based separations, CTC are captured depending on size, density, deformability or dielectrophoretic activity. Biological separation is based upon the fact that CTC, unlike hematopoietic cells, are epithelial cells expressing different types of surface proteins [61, 62]. The most common methods use antibodies against the surface marker epithelial cell adhesion molecule (EpCAM) to fetch CTC. EpCAM is well documented to be frequently present on the surface of CTC [63]. There are often other tumor cell markers used in combination with EpCAM to improve the sensitivity. There are also ongoing developments of different systems to enable the detection of CTC after EMT progression with mesenchymal markers and to identify EpCAM negative CTC [64, 65]. One of few commercial instruments approved for clinical use and the most well-known, CellSearch® utilize EpCAM+, and cytokeratins 8, 18+, and/or 19+ and CD45- to identify CTC. CellSearch is used in breast- prostate- and colorectal cancer to monitor oncological treatment. The method has

disadvantages though, both considering detection of EpCAM negative CTC and the diverseness of the tumor markers [66].

Last years the focus in CTC research has shifted from counting numbers of CTC and the connection with prognosis, towards identifying different subclones and CTC clusters to understand metastatic processes and biological functions of individual cells.



The metastatic cascade: The main steps of tumor spread. **a**. Intravasation: Tumor cells are first released from the primary tumor microenvironment, then traverse the interstitial connective tissue, and ultimately gain access to the circulation by penetrating the vascular basement membrane. **b**. CTCs escape from immune surveillance in the circulation: CTCs encounter immune cells through direct cell–cell interactions and are subject to immune-mediated elimination. **c**. Extravasation: In the process of extravasating to secondary locations, CTCs can directly interact with immune cells, supporting the formation of metastases.

Published in: Zhong, X., Zhang, H., Zhu, Y. *et al.* Circulating tumor cells in cancer patients: developments and clinical applications for immunotherapy. *Mol Cancer* **19,** 15 (2020). https://doi.org/10.1186/s12943-020-1141-9.

CTC clusters have been known for a long time, but the interest has increased markedly the last decade [67]. Clusters of CTC, also called circulating tumor microemboli, composed of cancer cells only or in combination with non-malignant cells, for example macrophages and fibroblasts, are even more extraordinary than single CTC in the blood. There are indications that CTC clusters have a greater metastatic potential and express more mesenchymal markers than single CTC [68-70]. CTC clusters, just like single CTC, detach from different parts of the primary tumor and enter the blood stream [71]. Due to strong epithelial cell-to-cell interactions in the cluster formations the CTC clusters are believed to be more protected and have a survival advantage[72]. The number of CTC clusters correlate with shorter progression free survival and shorter overall survival in several malignancies, among others PDAC [73-75]. Recently, in a study with an organoid transplantation model, malignant subclones of clustered colorectal cells were seen to promote the metastasis process through the creation of a microenvironment called fibrotic or pre-metastatic niche. In this supportive context even non-metastatic cells could start to spread [76, 77]. Further studies can hopefully gain more knowledge of the metastasis process of solid tumors.

Tumors are heterogeneous structures with many different subclones of cells with different attributes. CTC are shed of from various parts of the tumors and the analyses of CTC have helped to reveal the diverseness in cell populations of the tumors[78-81]. If CTC isolation is successful it is possible to do single cell analysis, where enormous amounts of information can be achieved. DNA, RNA, and proteins in the individual cells can be sequenced to provide knowledge of the genome, trancriptome and proteome. Proteins intracellular or on the cell surface can be defined, mutations mapped, rare cell populations uncovered and regulatory relationships between genes can be understood, to mention some of the opportunities [46, 62, 82, 83]. Single cell technology can support the development of personalized targeted cancer therapy. CTC subpopulations can be analyzed regarding gene expression and identify drug sensitivity. Knowledge of the HER2 expression pattern of CTC in breast cancer have been utilized to direct therapy decisions [84]. In pancreatic adenocarcinomas KRAS mutations of CTC and the primary tumor have been compared, showing a heterogenous pattern. Dissimilar KRAS mutations were verified to have differences in overall survival [85]. This kind of knowledge might in the future be helpful in personalized treatments.

Extracellular vesicles

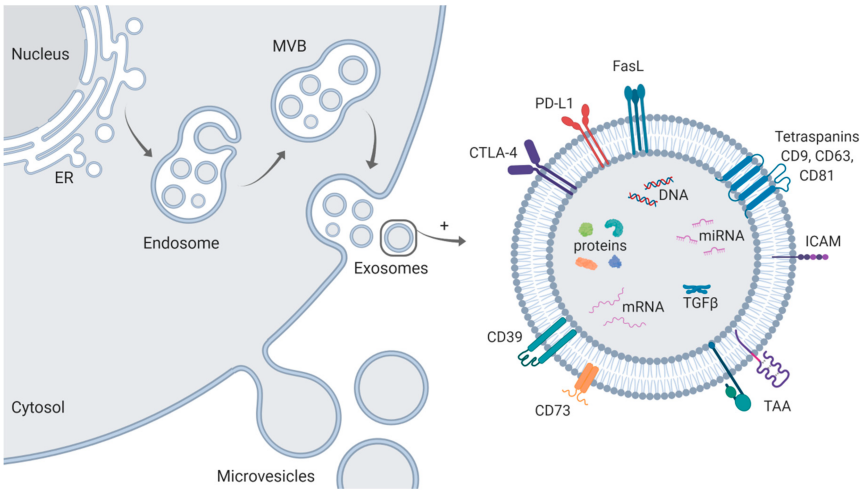
Extracellular vesicles (EVs) are nano-sized particles naturally released from mostly all cells in the body. They are surrounded by a lipid bilayer and have an important role in cell communication. Depending on size and biogenesis EVs can be divided into three groups; apoptotic bodies, exosomes and microvesicles [86, 87]. Apoptotic bodies have a diameter of 800 to 5000nm and are released from the plasma membrane of cells dying through programmed cell death, apoptosis [88]. Microvesicles are 50 to 1000 nm in diameter and are formed by sacs of the plasma membrane. Exosomes, with a diameter of 40 to 100 nm, are of endocytic origin created by inwards budding within the cells and then released by fusion with the plasma membrane [89-91]. EVs contain and transport cargo from the cells that secreted them. Proteins, lipids, enzymes, different types of RNA and DNA are shuttled in the EVs between cells. Distant cells enfold the EVs and their content can then influence the cells function and behavior [92]. EVs can affect the immune system, viral pathogenicity, pregnancy, cardiovascular diseases, the nervous system, cancer development and inflammatory conditions [87, 92-100].

EVs have been isolated from most of the fluids in the body; blood, saliva, urine, breast milk, ascites fluid, cerebrospinal fluid, bile, amniotic fluid, semen and broncoalveolar fluid [101-110].

Considering cancer more and more interesting discoveries emerge. Quite early the possibilities of EVs as biomarkers were recognized. EVs have cargo from the cells secreting them, so even tumor cells. This means they are carrying around specific tumor cell signatures. If EVs are isolated in blood, urine or other body fluids an analysis can reveal tumors presence in the body. The prospect of detecting a cancer with a blood test is referred to as a “liquid biopsy”, but identification in urine could be even less invasive [99, 111]. In pancreatic cancer exosomal Glypican-1 and an exosomal microRNA signatures have shown interesting results as biomarkers in blood, succeeding to separate malignant disease from benign, but further confirming studies are needed [112, 113].

By means of EVs tumor cells can transfer contents between them locally or to distant sites and to other cells. EVs with tumor content are called tumor-derived exosomes (TDEs) and are believed to constitute an important part in the cancer development. Metastatic attributes like growth factors, anti-apoptotic and mutant genes, enhanced migratory and invasive abilities and chemoresistance can be passed on [114-118]. Mesenchymal phenotypes can also be transferred from one cell to another by exosomes facilitating the cells

capacity of EMT [119]. The mesenchymal phenotype has increased migratory probability and invasiveness, evolved resistance to apoptosis and can produce extended quantities of extracellular matrix components [120].



Schematic representation of exosome biogenesis and molecular cargo. Exosomes are formed through inward budding of the endosomal membrane resulting in the formation of multivesicular bodies (MVB). Upon fusion of MVBs with the plasma membrane, exosomes are released in the extracellular space. In contrast, microvesicles are formed by simple budding of the plasma membrane. The molecular cargo of exosomes consists of proteins, miRNA, mRNA, DNA, and lipids. On their surface, they carry the tetraspanins CD9, CD63, and CD81, commonly referred to as "exosomal markers," adhesion molecules, which are specific to the cell of origin. Further, the presence of immune suppressive proteins such as CTLA-4, PD-L1, Fas-L, CD39, CD73, and TGFβ in HNSCC-derived exosomes has been reported.

Figure is created with BioRender and published in: Hofmann, L.; Ludwig, S.; Vahl, J.M.; Brunner, C.; Hoffmann, T.K.; Theodoraki, M.-N. The Emerging Role of Exosomes in Diagnosis, Prognosis, and Therapy in Head and Neck Cancer. *Int. J. Mol. Sci.* 2020, *21*, 4072. https://doi.org/10.3390/ijms21114072. Reprinted with permission CC BY 4.0 http://creativecommons.org/licenses/by/4.0/

The integration between the tumor microenvironment (TME) and the tumor cells is a prerequisite for tumor progression [121]. EVs influence the TME by promoting fibroblasts in the stroma to differentiate into cancer-associated fibroblasts (CAFs) [122, 123]. CAFs, having an essential function in the TME, in turn secrete non-TDEs that stimulate the cancer cells migration capacity, increase their invasive abilities and induce EMT [114, 122, 124, 125]. In pancreatic

adenocarcinoma CAFs exposed to gemcitabine release EVs that induce proliferation and therapy tolerance in the tumor cells [126, 127].

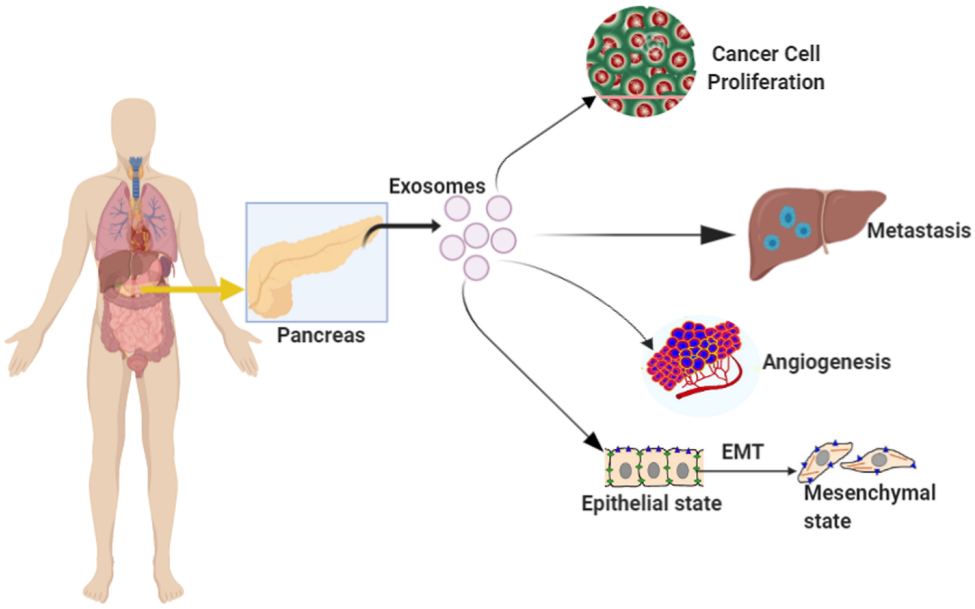
EVs also have important roles in angiogenesis and immunosuppression in the TME. By taking part in the angiogenetic processes in the tumor surrounding, TDEs contribute to an accelerated cancer growth[128, 129]. The metastatic potential of the tumor also becomes enhanced through a destruction of normal defense mechanisms and barriers in the vessels, which increase the vascular permeability and ease the passage of tumor cells into the blood stream [130, 131]

Cancer evolvement is also promoted by the TDEs multiple immune suppressive functions. Natural killer (NK) cells can be downregulated, apoptosis in T-cells induced and tumor opposing anti-bodies possibility to bind tumor cells impaired due to TDE activity [132, 133]. The functions of dendritic cells and macrophages may also be interrupted leading to tumor progression [134, 135].

Evidence is increasing that tumor cells create a pre-metastatic niche in distant organs, a microenvironment that facilitates further metastasis. EVs seem to have an important role in facilitating the development of the pre-metastatic niche [136, 137] by creating an immunosuppressive environment, inducing inflammatory processes, promoting vascular permeability and angiogenesis and remodeling the extracellular matrix (ECM) [131, 133, 138].

EVs have the potential to carry therapeutic agents to specific cells. Qualities like being natural transporters, having the capacity to circulate long times in the blood stream and their excellent biocompatibility make them perfect for the job [139]. This could revolutionize the cancer care, if for example chemotherapy packed inside EVs could target a specific type of tumor cells. The negative side effects of chemotherapy on non-tumor cells could then be decreased. At the moment there are several challenges left to be addressed. How to load the agents into the EVs and how to accomplish large scale production of EVs for clinical application are remaining problems that need to be solved [139, 140].

The future of EVs is exciting and the research area can hopefully contribute to further knowledge of cancer processes, identify tumor markers and develop targeted cancer therapy.



Highlights the roles of exosomes in pancreatic cancer progression that includes cell proliferation, metastasis, angiogenesis, and EMT.

Published in: Ariston Gabriel, A.N., Wang, F., Jiao, Q. *et al.* The involvement of exosomes in the diagnosis and treatment of pancreatic cancer. *Mol Cancer* 19, 132 (2020). https://doi.org/10.1186/s12943-020-01245-y.

1.1 THERAPIES IN PANCREATIC CANCER

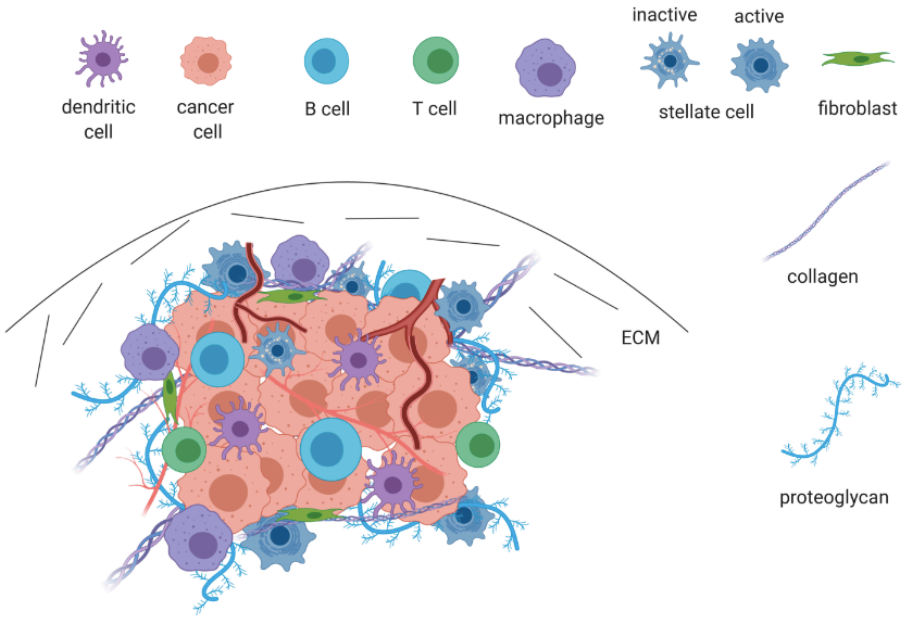
Chemotherapy in pancreatic cancer is used with the purpose to prolong survival. It is either utilized after surgery as adjuvant therapy or as palliative treatment, but neither is sufficiently effective. Neoadjuvant chemotherapy is foremost used in studies or as a bridge to surgery in treatment of locally advanced and borderline tumors [10, 141]. The treatments significantly improve the survival, but the prolonged lifetime is modest, at the best a few months in most of the patients [9].

FOLFORINOX, a combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin began to be more widely used in clinic about a decade ago with improved survival, both as neoadjuvant, adjuvant and palliative therapy [142-144]. The treatment is mainly suitable for a limited, younger cohort that can cope with the often prominent side effects [145]. Since the incidence of pancreatic cancer peaks around 70 years of age,[7] many of the cancer patients cannot tolerate FOLFORINOX. Though, if they are healthy without comorbidities, have Eastern Cooperative Oncology Group (ECOG) performance status score of 0 or 1 and achieve adjusted dosages, it is possible to threat even patients over 70 years of age with good response [146]. There are also other chemotherapy combinations used, with gemcitabine, cisplatin, epirubicin, nab-paclitaxel, erlotinib, bevacizumab, capecitabine and oxaliplatin that contribute to prolonged survival, but all combination therapies come to the price of worse side-effects [145]. The prospect of finding new more effective chemotherapies without severe side effects is not very likely. With certainty, other types of therapies are needed to seriously improve the prognosis.

Immunotherapy has revolutionized the treatment and improved the prognosis of many cancer types [147, 148]. The attempts so far in pancreatic cancer have been disappointing, which does not mean that there is no way forward [149]. The immunosuppressive tumor microenvironment (TME) in pancreatic adenocarcinomas is part of the problem [150].

1.1.1 THE TUMOR MICROENVIRONMENT

The TME in pancreatic cancer is highly immunosuppressive, which result in extensive resistance to conventional therapy generally and immunotherapy in particular. The stroma becomes dense and fibrotic during interactions between the cancer cells and the TME components facilitating tumor progression by diminishing the vascular perfusion, impeding the penetration of drugs and inhibiting the immune system response [151-153]. The stroma consists mainly of extracellular matrix (ECM) with its proteins, where different collagens are the most common [154]. Pancreatic stellate cells (PSCs) are abundant in the stroma of normal pancreas and produce collagen and



The pancreatic tumor microenvironment (TME). A complex ensemble of tumor cells, mesenchymal cells, inflammatory and immune cells, abnormal vascularity, and an excess of extracellular matrix (ECM). Hypoxia and excessive desmoplasia are the main features driving neoangiogenesis, immune suppression, and resistance to therapy.

regulate the synthesis of ECM [155]. Immune cells capable of destroying tumor cells like natural killer (NK) cells and cytotoxic T cells have been observed to be downregulated in TME, whereas immune cells with the capacity to promote tumor progression; myeloid-derived suppressor cells (MDSC) tumor-associated macrophages (TAMs), regulatory T cells (Tregs), fibroblasts and mast cells, become more and more prominent during the malignification process of the pancreatic tumor [153, 156, 157]. The TME created can protect the tumor cells from immune system attacks [153].

In the stroma of pancreatic adenocarcinoma, CAFs are frequently present. They can differentiate from mesenchymal stem cells (MSCs), PSCs and fibroblasts by EMT. CAFs are essentially important to the tumor progression and promote the generation of growth-, inflammatory- and angiogenetic factors[158, 159]. Cancer cell EMT and the metastasis processes are stimulated by CAFs [160]. CAFs also actively take part in the construction of the ECM to form the hard, fibrotic shell that function as a barrier, capable of resisting almost all sort of treatments [152, 153, 161].

1.1.2 IMMUNE SYSTEM BASICS

The immune system constitutes of many different components with various cell types and proteins. Their main purpose is to recognize and respond to foreign pathogens. All parts of the immune system cooperate to protect the body from intruders. Commonly the immune system is divided into two main parts according to their category of response; the acquired or adapted immune system and the innate or non-specific immune system [162].

The innate immune system is the first line defense and becomes immediately activated to attack pathogens when they enter the body to prevent spread. The defense consists among others of natural killer cells, macrophages, neutrophils, dendritic cells, mast cells and eosinophils and they are for example found in skin, hair, mucous membranes and cough. The immune response is unspecific and the attack is similar irrespective of the type of pathogen [163].

The adaptive immune response is the second line and highly specific to the pathogen. It takes up to four days to activate and consists of B and T lymphocytes that rapidly expand clonally and increase in numbers. They all have identical antigen receptors generated to fight the specific intruder. The adaptive immune system has a long-lasting memory by way of memory B

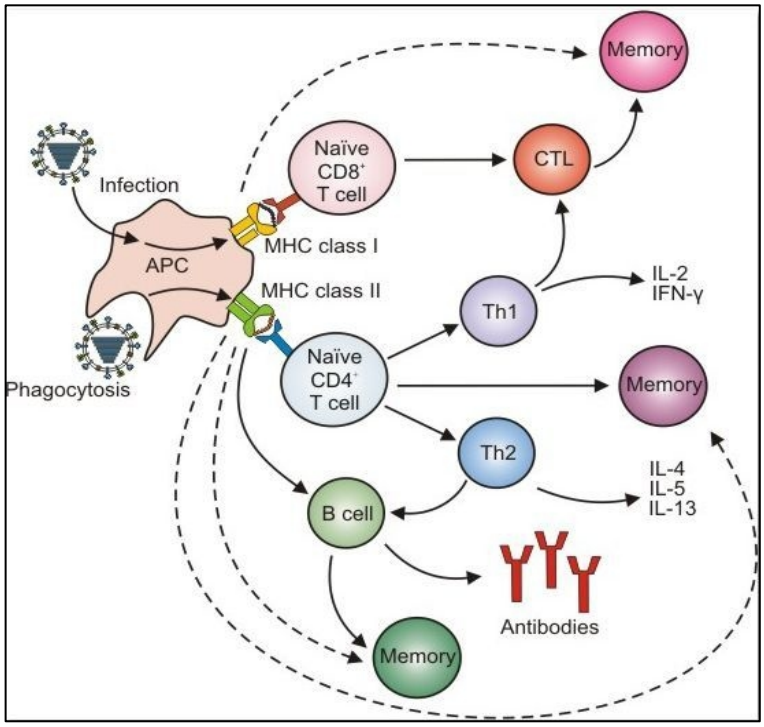
cells. The second time with the same exposure, the immune reaction is consequently faster. The B and T cells are besides in blood, positioned in among others pus, swelling and redness [164, 165].

B lymphocytes

B lymphocytes are bone marrow derived and become activated when an antigen, soluble or membrane bound, attach to their B cell receptor (BCR). B cells express immunoglobulin (Ig) receptors that can identify specific antigen types. Before activation the B lymphocyte can only express IgM, but thereafter the expression can be changed into IgA, IgD, IgE, IgG, or keep the IgM [166]. B cells have two types of immune responses, one without T-cells and one T helper cell dependent. The first response is fast, but less specific. The B cell can secrete IgM antibodies to attack the pathogen the first days until the specific defense is developed [167, 168]. During the interaction with the T helper lymphocyte the B cell attach with the Major Histocompatibility Complex (MHC) II to the TCR and with the (cluster of differentiation) CD40 surface protein to the T cell CD40L [169]. Cytokines secreted from T helper cells contribute to a proliferation in the B cells and determination of which isotope of Ig that shall be expressed. The B cells then differentiate into plasma cells or memory B cells. Plasma cells produce antigen specific antibodies and can continue with that for several weeks. They then move to the bone marrow and wait for new attacks from the pathogen, which they can respond to again quickly with new specific antibodies [170]. Plasma cells can be alive between a few weeks to many years [171]. Memory B cells stay in the circulation and if the same antigen as the original enter the body again, it binds to the BCR that has a high affinity for that typical pathogen. Thereafter the memory B cells differentiate into plasma cells and specific antibodies are secreted to fight the pathogen [172]. B cells also secrete cytokines that can affect T cells, dendritic cells and among others influence healing of wounds and tumor progression [170].

T lymphocytes

T lymphocytes can be divided into several different types of T cells and one way to classify them is in two main groups: CD4+ T cells and CD8+ T cells. CD8+ cells are called cytotoxic “killer” T lymphocytes. CD4+ can be further classified into to smaller groups, where the most important are helper T lymphocytes, and regulatory T lymphocytes [173].



Induction of humoral and cellular immunity. Induction of immune responses after a primary influenza A virus infection is indicated by solid arrows. The more rapid activation of virus-specific memory cell populations upon secondary encounter with an influenza A virus are indicated by dotted arrows.

Published in: Van de Sandt, C.E.; Kreijtz, J.H.C.M.; Rimmelzwaan, G.F. Evasion of Influenza A Viruses from Innate and Adaptive Immune Responses. *Viruses* 2012, *4*, 1438-1476. https://doi.org/10.3390/v4091438

All T cells have a T cell receptor (TCR) that can bind to MHC I or II receptors on cell surfaces of other cells. The T helper cells can attach to MHC II, present on antigen presenting cells (APC) and cytotoxic T cells bind to MHC I, present one all nucleus cells in the body. When a pathogen enters the body, APC have the capability to take up, process the pathogen into peptides and put it into the MHC II to present it on the cell surface. Dendritic cells, macrophages and B-cells can all function as APC and when they present the antigen, the T helper cells bind to the MHC II with the TCR. The T helper cell becomes activated, secretes cytokines, among others interleukin 2 (IL-2), that stimulate B cells, cytotoxic T cells and macrophages. T helper cells have

a co-receptor, CD4, which also attach to the MHC II to make the cell-to-cell adhesion more consistent. The CD40 ligand (CD40L), not to be confused with CD4, also expressed on the surface of activated T helper lymphocytes is important to the activation of the B cells. When the CD40L recognizes the CD40 protein on the B cell surface, their interaction lead to further B cell proliferation. There are also proteins on the surface of T helper lymphocytes that can stimulate the attachment to the APC, co-stimulators, whereof cluster of differentiation 28 (CD28) is one of the most important. CD28 binds to the B7 complex on APC, which leads to further stimulation of the T helper cell [(174)].

The cytotoxic T cells attach to the MHC I of cells in the body when they present a foreign antigen in the complex after being infected. To stabilize and improve the impact of the cell-to-cell adhesion, the cytotoxic T cell has a co-receptor, CD8 that attach to another locus of the MHC I. To avoid further spread of the pathogen and protect the body against viruses, bacteria and parasites, the cytotoxic T cell kill the infected cell, by inducing apoptosis [(175)]. Cytotoxic T cells also have co-stimulators like CD28, that can increase their efficiency, but they are not necessary for their activation [(176)].

The regulatory T cells (Treg), former known as suppressor T cells, are involved in the signaling deciding the amount of activity of the immune system. When an infection starts they signal to increase the activity and when the infection is under control, they communicate to decrease the immune response [(177)]. Tregs have impact on and can suppress both other types of T lymphocytes and B cells and this effect is essential for the balance of the immune system [(178)]. Autoimmune diseases are prevented by Tregs by establishing tolerance of bodily-specific proteins and Tregs are also important to inhibition of asthma and allergy reactions [(179)]. Tregs are activated via their TCR, but the exact mechanism is not fully understood. Just like T helper cells, Tregs use CD4 as a co-receptor but CD25, expressed on the Treg surface, are their most important co-stimulatory. CD25, constitute a part of the IL-2 receptor and IL-2 stimulation is subsequently mandatory for Treg proliferation and activity [(180)]. CD28, CTLA-4 and B7 are not believed to be involved in the activation [(181)]. Antigens are recognized by the Tregs where after the Tregs achieve the capability to inhibit cytotoxic and helper T cells by suppression of the IL-2 secretion and upregulation of IL-2 receptors. Tregs can suppress the surrounding cells both by direct cell-to-cell adhesion, secretion of cytokines, among others transforming growth factor beta (TGF-β), IL-10 and IL-35 and competitive binding to APC cells [(182)]. Studies have shown that many different types of antigen can activate Tregs, including

organ-specific antigen, self-antigen. How the Tregs know how to response to the various antigen types is not apprehended [183, 184].

Negative feedback mechanisms

There are negative feedback mechanisms controlling the T lymphocytes, all types, so they do not become overactive. Two of the most important control systems are induced by cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death protein-1 (PD-1). During the activation the T lymphocytes start to express both of these surface proteins. CTLA-4 attach to the APC in the B7 region just like CD28 and reduce the proliferation of the T helper cell and keep the process balanced [147, 185]. Presumably CD28 and CTLA-4 constitute a costimulatory-coinhibitory system that regulates this part of the immune response [186]. The IL-2 production and the expression of the IL-2 receptors become reduced and the cell cycle process inhibited, by the influence of CTLA-4 [187]. The inhibiting CTLA-4 effect of the cytotoxic T cells is partly due to inhibition of the dendritic cells. The dendritic cells also have B7 complexes and when the attachment to CTLA-4 occurs, the activity of the cytotoxic T cells decrease [187]. CTLA-4 seems to be less expressed on cytotoxic T cells compared to T helper cells. The CTLA-4 inhibiting effect on the cytotoxic T cells is probably also partly mediated by decreased T helper cell activity, though the T helper cells stimulate cytotoxic T cells by cytokine secretion [188]. Considering Tregs, they have a suppressive and regulatory function both by CTLA-4, but also independently. CTLA-4 also has inhibitive functions without connection to Tregs [189]. Several types of tumor cells also express CTLA-4 [190]. If CTLA-4 is blocked by antibodies an enhanced resistance to tumor cells can be seen, but also an augment of autoimmune diseases [191].

PD-1 can inhibit both the innate and adaptive immune systems and is expressed on active T and B cells, natural killer cells, macrophages and dendritic cells and can suppress their activity when binding to any of the ligands [192]. There are two ligands compatible with PD-1; programmed death protein -1 ligand (PD-L1) and PD-L2. Unfortunately tumor cells have been seen to express both PD-1 and PD-L1 to attempt to avoid the immune response [193]. Tumor infiltration lymphocytes (TILs) in malignant melanoma have been recognized with PD-1 and CTLA-4 proteins on their surfaces. This seems to be a way for the tumors to adjust the TME, leading to inhibition of the antitumor immune response [194]. PD-L1 is present on T and B lymphocytes and in macrophages and dendritic cells. The ligands upregulate when the cells are activated [195]. Even non-lymphoid cells like pancreatic beta cells, glia cells in the brain, endothelial cells in the heart and muscle

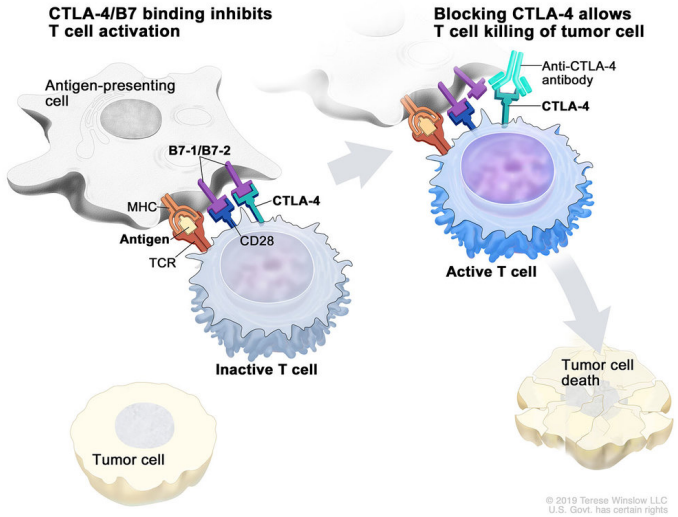
cells can express PD-L1. PD-L2 can be found on activated macrophages and dendritic cells. Both ligands can be found on various tumor cells [196].
PD-1 has, just like CTLA-4, great significance to the development of tolerance of bodily-specific proteins, to be able to avoid autoimmune reactions [196]. Expression of PD-L1 can help the tumor cells to inhibit the activity of cytotoxic T cells, induce EMT, promote metastasis and develop resistance to therapy [197].

1.1.3 IMMUNOTHERAPY

Immunotherapy in pancreatic cancer has been tried. So far, with limited success. Single therapies have not proved to be working at all, but combinations of treatments seem to be promising with a hard-patient selection. There are roughly five types of immunotherapy: immune checkpoints inhibitors (ICI), adoptive T-cell therapy (ACT), cancer vaccines, monoclonal antibodies and immune system modulators. In this thesis ICI and ACT are discussed.

Immune checkpoint inhibitors

The discoveries leading to the development of immune checkpoints inhibitors were in 2018 awarded with the Nobel Prize. The treatment has as a purpose to block the negative feedback mechanisms in the immune system.



Immune Checkpoint CTLA-4
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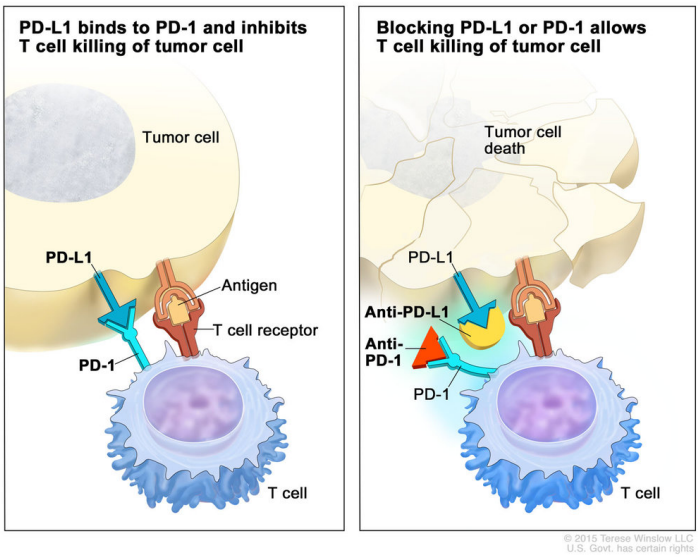
Antibodies against CTLA-4 and PD-1 are most common of the ICI and have been studied most extensively.

The main principle for the CTLA-4 ICI is that CTLA-4 antibodies bind to the CTLA-4 receptors on the T lymphocytes and block them. The negative feedback mechanisms do not function, and the T cell stays continuously activated. The effect of CTLA-4 antibodies is most substantial on T helper cells and Tregs, although CTLA-4 is present even on cytotoxic T cells. The expression of CTLA-4 seems to be lower in cytotoxic cell, which might be one of the reasons [188]. The T helper cells increase their activity due to the CTLA-4 receptor obstruction and the Tregs immunosuppressive capacity are inhibited [147]. The adverse effects of CTLA-4 ICI are massive with the wrong dosage. When mice lacking CTLA-4 receptors were observed, they died within three to four weeks, due to massive lymphocyte deposition in all organs. The lymphocytes infiltration was extensive in heart, lungs, liver, bone marrow and the lymph nodes and spleen were ten times larger than normal [198]. There after studies with the first CTLA-4 antibodies were performed in mice and it was proved that the adverse effects could be reduced by dosage adjustments [199]. The first CTLA-4 ICI clinical trial that convincingly could show a therapy effect came in 2003. Malignant melanoma was treated, and three out of fourteen patients had reduction in tumor size. Six patients got relatively severe autoimmune adverse effects with dermatitis, hypophysitis, enterocolitis, and hepatitis [200]. There is no selectivity in the CTLA-4 blockage, but all T cells are affected, and the autoimmune and hyperimmune reactions can become severe. Today treatments against the side effects, like steroids and immunomodulatory drugs are given, which can relieve the symptoms [201].

Regarding ICI with PD-1 and PD-L1, antibodies against both the ligand and the receptor can be used. Both the receptor and the ligand can be present on a relatively wide range of cells, why a blockage gives a broader spectrum of effects. Just like the situation in CTLA-4 ICI, PD-1 is expressed extensively in Tregs, meaning there is an effect with inhibition of the immune suppressive activity when PD-1/PD-L1 ICI treatment is started. PD-1 is predominantly active in a latter phase of the T cells development. CTLA-4 affects the activation, but the PD-1 foremost regulates the T cell effect in peripheral tissue and in the TME. An increased activity of natural killer cells in TME can also be seen after PD-1/PD-L1 antibody treatment and it is possible for B cells to enhance their antibody secretion [147]. The adverse events of PD-1/PD-L1 ICI are similar to CTLA-4 ICI and uppermost due to hyperimmune or autoimmune reactions. Dysfunction of the thyroid and endocrine pancreas and pneumonitis seems to be more common when anti-

PD-1s are used, whereas gastrointestinal symptoms, hypophysis and adrenal insufficiency more often occur with anti-CTLA-4 utilization. If the two ICI are combined the adverse event profile becomes aggravated [202].

There is a need of biomarkers that can predict the response of ICI, though just like most cancer treatments, only a limited amount of the patients will benefit from therapy. So far, no clinically useful biomarkers of CTLA-4 ICI have been detected, but there is research in progress. Considering PD-1/PD-L1 a high burden of mutated mismatch repair genes, called proficient mismatch repair (pMMR), results in accumulation of microsatellite sequences in the genome, microsatellite instability (MSI), which have been verified to correlate with the response to the anti-PD-1 therapy pembrolizumab [203, 204]. In the United States this drug was approved for treatment of unresectable or metastatic solid tumors, among others pancreatic cancer, with pMMR or MSI high phenotype and progression on standard therapy in 2017 [205]. In 2020 the approval was widened to also include patients with high tumor mutational burden (TMB)[206, 207]. TMB is a genetic signature, estimated by calculation of the number of somatic mutations per area in the genome. DNA sequencing is used to measure TMB [208]. This was the first time a therapy was approved based on the occurrence of a certain biomarker instead of the tumor type.



Immune Checkpoint PD-1
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High expressions of PD-L1 and PD-1 on tumors have been detected in many cancers. In malignant melanoma and non-small-cell lung carcinoma a correlation with response to ICI have been indicated. However, PD-L1 negative tumors can also respond to ICI, although less frequently. Considering connections between PD-1/PD-L1 expressions, ICI treatment and survival in malignant melanoma and non-small cell lung carcinoma the data are inconclusive. The more PD-L1 has been valued as a biomarker, the more inadequate the results have been. The PD-L1 expression has been described to vary considerable between different tumor types, different subgroups of tumors and tumor stages [209-211]. PD-1/PD-L1 expressions on TILs, CTC and exosomes have more recently gained increased interest and might in the future be able to add important information.

The TME are of great importance for the possibility to have effect of ICI. High levels of immunosuppressive cells and low numbers of natural killer cells and activated T cells could be seen in the TME of ICI non-responders [212, 213]. Unfortunately, pancreatic cancer has a verified unfriendly environment for the immune system to operate in. The characteristics of the pancreatic TME is believed to be one of the reasons for the limited effects of ICI seen in pancreatic cancer so far

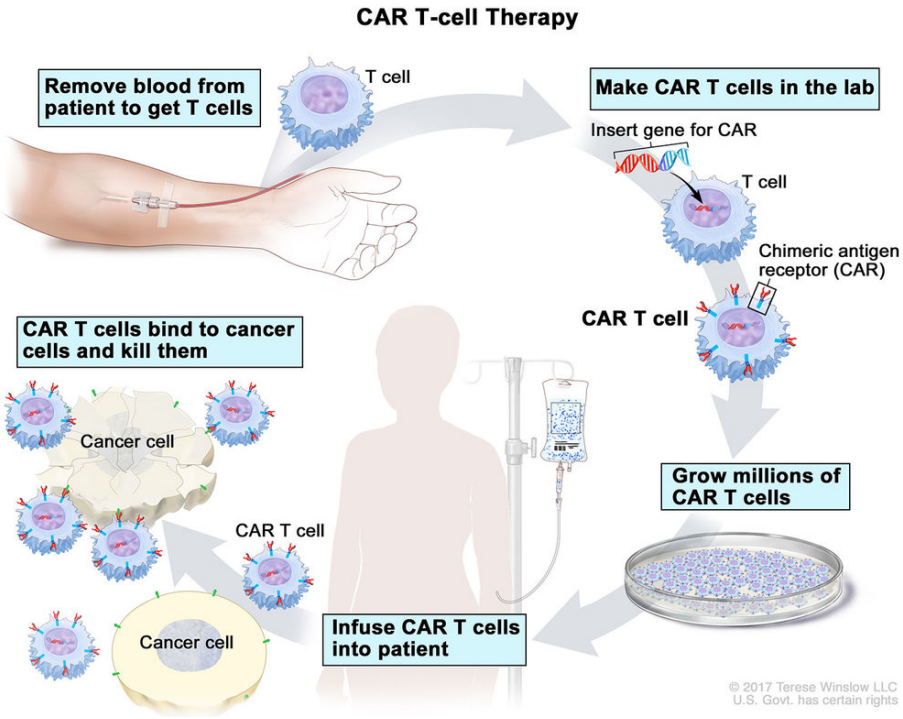
Adoptive T-cell transfer therapy

In adoptive T cell transfer therapy (ACT) the patient's own tumor infiltrating lymphocytes (TILs) are extracted from the patient's tumor. The TILs are then expanded *in vitro* together with IL-2, which stimulates them to grow. When the TILs have expanded and reached a number of around 10^{11}, they are infused into the patient. The TILs then attack the tumor cells and hopefully it results in a reduction in tumor size. ACT therapy with TILs has been successful in malignant melanoma, with up to 50 per cent of the patients responding. In ACT therapy with TILs, the antigens that the TILs respond to are unknown [214].

Considering solid tumors, the ACT therapy has not been very effective, at least partly due to the difficulties for the TILs to penetrate the tumor. Not many other cancer types besides malignant melanoma have had success with autologous TILs treatment. To try to improve ACT, therapies with known targets, tumor-associated antigens (TAA) have been tried. First proper TAAs must be identified. The TAAs need to be expressed specifically on tumor cells and not be present in healthy tissue. The TCR receptor can then be modified by genetic engineering to be able to recognize the specific antigen. It has also been possible to genetically engineer a special T cell receptor; chimeric antigen receptor (CAR) that in laboratories can be attached to the T

cells. The CAR then recognizes a TAA, which activate the T cell and induce the immune reaction [214, 215]. There is also a third way to use ACT, with targeted neoantigens. The neoantigens are formed when tumor cells mutate and express new proteins. These proteins are called neoantigens and are highly specific for the tumor cells and individual for every person. When neoantigens are identified, specific T cell receptors that recognize the antigen can be manufactured [216].

At the moment ACT has only been tried in studies and have shown promising results foremost in hematopoietic malignancies and malignant melanoma [217]. ACT can trigger severe side-effects, for example excessive inflammations, neurotoxicity and autoimmune reactions [218]. The ACT therapy is an individual therapy, that will require a lot of administration and organization to manage to set up for clinical use. The immunosuppressive TME is still a formidable challenge to overcome considering ACT therapy in tumors. So far there have not been any success in pancreatic cancer.



CAR T-cell Therapy
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1.2 PANCREATIC SURGERY

The most common localization of pancreatic adenocarcinomas is in the head, thereafter the tail and the body and in studies the frequencies range from 57 to 71 per cent, 16 to 26 per cent and from 13 to 17 per cent respectively [219, 220]. The operation of a tumor in the head of the pancreas, pancreaticoduodenectomy (PD), is extensive. Except from the pancreatic head, the duodenum, the external bile duct, the gallbladder and often part of the stomach are removed. The operation is called Whipple procedure. It is also common to leave the stomach and perform a pylorus preserving variant. Which one is best is not proven. There have been no differences in studies regarding overall long-term and disease-free survival [221, 222]. The latest Cochrane statement from 2016 summarized that the randomized controlled trials (RCTs) so far favored pylorus preserving PD regarding shorter operation time and less bleeding, but "standard" Whipple considering delayed gastric emptying. The final statement was though that the level of evidence was too low to draw any definite conclusions [223].

The next operative detail of the PD debated is the type of anastomosis. There are several different ways to construct a pancreatic anastomosis, but the divide is if the reconstruction is made towards the jejunum or the stomach. Even here the results so far in the RCTs performed, comparing complications of pancreaticogastrostomy and pancreaticojejunostomy, cannot prove any reliable differences, since no high-grade evidence is presented [224].

The morbidity is high after PD, well above 50 per cent, if both minor and major events are counted in [225-227] and the 90 days mortality rate is 3 to 4 per cent in high volume centers [228-230]. The most common complications are pancreatic fistulas, wound infections, postoperative bleeding, biliary leakage, lymphatic leakage and delayed gastric emptying [222, 226]. Postoperative pancreatic fistula (POPF) and postpancreatectomy hemorrhage (PPH) have the greatest impact on mortality [231].

1.3.1 POSTPANCREATECTOMY HEMORRHAGE

One of the most feared complications after PD is postpancreatectomy hemorrhage. The mortality rate of the most severe, grade C PPH, have been reported to be up to 50 per cent in the literature [232-234]. The incidence of PPH is between 3 and 10 per cent [235-237]. There is a clear connection between the occurrence of POPF and the development of late PPH [233, 238]. The problem is that this knowledge is only theoretically interesting and does not often help in the clinic, though the POPF seldom is known before the PPH C emerge. To be able to improve the survival rate and prevent the bleeding, other factors, predictive, needs to be defined.

To be able to compare surgical complications after operations of the pancreas, the international study group of pancreatic surgery (ISGPS) has formulated definitions of PPH and POPF, table 1,2 [239, 240]. Grade C POPF and grade C PPH is closely related, when a bleeding with sever clinical impact occurs.

Table 1. PPH grade A, B and C definition according to the ISGPS.

Grade	Time of onset. Location and severity.		Clinical condition	Diagnostic consequence	Therapeutic consequence
A	Early. Intra- or extraluminal. Mild.		Well.	Observation. Ultrasound. CT.	No
B	Early. Intra- or extraluminal Severe.	Late. Intra- or extraluminal Mild.	Often well. Very rarely life-threatening.	Observation. Ultrasound. CT. Angiography. Endoscopy.	Transfusion. Intermediate care unit or ICU. Therapeutic endoscopy. Embolization, relaparotomy for early PPH.
C		Late. Intra- or extraluminal Severe.	Severe. Life-threatening	Angiography. CT. Endoscopy.	Angiography and embolization, (endoscopy) *or* relaparotomy. ICU

Table 2. POPF definition according to the ISGPS.

	Biochemical leak (no POPF)	Grade B POPF	Grade C POPF
Drainage amylase > 3 times upper limit serum value	Yes	Yes	Yes
Drainage persisting > 3 weeks	No	Yes	Yes
Clinically relevant change of management due to POPF	No	Yes	Yes
Percutaneous or endoscopic intervention	No	Yes	Yes
Angiography due to POPF related bleeding	No	Yes	Yes
Reoperation for POPF	No	No	Yes
Infection related to POPF	No	Yes, without organ failure	Yes, with organ failure
POPF related organ failure	No	No	Yes
POPF related death	No	No	Yes

Late postoperative bleedings are connected to erosion of vessels due to leakage of pancreatic or biliary juices or infections in the abdomen [236]. Vessel erosion can lead to pseudoaneurysms and the most common site of bleeding is the hepatic artery and its branches [235, 236]. Sentinel bleed, warning bleed, is seen before severe PPH in between 30 and 100 per cent in studies [241, 242]. Angiographic intervention to control the arterial bleedings has improved the prognosis and reduced the mortality, compared to relaparotomy [243, 244]. Potential risk factors and predictive factors of PPH suggested in the literature are: age, BMI, male gender, intraoperative transfusion, portal venous-, arterial- or multivisceral resections, preoperative biliary drainage, history of abdominal surgery, preoperative low albumin levels, ASA $\geq$ 3, hypertension and nutrition status [233, 245-247].

2 AIMS

The overall aim of this thesis is to establish a platform for translational pancreatic cancer research to enable detection of potential tumor markers, evaluation of therapy responses and acquire a deeper knowledge of the mechanisms of tumor progress and metastasis and to address complications of pancreatic surgery.

The specific aims of this thesis are:

- To find a reliable method to perioperatively detect CTC in portal and peripheral artery blood and present a difference in the fractional uptake in the liver-lung compartments.
- To establish a PDX mouse model for pancreatic cancer research, where biopsies are xenotransplanted and TILs generated from the same tumor tissue, to enable evaluation of immunotherapy and combinatory treatments and in extension open for future clinical trials.
- To determine a trustworthy method for EV isolation from pancreatic adenocarcinoma tissue and assess the potential of EVs as biomarkers for pancreatic cancer.
- To identify predictive pre-, peri-, and postoperative factors of postpancreatectomy hemorrhage, a serious complication of pancreatic surgery.

3 PATIENTS AND METHODS

3.1 STUDY POPULATION

The patients included in the studies of this thesis are operated at Sahlgrenska University hospital. All of them were scheduled for pancreatico-duodenal surgery with curative intent, due to suspected pancreatic or periampullary cancer.

Ethical considerations

The ethical review board in Gothenburg approved all studies in this thesis (Paper I-IV). All of the patients included in the CTC, PDX and EV studies (Paper I-III) provided written informed consent.

3.2 ISOFLUX

To detect CTC, IsoFlux® (Fluxion Bioscience), a commercially available instrument was used. Briefly, combinations of antibodies are utilized to catch the CTC with an immunomagnetic enrichment method.

Portal venous and arterial peripheral blood was collected in ten patients perioperatively during pancreaticoduodenectomy (Paper I). Antibodies labeled with magnetic beads were added into the blood sample that was going to be analyzed. The tagged antibodies bind to the antigens on the CTC surfaces. The blood is then introduced in the IsoFlux machine and sent through a magnetic field. The magnetic labeled CTC get caught and separated from the rest of the blood cells. Epithelial cellular adhesion molecule (EpCAM), a well-known CTC marker, was used for the capture. The separated cells were mixed with buffer and stained with Hoeschst staining to identify nucleated cells, Fluorescein isothiocyanate (FITC) staining with cytokeratin (CK) 7, 8, 18, 19 antibodies to further verify CTC presence and Cy3 staining with cluster of differentiation 45 (CD45) antibodies to exclude leukocytes. The individual CTC were counted in fluorescence microscope. Cells with Hoechst+/CKs+/CD45- staining were determined as CTC.

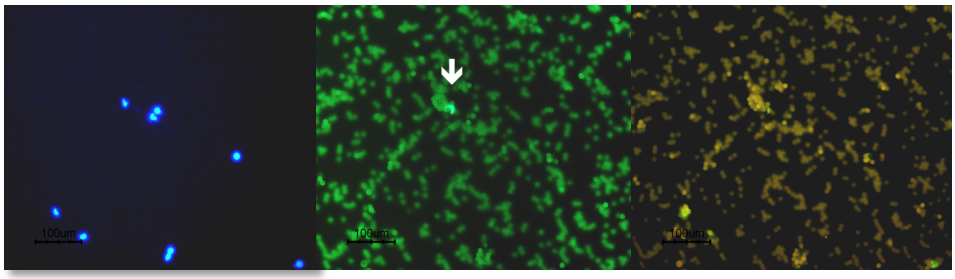


Figure. From right to left: Hoeschst staining, FITC (CK) staining and Cy3 (CD45) staining. Small green/yellow circles are magnetic beads. One CTC is seen in the FITC staining (white arrow).

3.3 FLUORESCENCE-ACTIVATED SINGLE CELL SORTING

Fluorescence-activated single cell sorting (FACS) is a flow cytometry-based cell sorting advice. Flow cytometry can be utilized to isolate different cell types and determine their volume and size. The method can simultaneously determine chemical and physical qualities of more than a thousand particles every second.
In FACS analysis fluorescent tagged antibodies are used to sort cells or other particles with high specificity. Arterial and portal perioperative blood samples from seven patients with periampullary cancer were mixed with the antibodies (Paper I). Blood from ten healthy blood donors, the control group, were also analyzed. After labeling, the cells are one by one sent through a laser beam where they are separated depending on how the light is reflected and bent away. The fluorescent light generates different charge in the cells and they then become sorted due to their differences.

FACS was in the CTC study used to evaluate different tumor markers capacity of CTC detection in periampullary cancer. EpCAM, MHC class I polypeptide-related sequence A (MIC-A), cluster of differentiation (CD) 34, CD 133, Vascular adhesion protein-1 (VAP-1), CK18, CK19 were validated.

3.4 PATIENT-DERIVED XENOGRAFT MODEL

When tissue from humans is transplanted into another species it is called patient-derived xenograft (PDX). The most common is xenografts from human to immunodeficient mice. In PDX models tumor tissue pieces or suspensions of the tissues are transplanted into mice, most often subcutaneously. It is also possible to implant the tissue into the same organ in the mouse as the original human organ; orthotopic transplantation [248]. The tumors growing are then serial transplanted into new mice. PDX models have been proved to be able to predict clinical prognosis and can be utilized to evaluate drug effects, detect biomarkers and study biological processes and to develop personalized therapies [249-251].

Though the mice in the PDX models have dysfunctional immune systems the possibilities to evaluate immune therapy have been limited. With the use of a NOG mice model expressing interleukin 2 (hIL2-NOG mice), PDXv2, it is possible to study adoptive T cell transfer therapy (ACT) [252]. Tumor tissue is transplanted to a hIL2-NOG mouse and tumor-infiltrating lymphocytes (TILs) derived from the same tissue are expanded. When the tumor is established in the mouse and has grown to a predetermined size, the TILs suspension is injected into the mouse. The tumor is considered to be responding to the ACT if a reduction in size can be measured [252].

In Paper II immunocompromised NOG mice were used to establish a PDX model for translational pancreatic cancer research. The succeeding models with synchronously generated TILs were serial transplanted into mhIL2-NOG mice models that were used to evaluate the response to ACT in pancreatic cancer. Between the steps of the serial transplantations the tumor tissues were paraffin-embedded and stained with H&E human cytokeratins, HLA 1 A,B,C and Ki67 to verify specificity with the original cancer tissue. Confirmation of identical tumor cells, perseverance of human cells and proliferation pattern were done. The expression of PD-L1 was valuated in the tumors of all patients by immunohistochemical analysis. KRAS mutation analyses of the tumor tissue were performed with Sanger sequencing in the succeeding NOG mice models.

3.5 ISOLATION OF EXTRACELLULAR VESICLES

When isolation of extracellular vesicles from tissues are performed there are briefly three main steps involved; treatment of the fresh tissues with enzymes, different steps of ultracentrifugation for separation of EVs and density differentiation to further purify the isolated EVs [253]. In Paper III tissues were perioperatively cut out from the pancreatic tumor specimen and immediately transported to the extracellular vesicle lab. Pieces of both tumor and non-tumor tissue from the specimen were used. First the tissues were sliced into smaller pieces and incubated in cell culture media with enzymes. Second the samples were filtrated to remove the tissue pieces before the filtrated solution was ultracentrifugated in several steps to separate larger and smaller vesicles, which were finally loaded on an iodixanol density cushion for additional purification.
The EV protein concentrations were determined and equal amount of protein from each sample were digested into peptides and labeled with tandem mass tags (TMT). Mass spectrometry (MS) analysis was performed to identify and quantify the protein content of the EVs.

In MS the components of a sample separates depending on their mass and electrical charge. The sample can be solid, liquid or a gas. First the sample evaporates into molecules in gas-phase and becomes ionized by the ionizing source, e.g. by laser or electron ionization. Thereafter the molecules accelerate into a beam, which passes through a magnetic field. Depending on their mass and electrical charge, mass-to-charge ratios, the particles will bend differently in the magnetism. A detector registers the course of the molecules and count the numbers.

Pathway analysis of the data was performed with database for annotation, visualization, and integrated discovery (DAVID).

3.6 PREDICTION OF POSTPANCREATECTOMY HEMORRHAGE

The study considering possible predictive risk factors of postpancreatectomy hemorrhage (PPH), Paper IV, is a retrospective matched control study. Patients operated with PD from 2003 to 2018 were included in the study. Operation codes for surgical procedures, ICD-10, were used to categorize the patients. Patients with multiorgan operations, transplantations and arterial resections were excluded to restrict the amounts of confounding factors. In total 517 patients were identified with the right criteria and 17 of these were confirmed with PPH C. The matched control group was randomized from the 500 remaining patients according to age, gender and year of operation. In total there were 68 matched control patients, four control patients for every PPH C patient. Suspected periampullary cancer or precancerous diagnoses were the criteria for surgery.

To assess predictive factors for PPH C, pre- peri- and postoperative data were registered. Preoperatively body mass index (BMI), cardiovascular disease according to the WHO definition, history of abdominal surgery, biliary stent, C-reactive protein (CRP) and ASA-score were validated as risk factors. Perioperative we investigated risks due to the amount of bleeding, type of pancreatic anastomosis and length of operation in minutes. The postoperative data registered were CRP, drain amylase and the presence of postoperative pancreatic fistula (POPF) or biliary fistulas. Additional collected data considering the pathology report, reoperations, angiographic intervention, mortality, sentinel bleed and when the postoperative bleeding occurred and from which vessel were also analyzed.

Statistical calculations of odds ratio and p-values were performed with conditional logistic regression in univariable analyses. The variables found to be significant were then assessed in a multivariable analysis. Two-sided statistical test was used and values below $p<0.05$ were considered significant.

4 RESULTS AND CONSIDERATIONS

4.1 CIRCULATING TUMOR CELLS - PAPER I

The portal venous and peripheral arterial blood from the ten patients with periampullary cancer included in the CTC study were ran through the IsoFlux® equipment. A difference could be verified between the quantities of detected CTC in the portal blood compared to in the arterial blood, with significant higher numbers in the portal blood. In two patients, CTC could not be verified neither in the portal or arterial blood. The pathological exam of these two patients revealed they were lymph node negative. The porto-arterial difference corresponds to a fractional uptake of forty per cent in the liver and lung parenchyma. The amount of CTC released in the circulation from the tumor during operation was also calculated and estimate to be 410 CTC per minute. A proliferation index was estimated, Ki-67, which was approximately eighteen per cent.

In the FACS analysis the CTC markers EpCAM, Mic-A, CD133, CD 34, CD 133, VAP-1, CK18 and CK19 were evaluated in the portal and arterial blood of seven patients with periampullary cancer. EpCAM and, Mic-A were positive in all the blood tests and CD 133 in all tests except for one, why those three markers can be regarded as relevant for CTC detection in periampullary cancers. In the control group no one was positive to any of the CTC markers.

The pathological exam verified seven patients to be pancreatic adenocarcinomas, two cholangiocarcinomas and one ampullary adenocarcinoma in the IsoFlux CTC group. In the FACS group there were six confirmed pancreatic adenocarcinomas and one duodenal adenocarcinoma.

Considerations

Relatively few CTC, maximum ten, were detected in the study, which is a common and known problem in CTC methodology. Often low amounts of CTC are detected almost irrespectively of the isolating method used due to low sensitivity [254]. The CTC are extremely rare compared to the amount of blood cells [255]. To find proper CTC markers are there for important. There are no standardized markers for CTC detection in pancreatic cancer. In our

FACS analyze EpCAM, MIC-A and CD133 were positive in nearly all blood tests, indicating they were present on the CTC surfaces. MIC-A and CD 133 were not used as markers in the IsoFlux analyze. Using improper markers may partly explain the low number of CTC detected. It should not affect the comparison of the CTC quantities done with the IsoFlux.

It is well known that CTC can escape detection when epithelial markers as EpCAM are used, through EMT [254]. There are also indications that MIC-A is downregulated in tumor cells after EMT [256], whereas CD 133 seems to be still present in pancreatic cancer [257]. There are markers identified that can detect CTC after EMT, though they are expressed on cell surface with mesenchymal phenotype. Vimentin is one of the most used and studied, in among others pancreatic cancer [258].

The porto-arterial difference of CTC verified in our study indicates a perioperative capture of CTC in the liver or the lungs. These findings support the theory of CTC subclones and clusters with metastasis capacity [76], but also an increased dissemination of tumor cells that might occur during surgery [259]. Several other studies have also detected higher numbers of CTC in portal blood compared to arterial and a correlation between the presence of CTC in portal blood and liver metastasis have been verified [260-262]. To catch, identify and analyze possible CTC clusters, with for instance single cell technology, is of great interest in the future. Even if the CTC detection methods never become sensitive enough to detect CTC in early cancers and function as tumor markers, much knowledge can be gained by analyzing individual CTC. The research field so far has increased the understanding of metastatic and tumor cells processes considerable. In pancreatic cancer more knowledge of therapy resistance and immunosuppression could help to improve the chances of survival.

4.2 PATIENT-DERIVED XENOGRAFT - PAPER II

In eleven out of twenty-nine patients, PDX models of the immunocompromised NOG mice were established. Six out of these also had TILs successfully expanded and was transplanted into hIL2-NOG mice, PDXv2. All these tumors grew up subcutaneously and TILs were injected in the mice. Three of the tumors were reduced in size after the autologous TILs treatment and considered to be responsive to ACT. In the other three no response could be seen. In every step of the serial transplantations the accuracy of the tissues was verified with immunohistochemistry staining.

The pathological examination of the twenty-nine tumors revealed that a majority, twenty-one were PDAC, three were ampullary carcinoma, two cholangiocarcinoma, one IPMN, one chronic pancreatitis and one adenosquamous carcinoma of the pancreas (ASCP). Of the three tumors responding to ACT, two were PDAC and one ASCP. The PD-L1 staining of the twenty-nine patients verified two strong PD-L1 positive tumors and one with moderate expression. All three were successful NOG mice models, demonstrating the possibility of PD-L1 positive tumors to be more invasive and perhaps establish themselves more efficiently. One of the tumors with strong PD-L1 expression was responding on ACT, but the one with moderate response did not show any regression after TILs injection. The second one with strong PD-L1 positivity did not have any autologous TILs expanded and subsequently the ACT response could not be tested.

The mutation analysis showed that all the eleven tumors established in NOG mice were KRAS positive.

Considerations

The aim of the PDX study to create a PDX model that can constitute a translational research ground for pancreatic cancer was accomplished. The mouse models were functional for general ACT studies. The take rate was 38 per cent in our study and that is well comparable to earlier studies [(263)]. Due to the dense and fibrotic stroma, the pancreatic tumors sometimes can be more difficult to grow in mice. It took several months, up to one year, for the tumors to establish and grow in the mouse models. The long-time span ranged from 60 to 400 days for the tumors to take and increase in size. The results indicated a possibility to identify patients where immunotherapy could be an option in pancreatic cancer. Even though the step between response in mouse model and response in a human is not insignificant. In specific circumstances it might be possible to achieve a treatment response of immunotherapy in pancreatic tumors. In three out of six, fifty per cent, of the hIL2-NOG mice models, a reduction in the tumor size could be seen after ACT. This opens for the opportunity to individualize treatment for pancreatic cancer. The PDXv2 model can also be used in future research to understand the background of and try to overcome the resistance to immunotherapy in the pancreatic tumors.

4.3 EXTRACELLULAR VESICLES – PAPER III

The pathology examination of the six patients included in the study identified three PDAC, one ampullary adenosquamous carcinoma, one fibrosis and one chronic pancreatitis. EVs were isolated from the tumor and non-tumor pancreatic tissues of the three patients with PDAC. Analysis of the EVs protein content with TMT mass spectrometry could verify 6861 proteins and the quantification was successful for 6113 proteins.

The DAVID analysis of the quantified EV proteins revealed that the accumulating cellular components were uppermost associated with the top GO terms “extracellular exosome” and ” membrane”. A considerably number of well-known EV proteins was identified among the quantified EV proteins from the pancreatic tumor and non-tumor tissue. All this combined indicate that the EV isolation process was successful and real EVs proteins were quantified. There was a difference in the protein expression between the tumor and non-tumor EVs. In the EVs from the tumor tissue 837 proteins were significantly upregulated and 173 downregulated, when tumor and non-tumor tissue derived EVs were compared. The upregulated EV proteins from the tumor tissue were associated with proteins known to be present in pancreatic cancer.

Considerations

Indeed, there were very few patients in this study, but the results are still interesting to discuss. First, EV isolation from pancreatic tissue was proved to be possible, which is the most important. There was a difference between the types of proteins upregulated in the EVs from the pancreatic tumor tissue compared to the non-tumor. The proteins themselves were different and the proteins from the tumor tissue EVs were associated with pancreatic cancer. This demonstrates an opportunity to separate tumor EVs from non-tumor, which is important, both in studies considering biological functions, but also when potential EV tumor markers are validated.

One of the most upregulated EV proteins in comparison between tumor tissue and non-tumor tissue, Anoctamin-9, is known to be overexpressed in pancreatic adenocarcinoma and is associated with tumor progression and worse prognosis [(264)]. Anoctamin-9 might have the right qualities to be valuable as a tumor marker.
There were also upregulated, and downregulated biological processes connected to the tumor tissue derived EV proteins of interest for further studies. The MHC-1/TAP pathway was downregulated, which in earlier

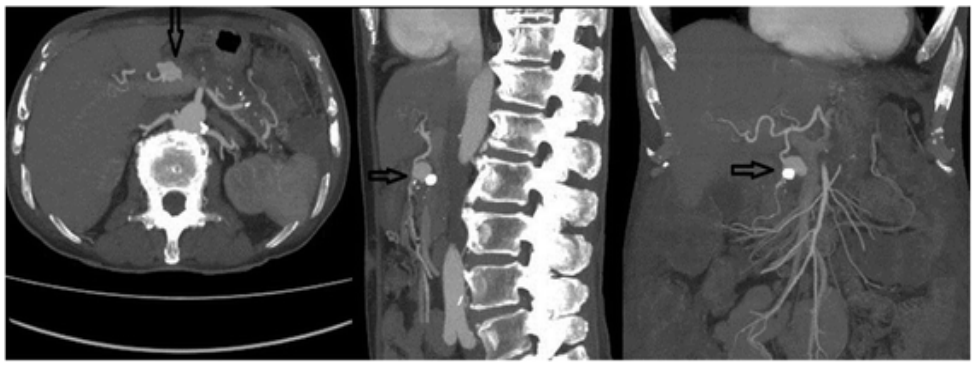
studies of pancreatic cancer have been seen to cause an impaired immune system [265]. "Nonsense-mediated mRNA Decay (NMD)", "Signal recognition particle (SRP)-dependent cotranslational protein targeting to membrane" and "Endoplasmatic reticulum (ER) to Golgi vesicle-mediated transport" have earlier been recognized to be involved in cancer progression. Although these complicated processes are not fully known, upregulation, as in our study, are connected to EMT, metastasis, aggressive growth and inhibition of tumor suppressor genes, which might impede the effect of immunotherapy [266-268].

The normal expression pattern of most proteins in EVs is not known, which make it difficult to determine if they are truly up- or downregulated. The same problem exists considering the biological processes, where it is unknown what an up- or downregulation in fact means. These problems are present in all EV studies though and the knowledge increase continuously when more research contributes. The findings in this study will be interesting to follow up in the future.

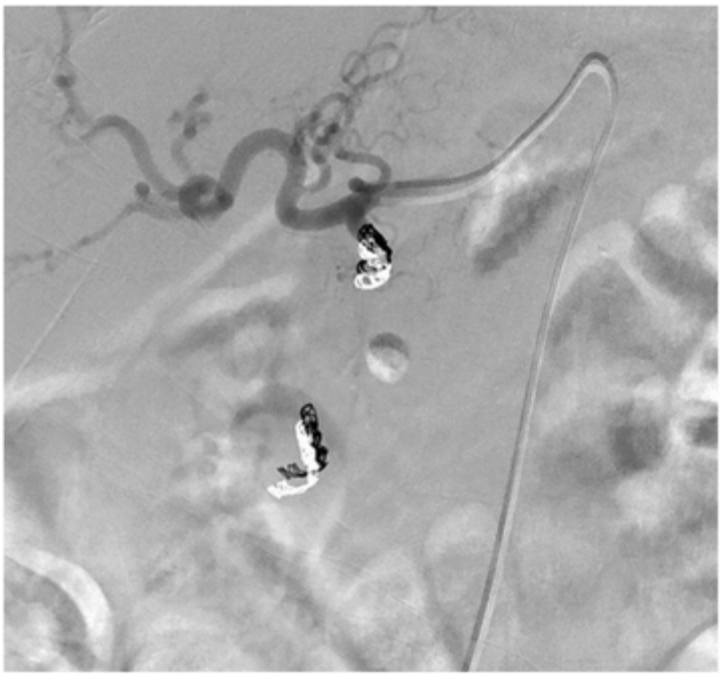
4.4 POSTPANCREATECTOMY HEMORRHAGE – PAPER IV

CRP postoperative day 5/6 with median 150mg/L were significantly associated with PPH C in the multivariable analysis. No one of the other evaluated factors could be proved significant. All patients in the PPH C group had POPF-C, but all of them were unknown when the bleeding started. Postoperative biochemical leak (amylase) and POPF-B were not associated with PPH C, since there were no significant differences between the groups. One third of the patients in the PPH C group had normal postoperative drain amylase values. In two patient's biliary leakage were the reason behind the PPH C, no POPF could be verified. No other casual factors besides bile and amylase leakage in the abdomen could be verified. There were no differences in the number of PPH C occurring in the patients depending on type of anastomosis; pancreaticogastrostomy or pancreaticojejunostomy.

The pathology report verified eight pancreatic adenocarcinomas, two cholangiocarcinoma, six ampullary adenocarcinoma and one duodenal carcinoma in the PPH C group.



Preoperative computed tomography angiography showing a pseudoaneurysm of the gastroduodenal artery of 24 mm (arrows). There is a clear calcification of 11 mm, compatible with calcified thrombosis. Transverse, sagittal and coronal views from left to right



Postoperative angiography after selective catheterization of the proper hepatic artery: complete exclusion of the pseudoaneurysm sac after coiling of the gastroduodenal artery.

Both illustrations above were published in: Abdelgabar, A., d'Archambeau, O., Maes, J. et al. Visceral artery pseudoaneurysms: two case reports and a review of the literature. J Med Case Reports 11, 126 (2017). https://doi.org/10.1186/s13256-017-1291-6 License permission: http://creativecommons.org/licenses/by/4.0/

The PPH C was verified between postoperative day 8 and 37, with a median of 16 days. A sentinel bleed occurred in thirteen of the patients and most often the hepatic artery and its branches were the source of the bleeding. In the PPH C group there were five patients (29%) who died within 90 days from the operation and in the control group four patients (6%) died. The remnant pancreas was removed in eleven of the patients because of completely detachment from the stomach or intestine, continuing bleeding or failure to establish proper drainage.

The mortality rate was lower in the PPH C subgroup treated with angiographic intervention (14%) compared to the subgroups with surgery (43%) or a combination of surgery and angiography (33%). The incidence of PPH C was 3%.

Considerations

CRP has been confirmed as a predictor of complication after pancreatic surgery in many studies. Connections between high postoperative CRP and fistulas and infections are acknowledged, often very early within the first three days [269-271]. One study has just like ours defined CRP 150g/L on day 5 as the most reliable day for detection of complications [272]. Computed tomography (CT) is most often used to verify different complications. Something that might be helpful considering defining the pathology is that the cut of is at day 5/6 when the computed tomography (CT) picture often is easier to distinguish from ordinary postoperative findings [273, 274]. A high quality CT scan is considered to be the best examination modality to detect postoperative complications [275].

Drains are often routinely placed in the abdomen during PD, to try to avoid or detect complications like POPF and PPH C. The advantage of drain usage is under debate, though randomized control studies have shown contradictory results [276, 277]. In this study drains could not prevent the emergence of PPH C and POPF-C. If the amount of PPH C were reduced or not due to drains is not known. The PPH C frequency of 3 per cent in this study is in parity with earlier reports [243, 278].

The lower mortality rate in the angiographic intervention subgroup compared to the emergency surgery group is consistent with other studies [244, 278]. Our findings support the recommendation of angiographic intervention as the first hand alternative to handle late postoperative bleedings [279]. Interventional radiology has shown excellent results in almost every area in has been

introduced in, whereas vessels, organs and cavities throughout the whole body can be reached [280].

It is known that soft pancreases are related to a higher risk of POPF [281] and there is a risk score, fistula risk score (a-FRS), using the pancreatic consistence, the width of the duct and BMI as risk factors [282]. In our study the width of the duct and consistence of the pancreas was not assessed and that is a limitation. Considering the pathology examination of the PPH C patients in our study, the ampullary adenocarcinomas were overrepresented compared to how common they generally are, six out of seventeen. There was also one duodenal carcinoma. One can speculate that, when the center of the tumor is at a relatively long distance from the pancreatic remnant, it might increase the probability of the remnant to be soft. There is thereby a possibility that soft pancreases might be overrepresented among the PPH C patients in our study.

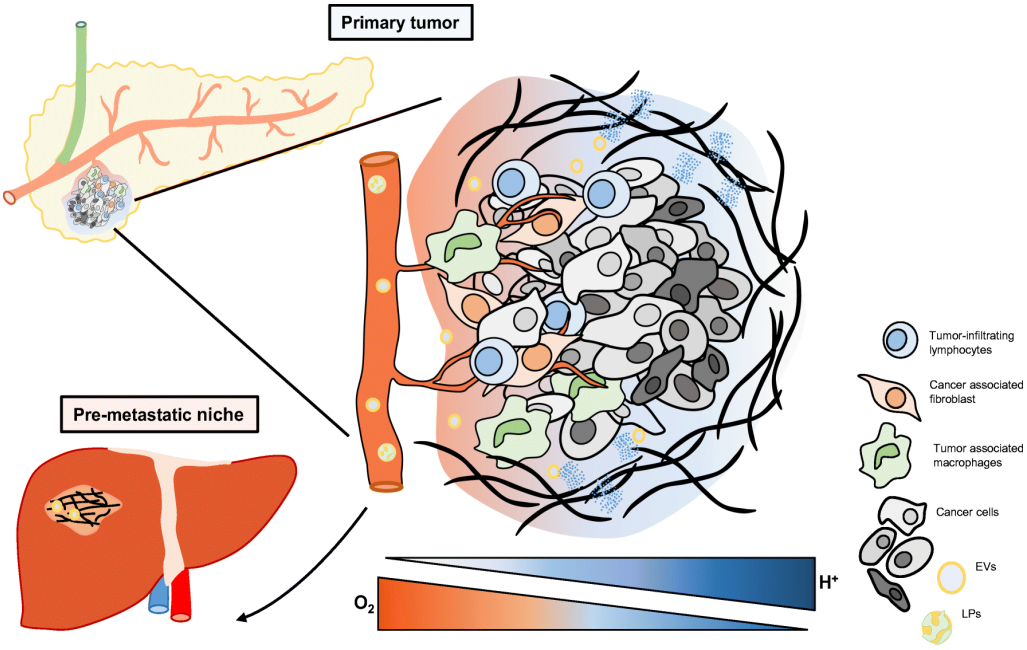
5 GENERAL DISCUSSION

The overall aim of this study to establish a translational network for pancreatic cancer research in order to improve diagnostics and treatment of pancreatic cancer was accomplished. This thesis may hopefully be a contribution to diagnostics improvements by identification of tumor markers for early detection and disease progression with the help of methods developed and results presented in the CTC and exosome studies. Treatment advancements can be achieved by testing potential therapies in the PDX models and by the survey of surgical complications to improve the caretaking of these patients.

The main problem is the devastating prognosis, the patients diagnosed with pancreatic cancer and everyone committed to take care of these patients, must face. The picture that has emerged during the work with this thesis is that it all seems to come down to the issue with the exceptional unfriendly TME that exists in pancreatic tumors. This thick, fibrotic and impermeable stroma, which obstruct any treatments and the immunosuppressive environment, where active immune cells becomes disarmed and aggressive tumor promoting cells can develop and take over [283]. Unfortunately, this is one of the attributes that characterize pancreatic cancer and makes it one of the most complicated malignancies to treat. This, in combination with difficulties to detect the disease in time does not improve the odds.

There is a possibility that the spread of tumors increases during the surgical exploration, which the result in the CTC study also might indicate. Several other studies have also been able to detect CTC in the portal blood and a connection with metastatic disease and prognosis has been verified [261, 262]. Perhaps an enhanced establishment of metastatic CTC clusters in the liver or lungs occur during surgery. If the spread is increased perioperatively because of intensification of tumor cells entering the blood stream, due to manipulation of the tumor is not proved, but it is described in the literature [284-286]. The extent of surgical trauma has been verified to correlate with the amount of CTC in the portal blood in pancreatic surgery [287]. Dissemination during surgery has also been seen, when port-site recurrences unfortunately have been noticed after laparoscopy [288, 289]. The increased inflammatory response and immune suppression that surgical trauma induce, might lead to a tumor spread both peri- and postoperatively. It is possible that, during this period of time, CTC obtain better preconditions to establish a functional pre-metastatic niche in the liver and lung compartments [290, 291]. Not a nice thought indeed, but still something that is necessary to consider. There are

indications that CTC prefer to disseminate to affected or damaged regions in the body, for example wounds, infections, inflammatory sites with tissue trauma like surgical areas [290, 292]. In the PPH C study, four patients in the control group died within 90 days from surgery, two of them due to recurrent disease with metastasized cancer. The patients with very early recurrence with extensive metastasis might be due to a surgical suppression of the immune system, which enable the cancer to spread fast.



Schematic overview of the tumor microenvironment. Cancer cells and their stroma together shape a complex environment, a process that is largely facilitated by adaptive responses to hypoxia and acidosis. A close interplay between stress-adapted cancer cells, ECM components, and stromal cells drives disease progression by tissue remodeling in the primary tumor as well as in distant tissues that form the pre-metastatic niche, here exemplified by pancreatic cancer dissemination to the liver. ECM extracellular matrix, EVs extracellular vesicles, LPs lipoproteins. Published in: Bång-Rudenstam, A., Cerezo-Magaña, M. & Belting, M. Pro-metastatic functions of lipoproteins and extracellular vesicles in the acidic tumor microenvironment. *Cancer Metastasis Rev* 38, 79–92 (2019). https://doi.org/10.1007/s10555-019-09786-5. License CC BY-4 http://creativecommons.org/licenses/by/4.0/

To try to prevent inflammatory and immunocompromising events and tumor dissemination, different strategies to avoid them have been carried out. With immune modulators like IL-2 and interferon alpha (IFN-α) less postoperative immune suppression have been seen [293, 294]. COX-2 inhibitors and β-blockers could both prevent surgically trigged immunodeficiency, local tumor infiltration and metastasis [295-297]. Neoadjuvant or close postoperative chemotherapy has also been tried with mixed results. Although some of the mentioned treatments seem promising, long-term outcomes have not been studied and that will be needed before clinical implementation is accepted. To introduce and establish an enhanced recovery after surgery (ERAS) protocol have been seen to reduce postoperative inflammation and has in follows up studies even been confirmed to improve overall survival of colorectal cancer [298, 299].

In the PDX study the tumors in the mice decreased in size after ACT treatment. Even though, there is a wide range between the reactions in mice and humans, it still gives some hope that during certain circumstances, when active or activatable immune cells still are present, immunotherapy could be effective.

The most extensive study of long-time survivors of pancreatic adenocarcinomas included 431 patients that had survived 10 years or longer after an operation with curative intention. Although the conclusion was that the patients' long-time survival was connected to pathologic T and N-stage; lymph node positivity and tumor size, many patients did not fit into that picture. Considering the T and N- stage, 56 per cent had T3 or T4 tumors, and only 18 per cent were T1. Regarding lymph nodes, 49 per cent were lymph node positive and the tumor size was smaller than 2 cm in only 20 per cent of the patients [300]. Accordingly, a relatively large amount of the patients had more advanced T-stage and lymph node spread among the 10 years survivors. There might therefor exist other underlying or contributing causes explaining the long-time survival. Patients with dominance of T and B cells in the tumors compared to immunosuppressive cells seem to have a better prognosis [301]. Long-time survivors of pancreatic cancer, with a median postoperative survival time of six years, were found to have infiltration of plenteous of cytotoxic t cells in the tumors and a presence of high numbers of neoantigen compared to the patients with a shorter survival time [302]. It creates optimism that it seems to be possible for the immune system to fight pancreatic cancer under certain circumstances.

What also looked promising is the future of EVs. The results in the EV study also pointed in the directions of immunosuppression. When the EV proteins

from the pancreatic tumor where compared with non-tumor tissue, the connected pathways were foremost involved in immune inhibition, tumor progression, EMT and metastasis. The same pattern over again. There are several potential exosomal tumor markers of pancreatic cancer already suggested. So far, no biomarker has fulfilled the criteria's to be used clinically, but there seems to be possibilities of it to happen in the future. Glypican-1 is one of the proteins, overexpressed on the surface of exosomes derived from pancreatic cancer cells that have been of most interest so far [112, 303]. Anoctamin-9, found to be upregulated in pancreatic tumor tissue EVs in our study, has been detected to be overexpressed in pancreatic cancer cells and also correlate with worse prognosis [264]. Mutations of the DNA in the exosomes and exosomal microRNA have also been of great interest. A combination of different EV surface markers, might otherwise be a way to success. Then not one protein needs to have both high sensitivity and specificity, since the combination may give more precise results. This has been tried and seems promising [304].

The progress the last years in nanotechnology is fascinating. The development of the mRNA vaccines, coated in synthetic lipid nanovesicles is a major step forward [305]. The technology to enclose medication into EVs is not here yet, but the proceeding progress in nanotechnology is promising. There are many advantages with natural nanotransporters, as EVs compared to synthetic. The natural are more immune friendly and can hopefully be selective in targeting, compared to the synthetic that is unspecific, at least so far. EVs are also naturally circulating in the blood and are stable there. They can penetrate natural barriers like the blood brain barrier, tissue-, cellular- and intracellular barriers, which often is problematic for synthetic nanotranporters. The EVs are natural transporters, deliverers and can release cargo in the cytoplasm of other cells [306]. They are well prepared for a future of targeted deliveries.

Considering PPH C, the need for a PPH C study with this focus can be discussed. The cause of PPH C were in this study exclusively fistulas with leakage of bile or amylase. It is not certain though that all undetected leakages create bleedings. Bleedings were seen in patients with remaining drains, POPF-B, in the PPH-C group and some patients with POPF-B and remaining drains, without bleedings, were among the patients in the control group. Other factors can be involved, although not verified in this study. Due to those facts, it was interesting to perform this study with the perspective and focus on PPH C instead of pancreatic fistulas.

Latent fistulas have been described in the literature before, when the drain amylase is normal, but a fistula develops or become symptomatic later. The symptoms were worse in these patients with more severe infections, bleeding and need of intensive care [307]. This is consistent with what happened to many of the PPH C patients in our study and constitutes the main problem, not to know who are leaking and which one of these patients will start to bleed. The CRP level determined in the PPH C study gives some directions. In our study 22 per cent of the patients had CRP levels above 150mg/l and 47 per cent of them had PPH C. There were 15 per cent in the control group that had CRP above 150mg/l and 53 per cent in the PPH C group. If this study is applied on the whole group of PD patients and everyone with CRP above 150mg/l would get a CT scan, it would be seven CT scans every year in average. Nowadays the numbers of PD have increased, why it would be approximately twenty CT scans per year to be able to detect and have a chance to avoid almost 50 per cent of the patients with future PPH C.

The pancreatic remnant was removed in the majority of the PPH C patients in our study, mainly of preventive reasons, to avoid further complications. The rescue operation in our center is performed when the bleeding complication is addressed, and the bleeding has stopped. Rescue pancreatectomy is often connected to high mortality in the literature, although it is mostly described due to POPF complications and not selectively PPH C [308, 309]. In our clinic the mortality has been directly connected to the bleeding complications and not the rescue afterwards. Our experiences of leaving the remnant in place have often been additional bleedings and infectious complications. It can be difficult to establish well-functioning drainage in the area of the pancreatic remnant, though it is placed an inaccessible part of the abdomen. The argument to remove the remnant is also that many of the patients are seriously ill after a bleeding episode and might not endure further complications. Arguments to retain the remnant are naturally the use of foremost the endocrine pancreas and to avoid an extra operation. The re-operations to remove the remnant are often difficult and surgically demanding. There is evidence that different mortality rates between hospitals not is due to the amount of complications, but failure to save the patients [310]. The failure to rescue has in studies been higher in low volume centers of pancreatic surgery compared to high volume centers [311]. In that perspective, except for prevention of complications, the most important is to have an action plan when complications occur.

6. CONCLUSIONS

The overall aim of the thesis to create a platform for translational pancreatic cancer research with collection of blood and tissue was accomplished.

- CTC was detected in blood perioperatively and the numbers of CTC were higher in portal blood compared to peripheral blood.

- PDX models, with NOG and hIL2-NOG mice, for pancreatic cancer research were established and the results indicates that immunotherapy in pancreatic cancer can be effective during certain conditions.

- Extracellular vesicles could be isolated from pancreatic tissue and have a potential to become useful tumor markers and to reveal information about the biological processes of tumor growth and metastasis.

- High postoperative CRP day 5 or 6 after operation can be a predictor of PPH C and this knowledge may hopefully lead to a possibility to avoid this complication.

7 FUTURE PERSPECTIVES

My dream is that during my lifetime the prognosis of pancreatic cancer will be the opposite of today; an eighty per cent curable disease. The research field of pancreatic cancer increases every year and we need to keep fighting to find a way to beat the odds we have now.

I believe that we need to penetrate the tumor microenvironment and break though the guards around the pancreatic cancer to be able to cure the disease. During the work with this thesis I have been increasingly convinced that this is the key to success. The immune system is almost neutralized by the pancreatic cancer in most patients and taken over by immunosuppressive cells. At the same time the immune system contains great forces that have proved to be able to make a difference in other diseases, when supported in the right way.

To be able to accomplish this, we need to know more about the mechanisms behind the immunosuppressive establishment and how the dissemination of the disease develops, though the tumor microenvironment changes during this process. The future for the CTC project is to isolate CTC and proceed with single-cell analysis. The prospect is to examine the possibilities to identify CTC clones with metastatic attributes. Single cell analysis could give information about mutations in the CTC and which type of proteins, receptors and genes they express. The information from these cells may contribute with knowledge considering the pre-metastatic niche formation and it might also be possible to understand how to inhibit them, by for example blocking their receptors.

Considering future studies with the PDX models, there are almost endless of opportunities. It is possible to combine the ACT with chemotherapy, other immunotherapies or cancer vaccines to optimize the treatment response and receive effect in more patients. The therapy can also be directed to the patients that seem to have better preconditions to respond to the therapy. This can be determined by immunohistochemistry of PD-L1 and MSI or RNA and genome sequencing, as indicated in our present study. We are also looking into the possibility to include patients with metastatic disease and favorable immune profile, PD-L1 or MSI high expression, in a clinical ICI trial.

In the EVs research project we first need to confirm our findings in a larger patient material, which is already ongoing, and the inclusion is recently finished. If adequate tumor markers or protein expressions significant of

pancreatic tumor tissue are identified, we will continue with EV studies of blood samples. To succeed with the recognition of pancreatic cancer tumor markers in blood, would make the dream of a liquid biopsy come true.

A great deal of interesting information regarding EVs, considering protein content and their associated biological pathways are also revealed in EV studies. We receive indications of their functions in the body, which contribute to the puzzle necessary to complete to understand the cancer process and then in the extension how to attack pancreatic tumors.

Maybe it will be possible to promote the immune system and to come around the immunosuppressive attributes of pancreatic malignancies eventually. Although the immunotherapy has not been successful so far, hopefully we can learn more to make it a useful treatment in the future.

I believe in the evolvement of all the methods described in this thesis. The extracellular vesicles have good chances to become both detectors of early cancer and deliverers of targeted therapy. CTC studies can give important information of tumor progression, the TME and the metastasis process. For research considering new treatments and detection of the tumor's characteristics, the PDX models are important.

To be able to work with pancreatic cancer, you must be optimistic and see the opportunities. There is so much to do and so much more to understand, and research left to be uncovered. There are so many patients that are fighting and have been fighting pancreatic cancer. We need to keep trying to beat the odds until we succeed.

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