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Analysis of tumor-specific mutations in liquid biopsies

Exploring the value of circulating tumor cells and circulating tumor DNA for early diagnosis in pancreatic cancer

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Für meine Oma

Summary

Pancreatic cancer (PC) is a devastating disease. Despite decades of research, resection of the tumor is still the lone curative treatment only feasible in early stages of PC. Diagnosis is usually at inoperable late stages due to unspecific symptoms and the lack of minimally invasive sensitive diagnostic techniques. Highly invasive tumor biopsy is the gold standard for diagnosis but difficult to obtain in PC. An alternative tool might be blood-based liquid biopsies which represent the disease systemically by minimally invasive blood draw. In contrast to tumor biopsies, liquid biopsy can be repeated and therefore might have the potential for sequential early diagnostics. A peripheral blood sample contains e.g. circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) originating from the primary tumor or metastasis. In this thesis, the diagnostic sensitivity of highly prevalent *KRAS* (Kirsten rat sarcoma viral oncogene homolog) mutations was evaluated in both ctDNA and CTCs in early stages of PC. In a first approach, after establishment of an optimized workflow isolation of synthetic mutant alleles spiked in plasma following subsequent mutation detection by digital PCR revealed that a minimum of 5 mutant alleles could be detected from plasma. Fifty samples each of plasma and matched tumor tissue from fifty PC patients diagnosed predominantly at early stages were analyzed for *KRAS* mutations. In 35% of cases the mutation detected in tumor was concordantly found in plasma. The analysis of ctDNA provides increased minimally invasive sensitivity as potential early diagnostic marker in PC. In a second approach, an EpCAM (epithelial cell adhesion molecule)-based (IsoFlux) and size-based method (automated filtration unit by Siemens) were compared for isolation of different PC cells whereby filtration was superior. A workflow for subsequent downstream analysis by digital PCR was established to detect mutant *KRAS* in isolated single CTCs. Frozen CTC-enriched diagnostic leukapheresis products (DLA) from patients diagnosed with PC were then analyzed for CTC number. 42% of patients were CTC-positive therein 40% in non-metastatic patients providing a storable medium of CTC-analysis. All tested CTCs were *KRAS*-negative concordant to the analyzed tumor. As a continuative excursus, CTCs were positively evaluated for monitoring and application to personalized medicine in advanced stages of lung cancer. Increased CTC numbers were obtained by an *in vivo* isolation technique. Additionally, mutations in CTCs for monitoring of therapies were successfully detected. In summary, these findings suggest ctDNA as a sensitive, minimally invasive early diagnostic marker for PC outperforming CTCs in terms of clinical utility and feasibility. The value of these findings especially in samples of apparently healthy individuals should be the aim of future research.

Zusammenfassung

Pankreaskarzinom (PK) ist eine fatale Erkrankung. Trotz jahrzehntelanger Forschung ist eine Resektion des Tumors bisher die einzige kurative Behandlung, die nur in frühen Stadien des PK möglich ist. Die Diagnose erfolgt meistens in inoperablen späten Stadien auf Grund von unspezifisch auftretenden Symptomen und Mangel an minimal-invasiven sensitiven Diagnosetechniken. Hoch-invasive Tumorbiopsie ist der Goldstandard für die Erstellung der Diagnose, welche allerdings in PK schwierig zu erhalten ist. Eine Alternative könnten Blut-basierte Analysen sein, welche die Krankheit systemisch durch minimal-invasive Blutentnahme repräsentiert. Im Gegensatz zu Tumorbiopsien kann die Flüssigbiopsie (Liquid Biopsy) wiederholt werden und hat daher das Potential für eine sequentielle Frühdiagnose. Eine periphere Blutprobe enthält z.B. zirkulierende Tumor DNA (ctDNA) und zirkulierende Tumorzellen (CTCs), welche vom Primärtumor oder einer Metastase stammen. In dieser Arbeit wurde die diagnostische Sensitivität von hoch-prävalenten *KRAS* (Kirsten rat sarcoma viral oncogene homolog)-Mutationen sowohl in ctDNA als auch in CTCs in Frühstadien des PK evaluiert. In einem ersten Ansatz ergab die Isolierung von synthetisch-mutierten Allelen, die in Plasma überführt wurden, mit anschließender Mutationsdetektion durch digitale PCR, dass fünf mutierte Allele über einen optimierten Workflow detektiert werden können. Fünfzig Plasma- und zugehörige Tumorproben von Patienten in überwiegend frühen Stadien des PK wurden auf *KRAS*-Mutationen analysiert. In 35% der Fälle wurde die Mutation im Tumor ebenfalls im Plasma detektiert. Die Analyse von ctDNA zeigt damit eine gesteigerte minimal-invasive Sensitivität zur potentiellen Frühdiagnose in PK auf. In einem zweiten Ansatz wurde eine EpCAM (epithelial cell adhesion molecule)-basierte (IsoFlux) Methode mit einer größenabhängigen Methode (automatisierte Filtration durch Siemens-Einheit) auf die Isolierung von PK-Zellen verglichen, wobei die Filtration überlegen war. Arbeitsschritte für anschließende Mutationsanalysen durch digitale PCR wurden etabliert, um mutiertes *KRAS* in isolierten Einzel-CTCs zu bestimmen. Gefrorenes Produkt der CTC-angereicherten diagnostischen Leukapherese (DLA) von PK-Patienten wurde auf CTC-Anzahl getestet. In 42% der Patientenproben konnten CTCs detektiert werden, wobei 40% in nicht-metastatischen Patienten isoliert werde konnten. Gefrorenes DLA-Produkt stellt damit eine lagerbare Quelle für CTC-Analysen dar. Alle getesteten CTCs waren übereinstimmend mit dem Tumor *KRAS*-negativ. In einem weiterführenden Exkurs wurden CTCs für das Monitoring und die Anwendung in personalisierter Medizin in fortgeschrittenen Stadien des Lungenkrebs positiv evaluiert. Erhöhte CTC-Zahlen wurden durch eine *in vivo* Methode erhalten. Zusätzlich wurden Mutationen in CTCs zur

Überwachung der Therapie erfolgreich detektiert. Zusammenfassend zeigen die Daten, dass ctDNA ein möglicher diagnostischer, sensitiver und minimal-invasiver Marker ist, der CTCs in klinischem Nutzen und klinischer Durchführbarkeit in PK überlegen ist. Der Wert dieser Ergebnisse sollte in weiterführenden Studien besonders an Proben von klinisch unauffälligen Personen vertieft werden.

List of abbreviations

BSA	bovine serum albumin
CA	carbohydrate antigen
CAPP-Seq	cancer personalized profiling by deep Sequencing
CD	cluster of differentiation
CDKN2A	cyclin-dependent kinase Inhibitor 2A
CEA	carcinoembryogenic antigen
CEP	centromere of chromosome
cfDNA	circulating free DNA
CI	confidence interval
CT	computed tomography
ctDNA	circulating tumor DNA
CTCs	circulating tumor cells
°C	degree Celsius
DLA	diagnostic leukapheresis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dPCR	digital PCR
EDTA	ethylenediaminetetraacetic acid
EGFR	epithelial growth factor receptor
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
EUS-FNA	endoscopic ultrasound fine needle aspiration

FBS	fetal bovine serum
FDA	Food and Drug Administration
FFPE	formalin fixed, paraffin embedded
FOLFIRINOX	leucovorin, 5-fluorouracil, irinotecan and oxaliplatin
HSA	human serum albumin
ISET	Isolation by Size of Tumor Cells
KRAS	Kirsten rat sarcoma viral oncogene homolog
M0	TNM-staging: no metastasis
M1	TNM-staging: distant metastasis
MEN1	multiple endocrine neoplasia 1
MET	mesenchymal-epithelial transition
µ	micro
min	minute/s
mL	milliliter
mm	millimeter
MRI	magnetic resonance imaging
ND	not determined
ng	nano gram
NIH	National Institute of Health
NSCLC	non-small cell lung cancer
OS	overall survival
%	percent
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDAC	pancreatic ductal adenocarcinoma
PSA	prostate specific antigen
RAS	rat sarcoma
SCLC	small cell lung cancer
SMAD3	mothers against decapentaplegic homolog 3
SMAD4	mothers against decapentaplegic homolog 4
TAM-Seq	Tagged Amplicon Sequencing
TKI	tyrosine kinase inhibitor
TP53	gene encoding protein 53
WGA	whole genome amplification

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1 Introduction

1.1 Cancer

Cancer is the second leading cause of death worldwide presenting with more than hundred different types (Stratton et al., 2009; World Health Organization, 2017). The majority of cancers derive from epithelial tissue: the most prominent types are breast cancer in woman and lung cancer in men (World Health Organization, 2017). Despite tremendous research on therapies, the number of diagnosed cases will rise over the next decades due to the aging societies and growing populations (Torre et al., 2016). Cancer develops from the uncontrolled proliferation of formerly healthy cells forming a tumor. A tumor is described as a malignant or benign mass of cells. In contrast to benign tumors, malignant tumors are able to spread systemically around the body by entering the blood stream and invading other tissues.

1.1.1 Carcinogenesis

The complex evolution from normal, healthy tissue to malignant, metastasized tumors is called carcinogenesis. Different models of carcinogenesis have been reviewed (Vineis et al., 2010). In this thesis, carcinogenesis is defined as the classic genetic, multistep process and the Darwinian cell selection of mutated cells (Figure 1).

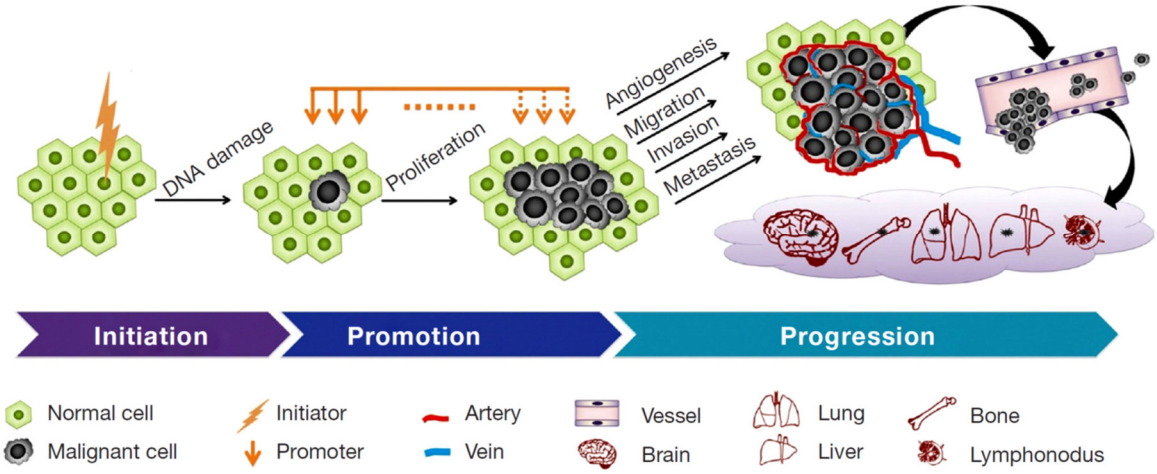

Figure 1: Carcinogenesis. Carcinogenesis is initiated by DNA-damaging initiators (Initiation). This DNA-damage is stably passed to daughter cells during mitosis. Once initiated cells are exposed to DNA-damaging promoters, carcinogenesis advances. The cells accumulate DNA mutations and the malignant transformation progresses (Promotion). The formed tumor can promote angiogenesis and subsequent migration from the primary site invading into distant organs leading to metastasis (Progression). This model shows a linear progress of the development. It is a classic theory to show the multi-event progress to tumor formation. However, the development towards malignancy is highly variable (modified after Liu et al., 2015).

Carcinogenesis is introduced by an initiator, e.g. chemical agents, physical exposure, or virus causing DNA damage by somatic mutations (Initiation) (Loeb and Harris, 2008). During mitosis, mutations are stably passed to the genome of daughter cells. The survival of the mutant cells is determined by Darwinian selection: only cells with survival benefit and autonomous growth advantages over surrounding cells are selected, thus resulting in clonal expansion of these cells (Stratton et al., 2009). Tumor formation progresses as other agents (Promotors) stimulate growth in initiated cells by e.g. alteration of gene expression or by binding to receptors (Promotion) (Liu et al., 2015). During promotion, somatic mutations accumulate leading to uncontrolled cellular growth (neoplasm) and clonal expansion of different subclones (Greaves and Maley, 2012). All malignant cells harbor the same characteristics like evading apoptosis, tissue invasion and limitless replicative potential (Hanahan and Weinberg, 2000; 2011). Malignant transformation progresses to a metastatic disease (Progression) which is the ultimate cause of cancer-related death (Fidler, 2003).

The transition of a healthy cell to a tumor cell is highly variable. Initiation and promotion are not always linear as shown in Figure 1. Recent publications have shown that activation of the early oncogene *KRAS* (Kirsten rat sarcoma viral oncogene homolog) is needed for both initiating and maintaining of the tumor (Collins et al., 2012). Therefore, the definitions of initiation and promotion often merge, as single steps do not always cover the complex carcinogenesis.

1.1.2 Cancer mutations

During carcinogenesis, somatic cancer mutations accumulate in the genome. Cancer mutations are grouped into two different types based on their consequences for the development of cancer: passenger and driver mutations (Stratton et al., 2009). Passenger mutations do marginally affect the progress of the disease. However, driver mutations are positively selected for growth advantages in the microenvironment and force the progression towards cell growth and malignancy.

Two types of mutations act as driver for malignant transformation: oncogenes and tumor suppressor genes. Oncogenes arise from activating mutations in their proto-oncogenes (gain of function). Oncogenes encode for proteins involved in excessive and uncontrolled growth like e.g. transcription factors, apoptotic regulation, growth factors and their receptors (Croce 2008). Mutations in proto-oncogenes are either point mutations

resulting in a slightly different protein that is constitutively active or gene amplification and chromosomal rearrangements, that result in the same protein but higher expression (Lodish et al., 2000). Point mutations and chromosomal rearrangements occur early during carcinogenesis, whereas gene amplification appears during progression of the disease (Croce 2008). Oncogenes are dominant meaning that one mutated allele out of the two is sufficient for induction or progression of cancer (Lodish et al., 2000). Mutations in the *RAS* (rat sarcoma viral oncogene homolog) gene are an example of an oncogene. *KRAS* is the most prominently mutated *RAS* gene in cancer types and occurs early during carcinogenesis (Baines et al., 2011) providing a possible mutation for early diagnostic.

Tumor suppressor genes express proteins involved in many cellular processes, e.g. cell cycle checkpoint control or control of mitogenic signaling (Sherr, 2004). Mutations in tumor suppressor genes are usually deletions or point mutations that result in no or a non-functional protein (loss of function). Mutations in tumor suppressor genes are recessive. One mutant allele only renders a cell more susceptible to malignancy but does not advance the disease like oncogenes. Only the mutation of both alleles causes tumor progression (Lodish et al., 2000). The most prominent tumor suppressor gene is *TP53* (gene encoding protein 53) (Hollstein et al., 1991).

In summary, activated oncogenes lead to uncontrolled cellular growth and inactivated tumor suppressor genes to loss of growth control. One prominent example is the early oncogenic driver gene *KRAS* - a *RAS* gene that is frequently mutated in several cancers and which might hold the potential for an early diagnostic marker.

1.2 Cancer biomarker

A biomarker is a molecule that can be found in tissue and body fluids like blood or urine allowing to draw conclusions about a person's health condition (FDA-NIH Biomarker Working Group, 2016). The variety among biomarkers is high including proteins, nucleic acids, antibodies or pattern of these (Henry and Hayes, 2012). The presence or absence of a biomarker results in subgrouping of patients in biomarker positive or negative groups which are then guided to treatment according to the biomarker test results.

Biomarkers are grouped into several categories (definitions according to the FDA-NIH Biomarker Working Group, 2016). A prognostic biomarker determines the likelihood of a clinical outcome like relapse or disease progression of a patient. One example is the increase of PSA (prostate specific antigen) after prostatectomy in serum of prostate cancer patients. The population of diagnosed patients with increased PSA concentration shows worse overall survival compared to individuals with no increase of PSA (Roberts et al., 2001; Stamey et al., 1987). The detection of a prognostic biomarker does not aid the physician to select a target-specific therapy, but might help to consider other therapy options. The earlier the detection of a prognostic biomarker or a trend towards worse overall survival (e.g. increasing PSA-concentrations), the earlier the chemotherapy can be intensified.

On the contrary, a predictive biomarker aids patient management and helps to predict the response to a specific targeted therapy in terms of benefit or adverse effects. Unlike biomarker negative patients, patients with biomarker positive test results might benefit from a specific therapy. For example, *EGFR* (epithelial growth factor receptor)-mutated lung or colorectal tumors can be treated with EGFR-targeted therapy like erlotinib, gefitinib or cetuximab, but EGFR-negative tumors might not be affected by that therapy. Additional detection of *KRAS* mutations in *EGFR*-mutated lung or colorectal cancer is associated with poor response to this targeted therapy (Eberhard et al., 2005; Lièvre et al., 2006). KRAS constitutively activates the EGFR pathway which cannot be controlled by EGFR-targeted therapy (Langer, 2011). Therefore, *KRAS* mutations are a negative predictive biomarker for the success of targeted therapy in lung and colorectal cancer.

A diagnostic biomarker is used for screening or the early detection of a disease. One example is the characterization of mutant DNA in stool for the detection of colorectal cancer (Imperiale et al., 2014). The detection of mutated DNA in stool is associated with the presence of colorectal tumors that shed DNA in stool and leads to the beginning of therapy. Other biomarker categories include safety biomarker (likelihood of adverse effects before and after exposure to a substance), risk biomarker (the potential of developing a disease in currently healthy individuals), pharmacodynamic/response biomarker (occurrence of biological response when exposed to drugs) and monitoring biomarker (serial measurements to determine status of disease) (FDA-NIH Biomarker Working Group, 2016).

Despite these definitions, characterization of biomarkers is often not strict in the literature and types of biomarker may overlay. For example, PSA is a prognostic biomarker in

diagnosed prostate cancer patients, but also a monitoring biomarker for the identification of the prognosis. Likewise, *KRAS* is a predictive biomarker for EGFR-targeted therapy but also has prognostic meaning as its detection is associated with disease progression (Eberhard et al., 2005)

Regardless of the tremendous amount of newly published biomarkers, only a minority of them are in routine clinical use. In fact, badly validated biomarkers or the absence of clinical utility often results in mistreatment and - in the worst case - harm for the patient (Henry and Hayes, 2012).

1.3 Early diagnosis in pancreatic cancer

Pancreatic cancer is a very fatal cancer type making it the fourth leading cause of cancer-related death in western countries (Siegel et al., 2016; Swords et al., 2016). The number of pancreatic cancer patients rises due to the demographic change in the aging society. By 2030, pancreatic cancer will be the second leading cause of death after lung cancer (Rahib et al., 2014). Ninety-five percent of pancreatic cancer patients suffer from PDAC (pancreatic ductal adenocarcinoma), a type of cancer rising from the ducts of the exocrine part of the pancreas, so that the majority of pancreatic cancers are referred to this group of diseases (Becker et al., 2014). The prognosis of patients diagnosed with pancreatic cancer is poor and has hardly improved in the last decade. The 5-year overall survival rate is 8% and therefore very dismal (Siegel et al., 2016). This is also highly dependent on the stage as prognosis is worse in late stages (Siegel et al., 2016). The main known risk factors positively associated with pancreatic cancer are family hereditary, cigarette smoking and diabetes mellitus (Everhart and Wright, 1995; Fuchs et al., 1996; Klein et al., 2004). Several precursor lesions are also known to lead to pancreatic cancer over years, such as chronic pancreatitis, pancreatic intraepithelial or cystic neoplasms (Compagno and Oertel, 1978; Lohr et al., 2005; Malka et al., 2002).

The fatal progression of the disease is mainly due to several factors: first, early stages of pancreatic cancer are asymptomatic. Usually, symptoms for pancreatic cancer occur late in the disease progress and are mainly unspecific like appetite loss, abdominal pain and weight loss, thus delaying the diagnosis (Holly et al., 2004). Accordingly, patients are diagnosed late in the progression of the disease - usually at distant stage III or IV (Siegel et al., 2016). The staging of pancreatic cancer refers to stage I to IV. Stage I tumors have

not spread to lymph nodes or impacted surrounding tissue. Stage IIA tumors have dispersed beyond the pancreas, but have not spread to lymph nodes, whereas stage IIB tumors have. However, stage I and II are considered to not have invaded blood vessels and therefore are early stages. Stage III and V (metastatic) are late stages. The tumor has already invaded neighboring vessels and might have spread into distant body parts leading to metastasis (Allen et al., 2017). Only about 10% of the patients are diagnosed at early, non-metastatic and local stage eligible for resection of the pancreas (Siegel et al., 2016). Although surgery is the only curative treatment today, the majority of patients relapse with metastatic disease within the first year after resection of the tumor (Oettle et al., 2013). This leads to the hypothesis that pancreatic tumor cells have spread early and systemically (Rhim et al., 2012; Takai et al., 2015).

Second, druggable targets are rare in pancreatic cancer so that treatment options are limited. The most frequent driver mutations in pancreatic cancer are *KRAS*, *TP53*, *SMAD4* (mothers against decapentaplegic homolog 4) and *CDKN2A* (cyclin-dependent kinase Inhibitor 2A). Several other genes are affected at a low frequency (<5%) resulting in multiple molecular alterations in the tumor (Bailey et al., 2016; Takai and Yachida, 2016; Waddell et al., 2015). Prevalence of *KRAS* mutations in tumor independent of stage is reported from 56-100% (Jones et al., 2008; Sanger Institute, 2017). Therefore, *KRAS* mutations represent the majority of genetic aberration in pancreatic cancer. The high prevalence supports the role of *KRAS* as early driver of neoplastic transformation during carcinogenesis and might provide a good target for early detection of the disease. Within the *KRAS* gene the majority of mutations in pancreatic cancer are mutated at Exon 2, with the most frequent mutations being at G12D (49%), G12V (32%) and G12R (13%) (Sanger Institute, 2017). If *KRAS* is mutated, the pathway cannot be regulated, resulting in uncontrolled growth and promotion of the tumor. Consequently, *KRAS* mutations are the main target for therapy. However, as *KRAS* has been considered a nonactionable oncogenic driver so far without effective therapy, survival of the patients has hardly improved within the last 30 years (Garrido-Laguna and Hidalgo, 2015; Sun et al., 2014). Recent studies on chemotherapy, e.g. albumin bound paclitaxel plus gemcitabine in metastatic PDAC, have only modestly extended the overall survival with partly severe side effects compared to standard gemcitabine monotherapy (Von Hoff et al., 2013). A study on combination chemotherapy called FOLFIRINOX (leucovorin, 5-fluorouracil, irinotecan and oxaliplatin) in advanced pancreatic cancer reported extended overall survival. Due to its toxicity, its usage is limited to patients with good constitution.

The survival advantage was 11.1 months compared to 6.8 months with gemcitabine monotherapy (Conroy et al., 2011).

Additionally, the tumor content of the pancreas is low (Biankin et al., 2012). The tissue is densely packed with stromal cells (Boyd et al., 2009). Because of this desmoplastic microenvironment today's drugs might actually not reach the tumor tissue appropriately. Therefore, the influence of the microenvironment on therapy success comes to the fore for therapy development (Feig et al., 2012).

1.3.1 Biomarkers in pancreatic cancer

The evolution from stage I to stage IV pancreatic cancer takes around one year only, but the progression from healthy tissue to neoplasm takes about a decade allowing a feasible window for early detection of cancer (Yu et al., 2015). Today's possibilities for detection of pancreatic cancer are imaging and biomarker analysis. Their potential for screening and early detection is described in the following section.

Pancreatic cancer is usually diagnosed by highly-invasive EUS-FNA (endoscopic ultrasound fine needle aspiration), CT (computed tomography) or MRI (magnetic resonance imaging). EUS-FNA seems to be the most sensitive technique with low false positive rate especially for smaller tumors, however, it is also the most invasive method (Kulig et al., 2005; Rosch et al., 1991). Screening individuals with a hereditary risk for pancreatic cancer has been proven to identify neoplasia early, although the false negative rate of fine needle aspiration is up to 23% (Canto et al., 2004; Woolf et al., 2013).

To date, no sensitive and specific blood-based biomarkers for early detection of pancreatic cancers are in clinical use. The ideal screening assay would be minimally invasive, accurate through all stages, sensitive and specific. One possible, widely used serum marker is CA19.9 (carbohydrate antigen 19.9 or sialyl Lewis antigen). It is a tumor-associated glycoprotein that can be detected in serum by immunoassays (Del Villano et al., 1983). In already diagnosed patients the sensitivity (elevated level and cancer, mean: 78.2%) and specificity (low level and no cancer, mean: 82.8%) are reliably moderate but not accurate for diagnosis (Poruk et al., 2013). One large study by Kim et al. (2004) including 71 000 individuals evaluated that measurement of CA19.9 is not useful for screening asymptomatic persons. Patients with elevated CA19.9 level were followed and only 4 of the cohort developed pancreatic cancer. The poor clinical utility

was also reported by other studies (Chang et al., 2006; Homma and Tsuchiya, 1991). Among high-risk patients, measurement of CA19.9 also failed to detect early stages of pancreatic cancer with small cancer (Locker et al., 2006). Additionally, elevated levels of CA19.9 were observed in plasma of pancreatitis patients resulting in false positives (Rocha et al., 2007). Furthermore, biliary epithelial cells secrete CA19.9 as well so that plasma of patients suffering from biliary construction show elevated levels (Lin et al., 2014). Ten percent of patients do not express a functional Lewis-antigen and are non-secretors (Ørntoft et al., 1996; Tempero et al., 1987). Furthermore, different cut off values for the identification of a true positive case complicate the evaluation as biomarker. Therefore, recommendations for the clinical use of this marker only refer to patients already diagnosed with pancreatic cancer following pancreatectomy, but not for diagnostics (Locker et al., 2006). Several other serum markers like CEA (carcinoembryonic antigen), CA-125, CA-242 have been assessed for pancreatic cancer management and diagnosis but evaluation as biomarker failed (Duraker et al., 2007; Ni et al., 2005).

As neither an imaging technique nor a sensitive biomarker is accessible to date for early diagnosis in pancreatic cancer, a strong medical need drives towards new reliable biomarkers.

1.4 Liquid Biopsy

Biopsy of the tumor tissue is today's gold standard for diagnosing, staging a disease and therapy selection. But especially for pancreatic cancer tumor tissue is difficult to obtain as patients might experience rare but severe complications such as pancreatic main duct injury (Chung et al., 2012). Furthermore, highly invasive tissue biopsies are not feasible for either screening and early diagnosis of healthy individuals, or multiple biopsies for monitoring of the disease over time.

One tool to overcome these challenges might be liquid biopsy. It is the minimally invasive analysis of circulating biomarker originating from the tumor in body fluids, e.g. blood by peripheral blood draw. Liquid biopsy represents the systemic disease and the "real time" status of the tumor. Liquid biopsies have already been shown to be utilized for stratification of patients, monitoring under chemotherapy or detection of response to

therapy (reviewed in Pantel and Alix-Panabières, 2013). As blood draws can be repeated multiple times, liquid biopsy provides a valuable tool for early diagnostics.

Whole blood contains different circulating biomarkers like cells, DNA, RNA, endosomes, and proteins that are still under evaluation for their clinical utility as biomarkers for diagnosis, prognosis, progression and response prediction. This thesis focuses on the comparison of circulating tumor DNA and circulating tumor cells as diagnostic biomarkers for the early detection of pancreatic cancer.

1.5 Circulating tumor DNA

1.5.1 Origin of circulating DNA

cfDNA (circulating free DNA) is isolated from urine or whole blood and was first described 1948 in plasma by Mandel and Metais (Mandel and Metais, 1948). cfDNA itself is not pathological but physiological, as it is also found in clinically healthy individuals and results from dying cells (Jahr et al., 2001; Page et al., 2013). Concentrations of cfDNA in healthy individuals are low and range from 3.6 – 10.4 ng/mL of plasma depending on the isolation methods (Suzuki et al., 2008; Yoon et al., 2009). cfDNA was also investigated in cancer patients (Leon et al., 1977; Stroun et al., 1987; Yoon et al., 2009). High levels of cfDNA were associated with overall tumor burden mainly in late, metastatic stages (Spindler et al., 2015; Yoon et al., 2009). Elevated levels of cfDNA as cancer biomarker are controversially discussed, as several parameters also increase the amount of cfDNA like exercising, inflammatory disease or myocardial infections (Breitbach et al., 2014; Chang et al., 2003; Zhong et al., 2007). This makes it difficult to determine a specific baseline related to cancer. Therefore, research focused on a plasma-based tumor source. Analysis of cfDNA revealed that genetic aberrations of the tumor were also found in plasma DNA (Vasioukhin et al., 1994). ctDNA (circulating tumor DNA) is defined as mutant DNA originating from a primary tumor or metastasis. It is a source of genetic information from the tumor co-isolated in a high background of contaminating wild-type cfDNA in the plasma.

Both cfDNA and ctDNA are reported to be released from the tumor, as depicted in the following Figure 2:

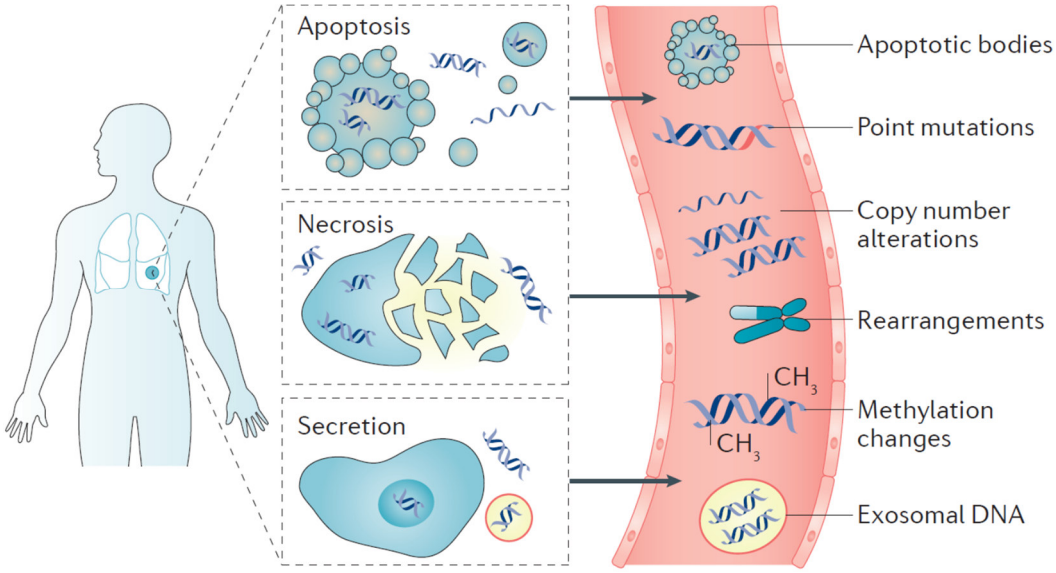

Figure 2: Mechanisms of DNA release into blood. Predominantly, apoptosis is published about cfDNA and ctDNA release in the blood. Necrosis and secretion of DNA have also been hypothesized but are a minor source of DNA to date (from Wan et al., 2017).

The predominantly published mechanism of DNA release in plasma is by apoptosis but is still under investigation. DNA is fragmented to chromatosome size of about 170bp (Jahr et al., 2001; Newman et al., 2014). This size is probably explained by a caspase-dependent endonuclease involved in the process of apoptosis cleaving DNA at specific sites (Lo et al., 2010; Mouliere and Rosenfeld, 2015). DNA released by necrosis results in larger genomic DNA (Wang et al., 2003). As shorter DNA fragments from plasma are observed, it is hypothesized that DNA is taken up by macrophages and partially digested (Diehl et al., 2005). Active release of DNA from cells, like secretion, is theorized as well but this still has not been proven in cancer patients (Anker et al., 1975). Efforts were made to trace back the size of the fragments to the existence of ctDNA in the background of cfDNA. But this resulted in contrary published results and has not been validated yet (Diehl et al., 2005; Heitzer et al., 2013; Mouliere et al., 2011).

1.5.2 ctDNA as biomarker

The use of ctDNA as biomarker has already been described as prognostic in several tumor types. Here, the presence of ctDNA correlated with overall survival or tumor burden (Bettegowda et al., 2014; Dawson et al., 2013). But the presence of ctDNA as prognostic marker is controversially discussed by several groups and is largely dependent on isolation methods, cohort of patients and analyzing methods (Camps et al., 2011; Nygaard et al., 2013). Furthermore, ctDNA has already been defined in feasibility studies as predictive marker and useful for treatment monitoring in e.g.

colorectal (anti-EGFR treatment and *KRAS*) and lung cancer (*EGFR*-TKI (tyrosine kinase inhibitor) treatment and *EGFR* T790M) (Diaz et al., 2012; Wang et al., 2014). ctDNA for screening purpose is a rising field of interest but has not been as extensively analyzed as in patients with metastatic diseases (Haber and Velculescu, 2014). One reason for this is the relatively low ctDNA allelic frequency or number of detected mutant copies in early stages compared to metastatic or late stages (Speicher and Pantel, 2014).

The detection of genetic alterations in ctDNA in concordance to the aberrations in the tumor has been shown for several solid tumor types, e.g. head and neck, lung, breast, liver, prostate and colorectal cancer (Bettegowda et al., 2014; Dawson et al., 2013). Thereby, different genetic alterations, like copy number changes (Chan et al., 2013; Heitzer et al., 2013), methylation changes (Balgkouranidou et al., 2014), single-nucleotide mutations or genetic rearrangements (Diehl et al., 2008; McBride et al., 2010; Yung et al., 2009) were identified in ctDNA isolated from plasma.

Circulating tumor DNA in pancreatic cancer

Mutation detection in circulating DNA isolated from plasma of pancreatic cancer patients has been described in the literature several times. In the late 90s, DNA with highly prevalent mutant *KRAS* was analyzed in pancreatic cancer proving the feasibility of ctDNA in pancreatic cancer (Castells et al., 1999). Since then studies on ctDNA analysis in pancreatic cancer have increased. In advanced cancer, ctDNA has been described as prognostic marker because the prognosis for patients with detectable mutations is worse compared to patients without ctDNA (Sausen et al., 2015; Tjensvoll et al., 2016). In other studies, no correlation between clinical parameters like overall survival, tumor size or tumor stage has been described (Maire et al., 2002; Uemura et al., 2004). Furthermore, in patients who were monitored for several months after resection of the tumor progression of the disease was detected earlier than by conventional radiological monitoring methods, providing ctDNA as powerful tool in cancer management (Sausen et al., 2015; Tjensvoll et al., 2016).

Publications about analysis of ctDNA in pancreatic cancer strongly focus on late stages. Detection of ctDNA was more successful in advanced stages and worse in localized early stages (Bettegowda et al., 2014; Calvez-Kelm et al., 2016). Few studies on early stage pancreatic cancer with limited success have been published to date (Bettegowda

et al., 2014; Sausen et al., 2015; Takai et al., 2015) describing a potential value of ctDNA. ctDNA as diagnostic tool is investigated in this thesis.

1.6 Circulating tumor cells

1.6.1 Origin of circulating tumor cells and epithelial-mesenchymal transition

Circulating tumor cells (CTCs) originate from the primary tumor or metastasis and are isolated from whole blood. No CTC is found in blood of healthy donors (Allard et al., 2004). CTCs originate from solid epithelial tumors. Therefore, CTCs harbor epithelial characteristics. The widely accepted definition of a CTC is EpCAM (epithelial cell adhesion molecule)-positive, cytokeratin (cellular skeleton protein of epithelial cells)-positive, leukocyte marker (such as CD45 (cluster of differentiation 45)-negative and nucleated (Allard et al., 2004). They are potentially also bigger in size than other blood cells (Stoecklein et al., 2016). Blood cells on the other hand do not express epithelial markers.

The mechanism of the entry of CTCs into the blood stream is still unclear. Two mechanisms are discussed: passive shedding or active release (Figure 3).

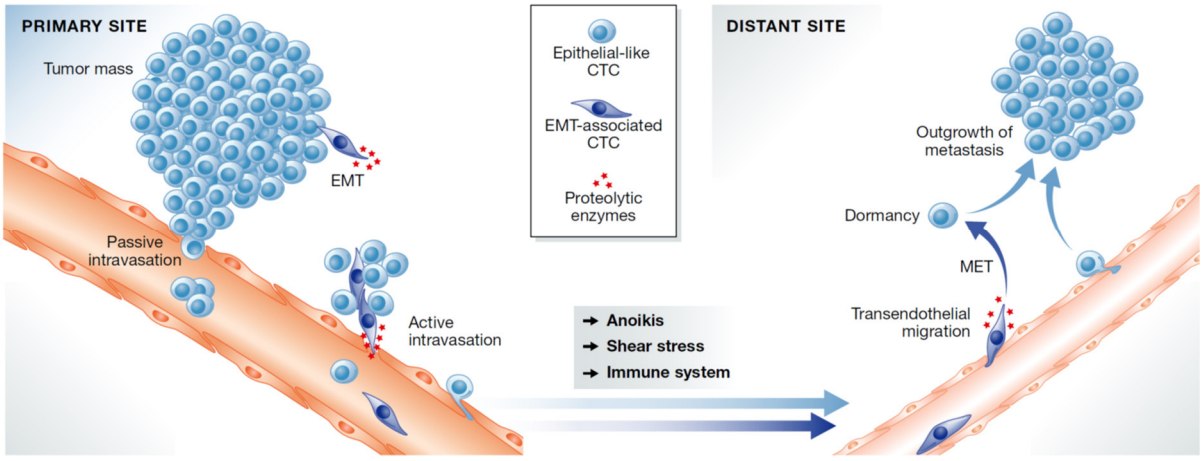

Figure 3: Origin of CTCs. CTCs originate from the tumor by passive or active intravasation (left). CTCs are either shed passively keeping their epithelial character or undergo EMT (epithelial-mesenchymal transition) to achieve mobile mesenchymal characteristics and evade the tissue. In the distance, CTCs might invade other tissue by MET (mesenchymal-epithelial transition) to develop metastasis (right) (from Joosse et al., 2015).

Cells might be shed mechanically and randomly in the blood stream by e.g. tumor growth or vessel acquisition (passive intravasation, Figure 3). Thereby, CTCs maintain their

epithelial character. If cells are released by active intravasation, an initial step called EMT (epithelial-mesenchymal transition) has to be initiated to migrate cells. During that process, cells lose their original, polar epithelia phenotype by detaching from the basal membrane and transition towards an invasive and motile mesenchymal phenotype (Figure 3 and 4).

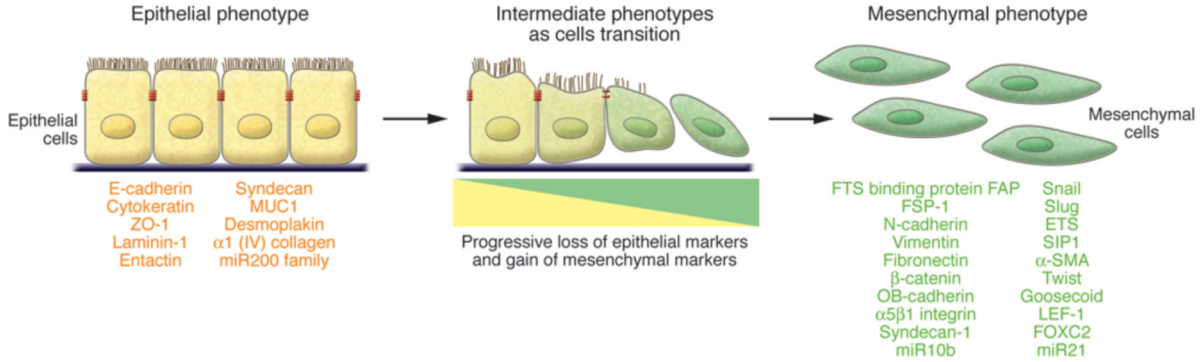

Figure 4: Processes of EMT. During EMT, the polar and immotile epithelial cells transition towards an intermediate phenotype. Epithelial markers like EpCAM or cytokeratin are downregulated and mesenchymal marker are upregulated until a mesenchymal phenotype is obtained (from Kalluri and Weinberg, 2009).

Thereby, EpCAM and cytokeratin expression is downregulated and mesenchymal characteristic occur like upregulated expression of vimentin (mesenchymal cellular skeleton protein) (Figure 4, Kalluri and Weinberg, 2009).

While circulating in the blood stream, survival of CTCs is challenged by several factors including the immune system and shear stress. Compared to the number of shed cells, only a minority of cells survive and invade distant tissue (Gupta et al., 2005; Luzzi et al., 1998). Analysis of developing metastasis *in vivo* after injection of a definite number of cancer cells revealed that only 0.01% of injected cells form micrometastasis (Fidler, 1970, as cited in Fidler, 2003). As a mesenchymal phenotype is not prone for uncontrolled growth, shed cells undergo a reverse mechanism called MET (mesenchymal-epithelial transition) (Celia-Terrassa et al., 2012; Tam and Weinberg, 2013). An epithelial phenotype is acquired leading to metastasis or new carcinoma (Chaffer et al., 2006; Thiery, 2002).

1.6.2 CTC isolation and detection

Usually 7.5 to 10 mL blood are used for the isolation of CTCs containing millions of other blood cells. Because of the tremendous numbers of contaminating blood cells (Figure 5) CTCs as rare cells have to be isolated by specific methods. Therefore, different methods have been developed using definite characteristics of the CTCs. The most established and published method is called CellSearch. It is an immunoaffinity technology positively enriching CTCs by the surface antigen EpCAM. Thereby, anti-EpCAM-coated magnetic particles isolate EpCAM-expressing CTCs from EpCAM-negative blood cells (Allard et al., 2004). CellSearch is also the only FDA (Food and Drug Administration)-cleared method for the isolation of CTCs of metastatic breast, colorectal and prostate cancer and their prognostic value to date (Janssen Diagnostics, 2017). The technology has been widely used as gold standard of CTC isolation and has been extended to the investigation of CTCs in other cancer types e.g. lung cancer (Krebs et al., 2011).

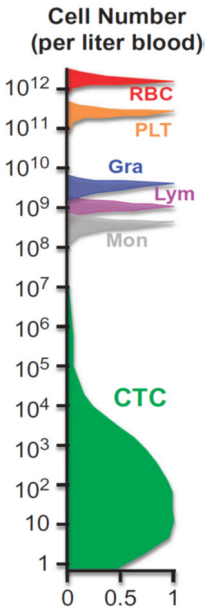

Figure 5: Frequency of rare CTCs compared to other blood cells. RBC = red blood cells; PLT = platelets; Gra = Granulocytes; Lym = Lymphocytes; Mon = monocytes (from Stoecklein et al., 2016).

After isolation, cells are detected by e.g. immunocytochemistry and counted (enumeration). Epithelial marker such as cytokeratin and EpCAM are used to discriminate CTCs from residual contaminating blood cells. Predominantly, cytokeratin 8, 18, and 19 are used for detection by the FDA-cleared CellSearch (Allard et al., 2004; Wit et al., 2015).

1.6.3 Challenges in CTC-isolation

As mechanisms of EMT during tumor progression have raised interest among CTC-research, doubts about the sufficient isolation of all subtypes of CTCs by EpCAM-based methods like CellSearch emerged (Gorges et al., 2012). This has led to a rising number of new technologies implementing other characteristics of CTCs including label-free methods like filtration (reviewed in Joosse et al., 2015). Filtration does not rely on EMT dependent surface markers but is based on the relatively bigger size of CTCs compared to most blood cells (Stoecklein et al., 2016). Size-based methods have shown improvement in both higher CTC numbers and isolation of subtypes of CTCs like EpCAM

low or negative CTC by several groups independent of stage of the disease (Khoja et al., 2012; Krebs et al., 2012; Lecharpentier et al., 2011; Wit et al., 2015). Other efforts to overcome the challenge of isolating all subtypes were serial isolation of CTCs first by CellSearch and subsequent processing of its waste through a sieve (Wit et al., 2015). Higher numbers of CTCs were obtained combining both methods showing the missing of subtypes by CellSearch. Publications of other techniques also emerged because of the limited success of label-dependent methods in early compared to metastatic stages (Alix-Panabieres and Pantel, 2016). Another issue is the discrimination between malignant and non-malignant disease by only number of CTCs. This might be difficult due to circulating epithelial cells in non-malignant disease that are stained by cytokeratin (Tanaka et al., 2009). This urges for additional downstream analysis to detect the true malignant origin.

Besides the challenge of capturing all CTCs of different stages, another obstacle is the amount of processed blood. 7.5 to 10 mL whole blood or less are used for capturing of cells (Coumans et al., 2012). To overcome this, a method for enrichment of leukocytes including CTCs called DLA (diagnostic leukapheresis) has been successfully suggested for the detection of CTCs (Fischer et al., 2013). During extracorporeal centrifugation of whole blood all constituents are separated by density. CTCs can be found in the layer of mononuclear cells. Leukapheresis is routinely used in the clinics for e.g. stem cell harvest (Malachowski et al., 1992).

Another technique to overcome the challenge of limited blood volume is an *in vivo* approach. A medical wire coated with anti-EpCAM antibodies is introduced into the patient's vein for 30 minutes resulting in the efficient passage of an estimated blood volume of 1.5-3 L (Saucedo-Zeni et al., 2012).

1.6.4 CTCs as biomarker

CTC counts determined by CellSearch are a validated prognostic biomarker in metastatic breast, colorectal and prostate cancer above a threshold of 3 (colorectal) or 5 (breast, prostate) CTCs per 7.5 mL of blood (Cohen et al., 2009; Cristofanilli et al., 2004; de Bono et al., 2008). Besides breast, colorectal and prostate cancer, the number of CTCs has also been published to be prognostic in e.g. lung and bladder cancer (Gazzaniga et al., 2012; Krebs et al., 2011). CTCs as source for possible predictive biomarker were described in isolated CTCs from e.g. breast, or colorectal cancer for late stages (Mostert

et al., 2013; Tewes et al., 2008). In addition to their prognostic value and their use for analysis of predictive biomarkers in the CTCs, CTCs themselves may be used as diagnostic biomarker. In breast cancer patients diagnosed with early stages of cancer CTCs were successfully detected (Nakagawa et al., 2007).

CTCs in pancreatic cancer

The clinical utility of CTCs in pancreatic cancer as biomarker has not been investigated as widely as in other indications like breast cancer. The original CellSearch manuscript reported the number of CTC-positive pancreatic cancer patients and the number of CTCs per mL blood itself as very low amongst all entities (Allard et al., 2004). This was also confirmed by other workgroups (Bidard et al., 2013; Khan et al., 2011). Increased CTC counts in pancreatic cancer isolated by CellSearch were correlated to worse overall survival (Kurihara et al., 2008). Nevertheless, the detection rates of CTCs in both operable and inoperable pancreatic cancer samples throughout all stages range from 5 to 100% (Bidard et al., 2013; reviewed in Nagrath et al., 2016). One *in vivo* study suggested an early loss of epithelial markers in pancreatic CTCs which might hinder the isolation by an EpCAM-dependent method (Rhim et al., 2012). Therefore, an antigen-dependent isolation approach might be difficult in pancreatic cancer and would also show an incomplete picture of the disease. The number of CTCs in early stages of pancreatic cancer was already reported as low (Ankeny et al., 2016). The diagnostic utility of CTCs is investigated in this thesis.

1.7 Excursus: Personalized medicine

1.7.1 Lung cancer

Comparison of ctDNA and CTCs for diagnostic utility in pancreatic cancer is the main topic of this thesis. As to date no actionable, feasible mutations have been found in pancreatic cancer, cancer management still relies on traditional chemotherapy. Personalized medicine has been a rising field to guide individual therapy, but is not feasible in pancreatic cancer to date. As an excursus to the research on CTCs as

diagnostic biomarker, CTCs are also evaluated in lung cancer as source of predictive biomarkers because targeted therapies are available.

Lung cancer is the leading cause of cancer-related death and will continue to remain so (Rahib et al., 2014; World Health Organization, 2017). Like in pancreatic cancer, surgery is the only curative treatment but only eligible in early stages. In more than 70% of the patients, surgery is not curative as patients present themselves with late stages (Travis et al., 2013). The 5-year overall survival rate is dependent on the stage: 58-73% (stage I) and 13% (stage IV) (Tanoue and Detterbeck, 2009). The absence of sensitive screening assays and unspecific symptoms cause the late diagnosis of lung cancer (Xiang et al., 2013). In patients with resected tumors the recurrence rate is already about 30% (Kelsey et al., 2009). Reasons for this might be on the one hand the early invasive characteristics resulting in early spread of tumor cells and on the other hand ineffective treatments that lead to progression of the disease.

The most common risk factor for lung cancer is tobacco (75-90%) (Sasco et al., 2004). Lung cancer is subgrouped into two different histological types: SCLC (small cell lung cancer) and NSCLC (non-small cell lung cancer). SCLC accounts for 15% of the tumors; 85% of cases are NSCLC, whereby the majority of them are adenocarcinoma (Herbst et al., 2008).

1.7.2 Tumor heterogeneity and therapy success

Surgery or other therapy recommendations depend on type of histology including molecular footprint of the tumor, staging, and general constitution of the patient (Lemjabbar-Alaoui et al., 2015). Biopsy of the tissue is the gold standard for diagnosis and treatment decisions. But a single tissue biopsy at the time of diagnosis only provides a snapshot of the biopsied tumor part (Gerlinger et al., 2012). During years of research, heterogeneity has become a challenge for therapy success. Heterogeneity is defined by phenotypic or genotypic differences between tumors (inter-tumor) and within a tumor (intra-tumor). Inter-tumor heterogeneity describes the variations between different tumors arising from the same tissue. Sequencing of lung tumor tissues has shown multiple and different genetic alterations (Ding et al., 2008). In contrast, intra-tumor heterogeneity is found within one tumor of one individual patient. This challenge of heterogeneity is partly overcome by subgrouping specific cancer types by tissue characteristics (like phenotypic (histological) or genotypic (mutations)) and guiding cancer management by personalized

medicine (Burrell et al., 2013). Adjuvant first line treatment of advanced lung cancer patients is platinum-based combination chemotherapy and/or radiation, but their clinical utility is restricted by severe side effects (Rajeswaran et al., 2008). Therefore, targeted therapies have been developed for patients who are positive for specific biomarkers. These therapies have a broader therapeutic window between side effects and response for the subset of biomarker positive patients.

Targeted therapy has mainly focused on NSCLC for the last years (Ridge et al., 2013). Since molecular aberrations in lung cancer were discovered, targeted therapy like anti-EGFR therapy evolved. Mutations are tested on tumor tissue at time of diagnosis which is obtained by highly invasive biopsy to stratify patients. Patients with *EGFR* mutant tumors especially with exon 19 deletions and activating L858R mutation benefit significantly from combination or monotherapy with targeted treatment by *EGFR* tyrosine kinase inhibitors (*EGFR*-TKIs) like erlotinib or gefitinib compared to standard first line therapy alone (Eberhard et al., 2005; Mitsudomi et al., 2010; Rosell et al., 2012). Progression free survival as well as reduction of symptoms but not prolonged overall survival are observed (Rosell et al., 2012). Additionally, anti-EGFR monoclonal antibodies like cetuximab binding to the extracellular domain of the EGFR are evaluated for treatment of confirmed EGFR-expressing lung cancer. Promising results are obtained in terms of progression free survival compared to chemotherapy (Pirker et al., 2009). Nevertheless, intra-tumor heterogeneity within a tumor of a single patient remains a major cause for resistance to targeted therapy. Intrinsic resistance describes the pre-existence of mutant clones responsible for therapy failure that might also be missed by the initial single tumor biopsy. Acquired resistance is the progression of the disease after benefiting from a specific therapy, as the resistant mutant clone grow *de novo* under therapy (Burrell and Swanton, 2014). Under chemotherapy, different clones with an alternative driver or a resistance mutation can expand rapidly under selective pressure of the therapy. Besides *EGFR* (26%), *KRAS* (21%) is amongst the most common oncogenic drivers in NSCLC (Sanger Institute, 2017). But mutations in *KRAS* are suggested as negative predictor of therapy (Eberhard et al., 2005; Sun et al., 2013). Mutant KRAS can be responsible for the poor response to EGFR-targeted treatment as it is downstream of EGFR resulting in autologous activation of the EGFR/KRAS pathway (see 1.2 Cancer biomarker). Patients with *KRAS*-positive tumors treated with *EGFR*-TKI might also experience reduced survival compared to standard chemotherapy (Eberhard et al., 2005). Another resistance mutation is *EGFR* T790M point mutation resulting in poor response to the EGFR-targeted therapy. After initial benefit of the EGFR-targeted

therapy, patients suffer a relapse under treatment. T790M mutation usually occurs *de novo* under treatment in about 50% of *EGFR*-TKI treated patients coexisting with the druggable *EGFR* target (Pao et al., 2005; Sequist et al., 2011). Newer studies also suggest that *EGFR*-TKI-naïve tumors can harbor T790M mutation that leads to initial resistance by selection of clones during treatment (Su et al., 2012). If a resistance mutation is detected, the result might lead to the change of therapy, e.g. to T790M-targeted therapy by Osimertinib (Cross et al., 2014). Additionally, under EGFR therapy, amplification of the MET-gene can accumulate which is also known as a reason for poor response to EGFR therapy (3% in therapy-naïve tumors to 21% after treatment, Bean et al., 2007). If detected, treatment might be changed to MET-targeted therapy like Crizotinib (Rodig and Shapiro, 2010; Tanizaki et al., 2011). Despite numerous resistance mutations in NSCLC, this thesis focuses on *KRAS* mutations.

Multiple re-biopsies need to be taken to identify resistance mutations under therapy. Traditional tissue biopsies are associated with significant risk. In patients with lung biopsy up to a third might suffer from pneumothorax (Laurent et al., 2000; Tomiyama et al., 2006). Tissue biopsy is not intended for serial biopsy and monitoring for adjusting treatment regimes. Therefore, potential targeted therapy for personalized medicine in lung cancer is available but the detection of resistance is still highly invasive.

1.7.3 Blood-based biomarker in lung cancer

To date, no evaluated reliable blood test is in clinical use to monitor lung cancer patients for e.g. acquired resistance to therapy. The earlier the mutations are detected under therapy, the earlier the change of the treatment for the benefit of the patient, thus revealing a high need for biomarkers. To date, no mutation-specific minimally invasive blood biomarker for the monitoring of lung cancer is in routine clinical use. Serum- or plasma-based minimally invasive biomarkers are not successful to date. CEA as serum biomarker has been analyzed in lung cancer in a variety of studies with different conclusions (Grunnet and Sorensen, 2012). Like in pancreatic cancer, analysis of CEA in lung cancer obtains false positive results or the results of the studies are not directly comparable due to different cut off values. Another serum-based biomarker is Cyfra 21-1. It is the fragment of cytokeratin 19 that is released into the blood during transformation of the cells and subsequent initiation of apoptosis. In patients with advanced lung cancer Cyfra 21-1 is detected by antibody staining with high specificity (94%) and high sensitivity (59%) in NSLCL but very low sensitivity in small cell lung cancer (19%) (Wieskopf et al.,

1995), thus leading to false negative results. The prognostic value of Cyfra 21-1 is debated, but was shown in advanced cancers (Ono et al., 2013).

No easily accessible blood marker to date has the potential of stratifying patients into different target specific treatment regimes. As liquid biopsy represents the systemic disease, it is therefore less prone to missing intra-tumor heterogeneity (Diaz and Bardelli, 2014). Liquid biopsy might be a useful source for genetic information of the tumor that can be easily accessed and repeated in lung cancer patients.

CTCs in lung cancer

The isolation of CTCs from lung cancer patients was described by several groups. Allard et al. published detection rates of ≥ 2CTCs of lung cancer sample in 20% of cases (Allard et al., 2004). Higher detection rates were achieved by size-based technologies (Hvichia et al., 2016; Krebs et al., 2012). One reason for that might be a subset of CTCs harboring epithelial and mesenchymal characteristics (Lecharpentier et al., 2011; Wu et al., 2015). Screening of individuals with precursor lesions for lung cancer have shown the potential of a minimally invasive early diagnosis in cancer patients (Ilie et al., 2014). CTCs in lung cancer have been described as successful prognostic marker. High numbers of CTCs were negatively correlated with progression free survival and overall survival by several groups especially under treatment (Hofman et al., 2011; Punnoose et al., 2012). Furthermore, molecular analysis of captured CTCs revealed information about *EGFR* mutations and response to therapy or therapy resistance (Breitenbuecher et al., 2014; Maheswaran et al., 2008).

1.8 Study design and patient samples

Two independent studies with human patient samples were performed to evaluate both CTCs and ctDNA as early diagnostic biomarker in pancreatic cancer. Another study was performed to describe improved sensitive detection of CTCs in lung cancer and their predictive potential by molecular characterization.

The first study evaluated the potential of *KRAS* mutations in ctDNA as diagnostic biomarker. Fifty, retrospectively collected matched tumor and plasma samples from pancreatic cancer patients and 20 healthy individuals were analyzed by digital PCR.

Patients were predominantly diagnosed with stage I and II (41/50). Tumor- and plasma-DNA was analyzed for highly prevalent *KRAS* mutations G12D, G12V and G12C, if applicable. Outcome was the detection rate of mutations in tumor positive and plasma positive samples as well as their concordances.

The second study addressed the feasibility of CTC enumeration in frozen DLA and their possible characterization by detection of *KRAS* mutations in metastatic and non-metastatic pancreatic cancer. Nineteen, retrospectively collected cryopreserved DLA products from pancreatic cancer patients (10 non-metastatic (M0) and 9 metastatic (M1)) were filtered for CTCs and analyzed by subsequent *KRAS* mutational analysis. The outcome was the number of CTCs and potential *KRAS* mutations.

As continuative excursus, another study was conducted to evaluate the significance of CTCs in lung cancer and the subsequent molecular characterization of the CTCs. Whole blood and captured cells on an *in vivo* wire were collected prospectively. The outcome is described as number of CTCs also during serial measurements (monitoring) and mutational characterization.

Ethical statements for all patient samples can be found in each manuscript.

2 Aim and objectives

Successful cancer therapy depends on early diagnosis followed by surgery and selection of the appropriate treatment. Current standard of diagnosis and determination of mutational landscape of tumors is highly invasive biopsy of the tissue. Minimally invasive blood-based liquid biopsies provide e.g. ctDNA and CTCs originating from the primary tumor or metastasis. However, there are still challenges in isolation and analysis of these cells and molecules as both are rare events in a high background of blood cells or nucleotides. Especially in pancreatic cancer, published studies strongly focus on late stages.

The aim of this thesis was to discuss ctDNA and CTCs as potential diagnostic biomarker in early stages of pancreatic cancer by optimizing the analysis of ctDNA and CTCs for early diagnosis. Two independent studies on samples of patients diagnosed with early and non-metastatic pancreatic cancer should be conducted and the outcome compared in order to conclude about a possible diagnostic marker in pancreatic cancer.

Digital PCR should be evaluated for the sensitivity and specificity for mutant alleles isolated from plasma or CTCs. For ctDNA, an optimized workflow for isolating fragmented DNA from plasma, enriching these fragments and subsequent detection of mutant alleles by digital PCR should be established to detect a limit of detection. To determine the diagnostic utility of ctDNA as early marker, this workflow shall then be applied to 50 tumor and matching plasma samples from patients diagnosed with early stages of pancreatic cancer.

The isolation of CTCs from frozen DLA samples of non-metastatic and metastatic pancreatic cancer patients should be evaluated by testing two different isolation approaches and subsequent molecular analysis. Pancreatic cancer cells as model shall be characterized and spiked into and recovered from whole blood and frozen DLA. Mutational downstream analysis by digital PCR aimed to determine true tumorous origin. The diagnostic utility should be tested on 19 frozen DLA samples from both metastatic and non-metastatic pancreatic cancer patients.

The outcome of both studies aimed to evaluate a possible biomarker for early diagnosis of pancreatic cancer.

Additionally, as an excursus to the diagnostic potential, CTCs isolated by an EpCAM-coated wire inserted into the vein of lung cancer patients shall be evaluated for sensitive detection, possibility of monitoring and characterization of mutations by digital PCR. Thereby, the feasibility of CTCs as monitoring and predictive marker shall be determined.

3 Manuscripts

3.1 Detection of *KRAS* mutations in ctDNA by digital PCR in early stages of pancreatic cancer

Nora Brychta, Thomas Krahn, Oliver von Ahsen

Clinical Chemistry Nov 2016, 62 (11) 1482-1491.

Name of the journal: Clinical Chemistry

Status: accepted

DOI: 10.1373/clinchem.2016.257469

Impact Factor (2016): 8,008

Authorship: First Author

Contribution: 80%

- establishment of workflow
- design and realization of experiments as well as analysis of data
- assistance with manuscript writing including visualization of data
- editing manuscript and data after review

URL: http://clinchem.aaccjnls.org/content/early/2016/08/30/clinchem.2016.257469

3.2 Isolation of Circulating Tumor Cells from Pancreatic Cancer by Automated Filtration

Nora Brychta*, Michael Drosch*, Christiane Driemel, Johannes C. Fischer, Rui P. Neves, Wolfram Knoefel, Birte Moehlendick, Claudia Hille, Antje Stresemann, Thomas Krahn, Matthias U. Kassack, Nikolas H. Stoecklein and Oliver von Ahsen

Name of the journal: Oncotarget

Status: under review, # 029460

Impact Factor (2016): 5,168

Authorship: Shared First Author (*)

Contribution: 40%

- Study conception
- Setup of IsoFlux-Experiments
- CTC-experiments
- Development of mutation detection
- Performance of experiments and data collection
- Writing manuscript and visualization of data

Isolation of Circulating Tumor Cells from Pancreatic Cancer by Automated Filtration

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Keywords: Pancreatic cancer, circulating tumor cells, *KRAS*, diagnostic leukapheresis, EMT

Abstract

It is now widely recognized that the isolation of circulating tumor cells based on cell surface markers might be hindered by variability in their protein expression. Especially in pancreatic cancer, isolation based only on EpCAM expression has produced very diverse results. Methods that are independent of surface markers and therefore independent of phenotypical changes in the circulating cells might increase CTC recovery also in pancreatic cancer. We compared an EpCAM-dependent (IsoFlux) and a size-dependent (automated Siemens filtration device) isolation method for the enrichment of pancreatic cancer CTCs. The recovery rate of the filtration based approach is dramatically superior to the EpCAM-dependent approach especially for cells with low EpCAM-expression (filtration: 52%, EpCAM-dependent: 1%). As storage and shipment of clinical samples is important for centralized analyses, we also evaluated the use of frozen diagnostic leukapheresis (DLA) as source for isolating CTCs and subsequent genetic analysis such as *KRAS* mutation detection analysis. Using frozen DLA samples of pancreatic cancer patients we detected CTCs in 42% of the samples.

Introduction

Pancreatic cancer is among the most lethal cancer diseases worldwide and the cases of newly diagnosed patients will raise further in the following years due to the demographic changes in the developed countries [1]. The 5-year survival rate of patients diagnosed with pancreatic cancer is still below 10% [2]. Major reason for this is the lack of effective screening for early detection and the usually late diagnosis due to nonspecific symptoms late in the progress of the disease. Nowadays, resection of the tumor is the only curative treatment. As metastasis occurs after initial tumor progression [3], early detection is of utmost importance for successful treatment. Pancreatic cancer seems to disseminate tumor cells relatively early as it has been shown that patients undergoing pancreatectomy for tumors smaller than 2 cm have less than 18% 5 years survival [4]. Even some patients after pancreatectomy for chronic pancreatitis develop disseminated pancreatic ductal adenocarcinoma (PDAC) although no tumors were found in the primary resectate [5]. However, until today no early screening tests are in routine clinical use [6]. CA19.9 is the only biomarker used to support diagnosis and response monitoring marker but is not sensitive enough for early detection [7].

One alternative diagnostic tool might be blood based biomarkers like circulating tumor cells (CTCs). The relevance of CTCs in pancreatic cancer has recently been reviewed [8, 9]. CTCs originate from the tumor, are shed from tissue into the blood stream and may be representative of the systemic disease [10]. On the other hand, CTCs are rare cells occurring in very low concentration in the peripheral blood which makes their detection challenging [11]. Several methods for isolation of CTCs have been used to isolate these cells utilizing different characteristics of the tumorous cells like surface marker or size [12]. One widely used surface marker is the epithelial cell adhesion molecule (EpCAM). EpCAM is expressed on cells derived from epithelial tumors including CTCs but not on regular blood cells such as leukocytes [13]. Since the EpCAM-based isolation of CTCs

(CellSearch) is FDA-approved for metastatic breast, prostate and colorectal cancer, it is the gold standard for CTC research and the number of CTCs was already described as prognostic for survival in these indications [14-16].

Although most pancreatic tumors are EpCAM-positive (96%) [17], the expression levels of EpCAM are heterogenous with only half of the tumors showing strong expression [18, 19]. This may explain why both the number of EpCAM-captured CTCs and also the number of CTC-positive patient cases is low [13, 20, 21]. The original publication on the CellSearch-Device describes the CTC-numbers in PDAC as lowest of all indications even in samples from metastatic patients compared to breast, colorectal or prostate cancer [13]. Although most studies showed comparably low numbers of CTCs in pancreatic cancer, the reported detection rates range from 5-100% depending on the volume of blood, isolation method and staining technique [20, 22-24]. This strong variability in the results hints to the strong need of better definition of CTC properties and careful validation of the technologies used.

A major obstacle for EpCAM based CTC isolation is the epithelial-mesenchymal transition (EMT) often observed with CTCs [25-27]. For pancreatic cancer this has already been described *in vivo* showing the loss of epithelial markers at an early development [28]. Therefore, an antigen-dependent approach for CTC isolation is especially difficult in pancreatic cancer [23, 29]. In addition to EMT other mechanisms of EpCAM downregulation such as internalization, proteolysis and promotor methylation have been described that may reduce the success rate of CTC isolation, as reviewed by Gires and Stoecklein [30].

Consequently, antigen-independent capturing strategies of CTCs emerged to overcome the challenge of detecting all phenotypic variants of CTCs. One possible alternative to the immune-affinity purification might be the filtration of CTCs. A pilot study by Khoja et al. already showed very promising results by using a filtration method (ISET) in

pancreatic cancer [31]. In order to increase throughput and standardize handling, automation is the ultimate goal for clinical devices. Therefore we tested an automated filtration-platform produced by Siemens [32].

Since the enumeration of CTCs solely based on EpCAM expression (or that of other epithelial markers like cytokeratins) may not be sufficient for unequivocal identification of cancer cells due to EMT, downstream analysis becomes important for defining truly tumor-derived cells. In pancreatic cancer, *KRAS* mutations are commonly used to detect malignant cells, because of their high prevalence of 57% [33]. This has been successfully been used for CTCs isolated by other techniques [22] but one group also reported non-matching *KRAS* status between tumor and CTCs in some cases [34].

In this work, we compare two different isolation techniques with automated devices (EpCAM dependent immune-affinity purification and filtration by size) for efficient capturing of cells in whole blood following *KRAS*-mutational analysis. A proof of concept study with frozen diagnostic leukapheresis samples was performed to determine the possibility of improved CTC detection in stored frozen patient samples.

Results

Here we compare two methods for isolation of pancreatic cancer circulating tumor cells. Cultured cells spiked in whole blood were used to test the performance of the methods. As EpCAM expression is highly variable within CTC-populations we selected three different pancreatic cancer cell lines that express different levels of EpCAM for the required spike-in experiments (Figure 1): Capan1 as high, BxPc3 as medium and Panc1 as low EpCAM expressing cell lines. The breast cancer cell line SkBr3 was additionally used as reference cell line as it was frequently used for the evaluation of analytical devices [32, 35]. Detection of CTCs is routinely performed by cytokeratin staining as

marker for the epithelial origin of the cells. Therefore, we also included cytokeratin stainings in Figure 1. In addition to differences in EpCAM expression, cytokeratin levels also vary between cell lines. Pancreatic cancer cells express predominantly cytokeratins 7, 8, 13, 18 and 19 (Supplementary Table 1). Most pan-cytokeratin antibodies like AE1/AE3 miss one or more of these cytokeratins. Therefore we developed an antibody cocktail that covers all the cytokeratins expressed in pancreatic cancer (Supplementary Table 2). By using this panel of cytokeratin antibodies, we enhanced the signal for all 4 cell lines tested to a similar fluorescence intensity level independent of the predominant cytokeratin isoform (Figure 1).

Next we determined the performance of two different CTC isolation methods by spiking cells into whole blood. We used an EpCAM-dependent immune-affinity approach and a filtration method to examine if the variable EpCAM expression in pancreatic cancer cells has an impact on the recovery rate. One, 3, 10 and 30 cells each were spiked in whole blood and subjected to the respective isolation procedures. The average recovery rate in the IsoFlux device is highly dependent on EpCAM expression (mean: 1-27%, Figure 2A and Supplementary Figure 1 for the individual numbers) compared to the size-dependent method which recovered in average 52-68% of the spiked cells (Figure 2B and Supplementary Figure 1 for the individual numbers). Therefore filtration gives a much more robust result throughout the variety EpCAM-expression in cell lines. Before subjecting the sample to the IsoFlux device, whole blood is separated by a Ficoll density gradient. This might result in a loss of CTCs which has been previously reported [13, 36]. As the used beads are rather big, the staining might be obscured by the beads (Figures 2C and Supplementary Figure 2) and hinder the detection. Figure 2C displays representative pictures of Capan1 cells isolated by the two different methods (see also Supplementary Figure 2 and 3 for all cell lines). Cells on the filters show better visual quality compared to the cells covered with magnetic beads which disturb the imaging. The detection rate of the breast cancer cell line SkBr3 was higher in both methods (mean

IsoFlux: 49%, mean filtration: 80%) owing to the very high EpCAM expression and the large cell size compared to the pancreatic cancer cell lines. We conclude that filtration is superior to surface antigen-dependent isolation of CTCs.

As whole blood samples cannot be stored and shipped easily and also to increase the amount of input material, we examined another clinical sample type, frozen apheresate, originating from diagnostic leukapheresis (DLA). DLA is a blood preparation highly enriched in mononuclear cells. It has been shown that in CTCs co-enrich in the mononuclear fraction [11]. In the present study 2mL of DLA product correspond to 60mL whole blood sample in terms of white blood cell counts. The DLA can be frozen and is therefore ideal for potential use in larger clinical studies. To test the performance of filtration after freezing and thawing of the DLA product we tested the recovery rate of Capan1 and Panc1 cells spiked in healthy donor DLA. The detection rate of the isolated cells was similar to those isolated from whole blood (Figure 3A and Supplementary Figure 4). When cultured cells were spiked in freshly prepared buffy coat, frozen, stored for several weeks and thawed using our established protocol the cell recovery was similar (Supplementary Figure 5). Representative pictures also show that cells stay intact during thawing (Figure 3B) as the morphology is similar to the cells isolated from whole blood (Figure 2C). Therefore the use of frozen DLA product as a source for CTC isolation is feasible.

After validation of input material and the preanalytical procedures we next used the filtration method to analyze DLA samples of pancreatic cancer patients. We detected CTCs in 42% (8/19) of the patient samples. We did not find a higher prevalence of CTC positivity in metastatic cases: 44% in M1 (4/9) and 40% in M0 (4/10) (Table 1). However, higher numbers of CTCs were found in patients diagnosed with distant metastasis.

In order to confirm the identity of the isolated cells as cancer derived CTCs we developed a method for mutational analysis of isolated CTCs picked from the filter

(Figure 4). After enumeration of the cells by fluorescence microscopy, we punched out single CTCs and subjected them to whole genome amplification (WGA) followed by digital PCR analysis for *KRAS* G12D and G12V. *KRAS* mutations were detected in both punches of whole blood and punches of DLA spiked with Capan1 (G12V) or Panc1 (G12D).

After establishing the dPCR analysis for isolated CTCs, we then used another aliquot of the same frozen pancreatic patients DLA to test the mutational analysis in patients CTCs. In this second round of analysis using only half of the input material, we detected CTCs in only 11% of the samples (2/19; M1 (22% (2/9)), M0 (0%, 0/10)). However, in the two samples of metastatic patients that gave the highest number of CTCs during the first test run, the presence of CTCs could be confirmed in similar numbers. In mutational analysis, both patients were *KRAS* negative while internal performance control for the mutation assays were positive (Table 2). For both patients, we sequenced the *KRAS* gene in primary tumor samples. Both tumors were wild-type for *KRAS* consistent with the CTC result (Supplementary Figure 6). In the samples of both patients we detected CTCs that were EpCAM-positive and EpCAM-negative showing the heterogeneity of CTC populations (Figure 5 and Supplementary Figure 7).

Discussion

In this study we show the superiority of filtering of CTCs in comparison to EpCAM-dependent capturing of CTCs. Using spike-in samples of well characterized cultured cells, we obtained very clear results showing that filtration is superior to EpCAM-dependent enrichment especially for EpCAM low expressing cells. However, using this approach for clinical samples we still obtained a relatively low frequency of CTC positive patients. We detected CTCs in 8/19 cases in the first round of analysis. In the second

analysis with less available input material, we could confirm the presence of CTCs in the two metastatic disease cases that also showed high CTC counts in the first analysis. The low frequency and number of CTCs despite the use of large amount of input material shows the limited clinical utility of this system in pancreatic cancer. Isolation of CTCs in pancreatic cancer has been recently reviewed [9]. This comprehensive overview reported a remarkable variation in detection rates and numbers of CTCs per ml between the different approaches tested. The reported detection rates range from 5% to 100% with a huge variability, essentially allowing no conclusion on the most suitable method. Interestingly the highest detection rates were reported using EpCAM-based methods.

The isolation of circulating tumor cells (CTCs) from patient samples faces several challenges: 1) Pre-analytical conditions (blood sampling, handling, storage and shipment), 2) Capturing of all phenotypical CTC subtypes, 3) Unequivocal definition of true CTCs by subsequent downstream analysis.

Ad 1) Eight to ten milliliter whole blood is the commonly used source for isolation of CTCs**.** The question on how to store unprocessed patient samples for later central analysis still remains unclear. One solution is the use of frozen diagnostic leukapheresis product, the enrichment of peripheral blood mononuclear cells. Diagnostic leukapheresis was already described as detection method for CTC as the number of CTCs and cases of CTC-positive patients increased also in early stages [37]. The use of DLA product as source for CTC isolation has been described and was also validated in this work (Figure 3). However, the way of blood sampling may not be optimal for pancreatic cancer. We hypothesize that peripheral blood from the cubital vein is not the optimal source for CTC isolation especially for pancreatic cancer because the CTCs from the pancreas will be transported to the portal vein first and have to pass the liver and may be filtered out already. This is supported by the fact that the 92% CTC positive cases were observed by Gall *et al.* using portal vein blood and the CellSearch platform [38]. In line with these

data, Catenacci et al. detected CTCs in 100% of portal vein samples (18/18) but only in 22% of the peripheral blood samples [39]. The fact that CTCs originating from the pancreas have to pass one more capillary system before entering the main circulation may be a reason for lower CTC counts in pancreatic cancer. Compared to other indications, pancreatic cancer showed the lowest CTC prevalence [13, 31]. Potentially the liver is especially effective in filtering out CTCs. This is supported by Bissolati and colleagues who found that CTC counts in portal-vein blood predicted liver metastasis [40]. In addition to blood sampling, consistent fixation protocols and preanalytics are necessary for optimal analytical performance and comparability of the results.

Ad 2) EpCAM low expressing cells are missed by EpCAM-dependent methods so that only the subpopulation of EpCAM high expressing CTCs is captured. In agreement with the earlier study of Akita et al who showed that half of the tumors express EpCAM in low or non-detectable levels [18], expression of the extracellular domain of EpCAM was found in only 79% of pancreatic tumor samples [41] and a detailed analysis in a different samples set showed that 29% of the tumors were negative and 36% only weakly expressing EpCAM [42]. The loss of epithelial antigens in a large fraction of pancreatic tumors is supported by the finding that E-Cadherin expression is lost in 53% of pancreatic tumors [43]. Filtration is an alternative to enrich larger tumor cells over the mostly smaller blood cells [44]. CTCs expressing EpCAM at variable levels are isolated by a filtration based method so that all CTCs with different surface marker should be isolated. The used filter contains pores with a size of 8µm. The performance of such device is also depending on cellular rigidity and applied pressure which may explain differences between the two different prototypes used in this study. Potentially the 8µm pore size may be too large for some pancreatic CTCs although it seemed suitable during the validation with cell lines. Spike-in cell lines do not reflect the patients CTCs but help to determine the technical performance of the method [11]. Recovery of CTCs by filtration might also be hindered by apoptosis as cells shrink during this process. This has

already been described in breast and prostate cancer [45]. Recently, isolation of pancreatic CTCs by both size and EpCAM-independent negative enrichment of pancreatic CTCs was described and showed very promising results [46].

Another filtration based system, the ISET method has been compared to the CellSearch system [31]. In that well designed study, ISET detected higher CTC counts compared to CellSearch but the CTCs detected by ISET were only characterized as CD45-negative larger cells. Although in this study, all tumors were EpCAM positive, the CTCs showed huge variability of EpCAM and even the cytokeratin expression varied dramatically, showing the difficult situation with the strong heterogeneity of real circulating tumor cells in contrast to cultured cells. Although ISET detected more CTCs than CellSearch, not even a trend for a correlation with progression free survival or overall survival was found. This is a strong argument against the clinical utility of the approach at least for pancreatic cancer. The lack of prognostic relevance together with the lacking consensus definition of a true CTC underlines the need for better characterization of CTCs in order to prove their tumor origin.

Ad 3) Concerning the clear identification of CTCs, it has to be pointed out that the majority of the CTC studies still rely on the visual identification of cells as stained or polymorph structures. Since staining intensity is highly dependent on the antigen expression and the quality of the used antibody there is no clear reference. Here, we tried to overcome the variety on staining be the usage of an antibody panel to increase staining and therefore facilitate the enumeration of CTCs. Although these improved methods showed benefits in studies with spiked cells, the number of CTC-positive patient samples remained low. EpCAM-independent methods should cope with CTCs originating of EpCAM-negative tumors or the loss of epithelial characteristics during EMT. However, also Cytokeratin loss may occur during EMT. For example, loss of Cytokeratin expression in Her2-FISH positive CTCs was described in the literature [47]. Recently

Gao et al., used CEP8 polyploidy as marker for pancreatic CTCs and found 96% of the isolated CTCs to be cytokeratin negative [46].

Therefore the clear identification of circulating tumor cells defined by other (more stable) features is required. Rhim and colleagues isolated epithelial cells in 8 of 11 patients with PDAC but detected the pancreatic specific Pdx-1 expression in only 29% of the cells isolated by EpCAM based microfluidics [48]. The identification of *KRAS* mutations in pancreatic CTCs should be a sensitive tool to prove at least their pancreatic origin although these mutations can already be detected in intra-epithelial neoplasias of the pancreas (PanIn) and do not prove the presence of cancer [49]. However, we did not find evidence for *KRAS* mutations in the two samples that were CTC positive. This finding was confirmed by sequencing primary tumor samples which turned out to be wild-type. We conclude that the 57% prevalence of *KRAS* mutations is already too low to use it as reliable marker for CTC confirmation. The high detection rate of 88% recently published for CEP8 polyploid CTCs [46] hints towards use of cancer-specific biomarkers with higher prevalence for the identification of CTCs in contrast to the merely epithelial phenotype.

In conclusion, we showed the advantage of a size based method in isolation of CTCs from pancreatic cancer and the feasibility of frozen DLA for detection of CTCs with subsequent downstream analysis in patient samples. Clinical use of circulating tumor cells in pancreatic cancer still needs a lot of efforts in ways of blood sampling, standardization of the preanalytics, the isolation procedures and definition of true CTCs with clear prove of their tumor cell identity.

Material and Methods

Patients

Whole blood of healthy donors was collected in EDTA-vacutainer (BD) by Clinical Research Services Berlin GmbH, Berlin. Donors were clinically healthy without any known neoplastic or infectious disease and provided written informed consent to use their blood samples for research purposes.

Diagnostic Leukapheresis samples

Diagnostic Leukapheresis (DLA) was exactly performed as previously described [37]. Part of the DLA product was spun at 200 x g for 10 min and the supernatant was removed. The DLA product was adjusted to a white blood cell (WBC) concentration of $1x10^8$/mL with 5% human serum albumin (HSA, 20%, octopharma) in RPMI-1640. Aliquots of 1mL DLA product were mixed with 1mL freezing medium (4mL HSA, 4mL RPMI and 2mL DMSO), frozen in a freezing container at -80°C over night and then transferred to storage in vaporous phase of liquid nitrogen.

The use of DLA for CTC screening of an increased blood volume was approved by the ethics committee of the Heinrich Heine University Hospital Duesseldorf. All participating patients and healthy donors gave written informed consent.

Methods

Cell culture and spike in of cells

Cell lines used for this study were obtained from ATCC and DSMZ. Capan1 was cultured in RPMI1640 + 20% FBS + 1% Glutamin, BxPc3 in RPMI1640 + 10% FBS + 1%

Glutamin, Panc1 in DMEM + 10% FBS + 1% Glutamin and SkBr3 in McCoys 5a +10% FBS +1% Glutamin at 37°C and 5% CO_2. Prior spiking, cells were washed once with 1x PBS (Gibco) and detached at 85% confluence by Accutase solution (Sigma). Cells were spun 5 min at 127 x g, resuspended in PBS and strained through a 35µm strainer to enrich the amount of single cells.

0, 1, 3, 10 and 30 cells were transferred manually into 10mL whole blood of healthy donors collected in EDTA-vacutainer (BD) and processed. The same number of cells was spiked in frozen healthy DLA in the vial directly after thawing. Cells were also spiked in freshly prepared buffy coat containing the same cell count as DLA, frozen and thawed for analysis.

Fluorescence staining

Detached cells were counted by CASY (Innovatis) and fixed by addition of Transfix (Caltag Medsystems) at a ratio of 1:20 for 45 min. Cells were spun on slides by cytospin Universal 320 (Hettich Lab). In brief, 1 x 10^5 cells were used per cytochamber (3 Chambers per slide) and centrifuged for 7 min at 750 x g with break on. Liquid was removed and then spun again at 100 x g for 1 minute with break off. Slides were dried over night at room temperature. Cells were permeabilized by 0.5% Triton for 15 min, washed with 1 x PBS, blocked with 10% normal goat serum, 1% BSA, 0.05% Tween and 0.5% blocking solution® (Candor) and stained for 45 min by either AE1-AE3 (1:100) or by the anti-Cytokeratin Alexa Fluor 488 panel (Supplementary Table 2, (AE1-AE3, C-11, A53B/A2, DC10, LPK5, each 1:100)) as well as by anti-EpCAM-Alexa Fluor 555 (VU1D9, 1:50). Cells were washed by PBS and nuclei were stained by HOECHST 33342 for 1 min following a wash step. Slides were dried at 60 °C for a maximum of 5 min and mounted

with Prolong Diamond antifade mountant (Thermofisher) and examined by an epifluoresence microscope (Observer Z.1 Zeiss and AxioVision V 4.8.2.0 Software).

Isolation of cells from blood by filtration (Siemens)

Blood was processed according to the manufacturer's instructions as described in [32]. In short, Transfix was added to 8-10mL whole blood (1:20) and incubated for 1h. Prefixed whole blood was transferred to a 50mL conical tube, EDTA-vacutainer was rinsed with PBS twice to a final volume of 20mL and subjected to the filtration device. Diluted blood was filtrated and the filter was rinsed with PBS. Cells on filter were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (Electron Microscopy Sciences), permeabilized with 0.5% Triton-X 100 (Sigma) and blocked as previously described. Cells were stained using the anti-Cytokeratin-Alexa Fluor 488-panel (Supplementary Table 2), anti-leukocyte-Alexa Fluor 647- panel (CD45 (MEM 28), CD66b (G10F5), CD3 (Sk7), CD14 (G1D3), CD68 (KP1), each 1:50), anti-EpCAM-Alexa Fluor 555 (VU1D9, 1:50) and Hoechst 33342. Stained filters were analyzed manually by Observer Z.1 (Zeiss) and AxioVision V 4.8.2.0 Software.

Isolation of cells from blood by IsoFlux (Fluxion Biosystems)

Blood was processed according to the recommendations by Fluxion Biosystems. Briefly, whole blood was separated by overlaying onto Ficoll (GE Healthcare). Blood and Ficoll were spun at 800 x g for 30 min with breaks off. Buffy coat and plasma were transferred to a new tube and centrifuged at 280 x g for 10 min. Supernatant was removed and the pellet was loosened and transferred to a 2mL tube. EpCAM-coated beads were added. Cells and bead mixture were incubated for 2 h with overhead rotation. The bead

suspension was then loaded into the cartridge and cells were isolated automatically by the device. After isolation cells were stained as previously described.

Isolation of cells from frozen DLA

Frozen DLA was thawed at 37°C in the cryovial and transferred to a 50mL tube with 1000U of Cyanase (Ribosolution) and 1mL pre-warmed thaw-solution (1:10 CTL-Wash Supplement in RPMI-1640) added dropwise to the DLA sample. In total 15.8mL Thaw Solution were added to a final volume of 20mL as follows: First, 9mL Thaw solution were slowly added dropwise. Cell suspension was incubated for 10 min at room temperature and cells were counted by Türks-Staining (Merck). Then, 6.8mL Thaw solution and 20µL cyanase inactivating protein (Ribosolution) was added (see also Supplementary Figure 8); tubes were incubated at room temperature for 20 min. 2mM EDTA (Life Technologies) and 1:20 Transfix was added. Tubes were incubated for 1 h and then directly subjected to the filtration unit. For the first round of analysis (experimental setup A), an early prototype of the Siemens Device was used. For experimental setup B, we had to use a second-generation filtration device. Cells were filtered and stained as described above and in [32].

Mutational analysis of isolated cells by digital PCR

Isolated cells were analyzed manually by fluorescence microscopy using a Zeiss Observer Z.1 and punched from filter with a 2mm Biopsy punch (pfm medical). The punch was controlled for presence of the CTC, transferred to a thin walled PCR tube and frozen until further use. Once shortly thawed, cells on punshes were subjected to whole genome amplification (Ampli1 WGA Kit, Silicon Biosystems), 1x for whole blood samples and 5x for DLA-samples. WGA-DNA solution was purified by Amicon Ultra 0.5mL

centrifugal filter (Merck Millipore)**.** In brief, WGA solution was added to the filter, washed twice with TE and eluted. Pure DNA was quantified by Broad Range High sensitivity DNA Assay and Qubit (Life Technologies). 50ng DNA was used for *KRAS* G12D and G12V by digital PCR (Quantstudio 3D, Thermofisher) as described in detail [50].

Tumor DNA preparation

Tumor tissues were marked on standard H&E-stained histologic slides. Afterwards, unstained serial sections of tumor tissues were mounted onto glass slides and macrodissected for DNA extraction. Every macrodissected tumor sample was cross-checked confirming that the percentage of tumor tissue was at least 80%. The extracted tumor cells were dissolved in a total volume of 190 µL digestion buffer (DNA tissue mini kit, Qiagen) and were treated with proteinase K overnight at 56°C. DNA purification was achieved using a nucleic acid robot device (BIO 101, Qiagen).

Sequence analysis

For sample 5539, PCR amplification was done in a total volume of 20µL containing 20ng genomic DNA, 0.2 mmol/L deoxynucleotide triphosphate, 0.5 units of Taq polymerase (HotStar Taq, QIAGEN), and the following k-ras primers: Fwd_5′-AGGCCTGCTGAAAATGACTGAA-3′, Rev_5′-AAAGAATGGTCCTGCACCAG-3′. Cycle sequencing analysis of PCR fragments was done with the BigDye Terminator system (PE Biosystems) using amplification primers for bidirectional sequencing. The reaction products were analyzed on an ABI PRISM 3700 sequencer (PE Biosystems).

For sample 5903, sequencing was done at GATC Biotech (Konstanz, Germany) using DreamTaqGreen Master Mix (ThermoFisher). Primers used:

5`CCTTATGTGTGACATGTTCTAATATAGT, 3` TTGGATCATATTCGTCCACAA. (167 bp PCR product, Tm 57.5°C). PCR conditions: 0.2µM Primers each, 0.5µL DNA Template. Cycling conditions: 95°C for 3 min; 95°C for 30 sec, 57.5°C for 30 sec, 72°C for 20 sec, repeat 30 times and final elongation at 72°C for 5 min.

Abbreviations

CTC, circulating tumor cells; DLA, diagnostic leukapheresis; EpCAM, Epithelial cell adhesion molecule; PanIn, Pancreatic Intraepithelial Neoplasia; PDAC, Pancreatic ductal adenocarcinoma; PFS, Progression-free survival; OS, Overall survival; EMT Epithelial Mesenchymal Transition, *KRAS*, Kirsten rat sarcoma viral oncogene homolog

Author Contributions

Nora Brychta: Study conception, Setup of IsoFlux-Experiments, CTC-experiments, Development of mutation detection, Performance of experiments and data collection, writing manuscript and visualization of data

Michael Drosch: Development of antibody-panels, Development of Siemens-Workflow, CTC-experiments, writing of manuscript

Christiane Driemel: Data collection, data analysis and CTC experiments

Rui P. Neves: Data collection, data analysis and CTC experiments

Johannes C. Fischer: DLA, data collection and data analysis

Irene Esposito: Sequencing of primary tumor samples and data collection

Wolfram Knoefel: Patient selection and recruitment, data analysis

Birte Möhlendick: Mutational analysis of tumor tissue

Claudia Hille: Data acquisition using Siemens

Antje Stresemann: Data review and writing the manuscript

Thomas Krahn: Funding acquisition, study design and writing the manuscript

Matthias U. Kassack: Writing the manuscript, Supervision

Nikolas H. Stoecklein: Study conception, Project administration, data review and analysis, writing the manuscript

Oliver von Ahsen: Study conception, Supervision, Project administration, writing the manuscript

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Conflicts of Interest

The authors have no conflicting interests to disclose.

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Tables

PID	Tumor type	Tumor stage	Isolated CTCs
5447	pancreatic	T3N1M1	0
5549	pancreatic	T3N1M0	2
5580	pancreatic	T3N1M1	0
5714	pancreatic	T3N1M0	0
5751	pancreatic	T3N1M0	0
5792	pancreatic	T3N1M1	1
5803	pancreatic	T3N1M1	2
5804	pancreatic	T3N2M0	2
5902	pancreatic	T3N1M0	0
5904	pancreatic	M1 (TxNx)	7
5931	pancreatic	T3N0M0	0
6012	pancreatic	T3N1M1	0
6017	pancreatic	M0	0
6033	pancreatic	T3N1M0	1
6098	pancreatic	M1	0
6104	pancreatic	T3N1M0	0
5689	pancreatic	T3N1M0	2
6016	pancreatic	M1	0
5539	pancreatic	M1	7

Table 1: Number of detected CTCs isolated from frozen DLA of pancreatic cancer patients in sample set A (2 vials). Overall detection rate of CTCs was 42% (44% M1, 40% M0). PID = patient identification number.

PID	Tumor type	Tumor stage	Isolated CTCs	Detected mutation	
				in CTCs	in tumor
5447	pancreatic	T3N1M1	0	NA	NA
5549	pancreatic	T3N1M0	0	NA	NA
5580	pancreatic	T3N1M1	0	NA	NA
5714	pancreatic	T3N1M0	0	NA	NA
5751	pancreatic	T3N1M0	0	NA	NA
5792	pancreatic	T3N1M1	0	NA	NA
5803	pancreatic	T3N1M1	0	NA	NA
5804	pancreatic	T3N2M0	0	NA	NA
5902	pancreatic	T3N1M0	0	NA	NA
5904	pancreatic	M1 (TxNx)	8	wildtype	wildtype
5931	pancreatic	T3N0M0	0	NA	NA
6012	pancreatic	T3N1M1	0	NA	NA
6017	pancreatic	M0	0	NA	NA
6033	pancreatic	T3N1M0	0	NA	NA
6098	pancreatic	M1	0	NA	NA
6104	pancreatic	T3N1M0	0	NA	NA
5689	pancreatic	T3N1M0	0	NA	NA
6016	pancreatic	M1	0	NA	NA
5539	pancreatic	M1	12	wildtype	wildtype

Table 2: Number of detected CTCs isolated from frozen DLA of pancreatic cancer patients for mutational analysis in sample set B (1 vial). Overall detection rate of CTCs was 11% (22% M1, 0% M0). PID = patient identification number.

Legends for figures

Figure 1: Cytokeratin expression in pancreatic cancer. Pancreatic cancer cell lines Capan1, BxPc3, Panc1 and breast cancer cell line SkBr3 were characterized by immunofluorescence staining anti EpCAM (Alexa Fluor 555, orange) and anti-Cytokeratin (Alexa Fluor 488, green). Nucleus was stained by Hoechst 33342 (blue). Scale bars represent 20µm.

Figure 2: Recovery rates by filtration and EpCAM-dependent isolation method of spiked cells in whole blood. (A) Recovery rates by EpCAM dependent magnetic bead isolation is lower in pancreatic cancer cells compared to SkBr3. (B) The filtration method shows high recovery rates independent of the EpCAM-expression. (C) Representative pictures of Capan1. The filtration also helps to identify morphology whereas the beads overshadow the signal of the immunofluorescence staining. Cells were stained by anti-Cytokeratin (green), anti-leukocyte panel (magenta) and nucleus (blue). Scale bars represent 20µm.

Figure 3: Recovery rates by filtration of spiked cells in frozen DLA. (A) Recovery rates of the thawed DLA are similar to the results of filtration of whole blood. (B) Representative pictures of Capan1 isolated from frozen DLA by filtration. Cells were stained by anti-Cytokeratin (green), anti-EpCAM (orange), anti-CD panel (magenta) and nucleus (blue). Scale bars represent 20µm.

Figure 4: Mutation detection of CTCs in whole blood and frozen DLA. (A) Detected cells were punched from filter and relocated on the punch before WGA. (B) Amplified DNA was analyzed by digital PCR for *KRAS* G12D and *KRAS* G12V. Note higher background of leukocytes in DLA. Scale bars represent 200µm (filter) and 20µm (single cell).

Figure 5: Detection of CTCs in frozen DLA samples from 19 pancreatic cancer patients. Representative pictures of isolated cell from patient 5904 and 5593. Within one patient both EpCAM high and EpCAM low CTCs were detected (see also Supplementary Figure 5). Cells were stained by anti-Cytokeratin (green), anti-EpCAM (orange), anti-CD panel (magenta) and nucleus (blue). Scale bars represent 20µm.

Figure 1

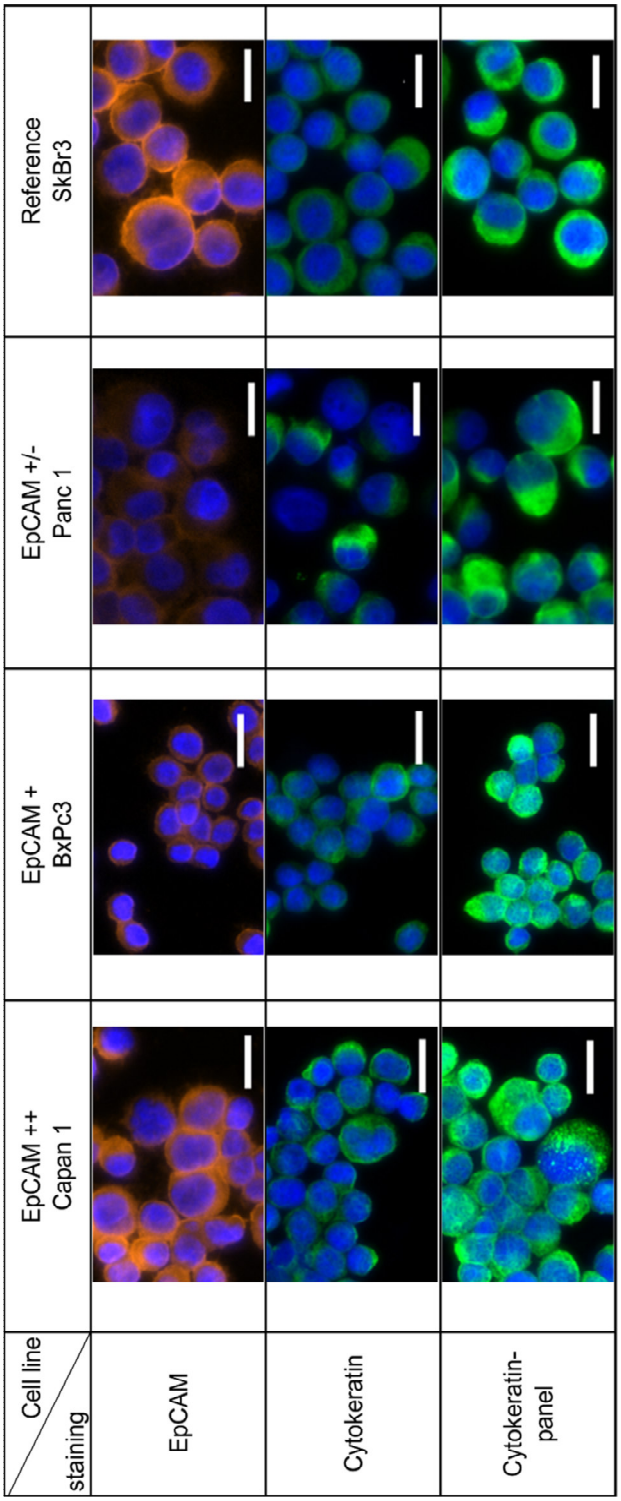

Cell line
staining
EpCAM ++
Capan 1
EpCAM +
BxPc3
EpCAM +/-
Panc 1
Reference
SkBr3
EpCAM
Cytokeratin
Cytokeratin-
panel

Figure 2

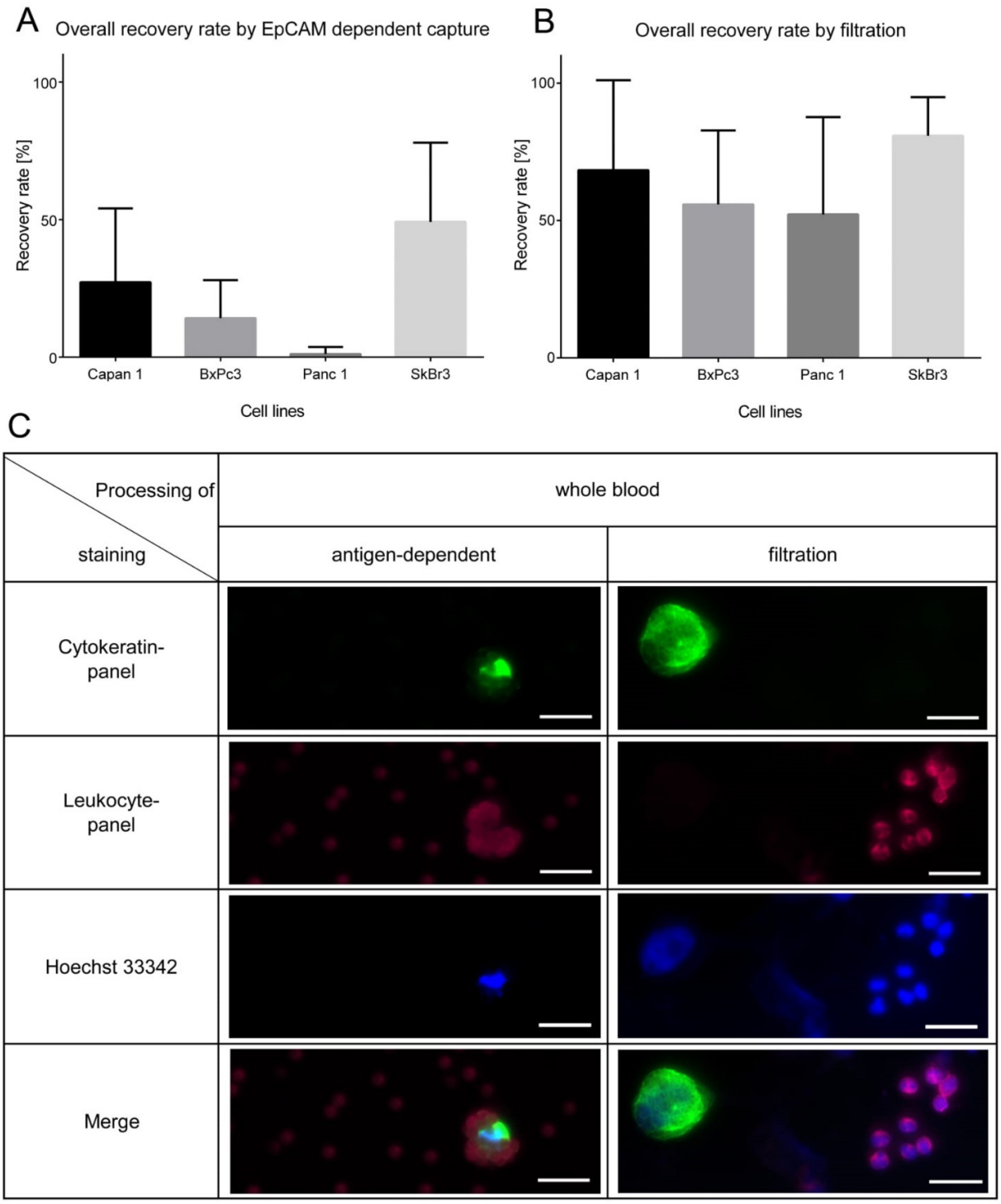

A
Overall recovery rate by EpCAM dependent capture
Recovery rate [%]
100
50
0
Capan 1
BxPc3
Panc 1
SkBr3
Cell lines
B
Overall recovery rate by filtration
Recovery rate [%]
100
50
0
Capan 1
BxPc3
Panc 1
SkBr3
Cell lines
C
Processing of
staining
whole blood
antigen-dependent
filtration
Cytokeratin-panel
Leukocyte-panel
Hoechst 33342
Merge

Figure 3

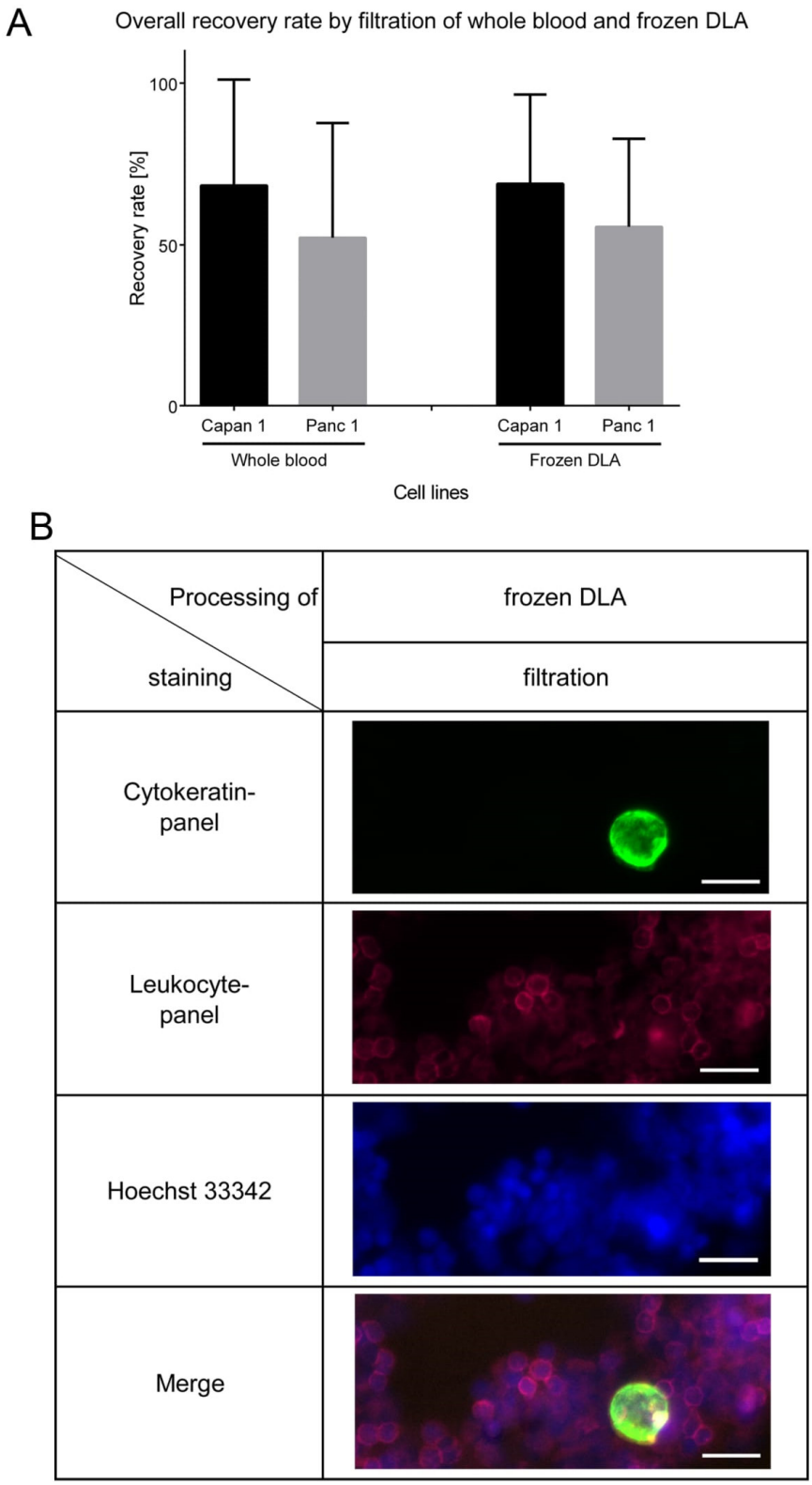

A
Overall recovery rate by filtration of whole blood and frozen DLA
Recovery rate [%]
100
50
0
Capan 1
Panc 1
Capan 1
Panc 1
Whole blood
Frozen DLA
Cell lines
B
Processing of
staining
frozen DLA
filtration
Cytokeratin-panel
Leukocyte-panel
Hoechst 33342
Merge

Figure 4

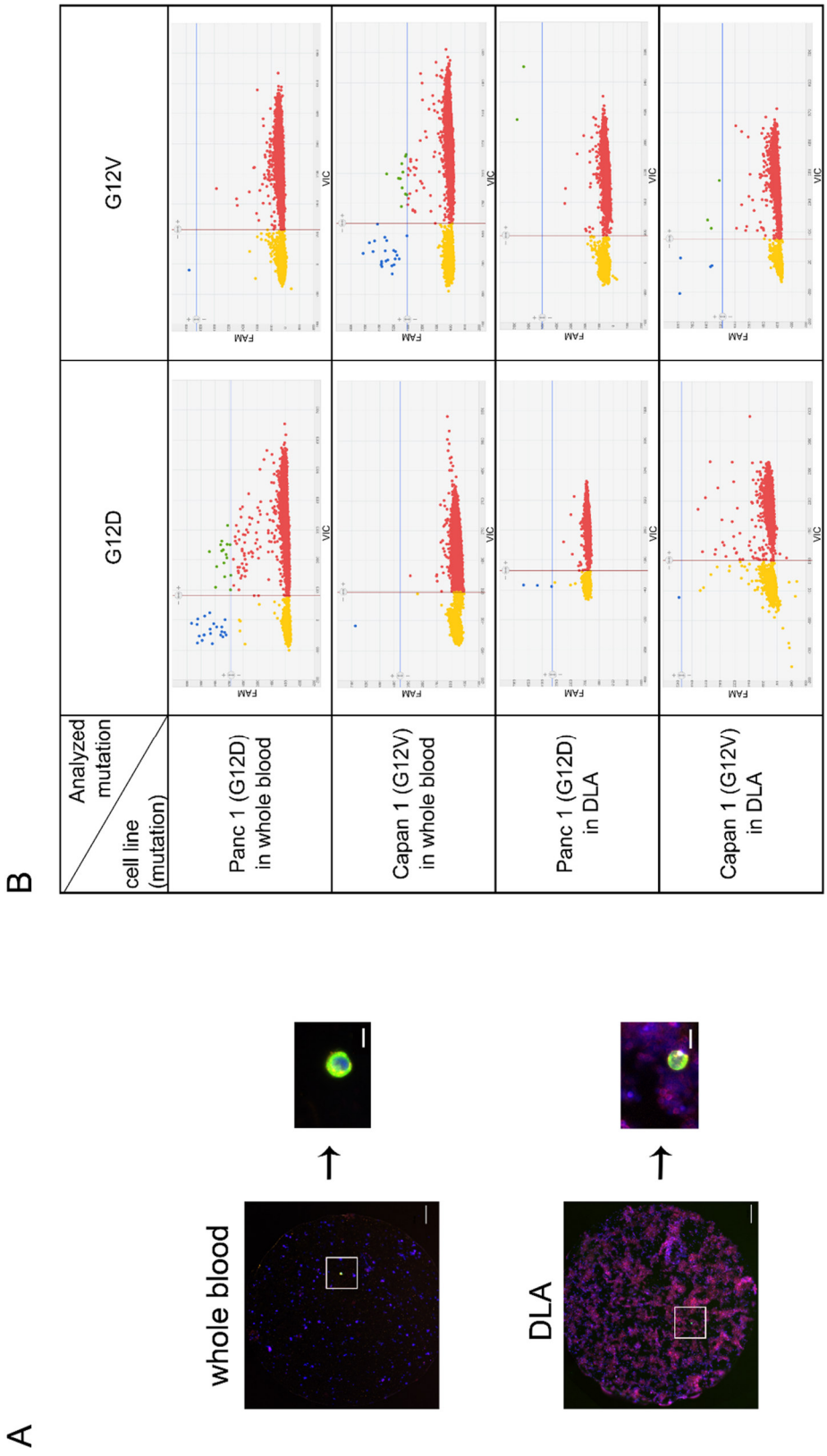
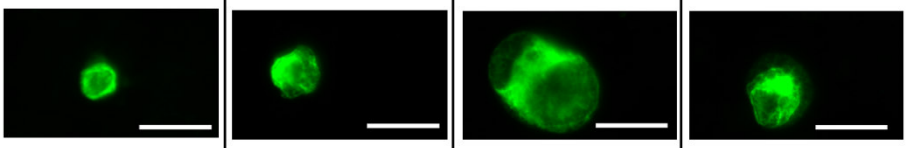
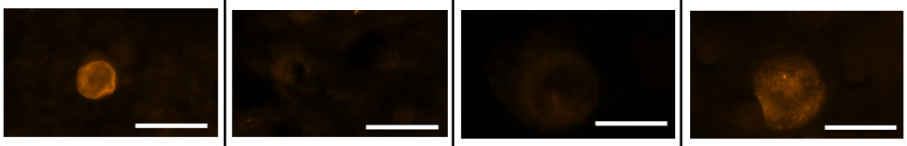
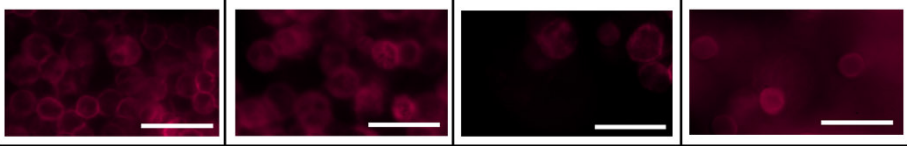
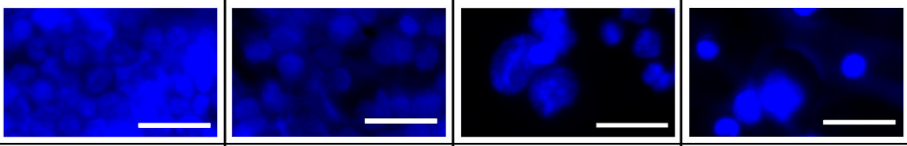
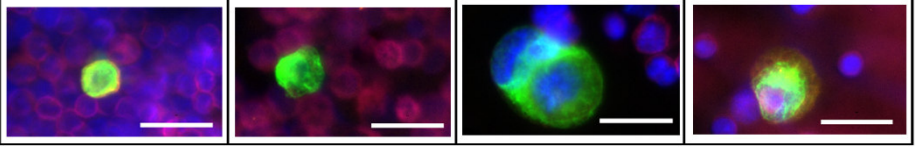
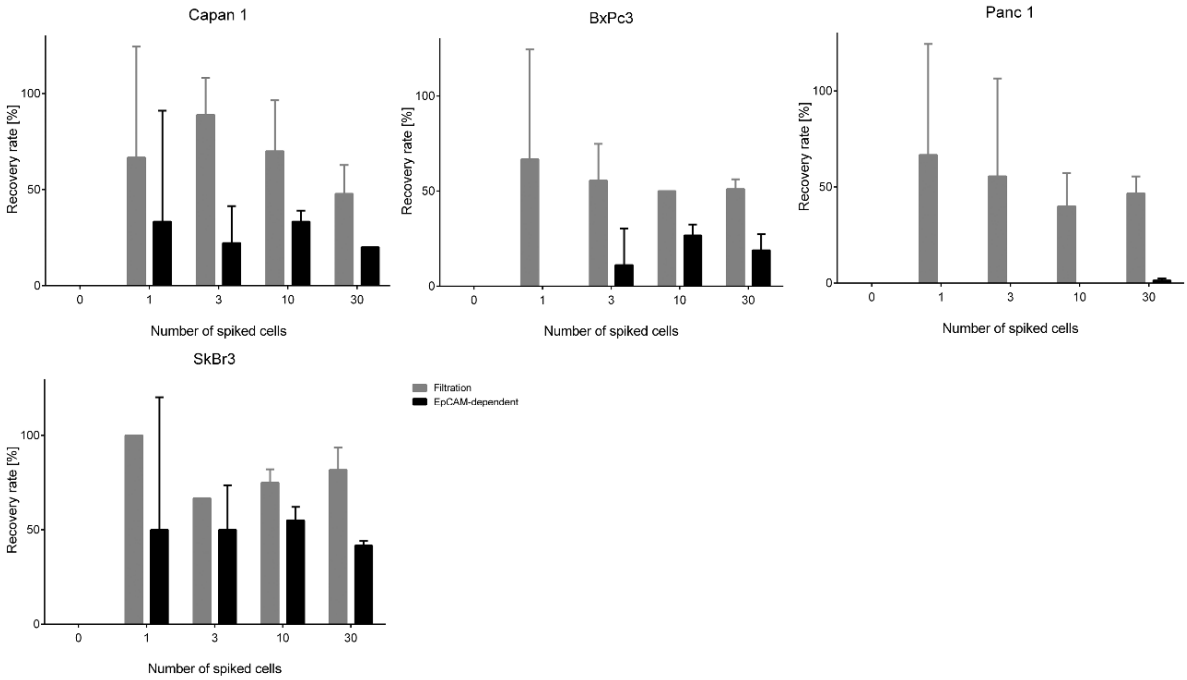

A
whole blood
DLA
B
Analyzed mutation
cell line (mutation)
G12D
G12V
Panc 1 (G12D) in whole blood
Capan 1 (G12V) in whole blood
Panc 1 (G12D) in DLA
Capan 1 (G12V) in DLA
FAM
VIC

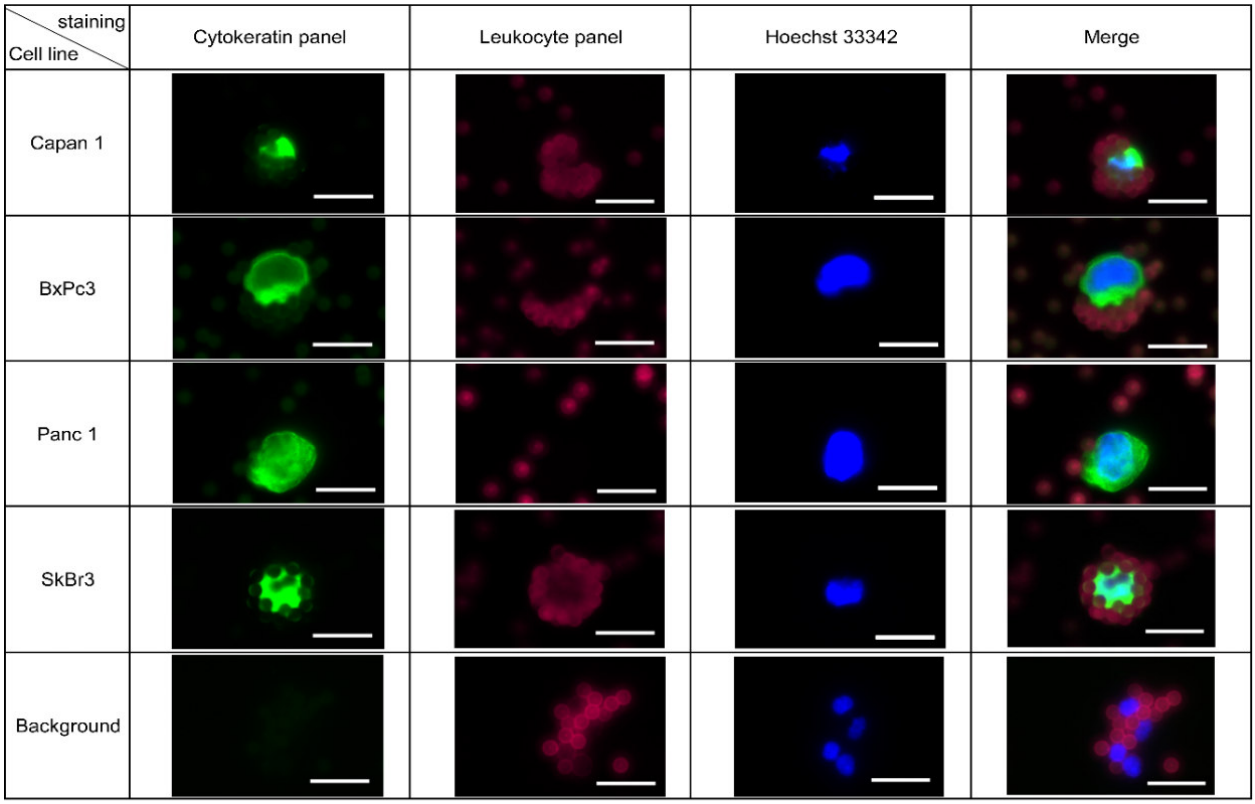
Figure 5

Filtration of / staining	DLA from pancreatic cancer patient 5904		DLA from pancreatic cancer patient 5539	
	CTC 1	CTC 2	CTC 1	CTC 2
Cytokeratin panel				
EpCAM				
Leukocyte panel				
Hoechst 33342				
Merge				

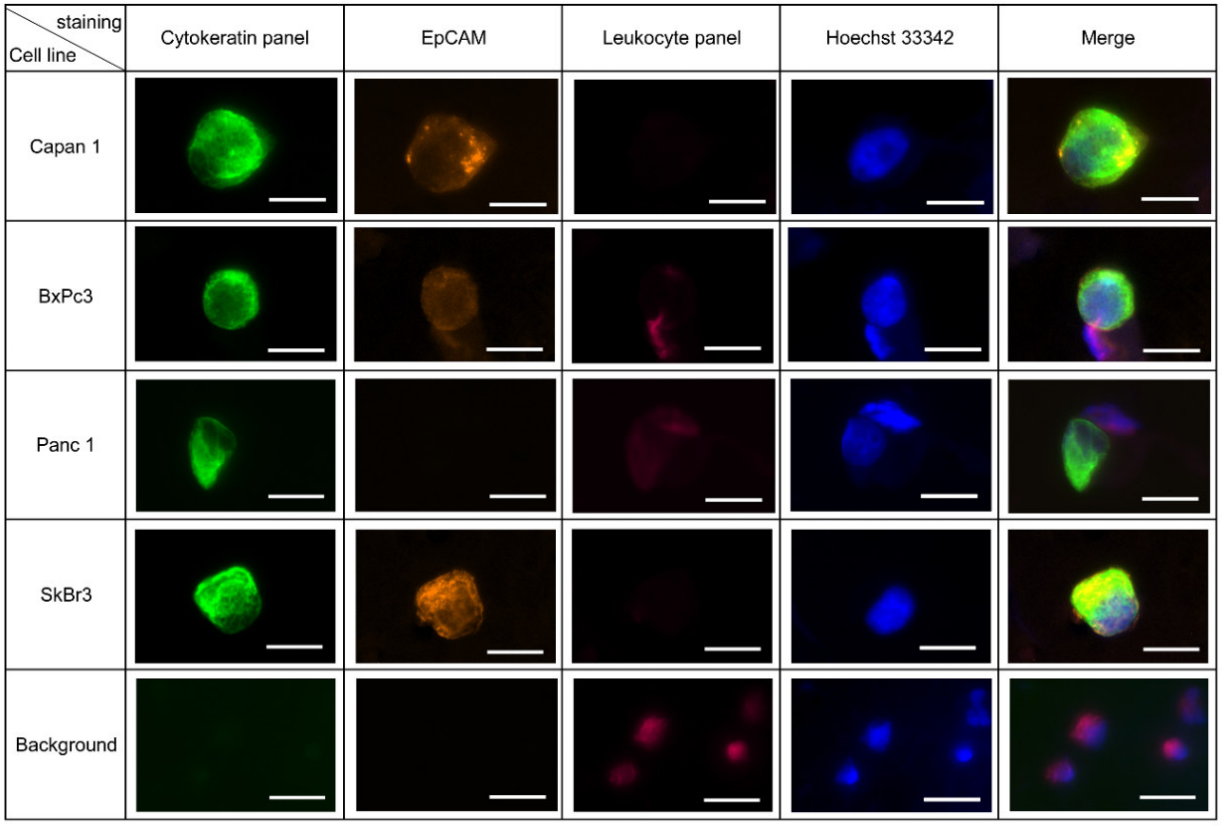
3.2.1 Supplementary Figure



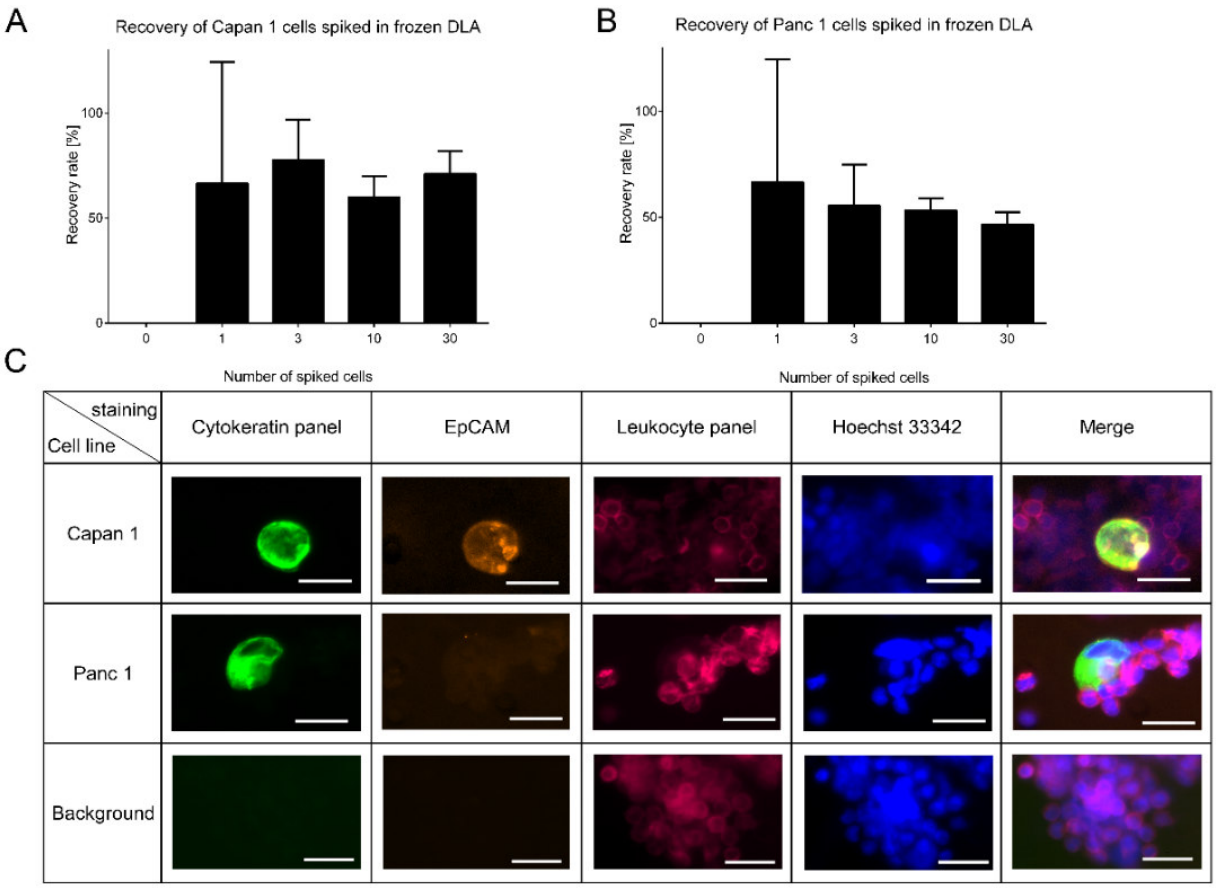
Supplementary Figure 1: Recovery rates of spike in experiments in whole blood.
1, 3, 10 and 30 Capan 1 (EpCAM high), BxPc3 (EpCAM medium) and Panc 1 (EpCAM low) as well as breast cancer cell line SkBr3 (EpCAM high) were spiked into whole blood and isolated by filtration (grey) and EpCAM dependent capture (black).



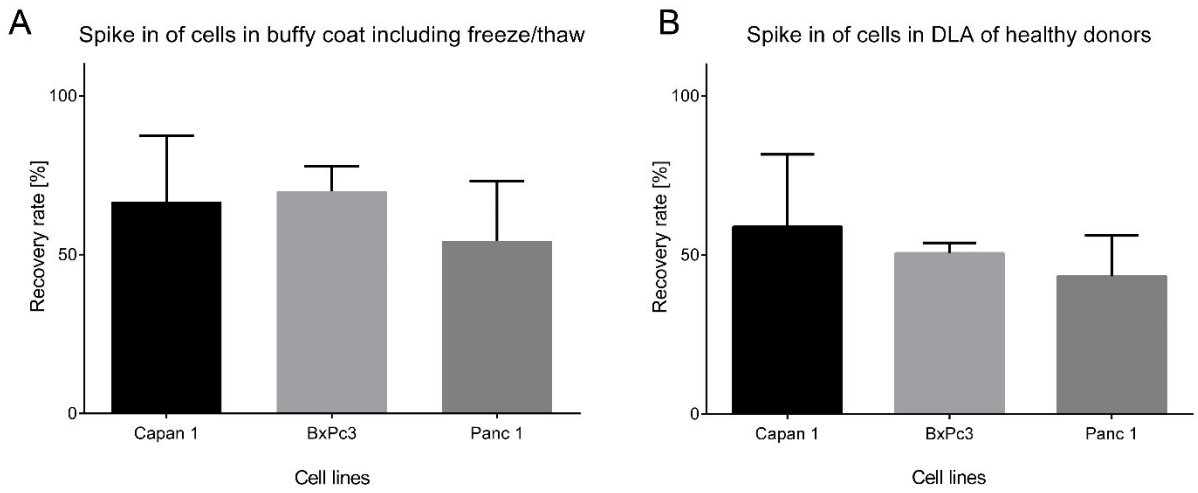
Supplementary Figure 2: Representative pictures of isolated cells from whole blood by IsoFlux. Scale bar represents 20µm



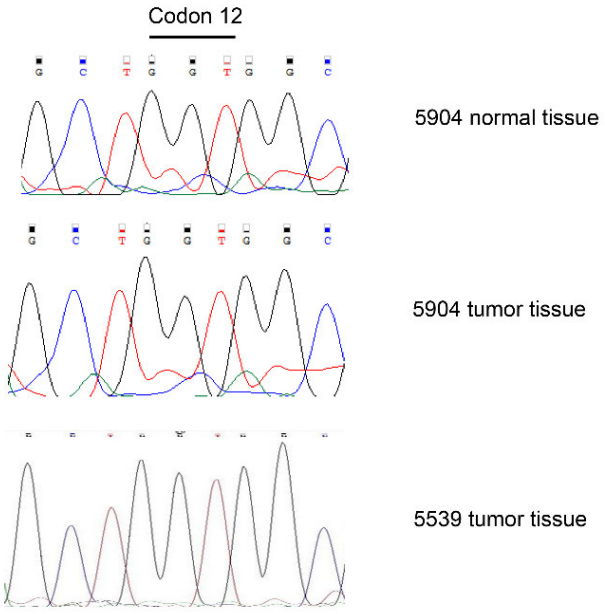
Supplementary Figure 3: Representative pictures of isolated cells from whole blood by filtration. Scale bar represents 20µm.



Supplementary Figure 4: Frozen DLA
(A) Recovery rate of filtration of spiked cells spiked into frozen DLA, (B) Representative pictures of isolated cells, (C) Representative pictures of cells isolated from frozen DLA



Supplementary Figure 5: No difference in recovery rate if samples were frozen
(A) Cells were spiked into freshly prepared PBMCs, frozen and thawn according to protocol
(B) Cells were spiked into thawn DLA from healthy donor.



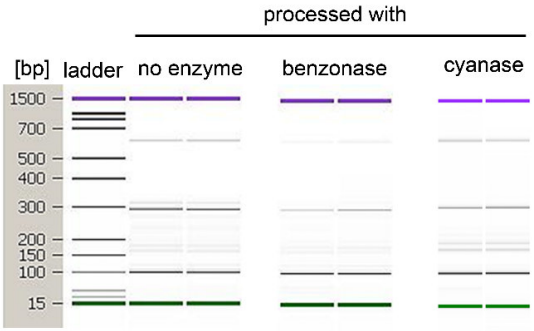
Supplementary Figure 6: Sequencing data for codon 12

CTC / sample	1	2	3	4	5	6	7	8
5904								

CTC / sample	1	2	3	4	5	6	7	8
5539								

CTC / sample	9	10	11	12
5539				

Supplementary Figure 7: Representative pictures of CTCs isolated from pancreatic cancer patients



Supplementary Figure 8: Effect on DNA-quality after thawing of samples using no enzyme, Benzonase (25U/mL) and Cyanase (50U/mL). Samples were processed according to protocol on the filtration device by using different or no enzyme. Isolated cells were punshed from filter and DNA was amplified by DNA. 1µL of WGA-DNA was used for quality control (QC). QC was conducted by amplifying four genomic loci of different length (91, 108-166, 299, 614bp). As benzonase activity is not inhibited during processing, DNA is digested as seen by faint bands especially at 614 bp. This is not seen in samples using cyanase as this enzyme can be inhibited during processing. Processing with no enzyme resulted in little recovery, benzonase and cynase in similar recovery.(data not shown).

3.2.2 Supplementary Tables

Cancer type / Cytokeratin	Pancreatic Cancer
1	
2	
3	
4	
5	
6	
7	x
8	x
9	
10	
11	
12	
13	x
14	
15	
16	
17	
18	x
19	x
20	

Supplementary Table 1: Cytokeratin expression in pancreatic cancer

(Min Jong Lee, Hye Seung Lee, Woo Ho Kim, Yunhee Choi and Mihi Yang (2003): Expression of Mucins and Cytokeratins in Primary Carcinomas of Digestive System. Mod Pathol; 16(5):403-410)

Antibody / Cytokeratin	AE1-AE3	C-11	A53-B/A2	DC10	LP5K
1	x				
2	x				
3	x				
4	x	x			
5	x	x			
6	x	x			
7	x				x
8	x	x			
9					
10	x	x			
11					
12					
13		x			
14	x				
15	x				
16	x				
17					
18		x		x	
19	x		x		
20					

Supplementary Table 2: Cytokeratin antibody panel

3.3 Enumeration and Molecular Characterization of Tumor Cells in Lung Cancer Patients Using a Novel *In vivo* Device for Capturing Circulating Tumor Cells

Tobias M. Gorges, Nicole Penkalla,Thomas Schalk, Simon A. Joosse, Sabine Riethdorf, Johannes Tucholski, Klaus Lücke, Harriet Wikman, Stephen Jackson, Nora Brychta, Oliver von Ahsen, Christian Schumann, Thomas Krahn, and Klaus Pantel.

Clin Cancer Res. 2016 May 1;22(9):2197-206.

Name of the journal: Clinical Cancer Research

Status: accepted

DOI: 10.1158/1078-0432.CCR-15-1416

Impact Factor (2015/2016): 8,738

Authorship: Co-Author

Contribution: 5%

- digital PCR experiments and analysis and visualization of data

URL: http://clincancerres.aacrjournals.org/content/22/9/2197.long

4 Overall discussion

Pancreatic cancer is a dismal disease with poor prognosis for the patient. Early diagnosis might lead to surgery - the only cure to date - and better prognosis for the patient if sensitive screening methods were available. Although the medical need for early diagnosis is tremendous, no biomarkers e.g. blood-based are in clinical use for screening purposes. The reason is the insensitivity of such biomarkers and their lack of specificity (see Introduction). Alternatives have emerged based on minimally invasive liquid biopsy like the analysis of ctDNA and CTCs which may have the potential for early diagnosis and screening.

In the following discussion, the potential of ctDNA and CTCs and their comparison as early diagnostic marker in pancreatic cancer is debated. An additional excursus to personalized medicine shall reveal the usage of CTCs in lung cancer.

4.1 Detection of mutations in liquid biopsies in early stages of pancreatic cancer

4.1.1 Circulating tumor DNA

After the discovery of physiological cfDNA in plasma, efforts were made to draw conclusions from cfDNA concentrations about a possible malignancy. However, the use of cfDNA alone as a diagnostic marker to detect a primary disease is difficult due to several reasons detailed in the following paragraph. Throughout publications about circulating DNA it is a familiar problem that differences in preanalytical sample handling, e.g. the absence of a standard protocol, impede comparison of results from different studies. The impact was already shown in several studies: Fleischhacker et al. (2011) described different isolation methods and the resulting varying cfDNA amounts. Sherwood et al. (2016) described the influence of using different preservative tubes on the amount of isolated cfDNA and detection of *KRAS* mutations. Furthermore, no coincident opinion determines the use of serum or plasma. The latter is used more frequently in studies, as it was shown that the amount of contaminating genomic cfDNA is higher in serum than in plasma, probably due to lysis of cells during coagulation of blood (Lee et al., 2001). Moreover, the use of different plasma volumes resulted in

different amounts of cfDNA, thus leading to different conclusions about significances of the results (see 1.5.1 Circulating tumor DNA). These methodological differences complicate the validation of cfDNA as biomarker. Additionally, physical activity and several inflammatory diseases also increase cfDNA-content with no sign of cancer (Breitbach et al., 2014; Chang et al., 2003; Zhong et al., 2007). Therefore, the tumorous origin of cfDNA namely ctDNA has to be proven for its use in cancer diagnostics.

KRAS mutations in ctDNA have been successfully detected in cancer types like colorectal cancer as early detection of minimal residual disease post-surgery was associated with negative prognosis (Kidess et al., 2015; Tie et al., 2016). The occurrence of mutant ctDNA after pancreatectomy in late stage patients could be detected before imaging techniques were able to detect the relapse (Sausen et al., 2015). These studies already proved the importance of *KRAS* mutations in ctDNA analytics.

To date, most studies analyzing matched plasma and tumor samples from pancreatic cancer patients strongly focus on advanced, undruggable late stages. *KRAS* detection rates in plasma samples from advanced pancreatic cancer patients were successfully reported ranging from 26 to 88% (Bettegowda et al., 2014; Calvez-Kelm et al., 2016; Earl et al., 2015; Pietrasz et al., 2016; Takai et al., 2015; Tjensvoll et al., 2016). Where tumor tissue was taken as positive control, concordances of tumor and plasma harboring the same *KRAS* mutation were achieved by 35-100% in advanced pancreatic cancers (Hadano et al., 2016; Kinugasa et al., 2015; Pishvaian et al., 2016; Uemura et al., 2004). In early stages of pancreatic cancer, Bettegwoda et al. (2014) reported 48% detection rates of ctDNA in plasma by e.g. BEAMing, but no tumor tissue was analyzed to confirm the results obtained from plasma. BEAMing is a highly sensitive method to detect low frequencies of mutant alleles. This technique combines a water-oil emulsion PCR on magnetic beads and detection by flow cytometry (Dressman et al., 2003). Sausen et al. (2015) described 43% mutation detection rates in plasma concordant to matched pancreatic tumor by digital PCR using high plasma volumes. In other studies, specifically early stages of pancreatic cancer mutations could not be detected sufficiently (Marchese et al., 2006; Takai et al., 2015). In summary, the concordances of mutations detected in matched plasma and tumor in advanced stages of pancreatic cancer are high. In contrast to that, very few positive results were obtained from localized or early stages of pancreatic cancer to explore the possibility and limitations of an early diagnostic marker.

In this work, matched tissue and plasma from pancreatic cancer patients were analyzed for *KRAS* mutations by highly sensitive chip-based digital PCR (dPCR) (Brychta et al.,

2016). Digital PCR is a technique to identify single mutations in a highly sensitive way by distributing the reaction into compartments. The limit of detection by dPCR was determined as five mutant alleles in a background of 50 ng wild-type DNA. *KRAS* mutations were concordantly found in tumor and plasma in 36% (*KRAS* G12D), 50% (*KRAS* G12V) and 0% (*KRAS* G12C) of the cases. The overall detection rate was 35% by using only 2 mL plasma and an optimized protocol for isolation of cfDNA and detection of mutant ctDNA by dPCR. These results are highly favorable as they show the highest diagnostic sensitivity and concordance to tumor tissue with limited plasma volume published to date in pancreatic cancer.

For the use in early diagnosis various aspects need to be considered, including prevalence of the mutation, used technology, allelic frequency and tumor content. Although mutations in *KRAS* occur early during carcinogenesis, pancreatic cancer is the cancer type associated with frequent *KRAS* mutations. In colon and lung cancer, the incidence of *KRAS* mutations is lower than in pancreatic cancer and therefore more difficult to evaluate as early diagnostic marker than in pancreatic cancer (colon: 30%, NSCLC: 21%, pancreas: 56%; Sanger Institute, 2017). This favors the identification of a diagnostic marker in pancreatic cancer, as one mutation might detect a majority of pancreatic cancer cases. Fifty patients are a small cohort, enough however, to detect significances in pancreatic cancer using highly prevalent *KRAS* mutations (Brychta et al., 2016). The detection rates and significances need to be validated in further studies with higher numbers of patients. As pancreatic cancer can occur in different areas of the pancreas, e.g. head, body or tail, equal numbers of patients with the disease in different areas need to be recruited. Also, higher numbers of patients with different types of histology, like neuroendocrine tumors, need to be included into the study. Especially in neuroendocrine tumors the prevalence of *KRAS* mutations is seemingly very low (2%, Sanger Institute, 2017) and therefore detection of *KRAS* as diagnostic marker has very limited clinical value. Other, more frequent mutations have to be used as diagnostic markers in this cancer subtype. Alterations in *MEN1* (multiple endocrine neoplasia 1) gene seem to be frequently detected in neuroendocrine tumors (44%, Jiao et al., 2011) and might be evaluated as a more useful marker.

The detection of mutant alleles is dependent on the sensitivity of the technique (Figure 6). In order to detect low allelic frequencies, highly sensitive digital PCR is favored over e.g. multipanel detection by sequencing techniques. The highest detection or concordances were obtained by highly sensitive digital PCR whereas sequencing

methods including targeted sequencing lacked sensitivity and failed to detect low allelic frequencies especially in early stages of pancreatic cancer (Takai et al., 2015).

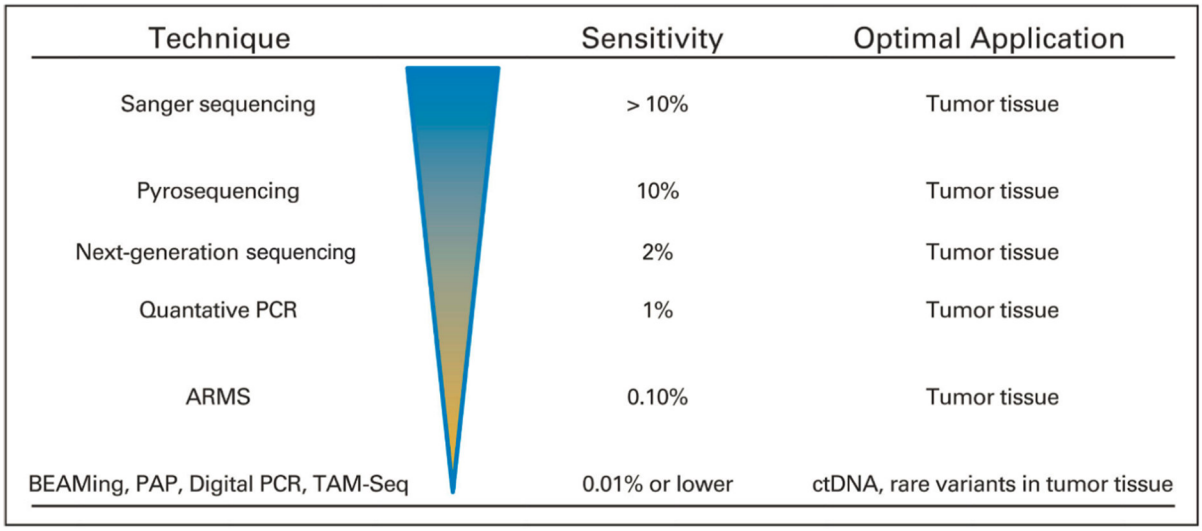


Figure 6: Sensitivity of technique determines the application. Only very sensitive techniques can be used for the detection of ctDNA in plasma. ARMS: amplification refractory mutation system; BEAMing: beads, emulsions, amplification and magnetics; PAP: pyrophosphorolysis-activated polymerization; TAM-Seq: Tagged Amplicon Sequencing (modified after Diaz and Bardelli, 2014).

One reason for the failure of sequencing especially in early stages might be the low amount of ctDNA in plasma. It is described that plasma of patients diagnosed with metastatic stages contains higher mutant allelic frequencies in metastatic patients' circulation than patients in early stages (Diehl et al., 2005). Compared to failure of sequencing in early stages of pancreatic cancer, Chen et al. and Guo et al. reported high concordances of ctDNA and DNA isolated from tumor of 50.4% and 78.1% by targeted sequencing including *KRAS* in early stages of lung cancer (Chen et al., 2016; Guo et al., 2016). This shows a general feasibility of sequencing in early stages of cancer except for pancreatic cancer. Detection of ctDNA in pancreatic cancer might be impeded by the unfavorable tumor environment. Low tumor content and collapsed vascularization might result in reduced circulation of ctDNA (Brychta et al., 2016; Provenzano et al., 2012).

As the detection of *KRAS* mutations is successful in early stages of pancreatic cancer, but does not cover 100% of the patient population, a panel of frequently occurring mutations might increase the detection of all subtypes and allow a complete coverage of the population. The simultaneous detection of multiple mutations by digital PCR is not feasible. Today's readily available methods like sequencing present a sensitivity of 2% (Figure 6). Compared to detectable 0.01% by digital PCR, sequencing is far from being used in early stages of pancreatic cancer. Additionally, the first step of targeted sequencing including promising highly-sensitive TAM-Seq (Tagged Amplicon Sequencing) is a pre-amplification step (Forshew et al., 2012). Pre-amplification might

include a polymerase-bias that shifts allelic frequencies or results in the loss of one allele due to uneven coverage. The usage of sequencing also requires target-specific optimization of the workflow (Aird et al., 2011; Forshew et al., 2012). Moreover, one study reported the calculated error rate of polymerases in highly sensitive BEAMing including a pre-amplification step. The error rate of polymerases might lead to false positive results if 0.01% allelic frequencies are considered as tumorous (Li et al., 2006). A debate has emerged if low allelic frequencies can be considered as a positive result. In pancreatic cancer, the tumor content especially in early stages is very low (see 1.3 Early diagnosis in pancreatic cancer), so low allelic frequencies are expected. This is also confirmed by the data of the present study using chip-based dPCR without pre-amplification (Brychta et al., 2016).

The major improvement of diagnostic sensitivity for early detection of pancreatic cancer achieved in the thesis also emphasizes the question about true results of very low mutant allelic frequencies in healthy individuals. The more sensitive a method is, the more likely mutations will be identified in clinically healthy individuals. Few studies have included healthy individuals and analysis of their plasma. Especially with highly-sensitive digital PCR positive mutant alleles can be detected in healthy individuals but are excluded from the study by threshold setting (Brychta et al., 2016; Hadano et al., 2016; Kinugasa et al., 2015). The usage of other high-fidelity polymerase might increase diagnostic specificity and lower false positive results even in healthy individuals (McInerney et al., 2014). If ctDNA is detected in healthy individuals but conventional imaging techniques do not show signs of malignancies, a clinical strategy needs to be identified to interpret and act on the results. Additionally, it is published that inflammatory diseases are precursor lesions to pancreatic cancer (Becker et al., 2014). Some patients with chronic pancreatitis already harbor *KRAS* mutations in lower allelic frequencies but their long-term effects are unclear. One study followed patients diagnosed with chronic pancreatitis and positive *KRAS* detection in serum for three years with no evidence of pancreatic cancer (Maire et al., 2002). As already diagnosed pancreatic cancer patients were analyzed in this study, other patients with precursor lesions need to be included in further studies as additional control group besides the healthy individuals.

To date, single mutation analysis like dPCR remains the only successfully applicable technique in pancreatic cancer as described in this work. During the years other sensitive techniques have evolved that might also detect early stages of all subtypes of pancreatic cancer. The absence of a preamplification step in third-generation sequencing like nanopore sequencing or the use of CAPP-Sequencing (cancer personalized profiling by

deep Sequencing) might also increase sensitivity of sequencing in the near future in order to also detect short fragmented ctDNA from cancers with low tumor content like in pancreatic cancer (Cheng et al., 2015; Newman et al., 2014; Norris et al., 2016). One potential hotspot panel for detecting different subtypes of pancreatic cancer might be *KRAS*, *TP53*, *SMAD3* (mothers against decapentaplegic homolog 3), and *SMAD4* *CDKN2A* and *MEN1* (Sanger Institute, 2017).

The 35% detection rate of *KRAS*-positive tumors and ctDNA reported in this thesis is already promising but the use of more plasma volume might further increase the diagnostic sensitivity to decrease the rate of undetected cases (false negative rate). 50 ng of DNA were used for dPCR analysis in this work. Studies with sequencing methods using the same amount of DNA failed due to difficult sequencing coverage of small allelic fractions (Takai et al., 2015). A higher analyzable plasma volume might also be useful for sequencing as isolated DNA-amount is proportional to plasma volume. This trend can already be inferred by the results published to date (Bettegowda et al., 2014; Sausen et al., 2015).

In short, the diagnostic sensitivity could be increased to successfully detect early stages of pancreatic cancer. These results open a new diagnostic possibility to minimally invasive diagnosis in pancreatic cancer.

4.1.2 Circulating tumor cells

Since the FDA clearance of the CellSearch system for prognosis, research on CTCs has exploded especially in colorectal, prostate and breast cancer (Cohen et al., 2009; Cristofanilli et al., 2004; de Bono et al., 2008). To date, the enumeration of CTCs is the major focus of research which has resulted in a tremendous number of papers in e.g. breast cancer, but also in other indications like pancreatic cancer.

Unfortunately, the absence of a standard for CTC-detection results in different CTC-isolation techniques with diverse fixation protocols and staining techniques. This has caused a high number of non-comparable publications. Therefore, the reported detection rates of CTCs in pancreatic cancer range from 5-100% using different methods through all stages with contradictory clinical conclusions and no clear definition of false positive or false negative results. Interestingly, for the detection rate of the low 20% and the high 100% both EpCAM-dependent methods were used in metastatic pancreatic cancer patients (Earl et al., 2015; Nagrath et al., 2007). Even use of the same semi-automated

CellSearch method and similar patient populations do not lead to comparable results. Earl et al. (2015) detected CTCs in 20%, Khoja et al. (2012) in 40% and Kurihara et al. (2008) in 42% of patients by CellSearch in metastatic pancreatic cancer patients.

The reported number of CTCs in pancreatic cancer is usually low with few exceptions especially in early, non-metastatic stages (Ankeny et al., 2016). No threshold is set and even one possible CTC is counted as positive result as reported by several studies (Ankeny et al., 2016; Bidard et al., 2013; Kurihara et al., 2008). This might include a strong bias towards higher detection rates and false positives as it depends on the experimenter's eye and the definition of a CTC which is a cytokeratin- and EpCAM-positive, nucleated cell as well as negative for CD45 in most studies (Ligthart et al., 2011). No fully automated devices for CTC enumeration are available to date which might further standardize CTC-analysis. High detection rates are also often described in publications authored by researchers of the company or institute providing the isolation device (Harb et al., 2013; Nagrath et al., 2007). No obvious trend of preferred isolation method is seen using CTCs in pancreatic cancer. The majority of studies have used CellSearch with varying success, probably due to the FDA clearance in other indications.

In this thesis, filtration and EpCAM-dependent method were compared for best detection rate of pancreatic circulating tumor cells from whole blood. EpCAM-dependent methods were shown to be vastly dependent on EpCAM expression (Recovery of EpCAM high cell line: 27% vs. EpCAM low cell line: 1% by IsoFlux; Brychta et al., 2017, under review) and therefore not suitable for pancreatic cancer. Filtration was superior to the antigen-dependent method especially in EpCAM-low expressing cells (IsoFlux: 1%; Filtration: 52%), a finding which has also been reported by other groups (Hofman et al., 2011; Khoja et al., 2012; Wit et al., 2015). As early dissemination of cells from the tumor in pancreas as well as loss of epithelial phenotype by EMT was shown early *in vivo* (Rhim et al., 2012) a size-dependent method might be superior to an antigen-dependent technique especially in early stages. This is already supported by the low amount of CTCs in locally advanced cancer (5%, Bidard et al., 2013) compared to metastatic cancer obtained by EpCAM-dependent CellSearch.

Pancreatic CTCs were successfully isolated by filtration from blood and frozen product of diagnostic leukapheresis. Using frozen DLA product CTCs were detected in 44% of patients with metastatic disease (M1) and 40% of non-metastatic patients (M0) providing a storable medium for characterization of pancreatic CTCs. Leukapheresis is a CTC-enrichment method and might overcome the drawback of CTC-analysis: limited analyzed

blood volume and limited CTC counts. During leukapheresis blood is centrifuged extracorporeally, leaving CTCs in the mononuclear layer. The use of DLA for CTC enumeration from pancreatic cancer has already been positively evaluated for freshly prepared DLA product (Fischer et al., 2013).

In this thesis, a very high number of mononuclear cells (2 x 10^8 cells per mL) had to be used for detection of CTCs especially in early, non-metastatic stages. Fischer et al. also reported promising CTC counts isolated from frozen DLA from breast cancer patients with no enrichment like filtration but sedimentation on a glass slide. This technique showed higher numbers of CTCs than with fresh DLA product and CellSearch enrichment in breast cancer. This leads to the conclusion that both filtration and EpCAM-dependent methods are still not satisfactory for the isolation of CTCs if unenriched methods show superior results even on cryopreserved samples. Apart from EMT for downregulation of epithelial marker, another reason for low CTC detection rates might be the size distribution of CTCs. The size is not uniform and differs from that of cultured cells, as cells isolated from patients are probably smaller (Park et al., 2014; Stoecklein et al., 2016). Therefore, CTCs might slip through the pores of the filter and be missed by staining and detection.

The currently accepted usual definition of the CTC is a CD45-negative, EpCAM- and cytokeratin-positive nucleated cell that may also lead to problems. In blood from patients with pancreatic cysts cytokeratin-positive circulating cells were isolated but no evidence of cancer was seen by the authors to date (Rhim et al., 2014). Furthermore, only few studies have included healthy donors to study the amount of false positive cells using their staining or isolation method (absent in e.g. Bobek et al., 2014; Khoja et al., 2012). In the current work, the detection of CTCs still relies on the epithelial marker cytokeratin. Cells might not be detected as they express cytokeratin only to a minor extend or not at all. A study staining cells by the EMT-independent marker CEP8 (centromere of chromosome 8) already showed promising result in detecting CTCs in pancreatic cancer (88%, Gao et al., 2016).

In CTCs isolated from pancreatic cancer patients, the population of CTCs was heterogenous in this work: both EpCAM-positive and EpCAM-negative CTCs were isolated from one patient. Taking into account the early spread of cells and the devastating prognosis of the disease, this might suggest that pancreatic circulating tumor cells are both EpCAM-negative and smaller than pore size and therefore very prone to

metastasize. The clinical relevance of these findings still has to be proven. In lung cancer, EpCAM-negative CTCs showed no prognostic relevance (de Wit et al., 2015).

The previously listed discrepancies for CTC enumeration urge a better definition of CTCs. One step towards this might be additional analysis of the CTCs like detection of mutations to prove their tumorous origin. The optimization of the of staining of filtered cells and subsequent *KRAS* mutational analysis of cells even isolated from frozen samples in this thesis opens a new path for confirming the source of epithelial cells and might help guiding early diagnosis in future studies. Unfortunately, in this study only 2 CTC positive cases could be investigated for *KRAS* mutations. *KRAS* mutations were not detected being concordant with the wild-type tumor status proving the technical feasibility (Brychta et al., 2017, under review). This number of analyzed CTCs, however, is simply too small to allow any clinical conclusion. In other studies, *KRAS* was detected with a high incidence up to 57% in pancreatic cancer (Earl et al., 2015; Kulemann et al., 2016). In those studies, CTCs were not stained before analysis to confirm presence of the CTCs, but blood was processed and directly subjected to preamplification and sequencing. Additional steps like staining of cells might be responsible for the loss of CTCs.

Although a stronger, tumor-specific definition of CTCs is highly needed, a *KRAS* wild-type tumor will not be detected by single mutational analysis alone. This hinders the screening of healthy individuals as the false negative rate might be high. As already discussed under chapter 4.1.1 Circulating tumor DNA, a panel of mutations might help to identify all subtypes of pancreatic cancer for diagnosis. Court et al. (2016) used modestly sensitive sanger sequencing and detected *KRAS* mutations already in 27% of single CTCs. In this study, amplification is stated as the main introducer of bias. Therefore, advanced methods of sequencing (e.g. third generation sequencing without amplification) might be a promising tool for diagnostics.

As one advantage of circulating cells is their integrity and possibility of other analysis like proteins or RNA, further analysis of CTCs by other pancreatic cell-specific markers might further improve diagnostics. Analysis of the MUC-protein family or RNA analysis in pancreatic circulating cells have already shown promising results in exploiting CTCs as biomarker (Dotan et al., 2016; Yu et al., 2012).

In summary, CTCs were successfully isolated by filtration of frozen DLA from both metastatic and non-metastatic pancreatic cancer patients. No *KRAS* mutations were detected due to wild-type tumors, but the established workflow for subsequent mutational

analysis could reveal the true tumorous origin of these cells and direct identification of CTCs in the future.

4.2 Comparison of CTCs and ctDNA as potential diagnostic marker for the detection of pancreatic cancer in early stages

To this day, only few studies have described the potential of circulating tumor DNA and circulating tumor cells as diagnostic tools for early detection of cancer. One incomplete study was performed to identify the best biomarker in pancreatic cancer (Earl et al., 2015). Therein, CTCs were isolated in a minority of patients in late stages whereas *KRAS*-mutant ctDNA was detected in more cases leading to the conclusion that *KRAS* mutations in plasma are a better biomarker for the detection of all stages of pancreatic cancer. This quintessence was also concluded by other researches investigating the potential of CTCs and ctDNA in other entities. ctDNA also seemed to be more sensitive compared to CTCs (enumeration and molecular characterization) also in advanced stages of lung cancer (Freidin et al., 2015; Guibert et al., 2016; Punnoose et al., 2012).

In this thesis, a comparison of CTCs and ctDNA is conducted in terms of early detection of pancreatic cancer, however, in different patient cohorts. Table 1 shows the results obtained in this thesis compared to the serum biomarker CA19.9 for early diagnosis in non-metastatic pancreatic cancer.

	CA19.9		Detection of *KRAS* in ctDNA	CTC enumeration (Detection of *KRAS*)
Medium	serum		plasma	DLA-product
Analyzed medium	1 mL		2 mL	2 x 10^8 cells/mL
Sensitivity [%] (Positive event and cancer)	78.2*	NA in early stages†	35	40 (0)
Specificity [%] (Negative event and no cancer)	82.8*		100	100
Clinical relevance in early stages	no		yes	no
Limitations	~ 10% of patients are non-secretor**		*KRAS* wild-type tumor not detectable	
	Elevated levels in patients with biliary construction and pancreatitis***		Threshold setting necessary for *KRAS* analysis in healthy individuals	

Table 1: Comparison of biomarkers for the early detection of pancreatic cancer. CA19.9 is compared to *KRAS* detection in ctDNA and CTCs. In terms of specificity, both ctDNA and CTC outplay CA19.9. Sensitivity of *KRAS* detection to determine true tumorous origin is higher in ctDNA. NA = not applicable; * for already diagnosed pancreatic cancer patients (Poruk et al., 2013); † from (Kim et al., 2004); ** from (Ørntoft et al., 1996); *** from (Lin et al., 2014; Rocha et al., 2007).

The CA19.9 assay shows a calculated sensitivity of 78.2% and a specificity of 82.8% (Poruk et al., 2013). These results are not confirmed in the screening of healthy individuals as both false positives are obtained and the prediction rate for pancreatic cancer of healthy individuals is low (Kim et al., 2004). Additionally, CA19.9 levels might not be elevated if the tumor is small (Locker et al., 2006). However, elevated levels of CA19.9 are also obtained from patients suffering from biliary construction or pancreatitis (Lin et al., 2014; Rocha et al., 2007). Furthermore, the percentage of non-secretors for CA19.9 is high which makes it difficult to differentiate between a non-secretor and a negative healthy value (Ørntoft et al., 1996). Compared to CA19.9, both circulating biomarkers are very specific. Moreover, the sensitivity of the detection of pancreatic cancer by *KRAS* analysis of ctDNA and the enumeration of CTCs is high in early stages. *KRAS* mutations were not detected in CTCs from pancreatic cancer patients being fully concordant to the *KRAS* wild-type tumor. As already previously discussed, the potential of detecting all types of pancreatic cancer in ctDNA by sequencing might further increase sensitivity of detection of pancreatic cancer in early stages. However, the high diagnostic specificity with increased sensitivity achieved in this thesis is a major advantage towards

analytical and clinical utility especially in early stages and screening of healthy individuals.

In this work, *KRAS*-mutant ctDNA was successfully detected in 2 mL of plasma in early stages of cancer indicating a possible early diagnostic marker. CTCs in DLA-product are isolated by filtration only if high numbers of mononuclear cells were used. For early detection or screening of healthy or high risk individuals this might be complicated. CTC isolation especially in earlier stages due to sparse numbers of CTCs or their escape from EpCAM-dependent or size-dependent capturing remains the major challenge and decreases their diagnostic sensitivity. Therefore, ctDNA as diagnostic biomarker for early stages of pancreatic cancer is positively evaluated in this thesis outperforming the use of CTCs for diagnostics.

One explanation might be traced back to the unfavorable microenvironment already at early stages. Densely packed stromal cells and decreased vascularization might hinder shedding of cells in the blood compared to small ctDNA molecules. There is also still the question of biological limitations of ctDNA and CTCs. Results were published that the numbers of both CTC and *KRAS*-positive cases are higher in portal vein or pancreatic juice (Berthelemy et al., 1995; Catenacci et al., 2015). Although the surgical drawing of blood from portal vein is not convenient and contradicts the original idea of liquid biopsy, this strengthens the hypothesis that all tumors release both cells and DNA into the blood stream. Due to the neighboring liver, circulating biomarkers are cleared by hepatic sequestration, possibly favoring CTCs over ctDNA. Therefore, the biological restrictions of the use of these markers still have to be investigated.

Additionally, mutational analysis of blood-based biomarker raises the general question which clone or mutation in ctDNA is detected in blood. As the tumor is heterogenic, it is not known if one particular subclone is more prone for shedding into the blood than another and therefore is identified by e.g. digital PCR or sequencing. Furthermore, it is arguable whether one measurement of ctDNA or isolation of one CTC reflects the whole mutational landscape of the tumor. One study reported that for the assessment of mutational status of *KRAS* in pancreatic cancer ten CTCs are necessary to reliably detect the mutation (Court et al., 2016). But especially in early stages of pancreatic cancer, the CTC number is low. This dilemma indicates that several serial measurements are necessary to cover the whole tumor spectrum. No such data are available on ctDNA-analysis.

Although ctDNA has been successfully evaluated for the detection of *KRAS*-mutant ctDNA in plasma of patients diagnosed with early stages of cancer, confirmation in a prospective trial will be required to prove clinical validity. This is of particular relevance for the interpretation of positive results in healthy individuals as the location of disease is unknown. A novel approach includes the unique methylation pattern of cfDNA tracing back to the origin of the tumor (Snyder et al., 2016). Epigenomic analysis and mutant ctDNA analysis of cfDNA would both answer questions about the location of the tumor and possible targeted therapy.

In summary, filtration of high input material of DLA to overcome the challenge of limited analyzable volume for CTC-isolation and subsequent molecular analysis was not successful as diagnostic marker for pancreatic cancer. Improved diagnostic sensitivity and utility was achieved using *KRAS* mutational analysis of ctDNA in early stages of pancreatic cancer.

4.3 Excursus: Potential of CTCs for personalized medicine

Although ctDNA was positively evaluated as a potential diagnostic marker outperforming CTCs in this thesis, one major advantage of CTCs is their potential use for testing a variety of predictive markers based on DNA-, protein- or mRNA-analyses.

As single cell molecular biology techniques and sensitivity of assays have improved in the last decades (Figure 6), analysis of CTCs for possible predictive markers for the initiation of minimal invasive personalized medicine have become popular. Detection of genetic aberrations in isolated CTCs with therapeutic relevance were already shown in e.g. *EGFR* analysis in NSCLC and colorectal cancer (Maheswaran et al., 2008; Mostert et al., 2013).

As previously described, a major drawback of CTC-analysis is the usage of limited analyzable blood volume and therefore limited number of CTCs. DLA seems to be a promising source in several entities (Brychta et al., 2017, under review; Fischer et al., 2013). Another approach to increase CTC numbers is the insertion of a wire coated with EpCAM-antibodies into the vein (Saucedo-Zeni et al., 2012). In this thesis, this approach was tested against the FDA-cleared CellSearch system in lung cancer patients (Gorges et al., 2016). CTCs were detected by immunofluorescence and enumerated. The *in vivo*

approach captured CTCs more sensitively and efficiently than the CellSearch system (*in vivo* wire: 58%, CellSearch: 27%) (Gorges et al., 2016). In patients diagnosed with non-small cell lung cancer the pre-operative detection rate of CTCs was even 72%. This result is highly favorable leading to increased detection of CTCs if higher blood volumes can be analyzed. Other studies using CellSearch reported detection rates of only 20%, 39% and 23% (Allard et al., 2004; Hofman et al., 2011; Krebs et al., 2011). The detection rate of CTCs isolated by the *in vivo* wire is also similar to antigen-independent methods like filtration (Krebs et al., 2012). Furthermore, as described in 1.5.2 circulating tumor cells, cells with epithelial character might be more prone for metastasis and therefore more suitable for biomarker assessment. Nevertheless, the relevance of different subtypes of CTCs still has to be proven.

Besides the increased detection rate of CTCs, extensive analysis of CTCs might be useful for stratification of patients, monitoring early detection of response or progression and especially to overcome the challenge of tumor heterogeneity. In this thesis, CTCs from two lung cancer patients with known tumor mutations were analyzed for the mutations *EGFR* and *KRAS*. The mutations were fully in concordance with the tumor mutations (Gorges et al., 2016). These results show the feasibility of mutational analysis especially important for personalized medicine.

In conclusion, using an EpCAM-coated *in vivo* wire in lung cancer patients, CTCs were captured more efficiently than by CellSearch and the number of CTCs was increased. Additional molecular characterization proving the presence of tumor mutations might overcome the challenge of heterogeneity leading towards a precise predictive marker.

5 Summary and Conclusion

Pancreatic cancer develops from slowly arising neoplasm over years to rapid progression of the disease to metastatic cancer in months. Therefore, the medical need for biomarkers in early detection of cancer is high. So far, no minimally invasive biomarker is clinically available to diagnose early stages of pancreatic cancer. Additionally, tumor biopsy in diagnosed patients is difficult to obtain and not feasible for

monitoring the disease. Circulating tumor DNA and circulating tumor cells might represent the systemic disease gaining popularity as minimally invasive biomarker.
In short, findings of this thesis are

- Feasibility of detection of *KRAS*-mutant ctDNA in early stages of pancreatic cancer with increased diagnostic sensitivity opening a new diagnostic window for early detection of pancreatic cancer.
- Advantages of marker-independent isolation method for the isolation of pancreatic circulating tumor cells and subsequent mutational analysis from frozen leukapheresis samples providing a storable medium for CTCs.
- Higher diagnostic sensitivity of ctDNA compared to CTC analysis in samples of early stages of pancreatic cancer patients
- Increased sensitive detection of CTCs by an *in vivo* device and the feasibility of downstream analysis for possible stratification of lung cancer patients for personalized medicine.

These findings lead to the conclusion that *KRAS*-mutant ctDNA in plasma of patients diagnosed with early stages of pancreatic cancer are a promising diagnostic marker that outperforms CTCs in terms of diagnostic sensitivity. But the clinical validity of these findings is still not proven. ctDNA analysis for diagnostic purposes has to be tested in prospective trials with a long follow up time to prove its value also in healthy individuals. Higher volumes of plasma and therefore higher amounts of analyzable DNA as well as the evolving sequencing technology may further increase sensitivity to detect low allelic frequencies in early stages of pancreatic cancer.

While the prognostic relevance of EpCAM-positive CTCs has been shown by CellSearch studies, the relevance of EpCAM-negative CTCs obtained by filtration still has to be confirmed. Although ctDNA shows relevant clinical feasibility, CTCs itself might add a broader understanding of phenotypic and genotypic characteristics. In this thesis, isolation and subsequent molecular characterization of CTCs from lung cancer already showed the promising potential of CTCs in personalized medicine.

Therefore, CTCs and ctDNA should be studied simultaneously in further prospective studies with long-term follow up.

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8 Declaration

Ich versichere an Eides statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Diese Promotion wurde bei keiner anderen Fakultät oder Universität eingereicht bzw. abgelehnt.

Düsseldorf, den 07.07.2017

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Nora Brychta