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Circulating and disseminated tumour cells as potential biomarkers for treatment response and disease progression in patients with locally advanced and/or metastatic pancreatic cancer

Master's thesis
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Kristin Reine
Master of Science in Biological Chemistry
Faculty of Science and Technology
University of Stavanger

University of Stavanger

HELSE STAVANGER
Stavanger University Hospital

Abstract

Introduction: Pancreatic cancer is the fourth most common cause of cancer related deaths in the Western countries. The poor prognosis of pancreatic cancer patients is often associated with early dissemination of the disease, late detection due to unspecific symptoms and chemotherapy resistance. There are two ways for tumour cells to enter the blood circulation, either by passive shedding of tumour cells from the primary tumour or by an active process called epithelial-to-mesenchymal transition (EMT). Circulating tumour cells (CTCs) that are resident in the bone marrow (BM) are called disseminated tumour cells (DTCs).

Around 85% of pancreatic cancers harbour point mutations in the *KRAS* gene, and these mutations represent highly tumour-specific traits that might be applied as surrogate markers for tumour cell detection. Hence, we wanted to use both *KRAS* mutations and four mRNAs as surrogate markers for CTC/DTC detection in blood (PB) and BM from patients with locally advanced and/or metastatic pancreatic cancer before and during chemotherapy.

Patients and methods: Six metastatic pancreatic cancer patients were included in the study and samples from nine healthy individuals constituted the control group. In the first part of the study we compared different strategies for enrichment of CTCs/DTCs, a manually prepared and a commercially RBC lysis buffer versus the Lymphoprep^{TM} protocol. A sensitivity analysis was performed to determine the detection limit of each mRNA marker with regard to the lowest amount of tumour cells detectable with RT-qPCR. The four mRNAs were also evaluated in six pancreatic tumour samples. Following tumour cell enrichment by Lymphoprep, CTCs and DTCs were detected indirectly using the epithelial-specific surrogate mRNAs CK8, CK19, EpCAM and CEACAM5, as well as *KRAS* mutations by real-time PCR.

Results: All the mRNA markers were highly expressed in the six pancreatic tumour samples compared to PB and BM samples from nine healthy individuals emphasizing their use as surrogate markers for CTC/DTC detection. Furthermore, our preliminary data show that we detect CTCs and DTCs in PB and BM samples obtained before treatment in 5/6 and 5/5 patients, respectively. Repeated blood sampling from three patients and BM samples from one patient, also confirmed the presence of CTCs and DTCs after initiation of the treatment. *KRAS* mutations were detected in CTCs and DTCs from 1/6 patients.

Conclusion: We detect CTCs and DTCs in PB and BM samples obtained both before and during gemcitabine treatment of metastatic pancreatic cancer patients with mRNA quantification and *KRAS* mutation detection by real-time PCR. However, inclusion of more patients is required to conclude on the clinical value of these data.

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Kristin Reine

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Abbreviations

ALDH	Aldehyde dehydrogenase
BCR	Breakpoint cluster region
BM	Bone marrow
CA 19-9	Carbohydrate antigen 19-9
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CK	Cytokeratin
CSC	Circulating stem cell
CTC	Circulating tumour cell
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
DTC	Disseminated tumour cell
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
LN	Lymph node
LOC	Lab-on-a-chip
MC	Mononuclear cell
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NTC	No template control
PanIN	Pancreatic intraepithelial neoplasia
PB	Peripheral blood
PNA	Peptide nucleic acid
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid

RT	Reverse transcriptase
TGF-β	Transforming growth factor beta

1 Introduction

1.1 Pancreatic cancer

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the Western countries. [1] In Norway the number of new cases of pancreatic cancer in 2010 was 650, which is equivalent to approximately 7 incidences per 100.000. [2, 3] In Europe, pancreatic cancer leads to approximately 40.000 deaths per year. [3, 4] This high death rate is caused by several factors, including a late diagnosis due to lack of symptoms at an early stage, extensive metastasis at the time of diagnosis and drug resistance. [5, 6] Accordingly, only a small number of pancreatic cancer patients are presented with operable disease at the time of diagnosis (approximately 5-15%). [5]

1.1.1 Symptoms

Early stage pancreatic cancer is generally clinically silent. Symptoms of pancreatic cancer first become apparent after tumour invasion of surrounding tissue or metastasis to distant organs. [1]

For tumours located in the head and body of the pancreas, symptoms are present due to compression of adjacent structures such as the bile duct, coeliac and mesenteric nerves, the pancreatic duct and the duodenum. Besides abdominal or mid-back pain, other signs connected to the disease may be the development of diabetes mellitus or malabsorption and weight loss. Pancreatic-duct obstruction may occasionally lead to pancreatitis. [5, 7] Digestive problems may occur if the cancer blocks the release of the pancreatic juice into the intestine. Venous thrombosis is not unusual and may be a presenting sign of malignant disease. [1]

1.1.2 Diagnosis

Diagnosis of pancreatic cancer is most frequently done by computed tomography (CT) and/or magnetic resonance imaging (MRI). CT scans are most often used to diagnose pancreatic cancer and to determine the extent of its spread. It shows the organs near the pancreas, as

well as lymph nodes (LNs) and distant organs the cancer may have spread to and, it provides information about the tumours relation to the surrounding vessels. MRI, on the other hand, is most suited for examining soft tissue and can sometimes provide much more tissue details compared to a CT scan. [3, 7-9] In some situations, a biopsy is required to confirm the diagnosis.[3, 8]

1.1.3 Disease staging

The stage of the disease, grade and resection margin status is defined from pathologic examination of the primary tumour. [4, 5, 7] The majority of pancreatic tumours are adenocarcinomas, which originate in the ductal epithelium. According to the American Joint Committee on Cancer tumor-node-metastasis (TNM) classification, the patients are pathologically classified according to the size of the primary tumour (T), regional lymph node involvement (N) and distant metastases (M). [10] T1 to T3 tumours are classified as resectable, while T4 tumours affect the coeliac axis or superior mesenteric artery and are classified as unresectable primary tumours. [1]

Invasive pancreatic cancers are believed to arise from non-invasive precursor lesions called pancreatic intraepithelial neoplasia (Pan-IN I-III), which reflects different grades of dysplasia in epithelium in the pancreatic ducts. The high-grade Pan-Ins may be converted to invasive pancreatic ductal adenocarcinoma. [11, 12] Although the Pan-INs are well characterized there are still no diagnostic criteria for classifying these premalignant lesions for an earlier detection of the disease. [3, 7, 8, 12, 13]

Additionally, the serum tumour marker carbohydrate antigen 19-9 (CA 19-9, also called sialylated Lewis antigen) is a biomarker proven to be useful for monitoring and early detection of recurrent disease in pancreatic cancer patients. Unfortunately, the CA 19-9 analyses have clear limitations because elevated CA 19-9 protein level also is observed in patients with non-malignant diseases, such as cirrhosis, chronic pancreatitis and cholestasis. New biomarkers and better staging of early pancreatic cancer are therefore highly needed.[7, 14]

1.1.4 Pancreatic cancer treatment

Today, no curative therapies are present for pancreatic cancer patients. [8] Accordingly, the vast majority of pancreatic cancer patients are today primarily treated with a palliative intent to reduce symptoms as well as to prolong life for some patients. Gemcitabine is generally advised as the standard first-line treatment for pancreatic cancer patients. [15] Gemcitabine is a deoxycytidine analogue that must be phosphorylated to become active (gemcitabine diphosphate and gemcitabine triphosphate). When activated, gemcitabine diphosphate inhibits ribonucleotide reductase and reduces the intracellular pool of deoxynucleotide triphosphate required for DNA synthesis. Gemcitabine triphosphate can be fused into an elongating DNA chain and result in premature chain termination. [5, 15] Although gemcitabine administration leads to a statistically significant longer progression-free and overall survival, the magnitude of the objective, radiographically measured response is rather small. [15] The pancreatic cancer drug resistance is believed to be caused by the very dense, fibrotic stroma surrounding the tumours (consisting of connective tissue, fibroblasts, leukocytes and blood vessels) and this stroma seems to be involved in the blockage of drug penetration and thus contribute to tumour survival. [7, 11, 16, 17] However, various combinations of drugs that result in "stromal collapse" are being tested in clinical trials and the results increase the optimism for a more effective treatment of pancreatic cancer patients in the future. [7, 18-20]

1.1.5 Survival

The overall 5-year survival rate for pancreatic cancer patients is less than 5%. [5, 21] Approximately 90% of the patients who present with advanced pancreatic cancer survive less than one year. About 80-85% of the patients with resectable disease experience disease relapse and die within 5 years of diagnosis. In average, metastatic patients treated with chemotherapy die within 5-6 months. [5-7]

1.1.6 Risk factors

Several factors contribute to an increased risk of pancreatic cancer. These include increasing age, diabetes mellitus, obesity, familial history of pancreatic cancer (genetic factors), chronic pancreatitis, chronic cirrhosis and cigarette smoking. [1, 5, 7]

Roughly 20% of pancreatic tumours arise due to cigarette smoking and genetic analyses have discovered an increased number of mutations in cancer-related genes correlated to smoking. [22] In addition to smoking, an important risk factor for pancreatic cancer is mutations. Patients with a familial history of pancreatic cancer constitute approximately 7-10% of the incidences. Germline mutations in the *BRCA2* gene are reported as the most known causes of inherited pancreatic cancer. However, germline mutations in the genes *PALB2*, *CDKN2A*, *STK11* and *PRSS1* are also associated with a significantly increased risk of pancreatic cancer. [1] Recent research has also shown an increased risk of pancreatic cancer in non-O blood group. [23]

1.1.7 *KRAS* mutations

Somatic mutations in the *KRAS* gene are present in 80-90% of pancreatic cancers. [7, 24] Transcription of a mutant *KRAS* gene results in an abnormal Ras protein locked in its active form, leading to an abnormal constitutive activation of proliferative pathways. [7] *KRAS* mutations seem to be present at an early stage of pancreatic cancer development, as it has also been detected in the premalignant Pan-IN lesions. [25] This suggests that *KRAS* activation may be one of the earliest genetic events leading to pancreatic cancer. [6, 8, 13, 25] Nonetheless, a prognostic value for *KRAS* mutations has not been demonstrated in pancreatic cancer patients. [26, 27]

1.2 Circulating tumour cells (CTCs) and disseminated tumour cells (DTCs)

1.2.1 Dissemination of tumour cells from the primary tumour into blood and bone marrow

Most cancer-related deaths are caused by dissemination of tumour cells from the primary tumour through the blood to distant organs to form metastases. Pancreatic tumour cells often disseminate to the liver, rarely to the lungs and skeleton. [3, 5] Dissemination of tumour cells may occur both hematogenously and lymphatically. By the lymphatic dissemination route the tumour cells may transit LNs before accessing the peripheral blood (PB). In contrast, other tumour cells appear to be able to enter the blood circulation directly, a process called haematogenous dissemination [6, 28-30], either by passive tumour cell shedding from the primary

tumour [30, 31] or by an active mechanism called epithelial-to-mesenchymal transition (EMT). Passive shedding is supposed to occur from the early stages of tumour formation and a large number of tumour cells can disseminate into the blood circulation in this way. [30, 32] EMT, on the other hand, is characterized by several molecular and cellular changes, where the tumour cells loose their differentiated epithelial features and obtain mesenchymal properties. Thus, EMT increases the motility and invasiveness of the tumour cells and is presumed to be required for invasion and metastatic dissemination of carcinoma cells. [14, 28, 30, 33-36] This has been confirmed in a recent study published by Yu and colleagues in Science [37] where they investigated the EMT process in CTCs from breast cancer patients. In this study the CTCs showed expression of both mesenchymal and epithelial markers. However, mesenchymal cells were highly enriched in the CTC population proposing an association between CTCs and disease progression. Yet, overt metastasis is only achieved by a minority (0.01%, [38, 39]) of these tumour cells, the so-called cancer stem cells (CSCs) or tumour-initiating cells [14, 40]. In pancreatic cancer different CSC populations have been identified. [41-44] These cells are assumed to survive in the blood stream (short half-life of CTCs [28, 45]), have the capacity of self-renewal and produce the heterogeneous lineages of cancer cells present in a tumour. [41, 46] The CSCs also seem able to persist in an inactive, non-proliferative dormant (G0) state for years. [47, 48] Cancer dormancy is clinically defined as a "recurrence of cancer, either locally or systemically, after a long period of time following successful treatment and removal of the primary tumour". [49, 50] The fact that CSCs show resistance to chemotherapy and radiation therapy makes the targeting of these cells especially challenging. [7, 41, 48-50] Furthermore, the mechanisms behind the transition of dormant tumour cells into proliferative cells again are largely unknown, but tumour cell interaction with the microenvironment, blood supply limitations or an active immune system is believed to be mechanisms involved in this process. [50] Tumour cells that are resident in PB are called circulating tumour cells (CTCs), while tumour cells, which home to the bone marrow (BM), are called disseminated tumour cells (DTCs). [6, 28-30]

The extravasation of tumour cells from the blood to form metastases in distant organs is thought to involve the reverse process of EMT, a process called mesenchymal-to-epithelial transition (MET). In this way the tumour cells are converted back to a more differentiated, epithelial cell state with similar phenotypes as observed in the primary tumour, thus making them capable of establishing new tumours. [28, 34, 35] A model of the tumour cell dissemination is presented in figure 1.1.

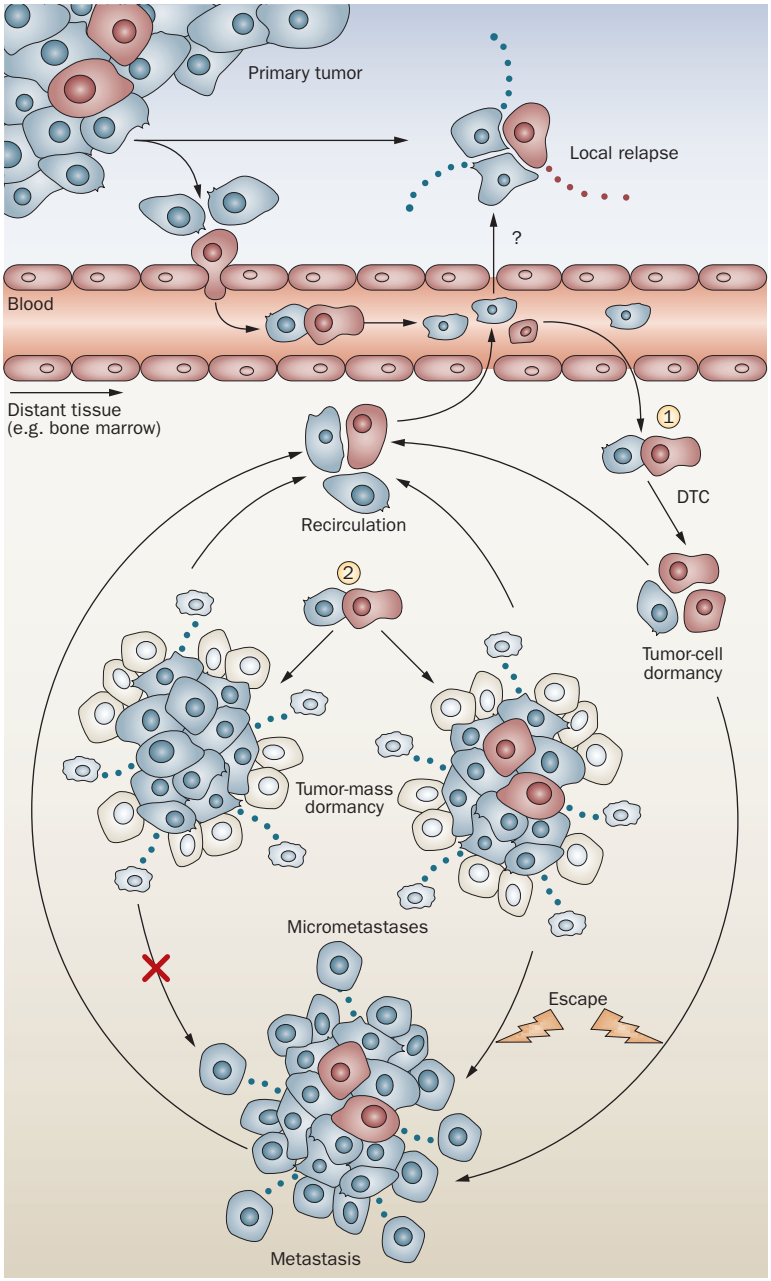

Figure 1.1: A model of tumour cell circulation and cancer dormancy. Tumour cells with stem cell properties (presented as red cells) and without stem cell properties (presented as blue cells) are released into the blood circulation from the primary tumour. In this figure the tumour cells metastasize to the BM (1) where they may transform into a dormant state or establish micrometastases (2). At each step of the metastatic cascade, the tumour cells can recirculate through the blood stream and in this way contribute to a heterogenic CTC population. The CTCs are also hypothesized to recirculate back to the primary tumour, thus enriching the tumour with more aggressive tumour cells. The figure is obtained, with permission, from Pantel *et al.* 2009. [29]

1.2.2 Genetic heterogeneity of circulating and disseminated tumour cells

Studies have revealed cellular heterogeneity within CTC populations. This is of great importance with regard to adjuvant therapy as the intention is to eliminate residual disease. [33, 51, 52] The heterogeneity within the CTC populations may be caused by both active (EMT) or passive tumour cells shedding from the primary tumour as well as from established metastases in distant organs. The results published by Yu *et al.* [37] seem to support these speculations as CTCs with both mesenchymal and epithelial mRNA markers expressed were found in the blood of the breast cancer patients investigated. Kim *et al.* [31] investigated whether CTCs derived from metastatic lesions could reinfiltrate their tumour of origin, and in this way enrich the primary tumour with more aggressive CTCs that have been adapted to new microenvironments (both in blood and in distant organs). This process known as "tumour self-seeding" seems to be driven by the CTCs' ability to sense the attraction signals from the tumour and then extravasate in response to these signals. [31] In another study by Klein *et al.* [51], screening of BM, PB and LN samples from breast, prostate and gastrointestinal cancers also showed high genetic differences among disseminated tumour cells, regardless of the cancer type. [51] Figure 1.1 also illustrates the process of tumour self-seeding by DTCs from the BM.

1.3 Methods for enrichment of CTCs and DTCs

CTCs/DTCs are present at a very low number in PB and BM (approximately 1 per $1x10^6$ leukocytes) [53], which make the detection of these cells difficult. Hence, the detection of CTCs/DTCs is usually performed with an initial enrichment procedure to increase the sensitivity. [29, 53, 54] The enrichment methods used are either based on morphological features (size or density) or selection of tumour cells by immunological techniques. [54] Commonly, enrichment of tumour cells is performed using density gradient centrifugation. The enrichment of mononuclear cells (MCs) (monocytes and lymphocytes), including tumour cells, is then done by centrifugation in an isosmotic medium (Lymphoprep™ (Axis-Shield PoC AS), Ficoll-HyPaque™ (Sigma-Aldrich) or OncoQuick® (Greiner Bio One). Red cell lysis is another approach, optimized for gentle lysis of erythrocytes and to have marginal effect on the leukocytes. Cell filtration, *i.e.* ISET (Isolation by Size of Epithelial Tumor cells)

on the other hand, is the most known membrane filter device used to separate the CTCs/DTCs according to size since CTCs/DTCs often are larger than leukocytes. [6, 53-55]

Immunomagnetic selection is used to achieve a more specific enrichment of CTCs. The principle of immunological capture techniques is that antibodies attached to paramagnetic beads bind to proteins present on the cell surface. When exposing the sample to a magnet, cells bound to the beads are separated from unbound material. By the use of antibodies against epithelium-specific antigens, as for instance the commonly used antigen epithelial cell adhesion molecule (EpCAM), [56] the tumour cells are captured by the magnet (positive selection). Another strategy is, however, to magnetically label the leukocytes by using antibodies against the leukocyte antigen CD (cluster of differentiation) 45 (negative selection). [29, 53, 54] Several immunomagnetic bead separation systems are commercially available as for instance the CellSearch® System (Veridex, the only FDA-approved system for breast, colon and prostate cancer), EasySep cell separation (StemCell Technologies), Dynabeads (Invitrogen™), magnetic-activated cell sorting system (MACS) (Miltenyi Biotec GmbH). Microfluidic methods are used to improve the sensitivity in CTC detection. [57] The CTC chip or the herringbone (HB) chip is the most acknowledged enrichment flow cell system. [6, 54, 58-60] A recently published study demonstrated the detection of both EpCAM positive and negative tumour cells. [57] They developed a multistage microfluidic device called the "CTC-iChip", which is used to detect CTCs from whole blood. The "CTC-iChip" strategies are either dependent or independent of tumour membrane epitopes and can therefore be used for different cancer types. [57]

1.4 Methods for detection of CTCs and DTCs

For detection of CTCs/DTCs in pancreatic cancer patients, two main methodological approaches are mostly used. These include: 1) immunological assays, the use of antibody-based detection techniques and 2) PCR-based techniques. [14] In immunocytochemistry, cells are attached to a solid support before fixation and staining for detection of CTCs/DTCs. The staining is performed by incubation with antibodies against different antigens, usually cytokeratins (CKs) and/or surface adhesion molecules like e.g. EpCAM. Enzymatic colour reactions or fluorescence are used to visualize the antigens and the detection and enumeration of CTCs/DTCs are done with fluorescence or light microscope. [6, 14, 29, 54, 61]

The PCR-based approaches detects highly expressed tumour-associated mRNAs as a measure for presence of CTCs/DTCs. [6, 29, 54] As tumour specific mRNA markers are lacking, epithelial-specific mRNAs, which are also expressed at low levels in normal blood cells, are frequently used for detection of CTCs/DTCs. However, a cut-off value for CTC/DTC positivity needs to be established from the highest mRNA level in the normal blood cells when using this strategy. [4, 6, 62] The PCR approach is indirect, as it detects elevated marker levels in cell lysates caused by the presence of CTCs/DTCs. Reverse transcriptase quantitative polymerase chain reaction, RT-qPCR, which enables quantitative detection of mRNA, is now the most frequently used PCR method. Some studies are based on the quantification of a single mRNA marker while other studies have increased the sensitivity for CTC/DTC detection by combining several mRNAs in multimarker assays (e.g. de Albuquerque and colleagues (ref. 73)). [14, 53, 54, 61, 62]

Peptide nucleic acid (PNA) clamp PCR is another approach for PCR-based detection of CTCs and DTCs. This method was first introduced in 1996 for detection of *KRAS* mutations in tumour samples. [63] The principle behind this method is that the PNA only binds to wild-type *KRAS* templates and in this way block primer annealing and amplification and enable the amplification of mutated *KRAS* templates. *KRAS* mutations are present in approximately 80-90% of pancreatic cancer patients. Thus, *KRAS* mutations may be used as a surrogate marker for CTC and DTC detection pancreatic cancer patients. [7, 24, 64]

1.5 Clinical implications of tumour cell detection in PB and BM of pancreatic cancer patients

Studies based on breast cancer and colorectal cancer patients show noteworthy indications that detection of CTCs in PB is related to a high risk of relapse. [65-69] Furthermore, CTC detection in breast cancer has been proven to be highly predictive of progression-free survival and overall survival. [67, 69] DTCs in BM have in many studies been shown to have prognostic value for several cancer types. [70-72] In pancreatic cancer, the number of relevant studies on CTC/DTC detection is few, but the clinical relevance of CTCs/DTCs has also in this respect been linked to poor prognosis in some of the studies. [6, 62, 73-83] Furthermore, the ability to monitor CTCs/DTCs and to molecularly characterize the tumour cells in PB and BM of cancer patients, can be a useful tool for guiding personalized therapy. [28, 29] Some of the

published studies with evidence of CTCs in pancreatic cancer patients did not evaluate the clinical associations, e.g. Ren *et al.* [84] and Zhou *et al.* [85] The studies mentioned below only include pancreatic cancer studies with survival analyses.

1.5.1 Evidence of clinical relevance of CTC detection in pancreatic cancer patients

Although the relevant studies are few, there are some evidence of clinical relevance of CTCs as a prognostic and predictive marker in pancreatic cancer patients. De Albuquerque *et al.* [73] recently published an evaluation of CTC detection by immunomagnetic enrichment followed by RT-qPCR using a multimarker mRNA panel consisting of the five markers, CK19, MUC1, EpCAM, CEACAM5 and BIRC5. Of the 34 pancreatic cancer patients (unresectable) included in this study, 16 (47.1%) were found to have elevated mRNA levels of at least one tumour-associated mRNA marker before systemic treatment. The CTC detection was associated with shorter progression-free survival ($p = 0.01$). [73]

Sergeant *et al.* [62] used EpCAM as a surrogate marker for quantification of CTCs with RT-qPCR in 49 pancreatic cancer patients, both resectable and unresectable. In preoperative PB samples EpCAM positivity were detected in 10/40 (25%) patients compared to 27/40 (67.5%) patients after pancreatic resection ($p < 0.0001$). Six weeks after surgery, 8/34 (23.5%) patients were EpCAM positive. 2/8 (25%) of unresectable patients were EpCAM positive. Although the study did not demonstrate significant associations between EpCAM positivity and disease-free and cancer-specific survival, tendencies were detected in the preoperative samples ($p = 0.28$ and 0.17, respectively). [62]

Khoja *et al.* [74] compared The CellSearch® system and the ISET technique for CTC detection in PB samples from 54 patients with newly diagnosed or progressive metastatic unresectable adenocarcinoma of the pancreas. ISET detected higher levels of CTCs than the CellSearch® system. However, only CTC detection with the CellSearch® system showed a nonsignificant trend towards shorter survival (decreased progression-free survival ($p = 0.13$) and overall survival ($p = 0.26$)). [74]

Kurihara *et al.* [75] investigated whether CTC detection could predict survival in 26 pancreatic cancer patients, both resectable and unresectable with the CellSearch® system. CTCs were

detected in 11/26 (42%) of the recruited patients and the median survival times of the CTC-positive *vs.* negative patients were 110.5 and 375.8 days, respectively. The study revealed a significant association between median overall survival time and CTC detection ($p < 0.001$). [75]

Hoffmann *et al.* [76] evaluated the diagnostic potential of CK19 mRNA detection in PB, BM and peritoneal lavage in resectable pancreatic cancer patients. The study included 37 pancreatic cancer patients and the authors compared RT-qPCR and nested PCR for CTC/DTC detection. CK19 mRNA positivity (above cut-off value) in PB and BM was detected in 24/37 (64%) samples and 0/37 samples with RT-qPCR, respectively. In 15 (40%) of the pancreatic cancer patients, disseminated tumour cells were detected in PB and BM and/or in peritoneal lavage by RT-qPCR. The CK19 mRNA levels in PB samples obtained 1 and 10 days after surgery tended to be lower than the levels in preoperative samples by RT-qPCR. The results showed that pancreatic cancer patients with at least one CK19 mRNA sample indicated a trend towards shorter survival ($p = 0.15$). [76]

Soeth *et al.* [77] evaluated the diagnostic value of CK20 RT-PCR for detection of disseminated tumour cells in preoperative PB and BM samples . Of the 172 patients that were recruited, a CK20 positive signal was detected in 81 (47.1%) in PB and/or BM. CTCs/DTCs were detected in 52/154 (33.8%) PB samples and 45/135 (33.3%) BM samples, respectively. Survival analysis revealed a statistically significant association between overall survival and the detection of CTCs and/or DTCs ($p = 0.05$). The mean survival time of CK20 positive patients in PB and/or BM were significantly reduced compared to CK20 negative patients (17.9 months *vs.* 26.1 months, respectively). [77]

Mataki *et al.* [78] detected CEA mRNA positive CTCs in PB by nested RT-PCR in 20 resectable pancreatic cancer patients. PB samples were obtained every 3 months after surgery. 6/20 (30%) of the pancreatic cancer patients were detected with CEA mRNA CTCs. In the overall patient group, which included ampullary and biliary duct cancers ($n = 53$), a higher CEA positivity rate was detected in 16 (75%) of the patients with relapse compared to 37 (5.4%) patients without relapse ($p < 0.0001$). CEA mRNA expression in PB may be an early indicator of relapse. [78]

Z'graggen *et al.* [79] used an immunocytochemical assay to detect tumour cells in PB and BM samples obtained from 105 pancreatic cancer patients. CTCs were detected in 26% of the PB samples and in 24% of the BM samples. CTCs were detected in 3/32 (9%) patients with resectable cancer compared to 24/73 (33%) in patients with unresectable disease ($p = 0.023$). There was a tendency towards a statistically significant association between CTC detection and disease progression ($p = 0.08$). [79]

1.5.2 Evidence of clinical relevance of DTC detection in pancreatic cancer patients

DTC detection in BM samples of pancreatic cancer patients is correlated with reduced overall survival. Detection of DTCs may therefore be advantageous as a prognostic marker. [77, 81-83] A study performed by Effenberger *et al.* [80] included one of the largest cohorts of pancreatic cancer patients with DTC detection in BM samples. All patients included in this study underwent pancreatic surgery and of the 175 pancreatic cancer patients, 24 (13.7%) patients had a positive DTC status detected by immunocytochemical cytokeratin assay. The presence of DTCs was associated with a significant decrease in overall survival ($p = 0.036$). [80]

Van Heek *et al.* [81] investigated the DTC status in 31 pancreatic cancer patients (resectable) with an immunocytological approach. CK positive cells were detected in 10 (32%) of these patients, indicating DTCs in their BM. Survival analysis further showed a reduced overall survival ($p < 0.04$) in this patient group. [81]

Roder *et al.* [82] analysed BM samples from 48 patients with resectable ductal adenocarcinoma of the pancreas. An immunocytochemical cytokeratin assay was used to detect DTCs and 25/48 (52.1%) of the patients were defined as DTC positive. By survival analysis it was demonstrated a statistically significant association between DTC positivity and decreased overall survival ($p < 0.03$). [82]

Vogel *et al.* [83] used immunocytochemistry to detect DTCs in BM from 71 patients who underwent surgery for adenocarcinoma of the pancreas. They found that 27/71 (38%) patients were DTC positive and among these patients there were a statistical trend towards reduced survival ($p = 0.06$). [83]

Other studies revealing DTC detection and insignificant association with progression-free and overall survival [86], and studies of gastrointestinal cancer patients [87] where only a few pancreatic cancer patients were included, are not mentioned in larger detail in this thesis. [86, 87]

1.6 Aims of the study

This master thesis is part of a larger project entitled "A novel therapy for locally advanced and/or metastatic pancreatic cancer based on nanoparticle albumin-bound paclitaxel and gemcitabine: Circulating tumour cells as a potential biomarker for treatment monitoring, -response and survival". The primary aim of the larger project is to investigate whether molecular detection of CTCs and DTCs can be potential biomarkers for treatment response in patients with locally and/or metastatic pancreatic cancer. However, specific aims for the present master thesis include:

- Comparison of different strategies for enrichment for CTCs/DTCs.
- Investigation of the epithelial-specific mRNAs CK8, CK19, EpCAM and CEACAM5 as surrogate markers for indirect detection of CTCs and DTCs by RT-qPCR.
- Investigation of *KRAS* gene mutations as a surrogate marker for CTC/DTC detection in pancreatic cancer patients.
- Comparison of the CTC/DTC levels in numerous PB and BM samples from the same patients.

2 Materials

2.1 Samples

Written informed consent was obtained from all participants included in this study and the regional ethical committee approved the project.

The patients (n = 6) included in this prospective study were consecutively recruited from September 2012 to April 2013. They were admitted to Stavanger University Hospital with advanced and/or metastatic pancreatic cancer. All the patients were treated with gemcitabine according to the national guidelines. [88] PB samples were obtained before initiation of treatment and every month for a maximum of two months during treatment with gemcitabine. Whole blood was collected into 9 mL EDTA tubes and the first few millilitres were discarded to prevent contamination of epithelial cells. BM samples (10 mL in EDTA) were drawn unilaterally from the posterior iliac crest under general anaesthesia prior to initiation of treatment and after one month of treatment. To avoid contamination of epithelial cells in the BM samples, a small incision with a scalpel was done prior to the BM sampling. The patient samples were either processed the same day as sampling or stored at room temperature until the next day.

One tumour tissue sample from a liver metastasis in one pancreatic cancer patient was included in the study as well as five human tumour tissue samples from adenocarcinoma of the pancreas (obtained from Asterand®).

PB and BM samples from nine healthy individuals constituted the control group in this project.

Table 2.1: Clinopathological parameters for the included pancreatic cancer patients.

Variable	Number of patients (n = 6)
Sex	
Male	5
Female	1
Pathology cell type	
Adenocarcinoma	6
Primary tumour localization	
Head	2
Body	1
Tail	3
Tumour size	
pT1	0
pT2	4
pT3	0
pT4	0
Unknown	2
Lymph node status	
pN0	0
pN1	2
pNX	4
Metastatic stage	
M0	0
M1	6
MX	0
Metastatic sites	
Liver	5
Lung	1
cTNM disease stage	
IA	0
IB	0
IIA	0
IIB	0
III	0
IV	6
Tumour grade	
I	1
II	2
III	1
Unknown	2

2.2 Cell culture

The human pancreatic tumour cell line AsPC-1 (Sigma-Aldrich®) originates from mouse xenografts where cells from a patient with pancreatic cancer were introduced. The cell line express carcinoembryonic antigen (CEA), human pancreas associated antigen, human pancreas specific antigen and mucin. [89]

2.3 Buffers and solutions

0.9 % NaCl

- 9 g NaCl
- dH_2O up to 1000 mL

5 x TBE

- 27 g TRIS Buffer
- 13.75 g boric acid
- 10 mL EDTA (0.5M)
- dH_2O up to 500 mL

0.5 x TBE

- 100 mL 5 x TBE Buffer
- dH_2O up to 1000 mL

0.5 M EDTA

- 186.1 g Ethylendiamin-tetra acetate x $2H_2O$
- dH_2O up to 1000 mL
- Adjust pH to 8.0

Hayem's staining solution

- 0.025 g Crystal violet
- 2.5 g acetic acid
- dH_2O up to 50 mL

RBC Lysis Buffer

- 8 g NH_4Cl
- KHCO3 (2M)
- 1.0 mL EDTA (0.1mM, 1 mL of 100 mM EDTA)
- dH_2O up to 1000 mL
- Adjust with $KHCO_3$ to achieve a pH of 7.2 – 7.6

2M $KHCO_3$

- 125.15 g $KHCO_3$
- dH_2O up to 250 mL

100 mM EDTA

- 37.224 g Ethylendiamin-tetra acetate x $2H_2O$
- dH_2O up to 1000 mL
- Adjust pH to 8.0

6x Loading Buffer

- 2.5 mL 99% glycerol
- 0.5 mL EDTA
- 2.0 mL dH_2O
- Bromophenol Blue

Agarose (3%)

- 1.5 g agarose
- 50 mL 0.5 x TBE
- dH_2O

1xPBS

- 5 Phosphate buffered saline tablets
- dH_2O up to 1000 mL

2.4 Reagents

Table 2.2: An overview of the reagents used in this study.

Reagents	Producer/ supplier	Product number	Area of utilization
5 x First Strand Synthesis (5x FSS Buffer)	Invitrogen™	P/N Y02321	DNase treatment and reverse transcription
RQ1 RNase-Free DNase	Promega	M610A	DNase treatment
RQ1 DNase Stop Solution	Promega	M199A	DNase treatment
Random Primers (1µg/µl random nonamer)	Invitrogen™	P/N 58875	Reverse transcription
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen™	P/N 100000840 -10777-019	DNase treatment and reverse transcription
25 mM dNTP: 2´Deoxyadenosine 5´-Triphosphate (dATP) 2´Deoxycytidine 5´-Triphosphate (dCTP) 2´Deoxyguanosine 5´Triphosphate) (dGTP) 2´Deoxythymidine 5´Triphosphate (dTTP)	GE Healthcare	 28406501V 28406511V 28406521V 28406531V	Reverse transcription
0.1 M DTT	Invitrogen™	P/N Y00147	Reverse transcription
M-MLV Reverse Transcriptase	Invitrogen™	28025-021	Reverse transcription
Lymphoprep™	Axis-Shield	1114545	CTC/DTC enrichment
Trypan Blue Solution (0.4%)	Sigma-Aldrich®	T8154	Determination of cell numbers
Hayem's dye: Crystal violet, Microscopy Cerstistain	Merck	1.15940.002 5	Determination of cell numbers
Agarose NA	GE Healthcare	17-0554-02	Gel electrophoresis
2-Mercaptoethanol (β-ME)	Sigma	M3148	CTC/DTC enrichment
Loading buffer (gel): Blue/Orange 6x Loading Dye	Promega	G190A	Gel electrophoresis
100bp DNA ladder	Promega	G2101A	Gel electrophoresis
GelRed™ Nucleic Acid Gel Stain, 10.000x in DMSO	Biotium	800-304 5357	Gel electrophoresis

2.5 Growth media for tumour cell line culturing

Table 2.3: Overview of the reagents included in the cell culture medium.

Reagents	Producer/supplier	Product number
RPMI 1640	Sigma	R0883
2mM Glutamine	Sigma	G7513
1mM Sodium Pyruvate (NaP)	Sigma	S8636
10% Foetal Bovine Serum (FBS)	Sigma	F7524
Penicillin Streptomycin (100x)	Sigma	P4333

2.6 Kits

Table 2.4: An overview of the kits used in this study.

Kit	Producer/ supplier	Product number	Area of utilization
AllPrep® DNA/RNA/Protein Mini Kit (50)	Qiagen®	80004	Isolation of nucleic acids
QIAamp® RNA Blood Mini Kit (50)	Qiagen®	52304	Isolation of nucleic acids
QIAshredder™	Qiagen®	79656	Homogenization
qPCR™ Core kit for SYBR® Green I	Eurogentec	RT-SN10-05	RT-qPCR
Agilent RNA 6000 Nano Kit	Agilent Technologies	5067-5111	For measurements of RNA quality/degradation

2.7 Primers and probes for RT-qPCR and the PNA clamp assay

All the primers were purchased from Eurofins MWG Operon. The PNA clamp was purchased from Eurogentec.

Table 2.5: Primers for amplification of CK8, CK19, EpCAM, CEACAM5 and BCR by quantitative real-time PCR. All primers were designed to span exon/exon boundaries.

Primer	Primer sequence		Product size, bp
CK8 - FF	forward	5'-CATGGGAGGCATCACCGCAG-3'	164 bp
CK8 - RF	reverse	5'-GCTCCAGGAACCGTACCTTGTC-3'	
CK19 - F	forward	5'-GATGAGCAGGTCCGAGGTTA-3'	96 bp
CK19 - R	reverse	5'-TCTTCCAAGGCAGCTTTCAT-3'	
EpCAM - FB	forward	5'-CGCAGCTCAGGAAGAATGTG-3'	88 bp
EpCAM - RB	reverse	5'-TGAAGTACACTGGCATTGACG-3'	
CEACAM5 - FC	forward	5'-GGGACCTATGCCTGTTTTGTCTC-3'	151 bp
CEACAM5 - RC	reverse	5'-GAGCAACCCCAACCAGCAC-3'	
BCR - sg1F	forward	5'-GCTCTATGGGTTTCTGAATG-3'	99 bp
BCR - sg1R	reverse	5'-AAATACCCAAAGGAATCCAC-3'	

bp = base pair

Table 2.6: Primers and probe for the PNA clamp assay.

Primer	Primer sequence		Product size, bp
KRAS-PNA-FB	forward	5'-GCCTGCTGAAAATGACTGAATATAA-3'	71 bp
KRAS-PNA-RB	reverse	5'-CGTCAAGGCACTCTTGCCTAC-3'	
PNA clamp	-	5'-CCTACGCCACCAGCTCC-3'	-

bp = base pair

3 Methods

3.1 Enrichment of CTCs and DTCs

Due to a low CTC and DTC concentration in PB and BM (1 per $1x10^6$ leukocytes), an enrichment step is essential to increase the sensitivity of the assay. [54] Traditional methods used for enrichment are Red Blood Cell (RBC) lysis buffer and density centrifugation. The RBC lysis buffer is optimized for gentle lysis of the erythrocytes with marginal effect on the leukocytes. [55]

The principle of density centrifugation is that the MCs, *i.e.* monocytes and lymphocytes, have a lower density compared to leukocytes and erythrocytes. Thus, the erythrocytes and leukocytes will sediment through the medium to the bottom, while the MCs, including tumour cells, will be retained at the sample/medium interface, see figure 3.1. [54, 90]

3.1.1 Enrichment of CTCs by RBC Lysis Buffer

- The PB samples were transferred into 50 mL Falcon tubes and added RBC lysis buffer in a ratio of 9:1 (e.g. 5 mL blood sample and 45 mL RBC lysis buffer).
- The cell suspension was vortexed and put on ice for 10 minutes to allow the red blood cells to lyse.
- The other cells were then pelleted by centrifugation at 370 x g (1400 rpm) for 5 minutes before the supernatant was discarded.
- The cells were washed with 5 mL recommended medium before centrifugation at 370 x g (1400 rpm) for 5 minutes. The supernatant was discarded and this step was repeated once more.
- The cells were then transferred to a new 15 mL Falcon tube (*i.e.* approximately 8 mL) and centrifuged at 370 x g (1400 rpm) for 5 minutes before the supernatant was discarded.
- The cell pellet was resuspended in 1 mL PBS added 2% FBS and 1mM EDTA.

- Counting of the cells was performed by the use of Hayem's staining solution, which lyses the red blood cells and stains the nucleus in the leucocytes purple.
- A centrifugation step at 370 x g (1400 rpm) for 5 minutes was then performed and the supernatant was discarded.
- The pellet was resuspended in 600 µl Buffer RLT (added 10 µl ß-Mercaptoethanol per 1 mL Buffer RLT), transferred into a new eppendorf tube and stored at -80°C for later use.

3.1.2 CTC and DTC enrichment by density centrifugation

- The PB and BM samples were transferred into a 50 mL Falcon tube and an equal amount of 0.9% NaCl was added (e.g. 9 mL blood sample, 9 mL 0.9% NaCl). The solution was mixed thoroughly.
- An equal amount (9 mL) of Lymphoprep was added to a new 50 mL Falcon tube and the NaCl-PB/NaCl-BM solution was carefully transferred onto the Lymphoprep media. The solution was centrifuged at 2000 rpm (755 x g) for 30 minutes.
- The buffy coat (MCs) was then carefully transferred to a new 50 mL Falcon tube followed by washing with 40 mL 0.9% NaCl. Centrifugation was then performed at 1100 rpm (228 x g) for 10 minutes and the supernatant was discarded.
- A second wash with 40 mL 1xPBS was then performed before a new centrifugation at 1100 rpm (228 x g) for 10 minutes and the supernatant was discarded.
- Finally, the cells were resuspended in 1 mL of 1xPBS.
- The number of cells in the sample was determined by counting using Hayem's staining solution, (47.5 µl Hayem's staining solution, 2.5 µl cell suspension), which lyse the red blood cells.
- The desired number of cells ($1x10^7$ cells) was transferred to new 1.5 mL eppendorf tubes, centrifuged at 1100 rpm (228xg) for 10 minutes and then lysed in either 600 µl (added to $5x10^6$ – $1x10^7$ cells) or 350 µl (added to < $5x10^6$ cells) Buffer RLT. The samples were thoroughly mixed to ensure a homogenised lysate before they were stored at -80°C for later use.

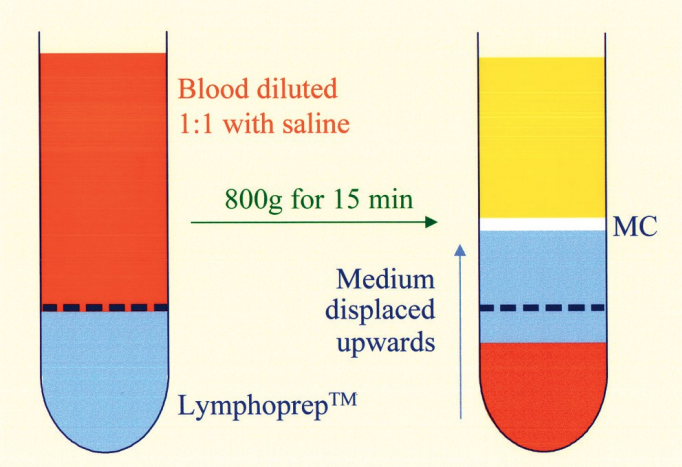

Figure 3.1: An overview of the Lymphoprep™ procedure used for tumour cell enrichment. The blood sample is diluted 1:1 with physiological saline and carefully transferred to a tube containing Lymphoprep. The mononuclear cells (MCs) are then separated from the blood cells by centrifugation and the cells can be transferred to a new tube for further use. [90] The figure is copied from the Lymphoprep™ protocol published by Axis-Shield and is available online. [90]

3.1.3 Determination of the cell concentration by the use of a light microscope

When determining the number of cells in a sample using a Bürker counting chamber, the cell suspension was diluted with either Hayem's staining solution (47.5 µl dye and 2.5 µl cell suspension) or 0.4% Trypan Blue (50 µl 0.4% Trypan Blue, 45 µl PBS and 5 µl cell suspension). The cells are counted using a light microscope. Hayem's staining solution lyses the red blood cells and the nucleus in the leukocytes is stained with a purple colour. In contrast, Trypan Blue discriminates between living and dead cells (dead cells absorb the dye and are seen as blue spots).

- The Bürker counting chamber is carefully cleaned and the coverslip is placed over the counting surface.
- The stained cell suspension is pipetted onto the Bürker counting chamber.
- Cells in A squares are counted using the 10x objective, see figure 3.2 below.
- The cell concentration is calculated as shown in *equation 1*, when the cell counting has reached > 200 cells.

Equation 1:

$$C = \frac{N}{a\ x\ V\ x\ D}\ x\ 1000 = cells/mL$$

C = cell concentration (cells/mL)

N = the number of cells counted

a = the number of squares (A) counted

V = volume of the counting chamber (0.1 µl)

D = dilution factor (1:20)

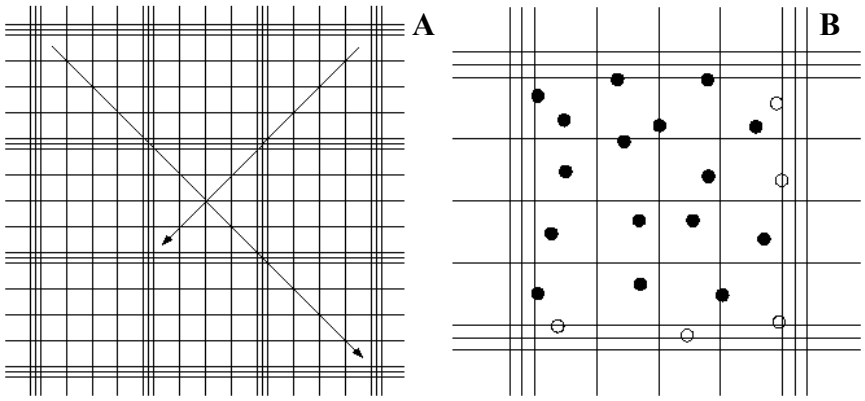

Figure 3.2: The image illustrates the 9 squares (A) in the Bürker counting chamber. When counting cells, one counts the cells within the A squares as well as cells lying on the top and left hand lines of each square. This is illustrated in the right picture **(B)** where all the cells marked in black are counted, while the cells marked in white are not.

3.2 Isolation of DNA/RNA and protein

3.2.1 AllPrep® DNA/RNA/Protein Mini Kit

The AllPrep® DNA/RNA/Protein Mini Kit is designed for purification of genomic DNA, total RNA and total protein content from the same cell or tissue sample. In the first step the samples are lysed and homogenized with a guanidine-isothiocyanate-containing buffer. This buffer inactivates DNases, RNases and proteases, to ensure isolation of intact DNA, RNA and proteins. The lysate is first transferred to an AllPrep DNA spin column, which enables selective and efficient binding of genomic DNA (15-30 kb). The flow-through from the AllPrep DNA spin column is used to isolate total RNA (> 200 nucleotides). The DNA spin column has a restricted capacity of $1x10^7$ cells. After washing, ready-to-use DNA is eluted from the column. [91] The flow-through from the AllPrep DNA spin column is added ethanol to get the suitable binding conditions for RNA before the sample is applied to an RNeasy spin column. Total RNA will bind to the column and after washing the RNA is eluted with RNase-free water. For protein purification an aqueous protein precipitation solution is added to the flow-through of the RNeasy spin column. Proteins are pelleted by centrifugation and intact proteins are redissolved in an appropriate buffer and ready for further use. An overview of the purification steps is presented in figure 3.3. [91]

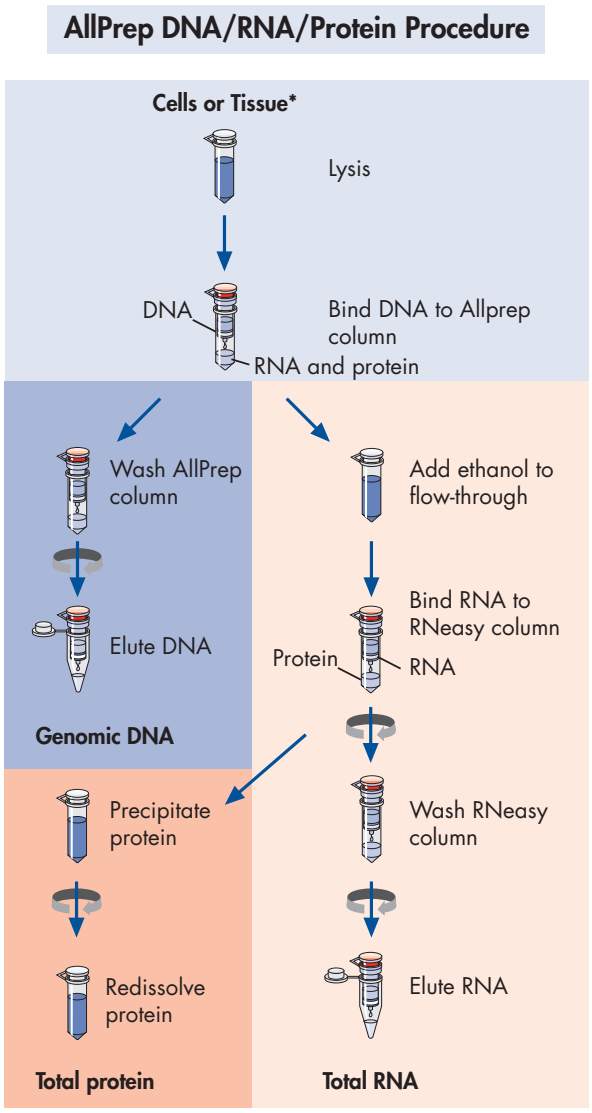

Figure 3.3: An overview of the DNA, RNA and protein isolation procedure. The figure is obtained from the AllPrep® DNA/RNA/Protein Mini Handbook. [91]

Procedure

- In brief, the cells were lysed in Buffer RLT according to table 3.1 below.

Table 3.1: Volumes of Buffer RLT to be added for lysing pelleted cells.

Number of pelleted cells	Volume of Buffer RLT
< $5x10^6$	350 µl
$5x10^6 – 1x10^7$	600 µl

- The lysate was homogenized through a QIAshreddder spin column and centrifuged at 17.000 x g for 2 minutes.

- Homogenized lysate was transferred to an AllPrep DNA spin column and centrifuged for 30 seconds at ≥ 8000 x g.
- The AllPrep DNA spin column was moved to a new collection tube and stored at room temperature (15-25°C) for later DNA purification steps.
- The flow-through was used for RNA purification.

Total RNA purification

- Either 250 µl 96-100% ethanol (if 350 µl Buffer RLT was used) or 400 µl 96-100% ethanol (if 600 µl Buffer RLT was used) was added to the flow-through from the previous step before the mixture was transferred to an RNeasy spin column and centrifuged for 15 seconds at ≥ 8000 x g. The flow-through was used for protein purification.
- The total RNA was washed several times before it was eluted by adding 40 µl RNase-free water to the spin column membrane and centrifuged at 1 minute at 8000 x g.
- The RNA samples were stored at -80°C.

In this study the flow-through used for protein purification was stored at -80°C for further use.

Genomic DNA purification

- 500 µl washing buffer was added to the AllPrep DNA spin column before the column was centrifuged for 15 seconds at 8000 x g.
- The DNA was washed with a second washing buffer before 50 µl preheated (70°C) elution buffer (EB) was added to the column. The column was incubated at room temperature for 2 minutes followed by the elution of DNA by centrifugation for 1 minute at 8000 x g. This step was performed twice (total volume = 100 µl).
- The DNA samples were stored at -80°C.

3.2.2 QIAamp® RNA Blood Mini Kit

QIAamp® RNA Blood Mini Kit may be used for isolation of total RNA from a maximum of 1.5 mL human whole blood (up to 0.5 mL whole blood equals $2x10^6$ leukocytes and from 0.5 - 1.5 mL whole blood equals $2x10^6$ – $1x10^7$ leukocytes). The principle of the method is first to lyse the erythrocytes, while the leukocytes are regained by centrifugation. The leukocytes are then lysed during the RNA isolation steps, under denaturing conditions where RNases are inactivated to ensure isolation of intact RNA. A high-salt buffering system is used in this process for binding of RNA longer than 200 bases to a silica-based membrane. The RNA is eluted from the membrane by addition of RNase-free water. [92] An overview of the RNA isolation is presented in figure 3.4.

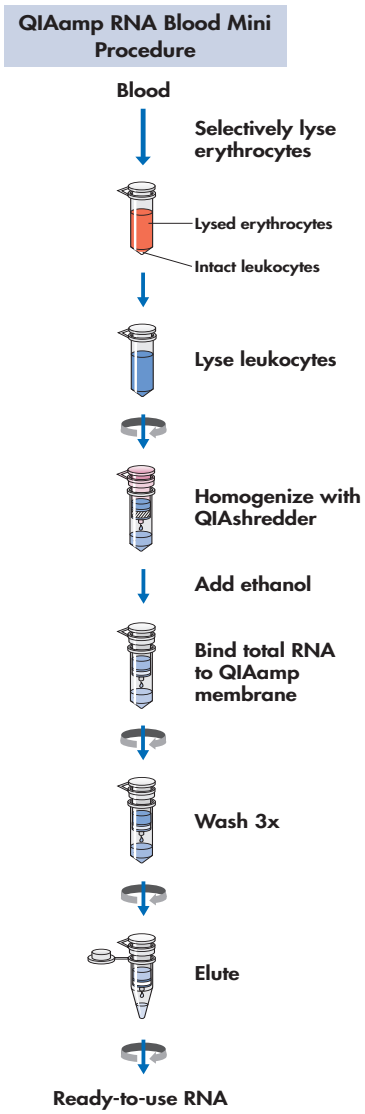

Figure 3.4: An overview of the procedure for isolation of ready-to-use RNA with QIAamp® RNA Blood Mini Kit. The figure is obtained from the QIAamp® RNA Blood Mini Handbook. [92]

Procedure

Enrichment of MCs

- One volume (1.5 mL) of whole blood was mixed with five volumes of Buffer EL and incubated on ice for 10-15 minutes before centrifugation at 400 x g for 10 minutes at 4°C.
- The supernatant was discarded.
- Two volumes of Buffer EL (3 mL) were then added and the pellet was resuspendeded quickly by vortexing followed by centrifugation at 400 x g for 10 minutes at 4°C.
- The pellet was resuspended in 600 µl Buffer RLT (added 10 µl ß-Mercaptoethanol per 1 mL Buffer RLT) per $2x10^6 – 1x10^7$ leukocytes for further RNA purification.

RNA purification

- Before purification the lysate was homogenized through a QIAshredder spin column by centrifugation at 17.000 x g for 2 minutes.
- One volume of 70% ethanol was added to the homogenized lysate and the sample was mixed before it was transferred to a silica-based membrane column and centrifuged for 15 seconds at ≥ 8000 x g.
- The total RNA was then washed several times before 40 µl RNase-free water was added to the column for elution of RNA by centrifugation for 1 minute at ≥ 8000 x g.
- All RNA samples were stored at -80°C for further use.

3.2.3 Isolation of DNA and RNA from tumour samples

When DNA and RNA are isolated from tumour biopsies it is necessary to homogenize the tissue before the nucleic acid purification procedure. Homogenization will shear high-molecular-weight components and create a homogenous lysate. [91]

Procedure

- The frozen tumour biopsy was transferred from liquid nitrogen to RLT lysis buffer and homogenized by an Ultra-Turrax T8 (IKE Works, Staufen, Germany) rotor-stator homogenizer before centrifugation at 17.000 x g for 3 minutes.
- The supernatant was then transferred into an AllPrep DNA spin column and centrifuged for 30 seconds at ≥ 8000 x g.

- Isolation of RNA/DNA was performed as described in section 3.2.1.

3.3 Concentration measurements on NanoDrop 2000c Spectrophotometer

The DNA and RNA concentration in a sample can be determined spectrophotometrically by measuring the absorbance at wavelength 260 nm. An optical density value of 1 corresponds to a concentration of 50 µg/mL for double-stranded DNA and 40 µg/mL for single-stranded RNA. The absorbance is however also measured at 280 nm for detection of possible protein contamination. Thus, the 260/280 ratio is used to define the purity of DNA and RNA. Highly purified DNA result in a ratio of approximately 1.8, while a ratio of approximately 2.0 is defined as pure RNA. The presence of protein or other contaminants in the samples will result in a lower ratio. [93]

Procedure

- 2 µl of "blank" sample (Milli-Q water for RNA measurements, Buffer EB for DNA measurements) was used to reset the instrument.
- 2 µl of fresh sample (RNA and DNA) was pipetted directly on to the bottom pedestal, the absorbance at 260 nm and 280 nm and the sample concentration as well as the sample purity was automatically calculated by the NanoDrop 2000c instrument.

3.4 DNA and RNA quality assurance by the use of Bioanalyzer 2100

Bioanalyzer 2100 (Agilent Technologies) is a microfluidics-based platform, which use the "lab-on-a-chip technology" (LOC) to perform analyses in a single chip. The LOC-technology reduces both the separation time and sample volumes. [94]

The chip contains micro-channels of glass, which create organised networks among several wells and before analysis the micro-channels are filled with both polymer and a fluorescence dye. Samples and a ladder with marker are loaded in each well. Fluorescence dye molecules are inserted into the DNA or RNA strands making them detectable with laser scanning. After loading of samples the negatively charged DNA and/or RNA molecules are separated according to size by migration towards the anode due to a voltage gradient. Small fragments

migrate faster than large fragments. The size and concentration of the DNA/RNA fragments correlates with the fluorescence detected. [94]

A software algorithm filters the data and presents the results as electropherograms (see figure 3.5) where fluorescence intensity versus migration time is plotted (the data can also be displayed as a densitometry plot, a "gel-like image".) The software automatically compares the unknown samples to the ladder, which contains a mixture of RNA of known concentrations, to determine the concentration of the unknown samples. The 18S and 28S ribosomal peaks are used for evaluation of the integrity of RNA and well-separated peaks indicate high quality RNA. The "lower" marker is used to align the samples and compensate for drift effects. [94, 95] The RNA integrity is presented as the RNA integrity number (RIN), which is scaled from 1-10, where 10 is the best. Samples with RIN values of 10 are considered as RNA samples of ideal quality. [94-97]

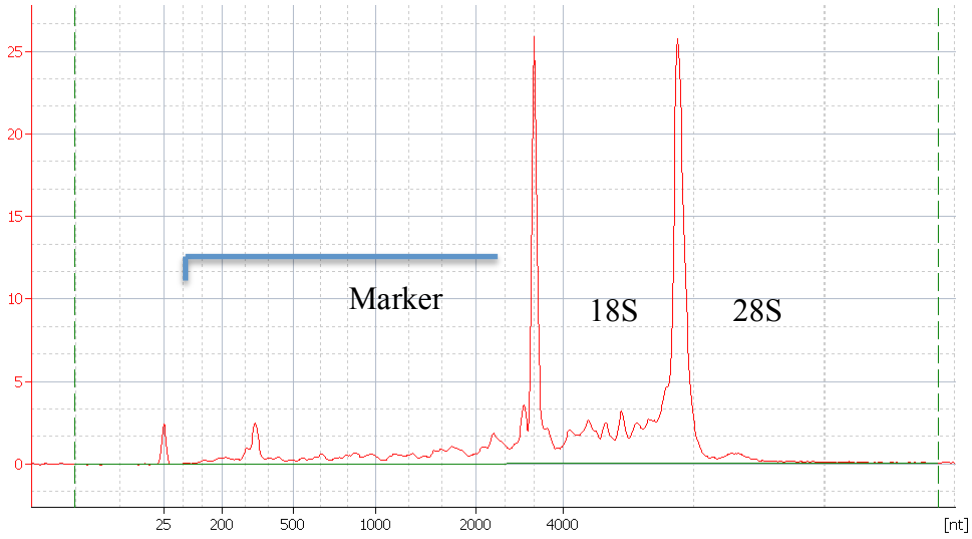

Figure 3.5: The figure shows a RNA electropherogram from the Bioanalyzer 2100 for a pancreatic tumour sample (PC6). The 18S and 28S ribosomal peaks are used for evaluation of the integrity of RNA and well-separated peaks indicate high quality RNA. The marker solution contains a 50 bp DNA fragment, which is used as a lower marker to align the samples within the same run. The marker is displayed as the first peak in the electropherograms. [94]

Procedure

The RNA 6000 Nano Kit was used in this procedure. The procedure was performed as described in Agilent RNA 6000 Nano Kit Guide. [95] A brief explanation is described below.

- 550 µl of RNA 6000 Nano gel matrix was passed through a spin filter and centrifuged at 1500 x g for 10 minutes. Aliquots of 65 µl were transferred into RNase-free microfuge tubes and added 1 µl of RNA 6000 Nano dye concentrate before the gel-dye mix was centrifuged at 13.000 x g for 10 minutes.
- Gel-dye mix (9 µl), Agilent RNA 6000 Nano Marker (5 µl), ladder (1µl) and samples (1 µl) was pipetted into marked wells on the chip. Fully prepared chip was vortex on a IKA vortexer at 2400 rpm at 1 minute.
- The chip was run at the Bioanalyzer 2100 within 5 minutes.

3.5 DNase treatment and reverse transcription

DNase treatment is performed on the RNA samples for degradation of contaminating single-stranded and double-stranded DNA. Reverse transcription results in single-stranded complementary DNA (cDNA), which is the template for tumour cell detection and characterization by RT-qPCR. Total single-stranded RNA is reverse transcribed by binding the M-MLV (Molony Murine Leukemia Virus) Reverse Transcriptase (RT), a recombinant DNA polymerase, to the random primers for initiation of the cDNA synthesis. RNaseOUT™ Ribonuclease Inhibitor is added to the mixture to protect RNA from degradation by RNases and will in this way improve the total cDNA yield. In addition, a negative control sample, without the addition of reverse transcriptase (NO RT), is included in every run for detection of inefficient DNase treatment and thus contaminating DNA in the RNA samples. [54, 98, 99]

Procedure

DNase treatment

- Thaw the RNA samples on ice.
- To 0.5 µg RNA sample add the following reagents:
  - 2 µl 5 x FSS Buffer
  - 1 µl RQ1 DNase
  - 0.25 µl RNaseOUT RNase inhibitor
- dH_2O is then added to a total reaction volume of 10 µl.
- Incubate the samples for 30 minutes at 37°C (water bath).
- Add 1.0 µl RQ1 Stop Solution to each tube.
- Incubate the samples for 10 minutes at 65°C to inactivate the DNase enzyme and then stop the DNase treatment.

Reverse transcription of DNase-treated RNA

- Add the following reagents to each tube with DNase treated RNA:
  - 0.2 µl 1 µg/µl random nonamer
  - 0.4 µl 25 mM dNTP
  - 0.4 µl dH_2O
- Incubate the RNA samples for 5 minutes at 65°C before cooling on ice for a minimum of 2 minutes. Then add the following to each tube:
  - 2.0 µl 5 x FSS Buffer
  - 2.0 µl 0.1 M DTT
  - 1.0 µl RNaseOUT RNase inhibitor
  - 2.0 µl dH_2O
- Incubated the samples for 2 minutes at 37°C (water bath).
- After this incubation, add 1.0 µl MMLV reverse transcriptase to each tube and incubate for 10 minutes at room temperature, following the reverse transcription at 37°C (water bath) for 1 hour.
- Inactivation of the enzyme is done by incubating the cDNA samples for 15 minutes at 65°C.
- Finally, 30 µl dH_2O is added for a concentration of 10 ng/µl (total volume of 50 µl).
- All samples were stored at -80°C for further use.

3.6 Quantitative real-time PCR

Reverse transcriptase quantitative polymerase chain reaction, RT-qPCR, allows the monitoring of PCR product formation during every PCR cycle, thus the name "real-time", by the use of different fluorescence chemistries (the DNA binding dye SYBR® Green I, hydrolysis probes, hybridization probes, molecular beacons among others). The principle of detection is that an increase in the fluorescence signal is directly proportional to the amount of target mRNA in the sample at the threshold value. [54, 100, 101] The threshold value is defined at the point where the mRNA target doubles for every cycle. The amplification is at this point described as 100% effective. The number of cycles at which the fluorescence exceeds the threshold is called the threshold cycle (C_t), crossing point (CP) or quantification cycle (C_q). [54, 100, 102] A low C_q-value reflects a high concentration of target mRNA present in the sample (see figure 3.6).

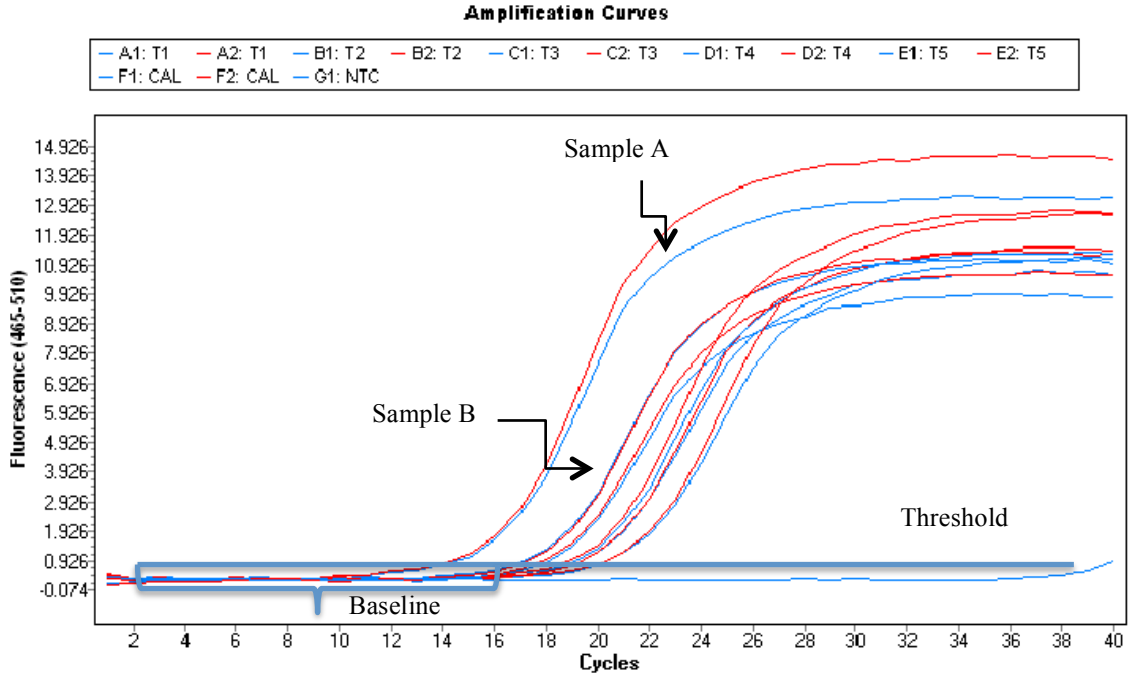

Figure 3.6: An amplification plot showing an increase in the fluorescence signal as a function of the number of PCR cycles. Sample A (C_q = 15.61) contains a higher amount of target mRNA compared to sample B (C_q = 18.29). The amplification plot is obtained from analysis of the tumour samples on the LightCycler® 480 real-time PCR instrument (Roche Applied Science).

When SYBR® Green I is used as the detection method the PCR products can be verified by dissociation curve analyses. As different PCR products have different melting temperatures (T_m), the dissociation curve analysis makes it possible to separate between specific and non-specific PCR products. [100-102] See figure 3.7 for an example of a dissociation curve.

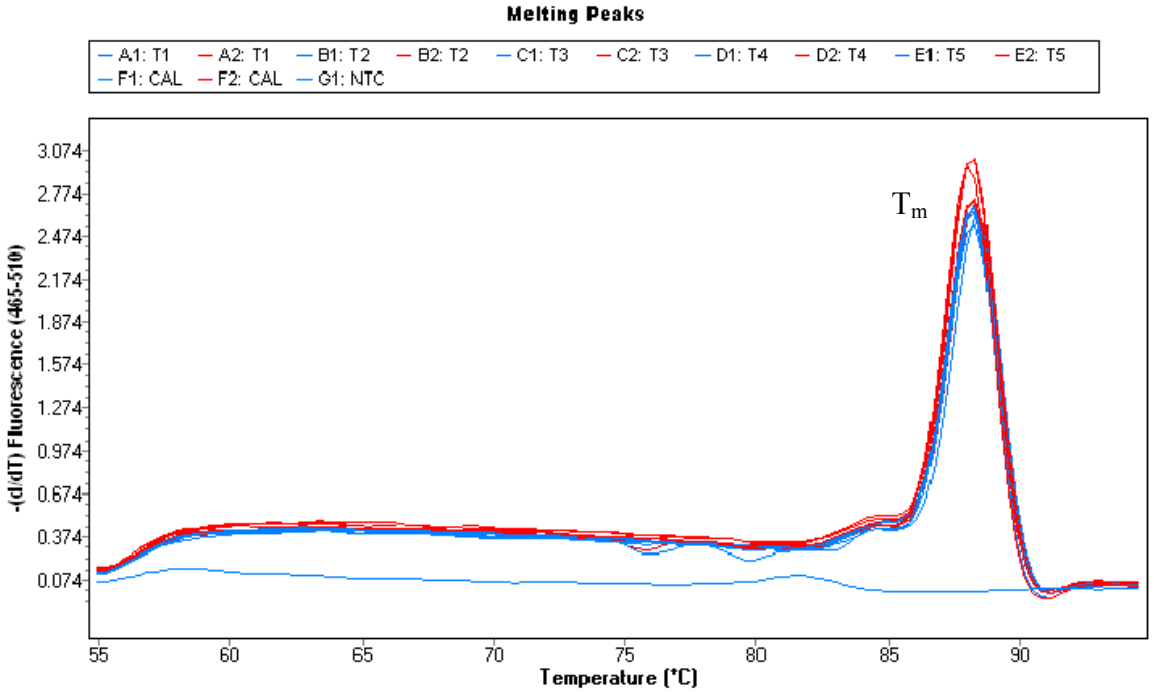

Figure 3.7: Dissociation curve analysis presented by the fluorescence as a function of temperature. The figure clearly shows that the correct PCR products have been amplified, this is demonstrated by similar melting temperatures (T_m), as expected. The dissociation curve is obtained from analysis of the tumour samples on the LightCycler® 480 real-time PCR instrument (Roche Applied Science).

Procedure

- Thaw all reagents on ice.
- Prepare mastermixes for each mRNA marker according to the volumes described in table 3.2 below.

Table 3.2: Mastermixes for quantification of CK8, CK19, EpCAM, CEACAM5 and BCR by RT-qPCR.

	CK8		**CK19**		**EpCAM**		**CEACAM5**		**BCR**	
Reagents	Final [conc.]	Vol. (µl)	Final [conc.]	Vol. (µl)	Final [conc.]	Vol. (µl)	Final [conc.]	Vol. (µl)	Final [conc.]	Vol. (µl)
dH_2O		17.38		16.13		16.51		17.01		16.88
10x PCR Buffer	1x	2.50	1x	2.50	1x	2.50	1x	2.50	1x	2.50
50 mM $MgCl_2$	1.50	0.75	2	1.00	1.25	0.63	1.25	0.63	2	1.00
5 mM dNTP-U-mix	0.2mM	1.00	0.2mM	1.00	0.2mM	1.00	0.2mM	1.00	0.2mM	1.00
10 µM F primer	0.10µM	0.25	0.3µM	0.75	0.3µM	0.75	0.2µM	0.50	0.15µM	0.38
10 µM R primer	0.10µM	0.25	0.3µM	0.75	0.3µM	0.75	0.2µM	0.50	0.15µM	0.38
1:200 SYBR® Green I *	1x	0.75	1x	0.75	1x	0.75	1x	0.75	1x	0.75
Hot Goldstar enzyme		0.12		0.12		0.12		0.12		0.12
Volume		23.0		23.0		23.0		23.0		23.0

Vol. = Volumes per reaction
[Conc.] = concentrations
* SYBR® Green I in dimethyl dulfoxide

- 23 µl of each mastermix was distributed to each well on the PCR plate before 2 µl (20 ng) cDNA was added.
- All samples were analysed in duplicates.
- No template control samples, containing 2 µl dH_2O instead of cDNA, were included for every mRNA marker in every run to assure no existence of non-specific PCR products.
- cDNA from the pancreatic cancer cell line AsPC-1 was used as a calibrator (positive control) and included for every mRNA marker in every run.
- Every run included the reference gene BCR used for normalization (see section 3.6.1).

- The PCR plate was placed in the LightCycler® 480 real-time PCR instrument (Roche Applied Science) and run at 95°C for 10 minutes to activate the enzyme, 40 cycles of 30 seconds at 95°C (denaturation) followed by 1 minute at 60°C (annealing and synthesis).
- The purity of the PCR products was verified by dissociation curve analysis at 95°C for 1 minute (denaturation) followed by 55°C for 30 seconds (renaturation) and 95°C for 30 seconds (denaturation).

3.6.1 Calculation of the relative mRNA expression level

In relative quantification all samples are normalized to a constantly expressed housekeeping mRNA (reference mRNA) to control for variations between the samples. A calibrator sample is also included to both correct for sample-to-sample variations and for run-to-run variation in the quantification process. [103] Mean Cq-values, based on two replicates analysed, are used in the calculations. Moreover, the mRNA levels were normalized against the BCR (NM_004327) mRNA levels and expressed in relation to the calibrator sample in this study. Relative gene expression was calculated for each mRNA marker using the $2^{-\Delta\Delta Cq}$ method as shown below (*equation 2* and *equation 3*). [102-104]

Equation 2:

$$R = 2^{-\Delta\Delta Cq}$$

R = relative mRNA concentration for target biomarker.
2 = the amplification efficiency where the template doubles in each cycle during exponential amplification. [102, 104]

Equation 3:

$-\Delta\Delta Cq = -(\Delta Cq_{sample} - \Delta Cq_{calibrator})$ where,

$\Delta Cq_{sample} = (Cq_{biomarker} - Cq_{reference})$ and $\Delta Cq_{calibrator} = (Cq_{biomarker} - Cq_{reference})$

3.6.2 Sensitivity analysis

A sensitivity analysis was performed to determine the lowest number of CTCs detectable by each mRNA marker with RT-qPCR. After enrichment of MCs from PB by Lymphoprep™, $2x10^6$ cells were distributed in 6 eppendorf tubes and added 0, 2, 20, 200, 20 000 and 200 000 AsPC-1 cells, respectively. Two replicates for each standard were prepared and quantified by RT-qPCR.

Procedure

- Enrichment of MCs was done by Lymphoprep™, as described in section 3.1.2.
- The amount of cells/mL was determined as described in section 3.1.3 and $2x10^6$ cells were distributed in 6 eppendorf tubes and added 0, 2, 20, 200, 20 000, 200 000 AsPC-1 cells, respectively. Two parallel series were made.
- DNase treatment and reverse transcription was performed as described in section 3.5.
- Finally, the level of each mRNA marker in AsPC-1 cells was quantitated by RT-qPCR described in section 3.6 and the sensitivity was determined for each mRNA assay.

3.7 Gel electrophoresis (3% agarose)

By agarose gel electrophoresis DNA fragments (e.g. PCR products) are separated according to size. When applying an electrical field across the agarose gel the DNA, which is negatively charged, will migrate towards the anode. The larger fragments migrate slowly because their progress is more impeded by the agarose matrix. The DNA bands, which consist of a collection of DNA molecules of identical length, on the agarose gel are either labelled or stained for visualization. [105, 106]

Procedure

- 1.5 g of agarose was dissolved in 50 mL of 0.5 x TBE Buffer, by boiling to compose a 3% agarose gel.
- The solution was poured into a tray and a comb was added for creation of the wells.
- The gel was stained with GelRed™. The gel was set to polymerize for at least 30 minutes before the comb was carefully removed.

- The samples were then added 6x loading buffer and loaded into the wells and separated by an electrical field of 80 V.
- Finally the PCR products were visualized by ultra violet light using the INgenius detector (Syngene).

3.8 Peptide Nucleic Acid Clamp PCR assay for *KRAS* mutations

In this study, genomic *KRAS* mutations in codons 12 and 13 were detected by a peptide nucleic acid (PNA) clamp method based on SYBR® Green I and a high-fidelity DNA polymerase. [64] The PNA clamp method is based on competitive binding of the PNA and the primer during the analysis. That is, the PNA suppress elongation of the wild-type templates by binding to wild-type *KRAS* templates and in this way block primer annealing (figure 3.8 A). In contrast, when a mutation is present it results in a mismatch between the PNA and the DNA, resulting in an unstable PNA/DNA complex, allowing the primer to bind to the DNA making elongation possible (figure 3.8 B) Thus, the binding of PNA to wild-type templates favour amplification of mutated templates. [64, 107]

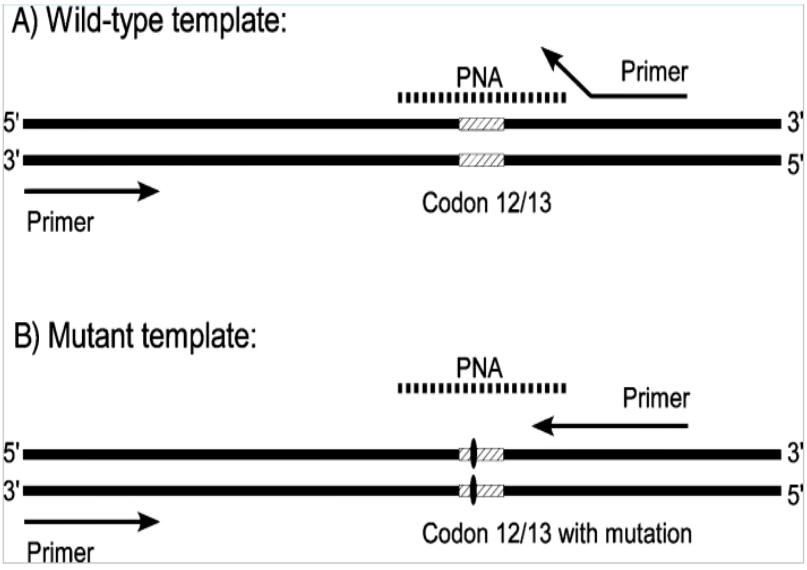

Figure 3.8: Schematic presentation of the PNA clamp assay. The primer and the PNA will bind competitively to part of the same DNA sequence. **A)** With wild-type *KRAS* as template, the PNA binds to the DNA and blocks primer annealing and elongation. **B)** When a mutation is present, there is a mismatch between the PNA and DNA leading to a weaker binding, allowing the primer to bind DNA and elongation can take place. The figure is obtained, with permission, from Gilje *et al.* 2008. [64]

Procedure

- Thaw all samples on ice and reagents at room temperature.
- Prepare PNA mastermixes according to the volumes described in the table 3.3 below.

Table 3.3: PNA mastermixes for Peptide Nucleic Acid Clamp PCR assay.

Reagents	Final [conc.]	Vol. (µl)
dH_2O		7.987
5x Phusion HF Buffer	1x	5.000
25 mM dNTP	0.2 mM	0.200
KRAS-PNA-FB primer	0.15 µmol/L	0.375
KRAS-PNA-RB primer	0.15 µmol/L	0.375
1:200 SYBR® Green I *		0.750
2 U/µl Phusion Polymerase	1x	0.250
Volume		15.0
100 µM PNA	0.25 µM	0.063

Vol. = Volumes per reaction
[conc.] = concentration
*1:200 SYBR® Green I in dimethyl sulfoxide

- 15 µl of PNA mastermixes were distributed to each well on the PCR plate before 10 µl (200 ng) DNA was added to each well.
- All samples were analysed in triplicates, both with and without PNA.
- No template control samples, containing 10 µl dH_2O instead of DNA, were included in every run to assure no existence of non-specific PCR products.
- DNA from the pancreatic cancer cell line AsPC-1 was used as a positive control and included in every run. In addition, a 1:1000 dilution of DNA from the colorectal carcinoma cell line LS174T (heterozygous GGT>GAT codon 12 *KRAS* mutation [c.35G>A]) in wild-type DNA was analysed in every experiment to monitor the sensitivity of the assay. [108] Wild-type DNA was analysed as a negative control.
- The PCR plate was placed in the Mx3000P real-time PCR instrument (Stratagene®) and run at 98°C for 30 seconds (initial denaturation and activation of the enzyme) followed by 45 cycles of 20 seconds at 98°C (denaturation), 10 seconds at 76°C (PNA annealing), 20 seconds at 60°C (primer annealing) and 72 seconds at 72°C (elongation). Fluorescence measurements for SYBR® Green I were completed at the end of the elongation step.

- The purity of the PCR products was verified by dissociation curve analysis at 95°C for 1 minute (denaturation) followed by 60°C for 30 seconds (renaturation) and 95°C for 30 seconds (denaturation).

For each sample, the relative ΔCq parameter was calculated as shown in below.

Equation 4:

$$\Delta Cq = Cq_{+PNA} - Cq_{-PNA}$$

Cq_{+PNA} and Cq_{-PNA} is the mean Cq-values based on the three replicates with PNA and without PNA, respectively. To obtain a more easily interpreted parameter, ΔΔCq was calculated.

Equation 5:

$$\Delta\Delta Cq = \Delta Cq_{wt,\ min} - \Delta Cq_{sample}$$

Where $\Delta Cq_{wt,\ min}$ denoted the lowest ΔCq measured for the control population. A sample was defined as positive for *KRAS* mutations when ΔΔCq > 0, *i.e.* that the ΔCq of the sample was lower than the $\Delta Cq_{wt,\ min}$. [108] For samples without an amplification curve, the Cq-value was set to 45, to not exclude these samples from further analyses.

3.9 Culturing of pancreatic cancer cells

3.9.1 Culturing of AsPC-1 cells

The pancreatic cancer cell line AsPC-1 was cultured in flasks containing RPMI 1640 (Sigma) supplemented with 2mM Glutamine (Sigma), 1mM Sodium Pyruvate (NaP, Sigma), 10% Foetal Bovine Serum (FBS, Sigma) and 5 mL Penicillin Streptomycin (Sigma). The cells were harvested by trypsination.

All practical work with cultured cells was performed with good sterile technique. UV light was used for disinfection of the sterile bench before and after use.

- Medium was preheated to 37°C before 10 mL was added to a 25 cm^2 flask.
- One ampoule with cells (2-4 x10^6 cells), stored in liquid nitrogen, was quickly thawed in a water bath at 37°C before the cells were transferred to the medium.
- The cells were grown at 37°C with 5% CO_2 for 2-3 days.

3.9.2 Subculturing of cells

- All reagents (phosphate buffered saline, trypsin and growth medium) were preheated to 37°C before use.
- The cells were examined in the microscope and split when they were 70-80% confluent.
- The medium was then removed and the cells were washed by adding 5 mL (to a 25 cm^2 flask) or 10 mL of 1xPBS (to a 75 cm^2 flask).
- After the washing, 1 mL (25 cm^2 flask) or 2 mL trypsin (to a 75 cm^2 flask) was added and the flask was incubated at 37°C with 5% CO_2 for approximately 5 minutes.
- 4 mL (25 cm^2 flask) or 3 mL (to a 75 cm^2 flask) of preheated medium was then added for deactivation of the trypsin in a total volume of 5 mL.
- After trypsination the cells were counted with Trypan Blue as described in section 3.1.3 and 2-4x10^4 cells were transferred to a new 75 cm^2 flask containing 25 mL of preheated medium.
- The cells were grown at 37°C with 5% CO_2.

4 Results

4.1 An overview of the methodological approach

In this study we wanted to investigate whether we could detect CTCs and DTCs in PB and BM samples from locally advanced and/or metastatic pancreatic cancer patients. Before the recruitment of the patient samples could start, parallel experiments with two red cell lysis buffers versus Lymphoprep™ for the enrichment of CTCs/DTCs, followed by DNA and RNA isolation with different kits were compared. A sensitivity analysis was performed to determine the lowest number of CTCs detectable by each mRNA marker with RT-qPCR. MCs, which include the tumour cells, were enriched from the PB and BM samples by density centrifugation using Lymphoprep™ before cell lysis and RNA and DNA isolation using AllPrep® DNA/RNA/Protein Mini Kit. Afterwards, the RNA samples were treated with RQ1 DNase before cDNA was synthesized using M-MLV reverse transcriptase. Quantification of the four epithelial-specific mRNAs CK8, CK19, EpCAM and CEACAM5 were then performed with the LightCycler® 480 real-time instrument using the qPCR Core Kit for SYBR® Green I. The mRNA concentrations were normalized against the BCR reference mRNA and expressed relative to the calibrator sample (AsPC-1 cell line) by the $2^{-\Delta\Delta Cq}$ method. For detection of *KRAS* mutations in codon 12 and 13 the DNA samples were analysed by the Peptide Nucleic Acid Clamp Assay. In PNA clamp PCR, wild-type specific PNA oligomers are used to suppress amplification of wild-type alleles during PCR. Any mutant allele will show unhindered amplification. An overview of the methodological approach is presented in figure 4.1.

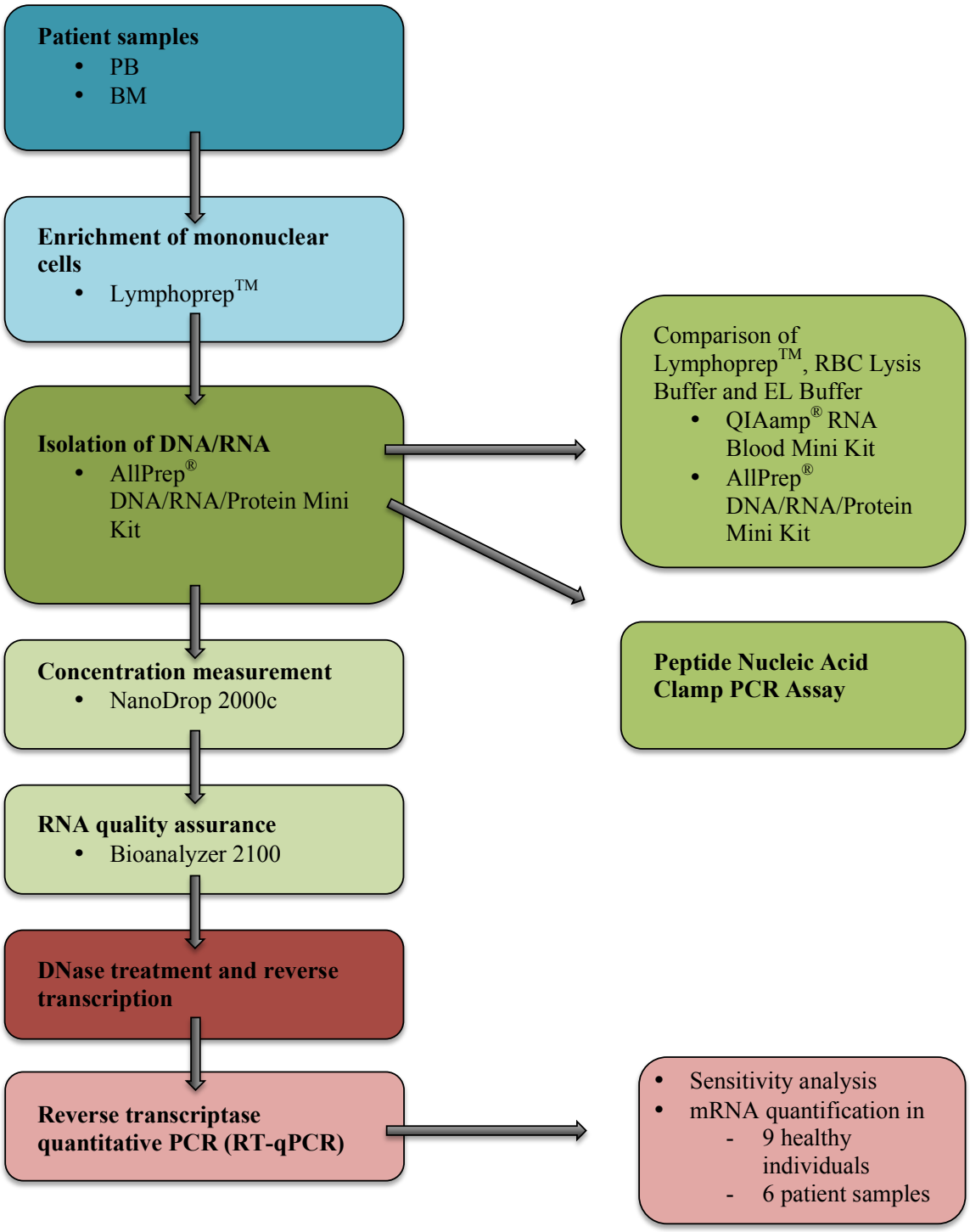

Figure 4.1: An overview of the different methodological approaches used in this study.

4.2 Optimization of methods for enrichment of CTCs and DTCs

Three different buffers (Lymphoprep™, RBC lysis buffer and EL buffer) were compared for enrichment of CTCs/DTCs followed by isolation of DNA and RNA using different kits (see section 3.2.1 and 3.2.2). Figure 4.2 shows an overview of the different methods used in the experiments.

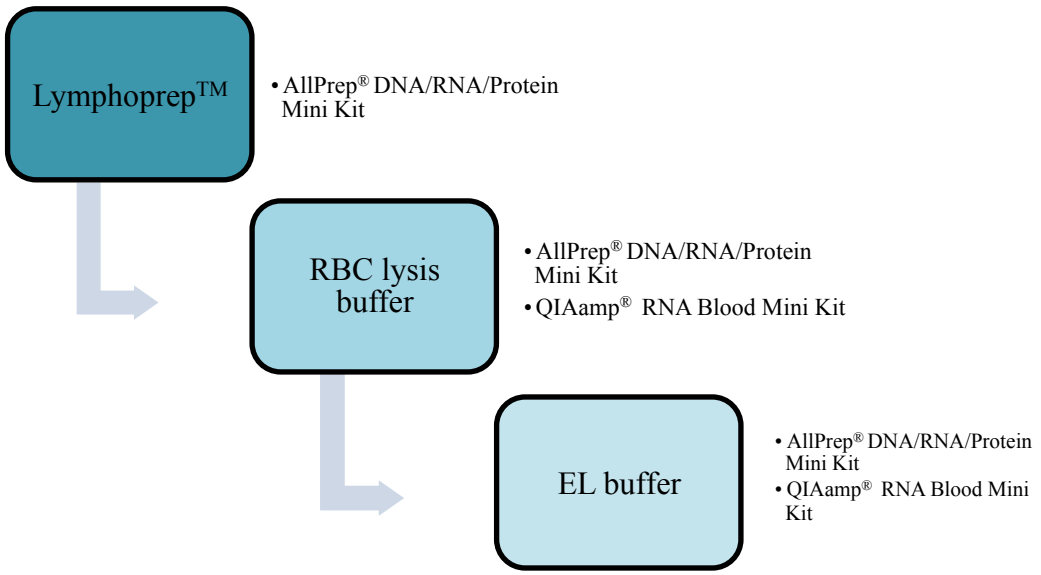

Figure 4.2: An overview of three different methods used for enrichment of CTCs and DTCs. Different DNA/RNA kits were used to verify the compatibility between the different enrichment methods and the DNA/RNA purification.

All experiments were performed by spiking 1000 pancreatic AsPC-1 cancer cells into 9 mL of PB in EDTA tubes (section 3.9.2), followed by tumour cell enrichment and DNA/RNA isolation. Before resuspension of the MCs in RLT buffer, the number of cells was counted (see section 3.1.3) to avoid overloading of the DNA/RNA column (the columns have a restricted capacity of $1x10^7$ cells). The low RNA concentrations (see table 4.1), measured by the NanoDrop instrument (section 3.3), revealed that neither RBC lysis buffer (section 3.1.1) nor EL buffer (section 3.2.2) is compatible with nucleic acid isolation using the AllPrep® DNA/RNA/Protein Mini Kit. In contrast, enrichment of MCs by Lymphoprep™ (section 3.1.2) followed by nucleic acid isolation using the AllPrep® DNA/RNA/Protein Mini Kit (section 3.2.1) did result in a sufficient amount of high quality RNA (table 4.1). This was also the case when QIAamp® RNA Blood Mini Kit (section 3.2.2)was used in combination with the EL buffer, a commercially available erythrocyte lysis buffer, for enrichment of MCs.

However, due to the capability of isolating DNA, RNA and protein from the same patient sample, the Lymphoprep™/AllPrep® strategy was chosen for further analyses in this study. Table 4.1 shows some relevant examples from the different experiments of RNA quantity and quality measured as the A_{260}/A_{280} ratio when different enrichment procedures and different kits for RNA purification are being used.

Table 4.1: Some examples of the RNA concentrations obtained in the verification of the most appropriate enrichment and isolation procedure to be used in this study. The ratio of absorbance at 260 and 280 nm is used to assess the purity of RNA and a ratio of ~2.0 is generally accepted as "pure" for RNA.

Enrichment method	Sample number	AllPrep® DNA/RNA/Protein Mini Kit		QIAamp® RNA Blood Mini Kit	
		RNA (ng/µl)	A_{260}/A_{280}	RNA (ng/µl)	A_{260}/A_{280}
Lymphoprep™	1	82.9	2.04	ND	ND
	2	90.0	2.04	ND	ND
	3	144.1	2.05	ND	ND
	4	137.0	2.07	ND	ND
RBC Lysis Buffer	5	1.6	1.44	88.4	1.92
	6	1.3	1.80	90.5	1.95
	7	1.6	1.67	33.1	1.88
	8	1.7	1.95	39.5	1.92
EL Buffer	9	3.7	2.05	84.4	1.97
	10	4.4	1.80	87.8	1.96
	11	ND	ND	70.8	1.98
	12	ND	ND	92.7	1.99

ND = not determined.

4.3 Determination of the sensitivity for CK8, CK19, EpCAM and CEACAM5 mRNA assays

To define the sensitivity of the four RT-qPCR assays, the detection limit of each assay was determined by mixing $2x10^6$ MCs from a healthy individual with 0, 2, 20, 200, 20 000 and 200 000 pancreatic AsPC-1 tumour cells, respectively. Two parallel dilution series were made for each marker. After DNA/RNA isolation with the AllPrep® DNA/RNA/Protein Mini Kit (section 3.2.1) and reverse transcription to cDNA (section 3.5), the four mRNA markers CK8, CK19, EpCAM and CEACAM5 were quantified in duplicates for each sample using the LightCycler® 480 real-time instrument (section 3.6).

For the mRNA markers CK8, EpCAM and CEACAM5, the lowest number of AsPC-1 cells detectable (higher marker mRNA level than in normal blood) was ≤ 200 per $2x10^6$ MCs (figure 4.3 A, C and D). For CK19 the sensitivity was ≤ 2 AsPC-1 cells per $2x10^6$ MCs (figure 4.3 B).

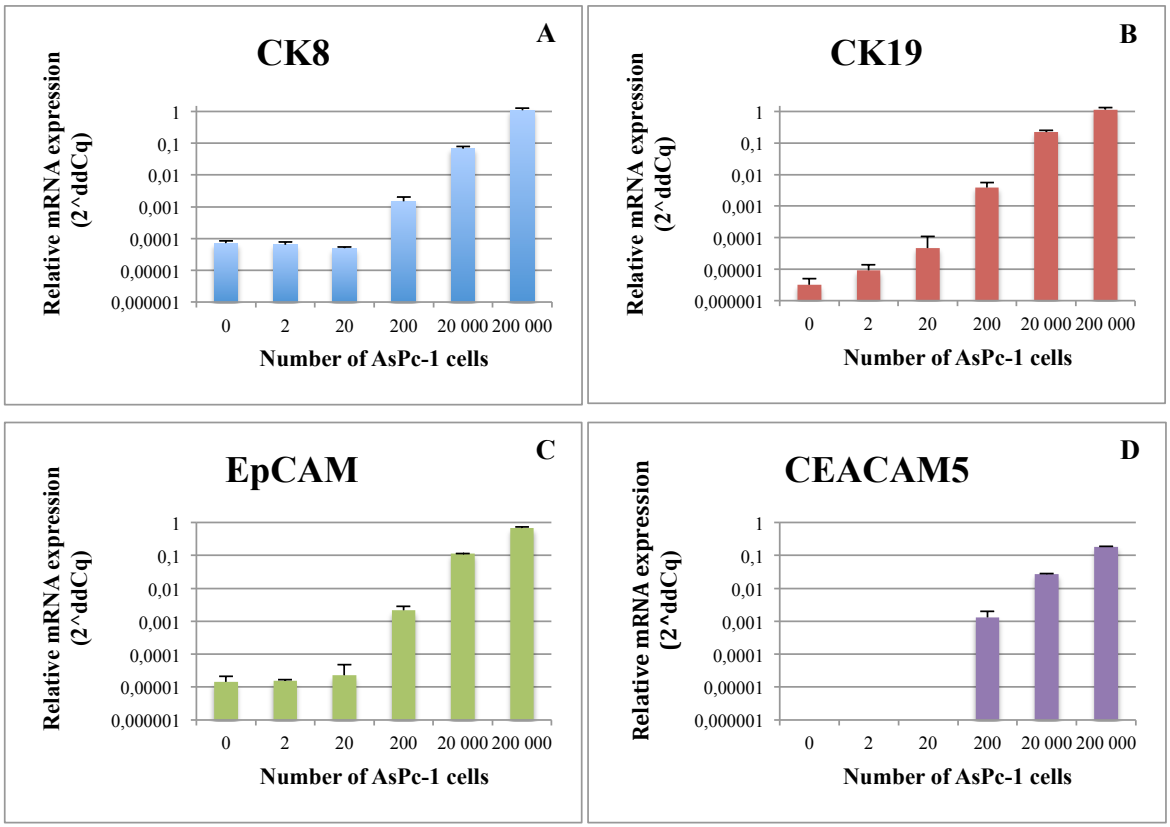

Figure 4.3: The assay sensitivity for CK8, CK19, EpCAM and CEACAM5 was determined by quantification of the four mRNAs in $2x10^6$ mononuclear cells (MCs) added 0, 2, 20, 200, 20 000 and 200 000 pancreatic AsPC-1 tumour cells. Samples added zero AsPC-1 cells show the levels of the epithelial mRNA markers in normal blood. The error bars are based on the standard deviation of two replicates. The figure shows a detection limit of **A)** ≤ 200 AsPC-1 cells for the CK8 assay, **B)** ≤ 2 AsPC-1 cells for the CK19 assay, **C)** ≤ 200 AsPC-1 cells for the EpCAM assay and **D)** a sensitivity of ≤ 200 AsPC-1 cells for the CEACAM5 assay.

4.4 Relative expression of CK8, CK19, EpCAM and CEACAM5 mRNA in PB and BM samples from healthy individuals

The levels of CK8, CK19, EpCAM and CEACAM5 mRNA in normal PB and BM were established from analyses of nine healthy individuals. For all the markers, the highest relative mRNA level in the control population was used as a cut-off value for normal mRNA

expression in further analyses. The cut-off values for CK8, CK19, EpCAM and CEACAM5 in PB were $3.34x10^{-5}$, $3.08x10^{-6}$, $2.30x10^{-5}$ and $2.45x10^{-6}$, respectively. For the BM samples the cut-off values were $3.92x10^{-5}$, $1.90x10^{-5}$, $1.30x10^{-2}$ and $4.09x10^{-6}$ for CK8, CK19, EpCAM and CEACAM5.

4.5 Evaluation of the mRNA levels in tumour samples from pancreatic cancer patients

To confirm that the mRNA markers chosen for this study are highly expressed in pancreatic tumour samples, and thus can be used as surrogate markers for the presence of tumour cells in PB and BM, we analysed the CK8, CK19, EpCAM and CEACAM5 mRNA levels in one liver metastasis from a pancreatic cancer patient and five tumour samples from adenocarcinoma of the pancreas by RT-qPCR. All tumour samples showed high levels of the four mRNA markers compared to the levels seen in PB and BM of healthy individuals (see figure 4.4). For CK8 mRNA, 6/6 tumour samples showed elevated mRNA levels compared to the normal expression in PB and BM samples in the control population. This was also the case for CK19 and EpCAM mRNA levels. Four of six tumour samples had a highly increased CEACAM5 mRNA level compared to PB and BM.

For comparison of the mRNA levels in tumours and the levels in normal PB and BM samples we calculated the specificity index for the different mRNA markers (table 4.2) with the equation shown below (*equation 6*).

Equation 6:

$$Specificity\ index = \frac{median\ value\ in\ pancreatic\ cancer\ tumours}{highest\ value\ in\ PB/BM} \quad 109$$

Table 4.2: Specificity indexes for the four mRNA markers in PB and BM.

	CK8	CK19	EpCAM	CEACAM5
PB	$7.05x10^{3}$	$1.41x10^{5}$	$1.25x10^{4}$	$3.10x10^{4}$
BM	$6.01x10^{3}$	$2.29x10^{4}$	$2.21x10^{1}$	$1.86x10^{4}$

The specificity indexes show that the CK19 assay had the best specificity in both PB and BM compared to the other markers. A specificity of $1.41x10^5$ means that, in average, one tumour cell can be detected among $1.41x10^5$ normal cells in PB and similarly detection of one tumour cell among $2.29x10^4$ normal cells in BM. The median tumour level of CK19, EpCAM and CEACAM5 in PB were more than 10^4-fold higher than the highest level in normal blood and indicates that the markers are suitable for CTC detection. In BM, the 10^4-fold higher level compared to the highest level in BM from the control population indicates that CK19 and CEACAM5 have the highest specificity in relation to DTC detection. [110]

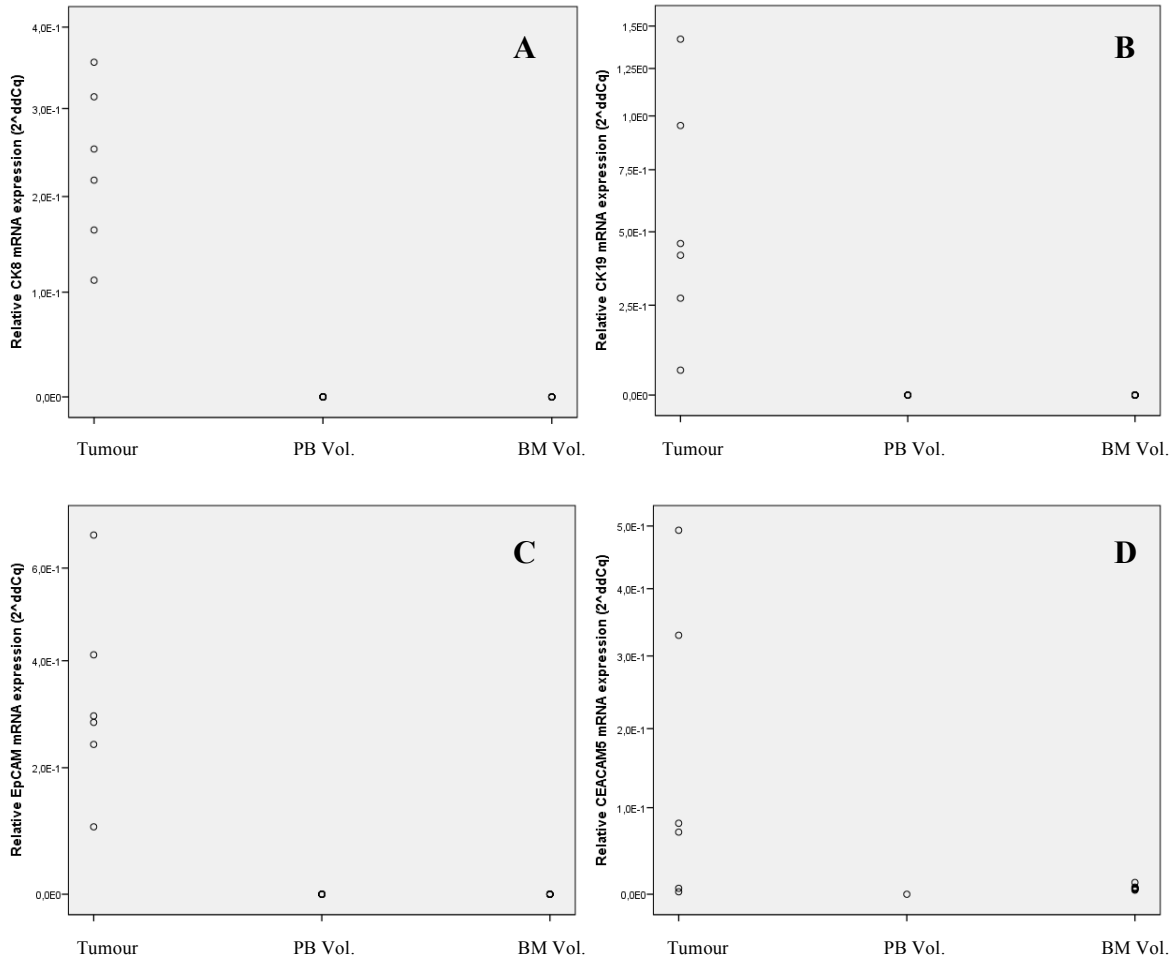

Figure 4.4: The relative mRNA expression in A) CK8, B) CK19, C) EpCAM and D) CEACAM5 in one liver metastasis from a pancreatic cancer patient and five tumour samples from adenocarcinoma of the pancreas compared to the mRNA levels in PB and BM samples from nine healthy volunteers.

4.6 RNA quality

We obtained PB samples before initiation of gemcitabine treatment from all the included patients (n = 6). However, only five patients accepted BM sampling before treatment start. Patient characteristics are listed in table 2.1 (section 2.1). A random selection of the patient RNA samples were analysed on the Bioanalyzer 2100 instrument (section 3.4) for quality assurance according to the RNA integrity, purity and concentration. The only pancreatic tumour tissue sample available was selected to see whether the isolation of DNA and RNA from tumour tissue gave high quality RNA. The RNA integrity numbers (scale 1-10, where 10 is the best) are presented in table 4.3.

Table 4.3: RNA integrity numbers (RIN) of randomly selected samples for RNA quality assurance with Bioanalyzer 2100.

Patient ID	RIN
PC1B1	9.5
PC1BM1	9.5
PC1BM2	9.6
PC2B1	9.5
PC3B1	9.5
PC3BM1	9.4
PC4B1	9.3
PC5B1	9.5
PC5BM1	9.1
PC6 tumour	8.3

PC = pancreatic cancer patient, B1 = PB sample before initiation of treatment, BM1 = bone marrow sample before initiation of treatment, BM2 = bone marrow sample taken four weeks after treatment start, PC tumour = pancreatic cancer patient tumour tissue from liver metastasis.

4.7 Detection of CTCs and DTCs before initiation of gemcitabine treatment

Five of the six patients showed elevated levels of at least one mRNA marker in PB and five of five patients in BM before initiation of the gemcitabine treatment, indicating the presence of CTCs and DTCs (see table 4.4 and 4.5). Compared with the highest normal CK8 level, 4/6 and 4/5 patients showed elevated CK8 mRNA level in PB and BM, respectively. Two of six patients had an increased CK19 mRNA level in PB, while none of the patients were detected with positive CK19 mRNA levels in BM. Increased EpCAM mRNA levels were demonstrated in 2/6 patients in PB samples and 1/5 patient in BM samples, respectively.

None of the patients were positive for CEACAM5 mRNA level in PB samples, however 1/5 patients was detected with an increased CEACAM5 mRNA level in BM samples. Of the four mRNA markers investigated, CK8 mRNA marker levels were demonstrated in 4/6 PB1 samples and 4/5 BM1 samples. We evaluated the combination of CK8, CK19, EpCAM and CEACAM5 mRNA detection.

4.8 Detection of CTCs and DTCs after treatment start

Three pancreatic cancer patients had repeated PB samples after start of gemcitabine treatment. We obtained three PB2 samples (4 weeks after treatment start) and two PB3 samples (8 weeks after treatment start). BM2 samples (four weeks after treatment start) were only obtained from one patient.

All three patients were defined as positive for at least one mRNA marker in PB2 (4 weeks after treatment start). One of the patients was also detected as CTC positive for one of the mRNA markers in PB3 (8 weeks after treatment start). The patients had different mRNAs expressed after initiation of the gemcitabine treatment (see table 4.4). Elevated CK8 mRNA levels were observed in 2/3 and 1/1 patients in PB and BM samples, respectively. Two of three patients had an increased CK19 mRNA level in PB, while 0/1 patient was detected with CK19 positive DTCs in BM. One of three patients showed elevated levels of EpCAM mRNA in PB samples, while 0/1 patient was positive for EpCAM mRNA level in the BM sample. Two of the three patients had an increased CEACAM5 mRNA level in PB and 1/1 patient in BM.

Of the included patients, one patient had PB2 and BM2 samples taken 4 weeks after treatment start. This patient was detected with both CTCs and DTCs (see table 4.5).

Table 4.4: Results from indirect detection of CTCs by quantitative real-time PCR in peripheral blood samples obtained before initiation of treatment (PB1), 4 weeks (PB2) and 8 weeks (PB3) of gemcitabine treatment from six patients with locally advanced and/or metastatic pancreatic cancer. mRNA positivity is indicated by X.

		PC1			PC2			PC3	PC4	PC5		PC6
	Marker	PB1	PB2	PB3	PB1	PB2	PB3	PB1	PB1	PB1	PB2	PB1
	CK8	x	x		x	x	x		x	x		
mRNA	CK19	x	x			x				x		
	EpCAM	x	x					x				
	CEACAM5					x					x	

PC = pancreatic cancer patient, CK8 = cytokeratin 8, CK19 = cytokeratin 19, EpCAM = epithelial cell adhesion molecule, CEACAM5 = carcinoembryonic antigen-related cell adhesion molecule 5.

Table 4.5: Results from indirect detection of DTCs by quantitative real-time PCR in bone marrow samples obtained before initiation of treatment (BM1) and after 4 weeks of gemcitabine treatment (BM2) from five patients with locally and/or metastatic pancreatic cancer. mRNA positivity is indicated by X.

	Marker	**PC1**		**PC2**	**PC3**	**PC4**	**PC5**
		BM1	BM2	BM1	BM1	BM1	BM1
	CK8	x	x	x		x	x
mRNA	CK19						
	EpCAM				x		
	CEACAM5		x			x	

PC = pancreatic cancer patient, CK8 = cytokeratin 8, CK19 = cytokeratin 19, EpCAM = epithelial cell adhesion molecule, CEACAM5 = carcinoembryonic antigen-related cell adhesion molecule 5.

4.9 Comparison of the mRNA marker levels in PB and BM samples drawn before and during treatment with gemcitabine

The three patients with repeated PB samples were both CTC and DTC positive for at least one mRNA marker in the PB and BM samples collected before chemotherapy (PB1 and BM1), respectively (see table 4.4 and 4.5). These patients were also positive for at least one mRNA marker in the PB and BM samples taken 4 weeks after treatment start. For PC1, the mRNA level of CK8, CK19 and EpCAM had increased 4 weeks after initiation of treatment (PB2). However, 8 weeks after treatment start (PB3), the mRNA levels of CK8, CK19 and EpCAM had decreased significantly. For PC2, both CK19 and EpCAM showed elevated mRNA levels after initiation of treatment (PB2). Furthermore, a highly increased level of CEACAM5 mRNA was detected in PB2. A significantly increased EpCAM mRNA level was observed in PB3, 8 weeks after treatment start. For PC6, a decrease in both CK8 and CK19 mRNA levels were observed in PB2. In contrast, the CEACAM5 mRNA level was strongly

increased. See figure 4.5 for the monitoring of the gemcitabine treatment by indirect CTC detection in repeated PB samples.

Only one of the patients had repeated BM sampling where BM2 was taken 4 weeks after treatment start. For this patient, PC1, CK8 and CEACAM5 mRNA levels were increased in BM2.

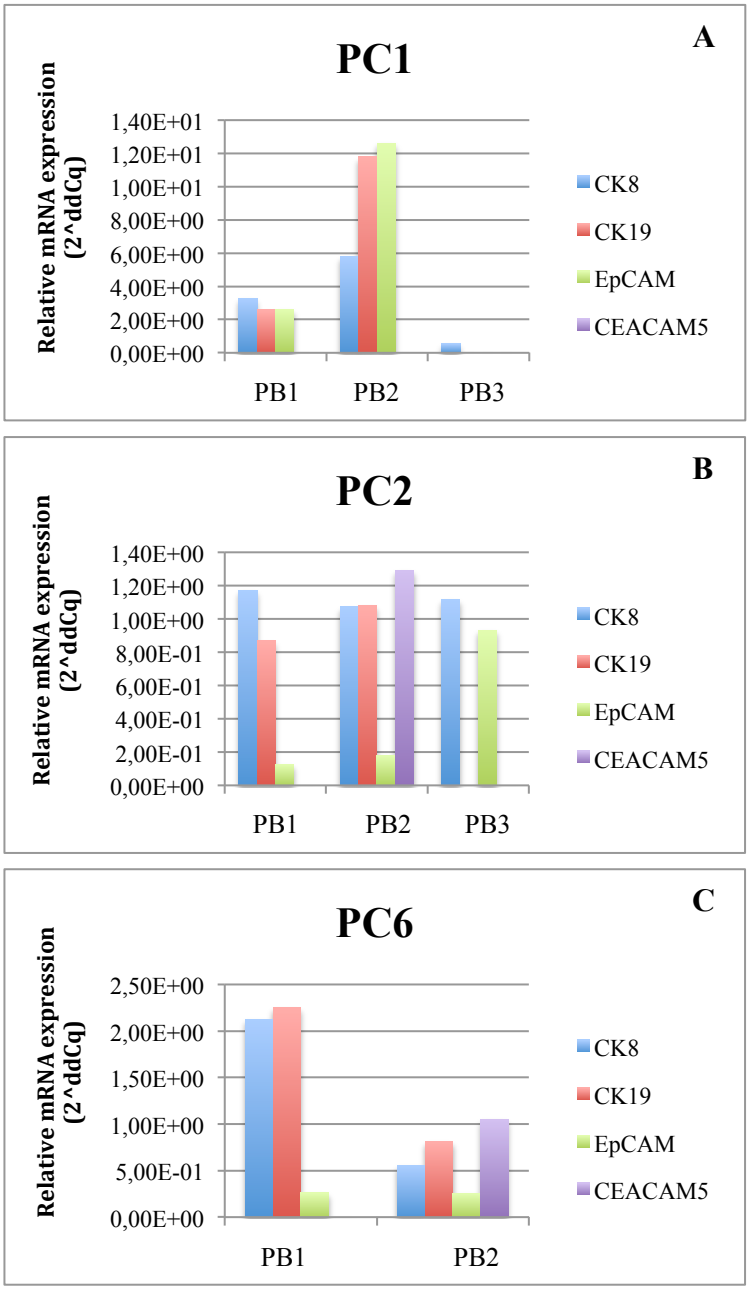

Figure 4.5: Monitoring of the gemcitabine treatment by indirect detection of CTCs in blood samples before initiation of treatment (PB1), 4 weeks (PB2) and 8 weeks (PB3) after treatment start for three pancreatic cancer patients A) PC1, B) PC2 and C) PC6. The data is normalized against the cut-off value for each of the mRNA markers. The figure shows the changes in the mRNA levels of the four biomarkers both before and during treatment.

4.10 Clinical outcome and mRNA-positive CTCs and DTCs

All the patients included in this study had metastatic disease established by CT scanning and by the end of this study five of six patients had died. Although the number of patients was too small for such analyses, we performed preliminary Kaplan-Meier analyses to obtain potential indications whether an increased CTC level in PB or DTC level in BM could be associated with earlier death. No significant association between the CTC/DTC level and overall survival was, however, not detected in this patient group so far.

4.11 Peptide Nucleic Acid Clamp PCR assay

4.11.1 Determination of a cut-off value for the PNA clamp PCR assay in PB and BM samples from healthy individuals

To determine the cut-off value for *KRAS* positivity, DNA samples from nine healthy individuals were analysed with the PNA clamp PCR assay. Afterwards the ΔCq-values and ΔΔCq-values (*equation 4* and *equation 5* in section 3.8) for both the healthy volunteers and the patient samples were calculated. The $\Delta Cq_{wt,\,min}$ denoted the lowest ΔCq measured for the control population, which was defined as 13.31 in this study. A sample was defined as positive for *KRAS* mutations when ΔΔCq > 0, which means that the ΔCq of the sample was lower than the $\Delta Cq_{wt,\,min}$.

4.11.2 Detection of *KRAS* mutations in PB and BM samples from pancreatic cancer patients with the PNA clamp assay

Due to a high frequency of *KRAS* mutations in pancreatic primary tumours we wanted to investigate whether the *KRAS* mutation status could be used as a surrogate marker for CTC and DTC detection. Of the six included patients, only one patient was demonstrated to be positive for a *KRAS* mutation in PB and BM samples by the PNA clamp assay. This patient had *KRAS* mutations detected in 3/3 PB and 1/1 BM samples. In addition, one tumour tissue sample (liver metastasis) from a pancreatic cancer patient was analysed and demonstrated to be positive for a *KRAS* mutation. Figure 4.6 summarizes the results from the PNA clamp

assay for both the PB and BM samples. The cut-off value for the PNA clamp assay was used to define *KRAS* positive patients. Patient samples above the horizontal line, *i.e.* the cut-off value, were characterized as samples positive for *KRAS* mutations. *KRAS* mutations were detected in all the PB and BM samples from PC2 as well as in the tumour tissue sample from PC6.

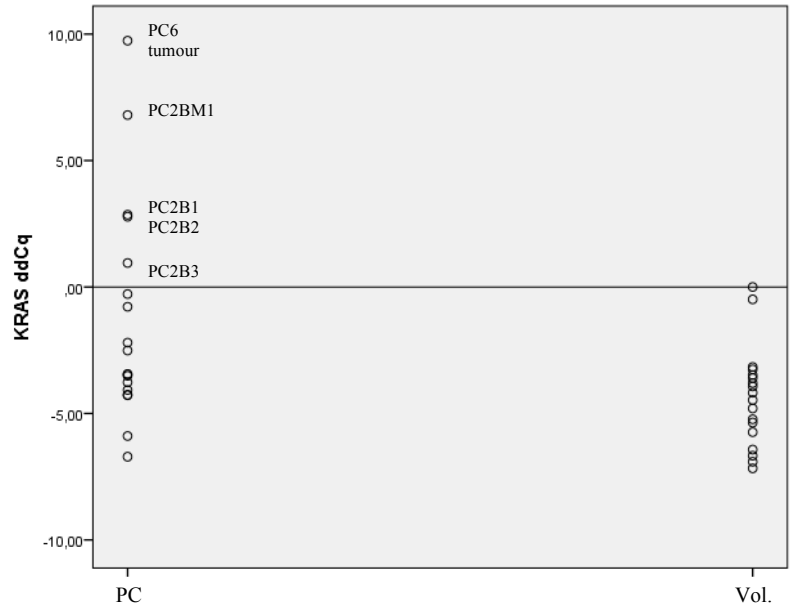

Figure 4.6: A dot plot for the *KRAS* ΔΔCq for pancreatic cancer patient samples (n = 18) and PB and BM samples from healthy volunteers (n = 18). PC = PB samples, BM samples and one tumour tissue sample from pancreatic cancer patients. Vol. = PB and BM samples from healthy volunteers. The horizontal line denotes the cut-off value used to decide whether the samples were positive or negative for *KRAS* mutations, *i.e.* the samples above the horizontal line are defined as positive, while the samples below are defined as negative.

5 Discussion

5.1 Enrichment of CTCs and DTCs

Due to the low number of CTCs in PB and BM (approximately 1 per $1x10^6$ leukocytes), optimal enrichment conditions are required to achieve efficient and reliable CTC/DTC recovery. Before the inclusion of patient samples could start we wanted to investigate whether RBC lysis buffer could replace Lymphoprep™ as a cheaper and more efficient method for enrichment of CTCs/DTCs. One hypothesis was also that the loss of CTCs would be smaller with RBC lysis buffer compared to density gradient centrifugations, with regard to the presence of tumour cell clusters.

Parallel experiments with a manually prepared RBC lysis buffer and the commercially available EL buffer (included in the QIAamp® RNA Blood Mini Kit) followed by nucleic acid isolation with AllPrep® DNA/RNA/Protein Mini Kit, gave lower RNA concentrations and lower A_{260}/A_{280} ratios compared to the Lymphoprep/AllPrep strategy (see table 4.1). This was despite the fact that the number of enriched cells was high with all three enrichment strategies (e.g. RBC lysis buffer: $2.2x10^7$ cells, EL buffer: $4.2x10^6$ cells, Lymphoprep: $6.5x10^6$ cells). The low A_{260}/A_{280} ratios indicated, however, the presence of protein or other contaminants that absorb strongly at or near 280 nm in the eluted RNA samples. [93] In contrast, enrichment with RBC lysis buffer and EL buffer followed by RNA isolation with QIAamp® RNA Blood Mini Kit gave high RNA concentrations and A_{260}/A_{280} ratios close to 2.0. Accordingly we concluded that neither RBC lysis buffer nor the EL buffer was compatible with the AllPrep® DNA/RNA/Protein Mini Kit. Since we preferred to purify genomic DNA, total RNA and total protein from the same patient sample, we chose to enrich CTCs/DTCs from patient samples by density gradient centrifugation using Lymphoprep™ followed by the AllPrep® DNA/RNA/Protein Mini Kit in the further analyses.

CTC clusters have been found in patients with advanced cancer [37, 58], an observation that is in contrast to the hypothesis that EMT results in migratory single tumour cells. Yu and colleagues found CTC clusters ranging from 4-100 tumour cells in PB from breast cancer patients with disease progression. [37] The tumour cells within these clusters expressed high

levels of mesenchymal markers and by staining these clusters, an abundance of CD61 positive platelets were observed. [37] This supported the hypothesis that platelets bound to tumour cells release transforming growth factor-β (TGF-β), thereby inducing mesenchymal transformation of epithelial cells in the PB. [111] It is thinkable, that these clusters, with enrichment methods as e.g. Lymphoprep™, can be lost during the procedure. A previous master student performed an experiment for evaluation of the loss of cell clusters with the Lymphoprep strategy. [112] Colorectal carcinoma cell line LS174T cells, which are rather sticky, were added to PB from a healthy volunteer to investigate whether the isosmotic medium used in Lymphoprep was capable of retaining the cell clusters. The observed cell clusters ranged from 2 to > 20 LS174T cells. Even though microclusters of different sizes were observed in this experiment, cell counting confirmed that the total loss of LS174T cells was small. [112] However, we cannot exclude the possibility that large CTC clusters in the PB from pancreatic cancer patients will be lost during the Lymphoprep procedure.

5.2 Determination of the sensitivity for CK8, CK19, EpCAM and CEACAM5 mRNA assays

The sensitivity analyses were performed to determine the detection limit of each mRNA marker with regard to the lowest amount of tumour cells detectable by RT-qPCR. Due to the expression of some mRNAs in normal blood cells a sample without any addition of AsPC-1 cells, sample zero, was included for each of the biomarkers. Thus, mRNA concentrations above the background level would reflect the presence of AsPC-1 tumour cells, which were mixed with $2x10^6$ MCs from a healthy volunteer. Due to the small number of parallel samples in this study it was not possible to perform any statistical analyses. The assessment of the sensitivity analysis was therefore only based on visual evaluation of the bar plots for each of the biomarkers. The detection limit for both the CK8 and EpCAM assay was between 20-200 tumour cells due to high background expression of these markers in normal blood. For CEACAM5, the lowest number of AsPC-1 cells detectable was ≤ 200 per $2x10^6$ MCs. In contrast to CK8 and EpCAM, there was no background expression of CEACAM5 in this experiment and a further optimization of this RT-qPCR assay could perhaps have increased the sensitivity. Mataki *et al.* [78] investigated the sensitivity of the CEA-specific RT-PCR assay and demonstrated that the assay could detect as low as 1 tumour cell per 10^7 MCs (10-fold dilutions of MKN-45 and MCF-7 gastric cancer and breast cancer cell lines in 10^7 MCs,

respectively). [78] The CK19 mRNA assay had the highest sensitivity among our assays of ≤ 2 AsPC-1 cells detectable per $2x10^6$ MC (figure 4.3). Hoffmann *et al.* [76] investigated the sensitivity of their CK19 assay and found that they could detect one tumour cell per 10^6 MCs (detected in experiments with CK19 expressing tumour cell lines CaPan2 and PANC1). The sensitivity for the CK19 assay in our study correlated with Hoffmann and colleagues. Thus, further optimization of the CK8, EpCAM and CEACAM5 assays in our study could preferably be investigated to increase the sensitivity of these assays for CTC and DTC detection.

5.3 Detection of CTCs and DTCs in pancreatic cancer patients before initiation of gemcitabine treatment

Technical difficulties in detecting CTCs and DTCs are both caused by the low numbers of these cells in PB and BM and the difficulty to distinguish them from normal epithelial cells and leukocytes. [113] In our study, analyses of nine healthy individuals revealed a relatively high level of EpCAM in the BM samples questioning the use of this marker for DTC detection. Furthermore, cellular heterogeneity resulting in expression of different mRNAs within a CTC/DTC population, make the detection even more challenging and the use of multimarker assays are preferable. [31, 52, 61] Only epithelial specific markers are used for detection of CTCs and DTCs in our study. The expression levels of epithelial antigens, (e.g. CKs and EpCAM) are, however, downregulated during EMT [113-115] and detection of CTCs/DTCs might preferably also include mesenchymal markers stimulated during EMT, as well as stem-cell markers, to increase the sensitivity for CTC/DTC detection. [28, 37, 114, 115] In several cancer forms, patients with a negative CTC status have been reported to develop metastasis and one possible explanation for this is that the cancer-initiating cells remain undetected by the existing detection methods. [115] The choice of broader range of mRNA markers for CTC/DTC detection is therefore necessary.

In this study, CTCs/DTCs were detected indirectly, using the epithelial-specific mRNAs CK8, CK19, EpCAM and CEACAM5 by RT-qPCR. Analysis of pancreatic cancer tumours confirmed that the mRNA markers are present at high levels in tumours compared to extremely low levels both in PB and in BM samples from healthy individuals, making them suitable as markers for CTC and DTC detection. Moreover, our preliminary results show that

we detect CTCs in PB samples obtained before gemcitabine treatment in 5/6 (83.3%) patients in our study. De Albuquerque *et al.* [73] demonstrated that 16/34 (47.1%) patients had CTCs in PB samples obtained before systemic treatment, with immunomagnetic enrichment followed by a multimarker RT-qPCR assay. In comparison to our results, de Albuquerque and colleagues also detected a high number of patients with a positive CTC status. They had higher cut-off values compared to our assays. [73] We have established cut-off values based on analyses of a low number of healthy volunteers and found lower cut-off values compared to de Albuquerque and colleagues. This may explain why we detected a higher number of CTC positive patients in our study. It is likely that some of our patients may be false-positive patients due to a too low cut-off value and high levels of the mRNA markers in normal blood. Sergeant *et al.* [62] used EpCAM as a surrogate marker for CTC detection by RT-qPCR and found that 10/40 (25%) patients were EpCAM positive before surgery. The use of only one biomarker for detection of CTCs may explain the low number of pancreatic cancer patients with a positive CTC status in this study. Furthermore, EMT and accordingly downregulation of EpCAM might also result in CTCs that remain undetected. [62] Khoja *et al.* [74] detected 93% CTC positive patients by ISET and 40% CTC positive patients by the CellSearch® system in PB samples obtained from patients with progressive metastatic or unresectable pancreatic cancer. The low number of CTCs detected with the CellSearch® system may be explained by EMT and a low number of cells expressing epithelial antigens. Furthermore, tumour cell clusters were detected with the ISET, but not with the CellSearch® system. [74] Kurihara *et al.* [75] detected a positive CTC status in 42% of patients with metastatic pancreatic cancer with the CellSearch® system, in correlation with Khoja and colleagues. [75] Soeth *et al.* [77] detected CK20 positive cells in preoperative PB samples by RT-PCR. CTCs were detected in 52/154 (33.8%) PB samples. [77] Z'graggen *et al.* [79] used an imunocytochemical assay based on epithelial-specific antigens for detection of CTCs in 105 patients. In PB samples, 24/73 (33%) patients with unresectable cancer were detected and defined as CTC positive. [79] Hoffmann *et al.* [76] investigated the CK19 mRNA level in resectable pancreatic cancer patients and CTCs were detected in 24/37 (64%) PB samples. [76] Although the results from our study reveal a lower number of samples with a positive CK19 mRNA level in PB (33.3%) compared to Hoffmann and colleagues, the CTC detection and the high specificity index would account for further investigation of the potential of CK19 mRNA detection in PB in pancreatic cancer patients. When comparing the numbers of CTC positive metastatic pancreatic cancer patients in the aforementioned studies, one would expect a range between 40-50% CTC positive patients as opposed to our study in correlation with de Albuquerque *et al.* [73], Khoja *et al.* [74] and Kurihara

et al. [75]. Sergeant *et al.* [62], Soeth *et al.* [77] and Z'graggen *et al.* [79] indicate a slightly lower percentage of CTC positive patients, however, most of these patients were diagnosed with resectable cancer, thus, one may expect a lower number of CTCs in PB in resectable patients compared to patients with metastatic pancreatic cancer.

Bone metastases in pancreatic cancers are rare, thus the relevance of DTC detection can be questioned. [80] However, BM is suggested to be a reservoir for DTCs and thus opens for the possibility of recirculation of tumour cells into other organs. [80] These results, obtained in mouse models, make it attractive to process further investigation of the role of DTCs in BM in pancreatic cancer. [4, 80] BM sampling is a much more invasive procedure compared to blood sampling, and PB samples are therefore much easier to obtain and cause less discomfort for the patients. The available studies on DTC detection in pancreatic cancer patients mostly include studies using an immunocytochemical approach for DTC detection and the analyses are often performed on BM samples taken prior to surgery.

In our study, we detected DTCs in BM in 5/5 (100%) patients. Effenberger *et al.* [80] detected DTCs in 24/175 (13.7%) in patients during surgery before tumour mobilization, which is a much lower number of DTC positive patients compared to our findings. Of the 24 patients with a positive DTC status, 13 (54.2%) experienced disease relapse. Moreover, they demonstrated that the DTC status was an independent prognostic factor for disease progression and overall survival. [80] Van Heek *et al.* [81] found DTCs in BM samples from 10/31 (32%) patients and a significant association with reduced overall survival. Roder *et al.* [82] detected CK positive DTCs in BM samples from 25/48 (52.1%) patients, while Vogel *et al.* [83] detected DTCs in BM samples from 27/71 (38%) patients and these data correlated with shorter survival. Soeth *et al.* [77] also detected CK20 positive cells in BM samples by RT-qPCR. They detected DTCs in 45/135 (33.3%) BM samples. Although there was a weak correlation between CTC/DTC detection and clinicopathological parameters, the detection rates of CTCs/DTCs increased with the tumour stage.[77] Hoffmann *et al.* [76] also analysed the CK19 mRNA levels in BM samples from resectable pancreatic cancer patients. However, none of the BM samples were detected with CK19 mRNA levels above the cut-off value. [76] In our study, the CK19 assay for both PB and BM was calculated with the highest specificity index compared to the median tumour level (table 4.2.) In correlation to Hoffmann and colleagues, none of our patients were detected with a CK19 mRNA level above the cut-off value. This may perhaps suggest that CK19 mRNA detection in BM samples from pancreatic

cancer patients may not be beneficial. Z'graggen *et al.* [79] detected DTCs in BM samples from 13/54 (24%) patients. They also found a concordance between PB and BM in 5/54 (9%) of the patients. They calculated the random chance of detecting tumour cells in both PB and BM to 7%, nearly identical to the 9% detected in the paired samples. [79] Our results revealed that all the patients (100%) were positive for both CTCs and DTCs, based on the criteria that at least one mRNA marker should have an increased mRNA level before it is defined as CTC or DTC positivity. However, we are aware of our shortcomings with a limiting patient and control population, thus, larger studies are therefore required to conclude the clinical relevance of DTC detection.

5.4 Monitoring of the CTCs and DTCs before and during treatment

Although the data are preliminary, we do detect persistent CTCs and DTCs in locally advanced and/or metastatic pancreatic cancer patients after initiation of gemcitabine chemotherapy. This is as expected because the gemcitabine treatment has been shown to be less effective.[15] However, the number of patients included in this evaluation is few as five of the included patients died during the recruitment period, and repeated blood samples was only obtained from three of the patients (median survival time for the pancreatic cancer patients was 37 days). Two of these patients, however, showed an increase in the mRNA marker levels in PB samples taken 4 weeks after treatment start followed by a decrease 8 weeks after treatment start. These results may be explained by an increased number of tumour cells shedding into the blood circulation from the primary tumour due the effect of the chemotherapy. As most of these tumour cells will be apoptotic and die, only the cancer-initiating cells will be able to survive and give rise to overt metastases [41, 46], this may be a possible explanation to the decrease in the marker level seen 8 weeks after treatment start.

Pancreatic CSCs represent phenotypically different cells and this is of great importance with regard to the treatment strategy. [42, 62] Intrapatient heterogeneity, *i.e.* genetic heterogeneity within an individual, has also been found, as different CSCs can be present at different times during disease development. [42] Yu *et al.* [37] compared the CTC features in pre- and posttreatment PB samples from ten breast cancer patients, five patients who responded to therapy and five that did not. In the patients with a treatment response, a decrease in the CTC numbers as well as a decrease in mesenchymal positive CTCs compared to epithelial positive

CTCs was detected in the posttreatment samples. Furthermore, the patients with progressive disease during treatment showed increased numbers of mesenchymal positive CTCs. [37]

Our preliminary results suggest that all the three pancreatic cancer patients investigated may have distinct CTC populations, explained by different mRNAs being expressed during treatment monitoring. Furthermore, when comparing PC1 and PC2 (figure 4.5) it might look like PC1 responded better to the gemcitabine treatment compared to PC2, but this is only a speculation based on the expectations that the number of CTCs will decrease if the chemotherapy is effective and that an increase in the number of CTCs reflects chemotherapy resistance. A larger number of patients would have to be included to conclude on the potential of using CTC and DTC detection in monitoring of the treatment response. Hoffmann *et al.* [76] detected CK19 mRNA levels in PB in 5/37 (13%) samples obtained the first postoperative day, and in 4/37 (10%) samples day ten after surgery. The CK19 mRNA levels in the PB samples obtained at 1 and 10 days after surgery tended to be lower than the levels in the preoperative samples. [76] Ren *et al.* [84] investigated PB samples from 41 pancreatic cancer patients obtained both before and one week after start of chemotherapy. CTCs were detected in 33/41 (80.5%) patients before therapy by immunocytochemistry. Only 12/41 (29.3%) patients were detected with CTCs after treatment start. A noteworthy decrease in the CTCs was demonstrated by apoptotic changes in the CTCs after treatment start. [84]

In our study, all the three patients who we obtained PB samples from after initiation of treatment were defined as CTC positive based on the expression of at least one mRNA marker. However, the number of patients is too small to conclude on the clinical relevance of our findings. Sergeant *et al.* [62], on the other hand, investigated the EpCAM mRNA level in 40 postoperative samples (day 1, day 7 and 6 weeks after surgery) and in addition, eight unresectable pancreatic cancer patients. An increase in the mRNA level was seen immediately after surgery and was detected in 27/40 (67.5%) patients. Six weeks after surgery, 8/34 (23.5%) patients were EpCAM positive. Two of the eight (25%) of unresectable patients were EpCAM positive. The postoperative samples did not reveal any significant associations between EpCAM positivity in PB and cancer-specific and disease-free survival. [62] Mataki *et al.* [78] also investigated the clinical significance of CTC detection in 53 pancreatic cancer patients by RT-PCR, as an indicator of relapse during follow-up, although after surgery. The CEA mRNA level was detected in 16 (75%) patients with relapse and was significantly higher than the CEA mRNA level in 37 (5.4%) patients without relapse. [78] Mataki and colleagues

therefore suggested the use of CEA mRNA level in PB by RT-PCR as a useful indicator of relapse. [78]

Only one patient had repeated BM sampling obtained 4 weeks after treatment start in our study. DTCs were detected in both of the BM samples and the mRNA marker expression seemed to differ between the samples. However, further analyses are needed to conclude on the value of monitoring CTCs and DTCs both before and during treatment in pancreatic cancer patients.

5.5 *KRAS* gene mutations as a surrogate marker for CTC and DTC detection

Around 85% of pancreatic cancers harbour point mutations in the *KRAS* gene, thus these mutations represent highly tumour-specific traits that possibly can be applied as surrogate CTC/DTC markers in pancreatic cancer patients. To our knowledge, no studies investigating *KRAS* mutations in CTCs/DTCs in pancreatic cancer patients have been published. However, a study performed on both non-metastatic and metastatic colorectal cancer investigated *KRAS* mutations in single CTCs by sequencing codons 12/13 from five patients. [116] A *KRAS* mutation was demonstrated in 5/15 (33%) CTCs from one patient. The *KRAS* mutation was also verified in DNA from the primary tumour. *KRAS* mutations in the primary tumours of the remaining four patients were detected, however, none *KRAS* positive CTCs were detected. [116] Another study performed on colorectal cancer patients detected *KRAS* mutations in tumours and in PB in 33 (43.4%) and 30 (39.5%) patients by gene expression array in 76 patients treated with chemotherapy, respectively. The results revealed that detection of *KRAS* mutations in CTCs had potential to detect metastatic colorectal cancer patients that would not benefit EGFR-targeted chemotherapy. [117] Both of these studies show that *KRAS* mutations can be detected in CTCs, which is in accordance with our results where 1/6 (16.67%) patients was detected with *KRAS* positive CTCs. This patient also had persistent *KRAS* positive CTCs detected after initiation of gemcitabine chemotherapy. This same patient was also detected with *KRAS* positive DTCs in the BM sample obtained before treatment start. For another patient, the PB sample analysed was not detected with *KRAS* mutations, however, *KRAS* mutations were detected in DNA from this patients' (PC6) liver metastasis. Gilje *et al.* [64] demonstrated the sensitivity of the PNA clamp PCR assay to be $1{:}10^4$. In comparison with Gilje *et al.* [64], the sensitivity of our assay was reduced and this may therefore explain the low

number of patients detected with *KRAS* mutations, although the high frequency of these mutations in pancreatic cancer patients. More samples are therefore needed to conclude the potential of using *KRAS* as a surrogate marker for disease progression and treatment monitoring in pancreatic cancer patients.

5.6 Conclusion and future perspectives

Our preliminary data show that we detect CTCs and DTCs in PB and BM samples obtained both before and during gemcitabine treatment of five metastatic pancreatic cancer patients. We use an indirect approach, which includes enrichment of MCs followed by tumour cell detection using mRNA quantification and *KRAS* mutation detection by RT-qPCR. However, inclusion of more patients is highly needed for assessment of the clinical impact of CTC and DTC detection. Moreover, analyses of a larger cohort of healthy volunteers could possibly have resulted in an increased cut-off value and a following decrease in the number of positive patients. It is therefore also desirable to analyse a higher number of healthy individuals to determine a definite cut-off value for CTC and DTC positivity and verify the positive patient samples. The use of immunomagnetic enrichment methods, in addition to gradient centrifugation, could also reduce the background level of the mRNA markers in future analyses.

Comparison of the CTC and DTC levels in numerous PB and BM samples from the same patients revealed that the pancreatic cancer patients investigated seem to have distinct CTC and DTC populations, explained by different mRNAs being expressed during treatment monitoring. Monitoring of the CTC and DTC level during treatment and molecularly characterization of single CTCs and DTCs, using single-cell PCR, to identify resistant cancer-initiating cells, would perhaps in the future help the clinicians to offer pancreatic cancer patients a more individualized treatment.

In some of the aforementioned studies, analyses of PB samples included both resectable and unresectable pancreatic cancer patients, while most of the studies on DTC detection involved resectable patients. In contrast, all the patients recruited to our study were patients with metastatic pancreatic cancer. It may have been interesting to investigate whether the high

number of CTC and/or DTC positive patients is equally represented in pancreatic cancer patients with non-metastatic disease.

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