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Clinical relevance of disseminated tumor cells in advanced pancreatic cancer

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Abstract

Introduction

Pancreatic ductal adenocarcinoma (PDAC), a particularly deadly form of pancreatic cancer (PC), continues to challenge modern medical science regarding diagnosis and treatment of the disease. The poor prognosis associated with PDAC is largely a result of its insidious pathogenesis. The symptoms of an adenocarcinoma of the pancreas in early stages are vague, if present at all, enabling it to grow and spread undetected. Related to this spread are disseminated tumor cells (DTCs), which are theorized to play an important role in metastatic growth These cells detach from the primary tumor, travel through the body's circulatory system, and established themselves in other tissues. The presence of DTCs in BM has previously been connected to a worsened prognosis for patients with breast cancer and colorectal cancer, and for PDAC. DTCs have also been observed to lie dormant in the bone marrow (BM) in other epithelial cancers, like breast cancer, before reactivating and proliferating again in later instances. The BM has thus been thought to work as a reservoir for these potentially metastatic cells. The purpose of this study was to identify potential biomarkers for DTCs and validate the prognostic information tied to the presence of DTCs in the BM of patients with advanced PDAC.

Patients and methods

74 BM samples were collected from 49 patients with locally advanced and/or metastatic PDAC before and two months after receiving chemotherapy. Samples were also collected from a healthy control group of 30 individuals. RNA from the samples was reverse transcribed and pre-amplified prior to analysis. DTCs were detected using real-time quantitative PCR (RT-qPCR) and a range of epithelial mRNA transcripts as biomarkers. Both known and novel biomarkers were utilized. The DTC-positive threshold was established using the highest measured level in the control group. Statistical methods and univariate survival analysis were performed to investigate prognostic significance.

Results

In pre-treatment BM samples, 13/49 (26.5%) were found DTC-positive by one or more biomarkers. In BM samples obtained during treatment, 7/25 (28%) were found DTC-positive by one or more biomarkers. A statistically significant relationship between the clinical T-stage and DTC-status in BM before treatment was found ($p = 0.04$).

In the pre-treatment BM samples, statistically significant relationships were observed for markers *KRT7* ($p = 0.04$) and *KRT8* ($p = 4E\text{-}5$) between DTC-positivity and reduced overall survival (OS). *SPINK1* ($p = 0.06$) was considered borderline significant. The observed relationship with OS for all markers combined was found to be statistically significant ($p = 0.002$). No significant relationship between DTC-positives during treatment and a reduced OS ($p = 0.5$) was found.

Conclusion

This study found that there is a negative impact on the prognosis of patients with advanced PC presenting with DTCs in BM samples collected before treatment. No negative impact on OS could be associated with BM DTC status determined during treatment. Transcripts *KRT7, KRT8* and *SPINK1* were confirmed as potential biomarkers for DTCs in the BM of patients with advanced PC.

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Abbreviations

BM	Bone marrow
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CT	Computerized tomography
CTC	Circulating tumor cell
dNTP	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
DTC	Disseminated tumor cell
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
HUH	Haukeland University Hospital
ICC	Immunocytochemical
KRT	Keratin
LAF	Laminar flow cabinet
LC480	Lightcycler® 480
mQ H_2O	Milli-Q water (Purified water)
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NaP	Sodium pyruvate
PACT-ACT	Pancreatic Cancer Treatment and Circulating Tumor Cells
PBS	Phosphate buffered saline
PC	Pancreatic cancer
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PET	Positron emission tomography
PMBC	Peripheral mononuclear blood cell
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative PCR
SUH	Stavanger University Hospital

Tables and figures

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1. Introduction

1.1. Pancreatic ductal adenocarcinoma

Pancreatic cancer (PC) appears in various forms and types, but almost all pancreatic tumors originate in the exocrine portion of the organ. The most common is pancreatic ductal adenocarcinoma (PDAC), which constitute about 85% of all exocrine PC's [1]. PDAC almost always arise from epithelial cells that line the ducts which transports the pancreatic juice into the duodenum. PDACs can also originate from acinar cells, the functional exocrine units of the pancreas. These are however extremely rare and account for approximately 1-2% of all pancreatic neoplasms [2]. PCs that arise in the endocrine part are also quite rare and more often less malignant, in addition to being easier to diagnose and treat [3]. Pancreatic ductal adenocarcinoma can thus in practicality be held responsible for the poor prognosis of PC as a whole and is generally referred to when using the term 'pancreatic cancer', as it shall likewise be in this thesis.

1.1.1. Epidemiology

PC is a notoriously lethal disease with a dismal record in relation to patient survivability. Based on GLOBOCAN's (Global Cancer Observatory) estimates from 2018, PC was diagnosed in more than 450 000 individuals globally, making it the 11th most prevalent cancer worldwide at the time [4]. The same year, around 430 000 succumbed to the disease, constituting 4.5% of all deaths caused by cancer worldwide [4]. In spite of the ever progressing understanding of the disease and advancement of medical technology, the incidence of PC is expected to increase in the future [4]. In Norway, 884 new cases were reported in 2019 while claiming 800 lives the same year, making PC responsible for 7.2% of Norway's cancer deaths [5]. The figures made it the fourth most lethal cancer in both sexes, and put the 5-year relative survival rate of PC in Norway at 13.1% [5].

While an exact cause of PC is yet to be uncovered, a range of risk factors have been identified; Lifestyle factors such as smoking, drinking, obesity, diabetes, dietary intake, and physical inactivity can play a part in its development [1], [4]. As with many cancers, the risk of developing PC increases with old age; Most patients are past 50 years old, and it rarely occurs in individuals younger than 40 [4].

1.1.2. Diagnosis and staging

The extremely poor prognosis of patients with PC is a result of the absence of clear, distinguishable symptoms during early stages of tumorigenesis, thus allowing the disease to grow and spread to other organs undetected. Common symptoms early in the disease are vague, and include abdominal pain, fatigue, loss of appetite and weight loss. In later stages, symptoms get more noticeable, such as jaundice, itchy skin, changes in coloration of stool and urine, and newly-found diabetes [6]. By the time symptoms are severe enough for most people to visit their physician, the cancer has usually metastasized to other organ tissues. At this point the alternatives for effective treatments are scarce.

To diagnose PC, a range of tests can be performed. Techniques include computerized tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) scans, endoscopic ultrasound, tissue biopsy and/or blood tests [7]. CT is the most widely available and validated technique for diagnosis and staging PC, as well as being the preferred method for determination of resectability [6], [8].

The clinical stages of PC are described using the TNM (Tumor/lymph node/metastases*)* classification system [9]. These stages grade the severity of the disease and are used to decide which treatment to offer. Table 1 shows the different clinical stages of PC, and what the characteristics of each stage are.

Table 1: Clinical stages of PC. *The stages of PC and stage properties, with description of relevant TNM classifications* [9].

Stage	TNM
0	Tis, N0, M0
IA	T1, N0, M0
IB	T2, N0, M0
IIA	T3, N0, M0
IIB	T1, B1, M0; T2, N1, M0, T3, N1, M0
III	T4, any N, M0
IV	any T, any N, M1

TNM Classification – T = Primary Tumor **N** = Regional lymph node **M** = Distant metastasis

Tis: tumor *in situ* **T0:** no evidence of primary tumor **T1**: tumor restricted to pancreas, ≤ 2 cm
T2: tumor restricted to pancreas, ≥ 2 cm **T3:** tumor extends beyond pancreas, excluding coeliac axis or superior mesenteric artery **T4:** tumor affects the coeliac axis or superior mesenteric artery
N0: no regional lymph-node metastasis **N1:** regional lymph-node metastasis
M0: no distant metastasis **M1:** distant metastasis

1.1.3. Treatment

Traditional treatments of PC include surgery, radiotherapy, and chemotherapy to improve life expectancy or relieve symptoms, with the only curative option being surgical resection of a localized tumor. However, around 80-90% of patients have unresectable growths when they are diagnosed [4].

In Norway, there is a national action program for the diagnostics, treatment and follow up of patients diagnosed with PC compiled by the Norwegian Directorate of Health [10]. According to this program, one of the most critical factors when assessing resectability is if the patient presents with metastases. The next criteria is the size of the tumor, and if it impacts on central vessels, primarily the superior mesenteric artery and the coeliac axis; This fact is also widely acknowledged internationally as discouraging for resective surgery [11]. The most common procedure for resection of a PDAC is the "*Whipple's surgery*", whereas a total pancreatectomy can be a surgical option in rare cases [12]. Neoadjuvant treatment is not a part of the standard curative treatment of tumors in Norway, while adjuvant treatment with chemotherapy is applied to most patients post-surgery to reduce risk of relapse [13].

For patients with non-resectable cancer, palliative chemotherapy is usually administered to relieve symptoms with the intent of both improving quality of life and improving life expectancy [14]. While there have been advancements in treatment options during the last decade in the form of multidrug approaches that prolong survivability for patients with advanced PC, the long-term prospects remain poor for these individuals [15], [16]. As of today, immunotherapy and targeted drugs show little to no benefit in PC, and no treatment represents a permanent remedy in patients with advanced disease [3]. Due to these vexing facts, it is of considerable interest to explore potential biomarkers that could lead to earlier diagnostics and prediction of the severity of the disease.

1.2. Disseminated Tumor Cells (DTCs)

Cancer cells may detach from a primary tumor and enter the circulatory system through passive shedding, or in relation to the process of epithelial-to-mesenchymal transition [17]. The cells are then denoted circulating tumor cells (CTCs), which may travel to and settle in tissues other than the origin site through the blood vessels or the lymphatic system and remain there as disseminated tumor cells (DTCs). In certain breast carcinomas, DTCs have been proven to possess the ability to go dormant and survive in the micro-environment of foreign tissue during and after a variety of treatments for potentially decades before being "reactivated" again and resuming their highly proliferative nature [18]. Clinical cancer dormancy has also been observed in other carcinomas, namely of the thyroid, kidney and prostate [19]. The biological mechanism behind this phenomenon is still unknown, yet potential factors have been hypothesized to be likely triggers.

These triggers could contribute to DTCs suddenly exiting their dormancy, based on clinical observations [18]. These DTCs may then in turn go on to develop metastases [20], [21], cementing their importance in contemporary studies aimed at understanding and combatting the disease.

1.2.1. Clinical relevance of DTCs in bone marrow

DTCs in bone marrow (BM) have been proven as an effective prognostic factor for patients with breast cancer, with probable ties to the fact that the disease commonly metastasizes to osseous tissue [22]. While indisputably of value in diagnostics when breast cancer is concerned, this method of prognostic analysis could be challenging when applied to PC as it rarely metastasizes to bone [23]. However, several studies suggest DTCs as a potential independent prognostic factor in operable PCs [24]–[27] in addition to similar findings regarding colorectal cancer, which also rarely spreads to bone [28], [29]. Perhaps the most important point on DTCs being of possibly huge clinical significance is their discussed dormancy, which effectively makes the BM act as a reservoir [30]. As such, proving the presence of DTCs in BM could be a critical factor in risk of recurrence when assessing patients that initially seems to have a relatively good prognosis. Based on these facts and implicating observations in other cancers, further investigations of DTCs clinical relevance and potential as biomarkers concerning PC is of particular importance.

1.2.2. Enrichment of DTCs

DTCs will usually be found in remarkably low concentrations if present in BM (1 DTC per $1 \cdot 10^5 – 1 \cdot 10^6$ normal cells) [31]. As a result, enrichment is needed beforehand [32]. Tumor cell enrichment is commonly carried out by either exploiting the target cells' physical qualities such as density in relation to other cells in sample content, most often blood cell. Gradient centrifugation of mononuclear cells is usually performed in an isosmotic medium like Lymphoprep, Oncoquick or Ficoll [33].

1.2.3. Detection of DTCs

For detection of DTCs, techniques based on ICC or polymerase chain reaction (PCR) can be utilized, as they are sufficiently sensitive and specific [34]–[36].

With ICC, the principle is to visualize specific proteins or antigens by targeting them with specific antibodies. This is normally carried out by fixating cells first to preserve the target antigen and cell morphology, using alcohol or formalin, before adding the primary antibody which attaches to it.

One can then add a secondary antibody modified with a fluorophore or an enzyme that can produce colored substrates, that attaches to this primary antibody, allowing it to be detected using fluorescence or light microscopy. Applied in this context, one can use this to determine if a sample contains a specific antigen which is absent in healthy BM, indicating the presence of foreign cells such as DTCs [36].

The aim of PCR-based methods is to amplify and detect the presence of epithelial transcripts in BM using molecular assays. The most common method is the real-time quantitative reverse transcriptase-PCR (RT-qPCR), which can be used to quantify levels of specific gene transcripts expressed in given samples [36], [37]. The technique relies on the reverse transcription of the target mRNA to cDNA before the RT-qPCR itself. The RT-qPCR uses fluorescing probes to measure the amplification of the product, if present, in real time [37]. Following this process, it is possible to calculate the relative levels of mRNA in the original sample [37]. If elevated in comparison to normal tissue, these levels may in turn indicate the presence of DTCs [20].

When aiming to detect DTCs in carcinomas, keratins (KRTs) are of particular interest. KRTs are a group of structural proteins that among other functions serve as part of the cytoskeleton, both in normal and cancerous epithelial cells [38]. Carcinomas are known to preserve expression of their specific KRTs to a large extent, despite undergoing cancerous transformation, which give them undeniable potential not only as markers for tumors, but also for DTCs [38]. Based on these implications, the usefulness of other epithelial proteins in similar experimental efforts should be investigated.

1.3. Purpose of study & experimental approach

The purpose of this study is to investigate the prognostic significance of DTCs in the BM of patients with locally advanced and/or metastatic PC. To achieve this, the use of both known and novel epithelial biomarkers will be combined with RT-qPCR to detect heightened levels of pancreas specific epithelial transcripts, which will then be compared to patient survival data.

This study is part of a larger clinical project called PACT-ACT (Pancreatic Cancer Treatment – And Circulating Tumor Cells), where one of the goals is to determine the clinical relevance of DTCs detected in BM samples from patients with advanced PC using both known and novel epithelial-specific transcripts as markers for DTCs. The samples were taken from patients prior to, and after two months of chemotherapy. Peripheral mononuclear cells, including the potential DTCs, have previously been enriched from the BM samples, lysed and frozen.

A previous student (D.Fostenes [39]) in the project has isolated and reverse transcribed RNA from the majority of the cell lysates, and looked for DTCs by measuring RNAs abundant in PCs but absent or at low levels in normal BM.

These RNAs can function as surrogate markers for DTCs, and *KRT8* was found to be promising after showing a strong correlation of RNA concentration to prognostic information.

A more recent student (T. Pedersen [40]) has evaluated all known KRTs further *in silico* following the success of *KRT8* to determine their potential as biomarkers. Among these, *KRT7* and *KRT18,* which had not been analyzed prior were determined to be promising candidates.

In this thesis sub-project of PACT-ACT, bioinformatic analyses of public RNA profiling data will be carried out to identify potential novel DTC biomarkers. These novel markers, in addition to *KRT7* and *KRT18* will be measured in all the collected BM samples using real-time quantitative PCR (RT-qPCR). In addition, more recently collected samples following the study of D. Fostenes will be analyzed for *KRT8, KRT19, EPCAM* and *CEACAM5* mRNA. Control samples from normal BM will be analyzed for comparison, with the purpose of determining elevated levels of the mentioned mRNAs in patient BM samples as indicative of the presence of DTCs. The indicated presence of these DTCs will then be compared with clinicopathological data for the patients by statistical methods. Updated survival data collected from the patients will be used in univariate survival analysis by Kaplan-Meier curves and logrank tests, investigating the prognostic value of DTC detection before start and after two months of chemotherapy.

2. Material

2.1. Patients

In this project, 74 BM samples were collected from 49 patients with locally advanced and/or metastatic PC receiving treatment at Stavanger University Hospital (SUH) and Haukeland University Hospital (HUH) in the period 2012 to 2020. In addition, BM samples were also collected from a voluntary control group of 30 healthy individuals. From all samples peripheral mononuclear cells were enriched by density gradient centrifugation, before being lysed, frozen and stored in the project biobank freezer.

2.2. Cell line for cultivation: AsPC-1

The cell line utilized in the project, AsPC-1 (Sigma-Aldrich, 96020930). The cell line was derived from nude mouse xenografts initiated with cells from ascites of a 62-year-old female Caucasian patient with PC. These cells produce carcinoembryonic antigen (CEA), human pancreas associated antigen, human pancreas specific antigen and mucin [41]. The cells were stored in a nitrogen tank.

2.2.1. Cell cultivation medium & subculturing reagents

The medium utilized in the cultivating part of the project was prepared using the reagents displayed in table 2. Subculturing reagents during and after splitting of cultures are also displayed in table 2

Table 2: Cell culturing reagents. *Reagents for the AsPC-1 cell cultivation medium and subculturing of cells.*

Reagent	Purpose	Quantity	Manufacturer	Product#
RPMI 1640	Medium	500 mL	Sigma-Aldrich ®	R0883
2mM Glutamine	Medium	5 mL	Sigma-Aldrich ®	G7513
1mM Sodium Pyruvate (NaP)	Medium	5 mL	Sigma-Aldrich ®	S8636
Penicillin Streptomycin (100x)	Medium	5 mL	Sigma-Aldrich ®	P4333
10% Fetal Bovine Serum (FBS)	Medium	50 mL	Sigma-Aldrich ®	F7524
Trypsin-Ethylenediaminetetraacetic acid (EDTA)	Cell split	Split dependent	N/A	N/A
Phosphate buffered saline (PBS)	Cell split	Split dependent	N/A	N/A

2.3. TaqMan® gene expression assays

The gene expression assays used in this study were of Applied Biosystems™ TaqMan® real-time PCR assays and are displayed in table 3.

Table 3: Gene expression assay list. *List of TaqMan® gene expression assays used in this studies experiments.*

#	Gene abbr.	Gene name	Assay ID	Manufacturer
1	*SPINK1*	Serine peptidase inhibitor, Kazal type 1	Hs00162154_m1	Applied Biosystems™
2	*PRSS2*	Serine protease 2	Hs00828418_gH	Applied Biosystems™
3	*REG1A*	Regenerating family member 1 alpha	Hs00984887_g1	Applied Biosystems™
4	*MUC1*	Cell surface associated mucin 1	Hs00159357_m1	Applied Biosystems™
5	*AGR2*	Protein disulphide isomerase family member anterior gradient 2	Hs00356521_m1	Applied Biosystems™
6	*TM4SF1*	Transmembrane 4 L six family member 1	Hs00371997_m1	Applied Biosystems™
7	*KRT7*	Keratin 7	Hs00559840_m1	Applied Biosystems™
8	*KRT18*	Keratin 18	Hs02827483_g1	Applied Biosystems™
9	*KRT8*	Keratin 8	Hs01595539_g1	Applied Biosystems™
10	*KRT19*	Keratin 19	Hs01051611_gH	Applied Biosystems™
11	*EPCAM*	Epithelial cell adhesion molecule	Hs00158980_m1	Applied Biosystems™
12	*CEACAM5*	Carcinoembryonic antigen related cell adhesion molecule 5	Hs00944025_m1	Applied Biosystems™

2.4. Kits

The kits utilized across the methods applied in this study are displayed in table 4.

Table 4: Kit list. *List of kits used in the methods applied in this study.*

Kit	Function	Manufacturer	Product#
Allprep® DNA/RNA/ Protein Mini Kit	Simultaneous genomic DNA, total RNA and protein purification	Qiagen®	80004
QIAshredder (250)	Homogenization	Qiagen®	79656
High Capacity cDNA Reverse Transcription Kit	Reverse transcription of total RNA to ss-cDNA	Applied Biosystems™	4368814
TaqMan® PreAmp Master Mix Kit	Preamplification of cDNA	Applied Biosystems™	4384267
TaqMan® Gene Expression Master Mix	Real-time quantitative polymerase chain reaction	Applied Biosystems™	4369016

3. Methods

3.1. Bioinformatic analysis of potential novel DTC markers

To identify potential novel DTC markers in PC, data of all protein-coding genes from *proteinatlas.org* [42] was acquired and imported to spreadsheets for sorting by relevant parameters using Microsoft 365® Excel® (2016). We first extracted the expression levels of all genes in normal tissue of the pancreas, peripheral mononuclear blood cells (PMBC) and BM, and levels of the marker RNA present in pancreatic tumor tissue. Ideally, the genes should exhibit high expression in normal pancreatic tissue and PC tissue, but low or preferably nondetectable levels in PMBC and BM, reason being that the sensitivity for DTCs in BM would be low if the background level of the transcripts in normal BM was too high. In addition to these parameters, genes that were more exclusively expressed in ductal, exocrine, epithelial cells were of particular interest given that PDACs arise in these cells.

The RNA concentration data (based on RNA sequencing) available for download [43] described the measured value of the normalized expression of transcript in healthy pancreas as NXP. NXPMBC stated the normalized expression in healthy PMBCs while NXBM was the normalized expression in healthy BM. FPKM stated the fragments per kilobase million in the BM of patients with PC. In the case where a transcript had levels measured in either PMBC's or BM, but still showed promise according to the other criteria, a calculated value NXP(NX/(PMBC+BM)/2) was utilized for comparison of these genes. The NXP value was divided by the mean of the measured levels in PMBC and/or BM divided by 2. These described measurements were then used as criteria when searching for potential biomarkers, by setting minimum or maximum values based on the data.

3.2. Cell cultivation

The cells from the AsPC-1 line were cultivated with the purpose of isolating the RNA, so that it may be used to test the amplification efficiencies of the new assays. Normally, the resulting RNA would also be prepared to serve as a calibrator sample for the RT-qPCR analysis. However, enough calibrator was prepared by a previous student working on the project [39].

Work surfaces and other equipment as necessary was wiped down with 70% ethanol disinfectant. Gloves and disposable lab coats were used, and sterile working techniques were followed for all processes.

3.2.1. Cell resuscitation

The cell culture AsPC-1 was stored in a nitrogen tank, where they are contained in a dormant state. Before cultivation, the cells need to be resuscitated to an active state. The protocol for resuscitation of the AsPC-1 cell line was as follows:

1. Sterile work environment was ensured by exposing the workbench to UV-light for 30 min. Cultivating medium (RPMI 1640, 2mM Glutamine, 1mM Sodium Pyruvate, Penicillin-Streptomycin solution, 10% Fetal Bovine Serum) was placed in heat locker for pre-heating by 37°C for 30 min.

2. T25 flask with 10 ml of pre-heated cultivating medium was prepared.

3. An ampoule of cells was retrieved from nitrogen cooler and thawed quickly by holding the bottom half of the tube submerged in a 37°C water bath. The tube was removed before thawing the sample completely, leaving a small clump of icy cells that continued to thaw while working quickly through the next point.

4. At the laminar flow cabinet (LAF) bench, the cap of the ampoule was wiped down before transferring the content carefully, dropwise to the prepared T25 flask using a 2ml pipette.

5. The cells were placed for incubation for 2-3 days at 37°C with a constant supply of 5% CO_2.

3.2.2. Cell splitting

When the cell culture grew, it was necessary to split the culture to facilitate further growth and maintaining the general health of the cells. The protocol for splitting of the AsPC-1 cell culture was as follows:

1. Cell culture density was examined by microscopy and controlled for potential contaminations or abnormalities. At 80-90% confluency, the cells were ready for splitting.

2. Cultivating medium, PBS and trypsin-EDTA was preheated to 37°C in a water bath, and the workbench exposed to UV-light for 30 min.

3. The medium in the cell culture was carefully pipetted out, before adding 5.0 mL (T25) or 10 mL (T75) PBS depending on flask size.

4. PBS was removed before adding 1.0 mL (T25) or 2.0 mL (T75) of trypsin-EDTA to detach the cells from the flask surface. The flask was then incubated at 37°C for 5 min.

5. Once cells were confirmed detached from the flask interior, 2.0 mL (T25) or 4.0 mL (T75) cultivating medium was added to inactivate the trypsin present.

6. 25 mL of pre-warmed medium was added to a new T75 flask. A pipette was used repeatedly multiple times back and forth to loosen potential cell clumps in the culture. The volume of cell-media mix was judged based on the concentration of cells in the original culture, and the noted culture generation ***P***. The mix was then transferred to the new flask.

7. The culture was incubated again 2-3 days at 37°C with a constant supply of 5% CO_2 until the next split or harvest.

3.2.3. Cell harvesting and lysis

Before processing the cells, the culture had to be harvested in a proper manner. The following lysis of the cells was necessary for use as template in the RT-qPCR or in a calibrator sample. The protocol for harvesting and lysis of the AsPC-1 cells was as follows:

1. Cultivating medium, PBS and trypsin-EDTA was preheated to 37°C in a water bath, and the workbench exposed to UV-light for 30 min. Cells were confirmed around 80% confluent before proceeding and examined for possible contaminations.

2. Steps 2-3 as described in 3.2.2 were performed.

3. A pipette was used repeatedly multiple times back and forth to loosen potential cell clumps in the culture before transferring the cell suspension to a 15 mL collecting tube.

4. 50 µL of the cell suspension was transferred to a microtube before preparing it for cell counting. Cell counting is described in section 3.2.4.

5. The cell suspension concentration was confirmed and noted for the following steps. If there were more than 1E7 cells present, the sample was split on multiple tubes.

6. The suspension was centrifuged at 300 x g (1200 RPM) for 5 min at room temperature.

7. Almost all the supernatant, save a tiny amount (about 10-20 µL) was removed by pipette. The tube was flicked multiple times to dissolve the pellet and resuspend the cells.

8. Appropriate amount of RLT buffer with β-mercaptoethanol was added to the suspension according to cell count, 350 µL per tube for <5E6 cells, or 600 µL per tube for 5E6-1E7 cells. A pipette was used to thoroughly mix and ensure proper lysis of the sample, by pipetting up and down about 10 times. The lysate was then ready for storage or isolation.

3.2.4. Cell counting

To assess the number of cells present in the samples, a Countess™ automated cell counter machine was utilized. To stain the cells for counting, trypan blue solution was used. The Countess™ cell counter worked by using image contrast analyzing algorithms to efficiently count live and dead cells with the help of the staining Trypan Blue.

The protocol for performing a cell count using this technology was as follows:

1. A 50/50 µL (1:1 ratio) of Trypan Blue solution and cell suspension was made, making sure that both suspension and Trypan Blue Solution was thoroughly mixed by pipetting up and down multiple times.

2. 10 µL of mixed solution was pipetted into a Countess™ chamber slide, letting it settle for about 30 seconds before inserting into the machine.

3. The Countess™ program has a range of settings that determine what size and shape objects should be considered cells. This was set for “Cell line” before capture. The values for concentration of living and dead cells were provided by the machine following analysis.

3.3. RNA isolation

To isolate total RNA from samples for later use in RT-qPCR, the AllPrep DNA/RNA/Protein Mini Kit from Qiagen was used. The kit is designed for purification of total protein, total RNA, and genomic DNA in the same procedure, simplifying the process by eliminating the need for sample separation. The lysate was acquired in the last steps of the cell harvesting procedure described in section 3.2.3..

The protocol was strictly following the kit handbook [44], with the following choices in certain steps:

- In step 6, depending on amount of RLT-buffer used in accordance with step 9 in the procedure described in section 3.2.3., 250 µL (For 350 µL RLT-Buffer used) or 400 µL (For 600 µL RLT-Buffer used) of 96-100% Ethanol was added to the flowthrough from the previous step 5 in the protocol. The BM samples in this experiment were prepared using 600 µL RLT-Buffer, therefore 400 µL 96-100% ethanol was used.

- In step 11, the RNeasy spin column was placed in a new 2ml collection tube, discarding the previous one and repeating the wash to eliminate possible carryover of Buffer RPE during elution.

- In step 12, 40 µL of RNAse-free water was used to elute RNA. Step 13 (repetition of step 12) was not carried out as the yield of RNA was not expected above 30 µg.

3.3.1. Measuring RNA concentrations

RNA concentrations in isolates were measured using a NanoDrop™ 2000c spectrophotometer. The device follows the principles of the Beer-Lambert Law, which correlates the attenuation of light with the characteristics of the subject that the light travels through. The instrument modifies the law to calculate nucleic acid concentration, using a pathlength between 1 mm to 0.05 mm with the generally accepted extinction coefficients of 40 ng-cm/µl for RNA [45] (Equation 1).

Equation 1:

$$C = \frac{A \cdot \varepsilon}{b}$$

Where:

C = Concentration of nucleic acid (ng/µl)

A = Absorbance in units of absorbance (AU)

ε = Wavelength-dependent extinction coefficient (ng-cm/microliter)

b = Pathlength (cm)

An absorption ratio of 260/280 was also calculated with the analysis to determine if the purity in the samples were satisfactory, where a value of ~2.0 is generally accepted as pure for RNA [45].

The protocol for measurement of nucleic acids using with the NanoDrop™ 2000c was in accordance with the handbook [45], with particular focus on instrument washing in between samples and blanking beforehand.

3.4. Reverse transcription of RNA to cDNA

Since a PCR-reaction functions with DNA and not RNA, it was necessary to reverse transcribe the RNA in the samples. This process is enabled by exploiting the enzyme *Reverse transcriptase*, naturally found in retroviruses, which generates cDNA from RNA. The reverse transcription in this thesis was carried out using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems™. The protocol was as follows, based on the kit user guide [46] :

1. Template RNA was thawed in preparation for the reverse transcription. In addition, the reagents provided in the kit was placed on ice for thawing and kept on ice with the RNA for the entirety of the experiment as far as practicality allowed it.

2. Master mix was prepared for *n+1* reactions, the excess prepared to mitigate potential loss of master mix during the experiment. The master mix was prepared from the following reagents and mixed thoroughly.

    - 2 µL 10x RT-buffer per. reaction
    - 0,8 µL 25x deoxynucleoside triphosphate (dNTP) mix per. reaction
    - 2,0 µL 10x random primers per. reaction
    - 1,0 µL RNAse inhibitor per. reaction
    - 3,2 µL Milli-Q water (purified water, mQ H_2O) per. reaction

3. 9,0 µL of the master mix was pipetted to each tube for their respective reactions.

4. 1,0 µL of Multiscribe Reverse Transcriptase was added to each tube, excluding any negative controls. For negative controls, 1,0 µL of mQ H_2O was added instead.

5. 1,0 µg RNA from sample was added to each tube. Depending on the RNA concentration of the sample, naturally the volume in µL was added.

6. Depending on µL RNA added, mQ H_2O was added to make total 20 µL of reaction mix. (10 µL – RNA µL added = µL mQ H_2O to add)

7. The tubes were placed in a thermal cycler and the following program initiated

    - 10 minutes at 25 °C (primer binding)
    - 120 minutes at 37 °C (polymerization)
    - 5 minutes at 85 °C (inactivation of reverse transcriptase
    - Cooling at 4 °C until collection

8. The resulting cDNA was transferred to freezable microtubes and stored in freezer.

3.5. Pre-amplification of cDNA

The cDNA produced from reverse transcription could be subject to a low number of copies of the target RNAs, given that the BM samples may have very low concentrations of these. However, an amplification in advance of the RT-qPCR before dilution could be performed to ensure an amount sufficient for subsampling. This in turn allowed for more runs of the eventual RT-qPCR.

The kit used for the pre-amplification was the TaqMan® PreAmp Master Mix Kit from Applied Biosystems™, and the protocol as follows based on the user guide [47]:

1. Assay mix was prepared with every assay of interest and diluted with mQ H_2O to make the finished mix 1:100 for each assay. The final volume was scaled to fit the number of reactions needed, as 12,5 µl of assay mix is needed per reaction. E.g., for 100 reactions, the final volume would be 1250 µl, with each assay making up 12,5 µl each.

2. Master mix was prepared for n+1 reactions using the following reagents:

    - TaqMan® PreAmp Master Mix (2x): 25 µl per. reaction
    - Assay mix: 12,5 µl per. reaction
    - mQ H_2O: 7,5 µl per. reaction

3. After thoroughly mixing, 45 µl of the master mix was pipetted to every reaction tube.

4. 5 µl of cDNA was added to the respective tubes for each reaction.

5. The tubes were placed in a Veriti™ 96-well Thermal Cycler and run through the following program:

    - 10 min at 95 °C
    - 14 cycles of 95 °C for 15 sec, then 60 °C for 4 min.

6. The resulting pre-amplified cDNA was diluted 20x using mQ H_2O, by transferring it to a microtube and adding 950 µl.

7. The samples were then used in RT-qPCR or stored at -20 °C for later use.

3.6. Real-time quantitative polymerase chain reaction (RT-qPCR)

To quantify the amount of target mRNA in the samples by using the pre-amplified cDNA, RT-qPCR was utilized. The RT-qPCR monitors the amplification of target cDNAs during the reaction, by using sequence-specific fluorescent reporter/quencher-probes added to the reaction. The probes are attached to oligonucleotides, which during the elongation step of the reaction degrade as hybridization takes place. The reporter part of the probe is then released and will while no longer in proximity of the quencher fluoresce and become measurable at that exact moment. As a result, it is possible to measure the accumulation of the amplified product as fluorescence increases as the reaction progresses [37].

Before carrying out the experiment, a threshold value of amount of fluorescence is selected. Once any given reaction well is measured to reach the threshold value, the amount of amplification cycles run up to that point is registered in a curve plot produced by the software accompanying the experiment machine. This value is denoted the C_q-value [48]. The results would reflect which samples contain a higher or lower level of amplification in relation to each other based on their respective C_q-values, which in turn correlate their relative concentrations of cDNA in the sample.

The RT-qPCR was carried out using a Lightcycler® 480 (LC480). The kit used for the RT-qPCR was the TaqMan® Gene Expression Master Mix, and the protocol on the following page based on the user guide [49]:

1. The assays that were to be used in the RT-qPCR were placed for thawing, and the LC480 machine was turned on beforehand.

2. Master mix was prepared for *n+1* reactions for each assay, with the following components:

- 2x Gene expression master mix – 12,50 µl per. reaction

- 60x Gene expression assay [Gene] – 0,42 µl per. reaction
  OR
  20x Gene expression assay [Gene] – 1,25 µl per. reaction

- mQ H_2O (60x) – 5,83 µl per. reaction
  OR
  mQ H_2O (20x) – 5,00 µl per. reaction

3. After thorough mixing, 18,75 µl of master mix was pipetted to each respective well on a PCR tray for the LC480.

4. 6,25 µl of pre-amplified cDNA was pipetted to each respective well.

5. The following program was run in the LC480 machine:

    - 50 °C for 2 minutes (UDG-probe activation)
    - 95 °C for 10 minutes (Polymerase activation)
    - 40 cycles of 95 °C for 15 seconds, followed by 60 °C for 1 minute.

6. The LC480 software would then display the results and setup of analysis with the desired thresholds could be performed through the program software.

For these experiments, the "absolute quantification / fit points" option was selected in the analysis using the software to calculate C_q-values. The noise-threshold was adjusted for each individual assay as necessary. The threshold to determine C_q-value was set to "2.2" for all markers.

3.7. Determination of PCR amplification efficiency

As a quality-assuring step, the amplification efficiencies for the potential DTC marker PCR assays were determined before including them in a pilot study. To determine amplification efficiencies, RT-qPCR was carried out with the pre-amplified cDNA from the AsPC-1 cell line, with a 4x dilution series (1:4, 1:16, 1:64 and 1:256). The LC480 software uses the standard curve method to calculate the efficiency from the resulting curve's slope (Equation 2).

Equation 2:

$$e = 10^{-\frac{1}{slope}}$$

Where:

e = Efficiency

Slope = The calculated slope of the standard curve with Ct-values on the Y-axis, and the logarithmic quantity on the X-axis.

The efficiency of the amplification could be defined as the amount of target gene products detected at the end of the cycle divided by the amount of target gene products at the start of the cycle. High quality amplifications run at e = 2.0, where the target product doubles each cycle, but values E>1.8 are generally acceptable [50]. The protocol was as described in 3.6., except for the samples being replaced with the dilutions of known concentration from the AsPC-1 cell line.

3.8. Calculating relative gene expression levels

The quantification of gene expression using the RT-qPCR-method described in section 3.6. would provide results which were likely subject to experimental variation. Multiple reaction plates across multiple runs were required to gather the necessary data from all samples in the patient and control groups. To nullify variation across the samples, transcript levels of housekeeping gene *BCR* (which is perpetually expressed in both normal and cancerous cells) were utilized to normalize all data to ensure reliable future calculations. Experiment plate-to-plate variation for all assays, including *BCR*, were corrected using a calibrator sample derived from the AsPC-1 cell line made by a previous student in the project [39].

Duplicates were prepared for each sample and the calibrator sample in each experiment, providing two C_q-values for each sample and calibrator. The mean value of these C_q-values was used in following calculations.

To calculate the relative gene expression levels the $2^{-\Delta\Delta Cq}$ method proposed by K.Livak and T. Schmittgen was used [51]. This method allows for effective relation of measured signal from target transcript in the samples to signals of a given reference gene (preferably a housekeeping gene), further allowing for fold change comparison with a calibrator. The formula derived in this method ultimately provides the dubbed "R-value" (***R***), the relative value for the measured amount of target mRNA, and can be formulated as described in Equation 3:

Equation 3:

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

Where:

$$\Delta\Delta Cq = \Delta Cq_{sample} - \Delta Cq_{calibrator}$$

$$\Delta Cq = Cq_{target\ gene} - Cq_{reference\ gene}$$

$$\boldsymbol{R} = R\text{-}value\ (Relative\ mRNA\ concentration)$$

3.9. Statistical analyses

To plot and identify DTC-positives in the pilot study and the complete RT-qPCR-experiments, both R statistical computing software and Microsoft 365® Excel® (2016) was utilized.

For analyses aimed at cross-comparing results of different gene assays and DTC-positive samples, R statistical computing software and IBM® SPSS® Statistics software was utilized.

To investigate the prognostic significance in terms of survivability of DTC-positive patients compared to DTC-negative patients, Kaplan-Meier analyses and Logrank-tests were carried out using R statistical computing software.

When calculating the statistical significance of the clinicopathological data compared to DTC-status in patients, 'Fisher's exact test' was performed. For continuous variables, the 'Mann-Whitney' method was applied.

For all analyses where a P-value was calculated, $P \leq 0.05$ was considered statistically significant.

4. Results

4.1. Project overview

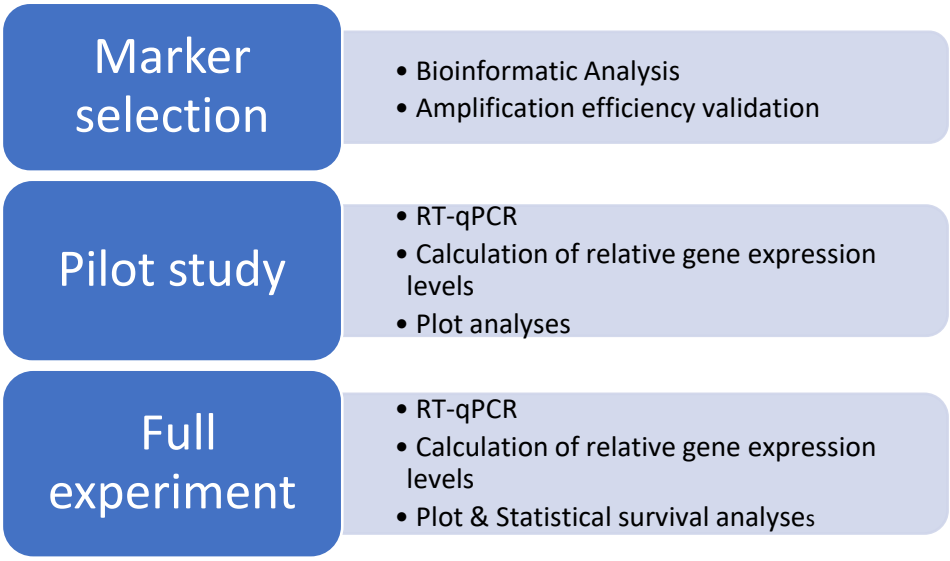

Figure 1: Project overview. *Overview of the main project tasks.*

The main body of the thesis work can be summarized in three sections: Marker selection, pilot study and full experiment. These sections and their content are displayed in figure 1.

4.2. Bioinformatic analysis of potential novel DTC markers

To identify potential novel DTC markers, we searched public gene expression data for transcripts with high levels in normal pancreas and PCs, but low or nondetectable levels in normal BM and peripheral blood. The bioinformatic analysis (described in section 3.1) yielded six promising candidate mRNAs for DTC marking after using a process of elimination by looking at the optimal levels and relationships of the variables (see Table 5). The principle was to sort the genes by concentration measures acquired from *proteinatlas.org* [43]: The normalized expression of the gene in normal pancreas (NXP), normal PMBCs (NXPMBC), normal BM (NXBM), and the detected fragments per kilobase million in BM of patients with PC (FPKM).

In addition, a practical value was calculated to better compare genes that had measurable levels in NXPMBC or NXBM, creating a fifth variable NXP(NX/(PMBC+BM/2)) for comparison purposes. As the variable name suggest, this value was acquired by dividing the NXP by the mean of PMBC and BM.

The other variables were then sorted by the following criteria using Microsoft 365® Excel® (2016):

- NXP > 50
- NXPMBC < 1,0
- NXBM < 1,0
- FPKM > 125

Table 5: Bioinformatic analysis results. *Results for bioinformatic analysis of gene expression data.*

#	GENE	NXP	NXP/(NX(PMBC+BM)/2)	NXPMBC	NXBM	FPKM
1	*SPINK1*	1767,1	35342	0,1	0	260,4
2	*PRSS2*	6267,9	41786	0,3	0	225,4
3	*REG1A*	1772,6	N/A	0	0	225,4
4	*MUC1*	74,2	134,9	0,2	0,9	148,2
5	*AGR2*	59,2	N/A	0	0	147,6
6	*TM4SF1*	54,3	90,5	0,9	0,3	146,1

NXP – *Normalized expression in healthy pancreas*

NXPMBC *– Normalized expression in PMBCs*

NXBM – *Normalized expression in normal BM*

FPKM – *Fragments per kilobases in BM of individuals with PC*

NXP/(NX(PMBC+BM)/2) – *Practical calculation to further compare transcripts with measured values in either the NXPMBC or NXBM category.*

4.3. Validation of amplification efficiencies

TaqMan® Gene Expression Assays for the candidate DTC marker transcripts were selected from the assay database of Thermo Fisher. If several assays were available for the same transcript, the recommended assays were chosen. Amplification efficiencies for each assay were determined by the standard curve method (section 3.7.). Example amplification curves and a standard curve are shown in figure 2 and 3, respectively. The assay amplification efficiencies were validated in accordance with the accepted values for efficiency and error of the standard curve provided by the LC480 software [50]. The amplifications were run for 40 cycles on 4x dilution series of the AsPC-1 cell line for the standard curves. All assays were approved (see Table 6). Assays used previously in the project were excluded as they had already been validated (*KRT7, KRT8, KRT18, KRT19, CEACAM5, EPCAM)*.

Table 6: Amplification efficiency validation results. *Amplification efficiency validation results for candidate DTC marker transcript assays.*

Gene Assay	Error	Efficiency
REG1A	0.09	1.86
AGR2	0.041	1.96
PRSS2	0.12	1.95
TM4SF1	0.036	1.99
SPINK1	0.021	2.05
MUC1	0.045	1.96

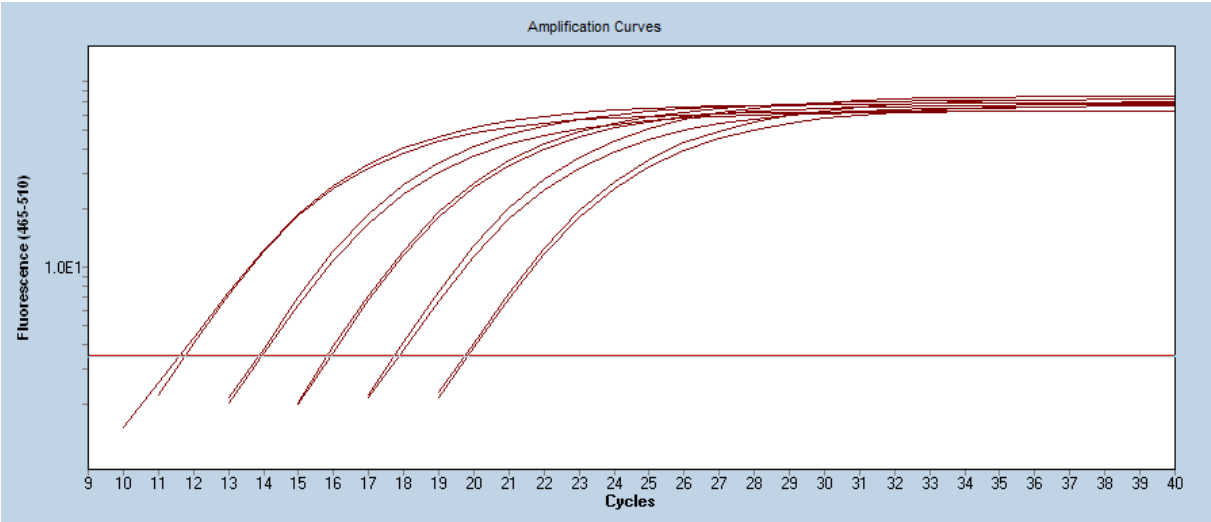

Figure 2: Amplification curve example. *Example of the amplification curves resulting from the AsPC-1 4x dilution series run to determine assay amplification efficiency of SPINK1. The horizontal line represents the set threshold for fluorescence measured to intersect with the curves to determine C_q-value as described in section 3.6..*

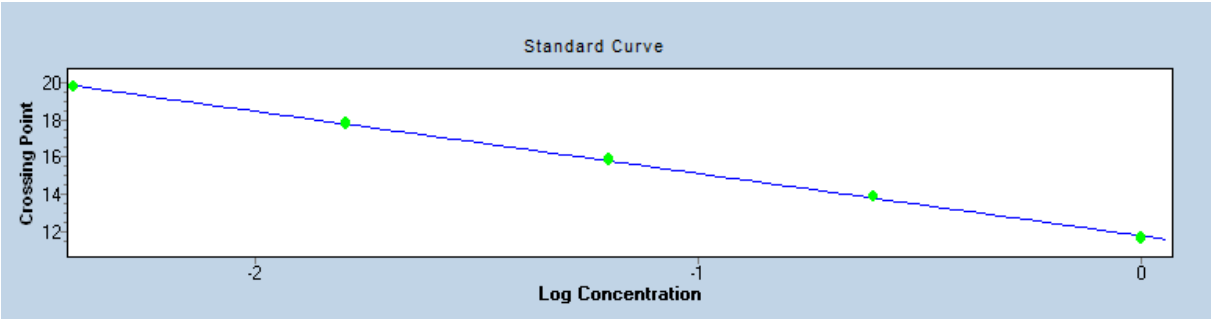

Figure 3: Standard curve example. *Example of the standard curve provided by the LC480 software using the results from the AsPC-1 4x dilution series run to determine assay amplification efficiency of SPINK1 as described in section 3.7. The green dots represent the mean of duplicate samples of each dilution, while the blue line shows the linear regression of these values.*

4.4. Pilot study of potential DTC markers

The relative levels of the candidate DTC marker transcripts were measured using RT-qPCR in 15 patient BM samples and 15 control BM samples as a pilot-study to further assess their utility before potential application in the full-scale experiment. The RT-qPCR, calculation of the relative levels and statistical methods used for this purpose are described in sections 3.6, 3.8 and 3.9.. The measured levels of the mRNA transcripts in BM samples for the candidate genes are displayed in Figure 4. *REG1A* and *PRSS2* are not included in the figure, as they had extremely high levels of background expression in normal BM. Legible and comparative plots could thus not be produced for these markers.

In the pilot experiment, conditions for a suitable marker were set to **1.)** Low or undetectable levels in normal BM (controls), and **2.)** High levels in one or more patients (assumed DTC-positives).

- *REG1A* and *PRSS2* runs were found at very high levels in control BM. As such, they were deemed unfit as markers.

- *TM4SF1*, *MUC1* and *AGR2* measured higher or similar relative concentrations in controls compared to patients, making them unsuitable as markers according to the results.

- *KRT18*, while displaying relatively high levels in both patient and control samples, had one extremely high measurement in the patient group, justifying including it in the full experiment.

- *KRT7* and *SPINK1* both showed relatively low concentrations in normal BM, making them adequately sensitive to positives and potentially suitable for marking. *KRT7* showed 3 instances of heightened levels in patients, and *SPINK1* showed 1 in the pilot study, strengthening interest in these assays. Thus, both *KRT7* and *SPINK1* were included in the full experiment.

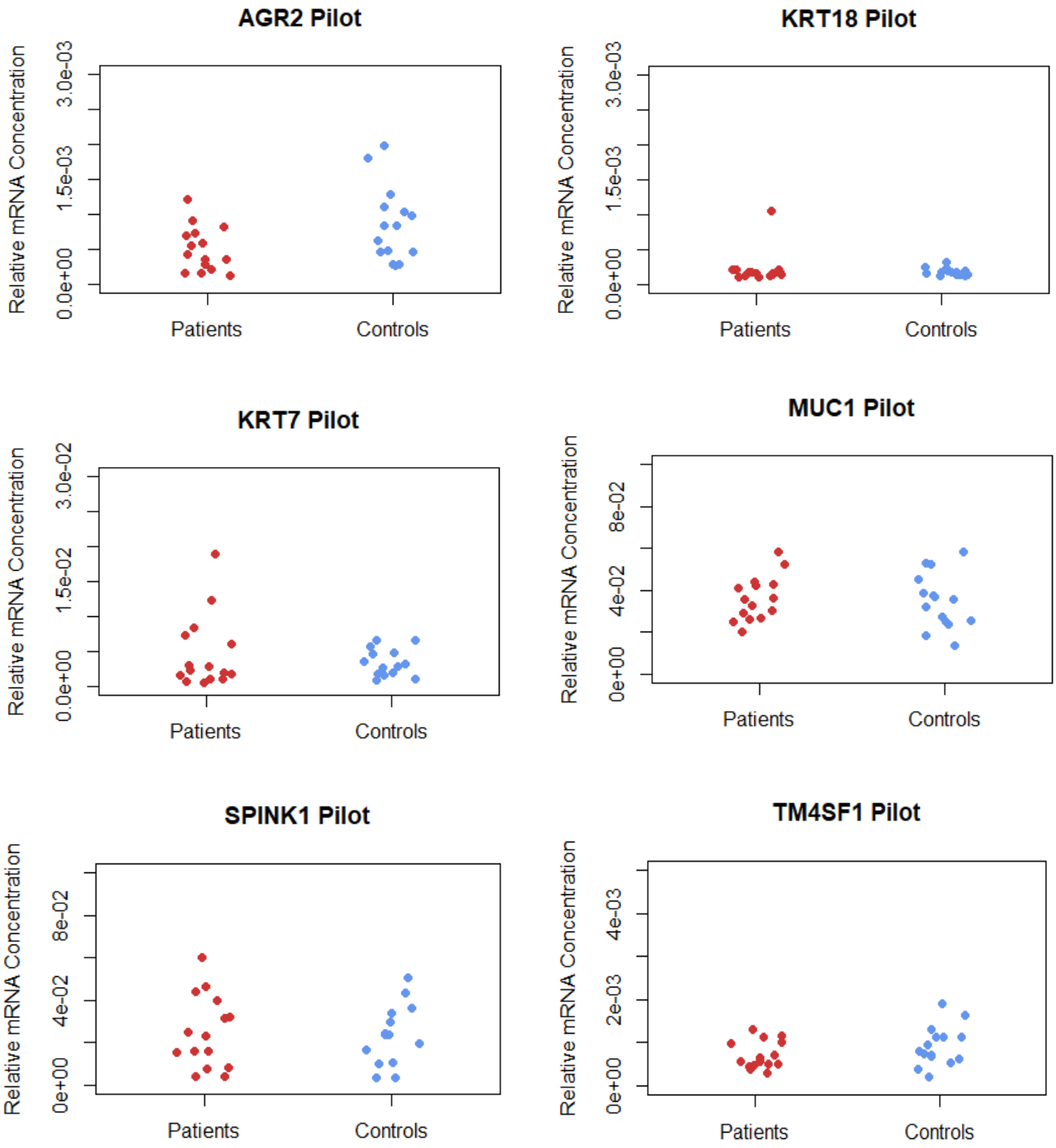

Figure 4: Pilot study BM marker levels. *Relative concentrations of candidate gene mRNA in patient (red) and control (blue) BM samples measured in the pilot study. Only samples with measurable levels are shown.*

4.5. DTC marker levels in the full PACT-ACT cohort

The relative concentrations of the included markers *KRT7* and *SPINK1* from the pilot study were measured in the BM of patients of the PACT-ACT cohort using RT-qPCR (section 3.6). The most promising DTC markers previously analyzed [39], *CEACAM5*, *EPCAM*, *KRT8* and *KRT19* were also measured in the recent sample additions from the updated patient cohort. Using the control group as a guideline, the thresholds for DTC-positivity were set to the highest level measured in the control samples from healthy volunteers. Any values exceeding this threshold were then counted as deviations from normal levels, and thus DTC-positive. In the pre-treatment samples, 13/49 (26.5%) were found DTC-positive, 4 of these by multiple markers, while during-treatment samples had 7/25 (28%) DTC-positives, all by single markers (see Table 7). Figure 5 on the following page graphically display the results.

Table 7: DTC-positives by marker and sample type. *The number of positives for each marker, in pre-treatment positive samples and during-treatment positive samples.*

Marker	Pre-treatment positives	During-treatment positives
CEACAM5	1	4
EPCAM	2	1
KRT7	4	0
KRT8	5	0
KRT18	2	0
KRT19	2	2
SPINK1	6	0
All markers	**22**	**7**

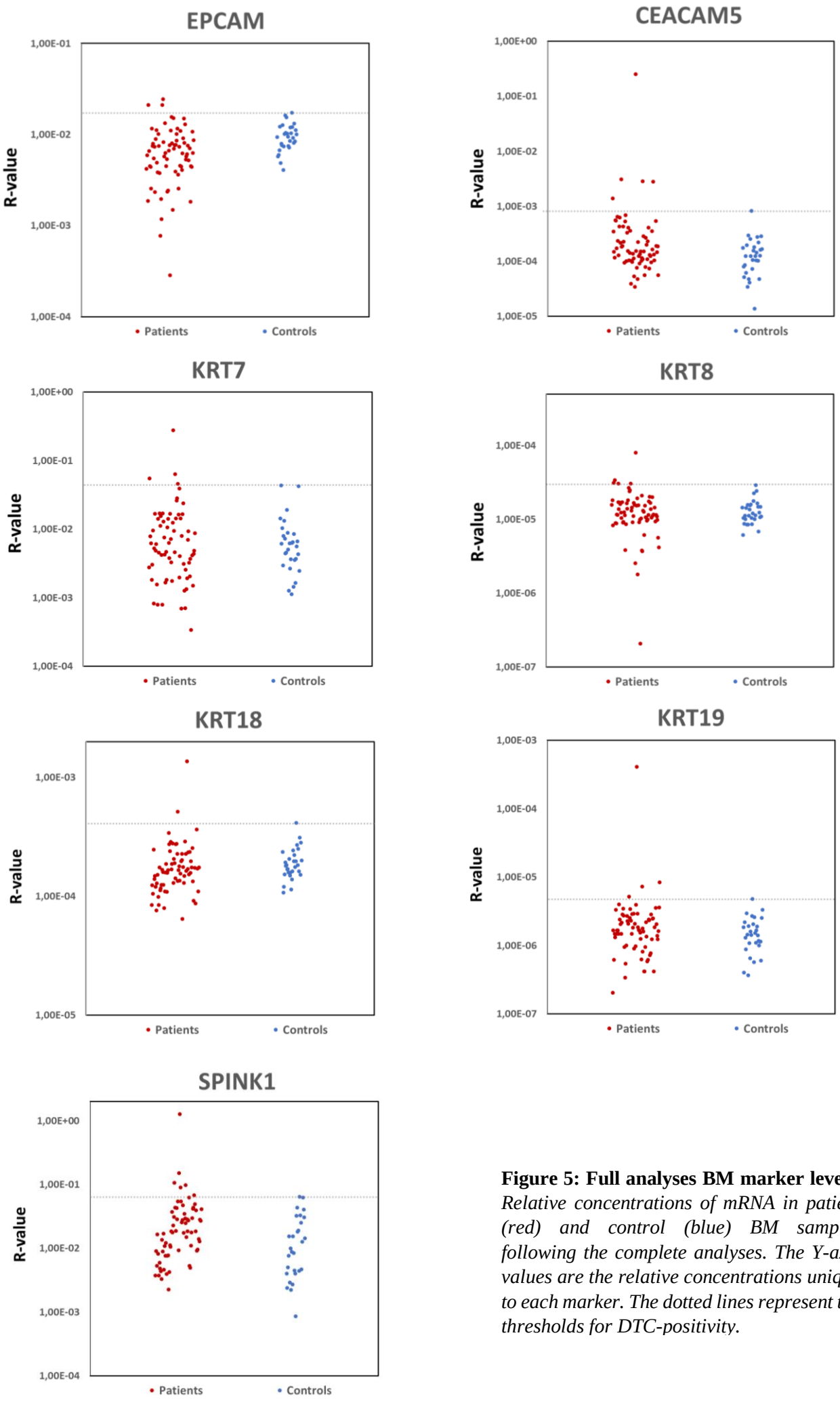

Figure 5: Full analyses BM marker levels. *Relative concentrations of mRNA in patient (red) and control (blue) BM samples following the complete analyses. The Y-axis values are the relative concentrations unique to each marker. The dotted lines represent the thresholds for DTC-positivity.*

4.6. Comparison of clinicopathological data and DTC-status

The clinicopathological data from the patients participating in the study was analyzed in comparison with the determined DTC-statuses in samples taken before treatment to investigate potential statistical significance (Table 8). The statistical calculations (p-values) used are described in section 3.9..

Table 8: Clinicopathological data compared to DTC-status pre-treatment. *Comparison of the clinicopathological parameters and patients DTC-statuses pre-treatment, with calculated p-values. Patients in the TX category in the T-stage and NX category in the N stage are not included in the test.*

Parameter	All patients (n=49)	DTC+ (n=13)	DTC- (n=36)	p-value
Mean age	64 (41-79)	62 (46-74)	65 (41-79)	0.4
Sex				
Male	29 (59%)	9 (69%)	20 (56%)	0.5
Female	20 (41%)	4 (30%)	16 (54%)	
Primary tumor location				
Head	20 (41%)	4 (30%)	16 (44%)	
Body	7 (14%)	3 (25%)	4 (11%)	0.6
Tail	7 (14%)	2 (15%)	5 (14%)	
N/A	15 (31%)	4 (30%)	11 (31%)	
Clinical T-Stage				
T1	1 (2%)	0 (0%)	1 (3%)	
T2	14 (29%)	7 (52%)	7 (19%)	**0.04**
T3	8 (16%)	3 (25%)	5 (14%)	
T4	20 (41%)	2 (15%)	18 (50%)	
~~*TX*~~	~~6 (12%)~~	~~1 (8%)~~	~~5 (14%)~~	
Lymph node status				
N0	19 (39%)	6 (45%)	13 (36%)	
N1	17 (35%)	4 (30%)	13 (36%)	0.6
N2	3 (6%)	0 (0%)	3 (8%)	
~~*NX*~~	~~10 (20%)~~	~~3 (25%)~~	~~7 (20%)~~	
M-Stage				
M0	11 (22%)	1 (8%)	10 (28%)	0.2
M1	38 (78%)	12 (92%)	26 (72%)	
Clinical stage				
III	11 (22%)	1 (8%)	10 (28%)	0.2
IV	38 (78%)	12 (92%)	26 (72%)	
ECOG-status				
0'	13 (27%)	2 (15%)	11 (31%)	0.6
1'	30 (61%)	9 (70%)	21 (58%)	
2'	6 (12%)	2 (15%)	4 (11%)	

Statistical significance was observed between DTC-status and clinical T stage (p = 0.04).

4.7. Survival analysis

To investigate the prognostic significance of the presence of DTCs in the analyzed samples, Kaplan-Meier curves and Logrank-tests were performed. The analyses aimed to determine if there is a statistical significance in the observed overall survival (OS) difference between DTC-positive patients versus DTC-negative patients for single markers and their combination. The measurement of relative mRNA levels and subsequent analyses were carried out on the BM samples taken from patients before treatment, and after two months of chemotherapy. Gene assays *CEACAM5, EPCAM, KRT7, KRT8, KRT18, KRT19* and *SPINK1* were analyzed.

Of the pre-treatment samples, 13/49 (26.5%) were found positive for one or more DTC-markers. Among the during-treatment samples 7/25 (28%) were positive for one or more DTC-markers.

4.7.1. Pre-treatment sample analyses

Analysis of the OS of the combined DTC-positive patients (patients positive for at least one DTC marker) compared to DTC-negative patients was carried out to evaluate significance of total DTC-status pre-treatment. The observed difference was statistically significant (p = 0.002) for pre-treatment samples (Figure 6). The following page shows single/multimarker results (Figure 7).

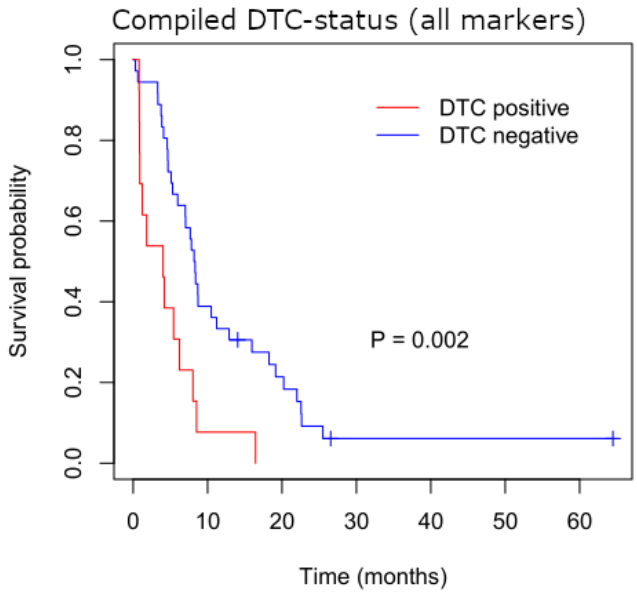

Figure 6: Overall survival according to pre-treatment DTC status (all markers). *Kaplan-Meier curve showing the OS of DTC-positive and DTC-negative patients, according to all markers combined. Censored patients are indicated by vertical ticks on the curves. P-value from the associated logrank tests is included inside the plot.*

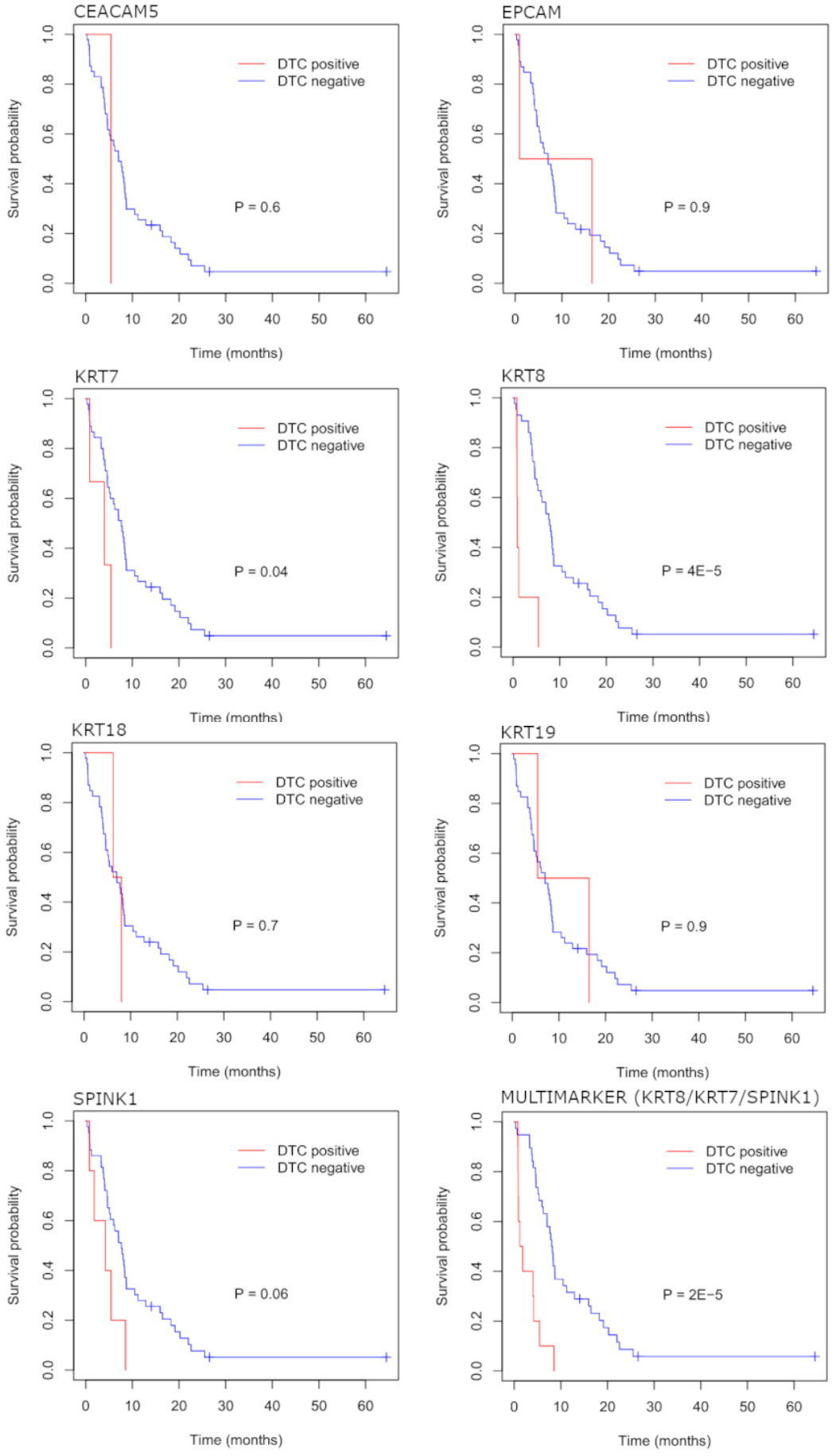

Figure 7: Overall survival according to pre-treatment DTC status (single/multimarker): *Kaplan-Meier curves showing the OS of DTC-positive and DTC-negative patients (for the marker in question), both according to single markers (CEACAM5, EPCAM, KRT18, KRT19, KRT7, KRT8 and SPINK1) and a multi-marker combination (KRT8/KRT7/SPINK1, bottom right). Censored patients are indicated by vertical ticks on the curves.*

The difference in OS between DTC-positives by single markers *KRT7* (p = 0.04) and *KRT8* (p = 4E-5) compared to DTC-negatives was considered statistically significant, while *SPINK1* (p = 0.06) was considered borderline statistically significant. Judging by its plot, *SPINK1* still shows a clear connection between heightened levels and a reduced OS. Based on these results, a combination of all three was put through to a separate analysis for evaluation. The resulting multimarker *KRT8/KRT7/SPINK1* had the strongest p-value (p= 2E-5).

4.7.2. During-treatment sample analyses

The samples collected during treatment that came out DTC-positive (7/25) did so for *CEACAM5*, *EPCAM*, and/or *KRT19* (Figure 8).

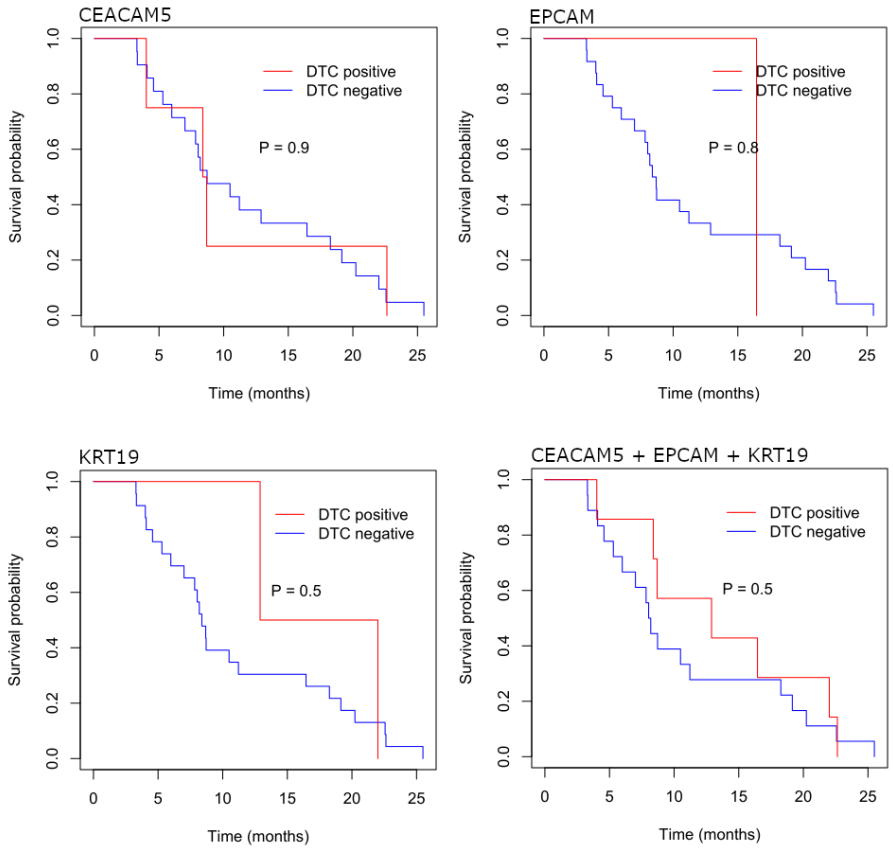

Figure 8: Overall survival according to DTC status during treatment. *Kaplan-Meier curves showing the OS of DTC-positive and DTC-negative patients, according to markers CEACAM5, EPCAM, KRT19 and the combined markers (bottom right). Censored patients are indicated by vertical ticks on the curves. P-values from associated logrank tests are include inside the plots.*

Following the analyses of samples during treatment, no statistically significant relationship between survival and heightened levels of the markers was observed.

5. Discussion

The role of DTCs in BM as an independent prognostic factor has previously been demonstrated in breast cancer [22], in addition to being implicated in PC [24]–[27], [34], [52]. Despite the rare incidence of bone metastases in PC patients [23], the results observed in the previous studies suggest that the clinical relevance and prognostic significance of DTCs in PC should be investigated further. In this thesis, the aim was to assess the connection between DTCs in BM and the survival of patients with inoperable PC. The analysis found that elevated levels of DTC-markers was linked to a lowered OS ($p = 0.002$) in patients before treatment. The results concerning patients during treatment showed no connection between DTC-positives and reduced OS ($p = 0.5$).

5.1. DTC detection

5.1.1. RT-qPCR vs. ICC

RT-qPCR was the chosen method for detection of DTCs in this study instead of ICC, which has previously been applied often in similar studies. One of the downsides of RT-qPCR is its indirect detection, as one does not observe and identify the cells as in ICC. Thus, one does not benefit from any advantages this might bring, like potentially differentiating between actual DTCs or other cell types. On the other hand, while ICC allows for this in some degree, any interpretation of said observations is more subjective and relies on whoever is assessing the images. ICC is also dependent on the antibodies applied, and the classic way of performing ICC often entails using a limited number of markers. Considering the latter facts, RT-qPCR was deemed a better alternative for this study.

The subject of ICC versus RT-PCR for this application is in present time debated. On PCR-based detection, Hugenschmidt *et al* make note of the fact that there may be a distinction in sensitivity and/or specificity related to the higher proportion of detected cases in studies that applied it, while also pointing at the lack of comparative studies between both methods [52]. The article compared several similar ICC studies as well, and argued that methodological differences and lack of standardization across studies that applied it may have played a role in the varying results observed between them [52]. In a large study with a cohort of 385 patients, Becker *et al* did find that in 73% (280/385) of breast cancer cases where DTCs were detected, both methods agreed [53]. It may be of value to conduct more such comparative studies down the line in the interest of working towards uncovering which method works best, and in which cases they do.

5.1.2. mRNA biomarkers

When deciding which mRNA transcripts would best suit our purpose to detect DTCs in the BM of patients with PDAC, a couple of facts had to be taken into consideration. Since PDAC mainly arises in epithelial ductal cells in the exocrine part of the pancreas, it followed that the biomarkers should be transcripts expressed in these cells. Previous studies that detected DTCs in BM mainly applied epithelial KRTs, which falls into this category of cells in combination with both ICC [24]–[27] and RT-qPCR [34], [54]. Such KRT species were thus included. Non-KRTs *CEACAM5* and *EPCAM* were mainly included to follow up on the work of D. Fostenes [39] as the PACT-ACT cohort had since expanded.

In the bioinformatic analysis (section 3.1.) step of this project, we wanted to explore other novel epithelial markers in addition to the already established KRTs, which resulted in a range of candidates also expressed in the exocrine ductal cells. We then narrowed down this selection further by excluding genes which had unfavorable levels of expression, meaning too high or too low, in either normal pancreas, BM, and PMBCs, or in the BM of patients with PC (section 3.1.). This resulted in the six candidates *REG1A, AGR2, PRSS2, MUC1, TM4SF1* and *SPINK1* (section 4.2).

The approved candidates (in addition to *KRT7* and *KRT18* which had been previously identified by T. Pedersen [40]; these were however not part of the bioinformatic analysis) were run through a pilot study before being included in the main experiment (section 4.2). Interestingly, while all the candidate transcripts from the bioinformatic analysis should in theory have been good matches for their purpose, only *SPINK1* qualified for the full experiment. This may reflect some differences between the sensitivity of the RT-qPCR method and the RNA-sequencing data from *proteinatlas.org* [43]. For example, the reported levels of normalized expression of *AGR2* in healthy BM and PMBCs according to *proteinatlas.org* were 0/0, meaning non-detectable. The pilot study results still indicated detectable levels in the control samples, even more so than in the patient samples, as it also did for multiple candidates. *REG1A* and *PRSS2* had such high background levels in normal BM, that it would take thousands of DTCs in a sample for detection, which defeats the purpose of a sensitive biomarker.

5.1.3. One-step vs. Two-step RT-qPCR

When using RT-qPCR to quantify mRNA, it is possible to do so in a one-step reaction or a two-step reaction. The former performs both the synthesis of cDNA from RNA and the following PCR amplification in the same run, while the latter separates these steps. Two-step RT-qPCR was applied in this study for multiple reasons. Most importantly, two-step protocols are more sensitive than one-step PCR [37], and considering that DTCs present in such a miniscule quantity in BM, the process of pre-amplifying (section 3.5) is ideal [55]. Pre-amplifying cDNA ensures a higher quantity of template from smaller sample sizes that can be consistently diluted to identical concentrations for several PCR-runs.

In addition, the reverse transcription (section 3.4) process facilitates storage for projects spanning longer periods seeing as DNA is more stable than RNA.

While one-step RT-qPCR is thought to reduce experimental variation [37], the mentioned advantages of the two-step process make it essential for this study. To avoid systematic error, the efficiencies of the gene assays used were also verified (section 4.3). If the amplification efficiency is sub-par, the lowest concentrations of target mRNA would not be amplified within the 40 cycles in each run. In addition, the $2^{-\Delta\Delta Cq}$ method used to calculate the relative mRNA-concentrations described in section 3.7 is somewhat reliant on the assumption that PCR efficiency is optimal [51].

5.1.4. DTC-status threshold

The risk of false-positives when determining DTC-status was considered before selecting the threshold distinguishing normal BM levels of mRNA from pathological levels. To better avoid these, the highest level observed in the control sample pool was selected as the threshold. The advantage of this method is that regardless of the relatively small control group (30), it reduces the risk for false positives in patient samples. However, the strategy may in turn give false negatives by excluding DTC-positives that otherwise may affect the results on the account of controls with aberrantly high levels that push the threshold. In a similar study, Sergeant *et al* [56] applied a cut-off on the upper-limit 95% confidence interval, which is a statistically sound method. If our study had a larger control group, this approach might have been more advantageous as it improves the odds of establishing a threshold that realistically exclude controls that deviate from wild type expression.

5.2. DTCs and clinicopathological data

Clinicopathological data for the patient cohort was compared with their DTC-status before treatment (section 4.6). Considering that the cohort only included patients with PC stage III or IV, the analysis may be limited in that regard. While there was no statistically significant association between DTC status and M stage ($p = 0.2$), there was an obvious trend (8% vs. 92%) that should be taken into consideration. The clinical T-stage parameter came out statistically significant ($p = 0.04$) in relation to DTC-status, the number largely attributed to the fact that clinical T4 patients represented only 2/20 (10%) of DTC-positive patients in its category. Surprisingly, this implies that patients with an advanced primary tumor is less likely to present with DTCs in BM, where one would perhaps expect the opposite.

Soeth *et al* on the other hand observed in their study that detection rates increased with tumor stage when applying nested *KRT20* RT-PCR, leading to the conclusion that the detection rates of DTCs are stage dependent [34]. It could be of interest to further investigate this relationship in future studies to understand why these results conflicted.

5.3. Prognostic value of DTC detection

In the current study, DTCs were detected in 13/49 (26.5%) patients before treatment that trended towards a reduced survival (section 4.7.). Similar findings of trends or statistically significant connections were reported by Effenberger *et al*, with 24/175 (13.7%) [24], Roder *et al* with 25/48 (52.1%) [25] and Vogel *et al* with 27/71 (38%) [27] DTC-positive patients. Some of these studies also included operable patients. These studies applied ICC to detect DTCs, using pan-keratin antibodies in BM samples. Looking at other studies where PCR methods were applied like in this thesis, Hoffman *et al* and Soeth *et al* had 25/37 (67%) [54] and 45/135 (33.3%) [34] DTC-positive patients respectively. Similarly, these RT-PCR studies used *KRT19* and *KRT20* to detect DTCs while observing a negative impact on patient prognosis. Soeth *et al* pooled survival data for both BM and/or venous blood samples [34], which may affect the comparison.

While the studies mentioned up to this point agree that there is a prognostic value to DTC detection using KRTs, Hugenschmidt *et al* concluded that there was no correlation to OS or disease-free survival (DSF) [52]. It is worth noting that this group, as well as others mentioned in the work did so after applying the broader range cytokeratin antibodies *AE1/AE-3* [52], while Effenberger *et al* and Vogel *et al* applied *A45-B/B3* directed at a smaller selection consisting of *KRT7, KRT18* and a heterodimer *KRT8/18* when they did find prognostic information [24], [27]. It may be that only certain subtype DTCs that express certain KRTs (or other epithelial transcripts for that matter) negatively impact prognosis. If this is the case, it explains why these more specific analyses give a higher accuracy on DTCs that carry prognostic information.

As mentioned in the introduction, the PACT-ACT project has previously had intriguing findings using *KRT8*, and the new results for *KRT7* add to the markers' potential application. *KRT18* and *KRT19* did not provide any insights in this experiment, and it could be that they are expressed at too high levels in healthy BM to achieve a sensitive enough DTC detection, at least concerning PDAC. Aside from the KRTs, *SPINK1*, which also gave prognostic information has previously been connected to pancreatitis, which is a PC risk factor, and implied in PC [57], [58]. One common denominator does however appear to be the KRTs role in this particular diagnostic pursuit regardless of detection method, which seems to further solidify their potential in the field.

5.4. Clinical relevance of DTCs during chemotherapy

While positives for DTCs were identified using *KRT7, KRT8, KRT18, KRT19, SPINK1, EPCAM* and *CEACAM5* in samples taken before treatment, only *KRT19, EPCAM* and *CEACAM5* measured above their control group thresholds in the samples taken during treatment. In contrast to the samples taken before treatment, no prognostic connection was found between DTC-positivity and these samples. There are a couple of ways to interpret these facts.

The most likely theory is that the high *CEACAM5*, *EPCAM*, and *KRT19* levels are not caused by the presence of DTCs, but other cell types with high levels of the measured transcripts caused by the disease or its side effects. BM cells, PMBCs or other epithelial cells could all be potential sources. Some of the pre-treatment DTC-positive patients by *KRT7, KRT8* and *SPINK1* succumbed to the disease shortly after pre-treatment sampling, making it so that no during-treatment sample could be collected. This does in part explain the lack of positives for these markers in the category, which in turn might explain why we didn't observe any prognostic significance there either. In the case of *SPINK1*, this might tie into the gene's link to pancreatitis, which could be deadly on its own. Nevertheless, the low number of positives in during-treatment samples, and their lack of prognostic value support the possibility that chemotherapy in many cases has had a destructive effect on the DTCs or their formation. Finding related documentation on the effect of chemotherapy on DTCs in BM to corroborate the theory was however challenging to come by, which makes it an interesting subject for future studies.

In a more improbable scenario, the persistence of positives by *KRT19, EPCAM* and *CEACAM5* across both sample categories is a result of them representing DTCs. However, if we assume that these DTCs do carry prognostic information, this conflicts with our observation that none of these markers identified patients with shorter overall survival, whereas *KRT7, KRT8* and *SPINK1* mRNA did. It could be that due to the heterogeneity and variation in tumor cells, subtype DTCs (as mentioned in section 5.3) which contain these transcripts at heightened levels are less indicative of a worse prognosis compared to other subtypes of DTCs that present with a different gene expression pattern.

5.5. Future perspectives

The findings of this study add to the consensus of DTCs holding promise as a prognostic factor in PC. It is a fair assessment that KRTs and other epithelial transcripts should be investigated further for their potential as biomarkers for DTCs in PC. There is no denying the connection between reduced OS and heightened levels of *KRT8* and *KRT7* in BM according to these experiments, even though *KRT18* and *KRT19* did not follow in that suit. In addition, *SPINK1* should also be considered for further experiments as it displays a similar connection, where an expanded experiment with a larger patient cohort could be of value.

The application and practicality of the methods described in everyday clinical setting is however questionable. While strides have been made in RT-qPCR technology the last decade, rendering it more cost-effective and quicker, the gravity and reliability of the prognostic value DTCs in BM seems to provide is still up for debate. Moreover, BM harvesting procedures can be quite unpleasant for the patient and are not usually routine during treatment, but if the value of DTC detection is confirmed to be great enough it could be in the future. As discussed in section 5.1.1., comparative studies of ICC vs. RT-qPCR could thus be of value to the field in this regard.

6. Conclusion

The relationship between the reduced OS of patients with locally advanced and/or metastatic PC and the presence of DTCs in patient's BM was investigated using both known and novel epithelial mRNA transcripts with RT-qPCR. The prognostic significance of these relationships was assessed using Kaplan-Meier curves and the associated logrank tests. Samples taken before and two weeks after chemotherapy were analyzed. Based on the results presented, we found that there was a negative impact on the prognosis of patients that present with DTCs in the BM before treatment. The prognostic effect was primarily based on the elevated levels of *KRT7*, *KRT8*, and *SPINK1* mRNA. The transcripts *KRT7*, *KRT8* and *SPINK1* show promise as novel biomarkers for DTCs in the BM of PC patients while providing significant prognostic information and should thus be considered for future study. On the contrary, DTC-positives identified by *KRT18*, *KRT19*, *CEACAM5* and *EPCAM* mRNA measurements did not show any association with a reduced OS in our study. Nevertheless, the findings of this study suggest that there is a clinical relevance of DTCs in the BM of patients with advanced PC.

7. References

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