Anna Tanem Stølan

MicroRNAs as potential biomarkers for prostate cancer

A RT-qPCR validation study of differentially expressed microRNAs in serum from pre- and post-operative prostate cancer patients

Master's thesis in Biotechnology
Supervisor: Sandra Amalie Dybos
August 2020

Master's thesis

NTNU
Norwegian University of
Science and Technology

Biobank1

Anna Tanem Stølan

MicroRNAs as potential biomarkers for prostate cancer

A RT-qPCR validation study of differentially expressed microRNAs in serum from pre- and post-operative prostate cancer patients

Master's thesis in Biotechnology
Supervisor: Sandra Amalie Dybos
August 2020

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

NTNU
Kunnskap for en bedre verden

Abstract

Prostate cancer is one of the most common types of cancers among men worldwide. The diagnostic process can be invasive, time consuming and often lead to diagnosis of cancers that are not destined to cause harm. Because of these issues there is a need of new biomarkers that are able to effectively detect whether the patient has prostate cancer. Among the potential biomarkers are the short and non-coding microRNAs, known for regulating gene expression. The regulatory role of microRNAs has been connected to various biological processes, such as cellular differentiation and apoptosis. Research shows that aberrant expression of some microRNAs can be used to separate cancer from healthy controls, and that their expression can be informative regarding status of the cancer.

The aim of this thesis was to validate, by real-time qPCR, the change in expression of circulating microRNAs shown by sequencing to have a significant change in expression following prostate cancer treatment. In the theoretical part of this study, a literature search was performed in order to identify microRNAs best suited as biomarkers for prostate cancer. By using the obtained information from the literature search in combination with resulting data from the sequencing project, six microRNA targets were selected for validation.

In the experimental part of the study, isolated RNA from serum samples collected from prostate cancer patients were reverse transcribed to cDNA and analysed by quantitative real-time polymerase chain reaction. Samples from some of the patients failed to give results and were omitted from the data analysis. Analysis of the data from the remaining samples successfully validated the significant change in expression following treatment for two of the targets. To determine whether it was degradation of isolated RNA that caused unsuccessful results for some samples, a repetition of the experimental procedures should be done. The microRNAs with a significant change in expression should be further validated and their regulatory role further explored.

The literature search revealed that even though there is a lot of research on microRNAs as biomarkers, there is limited overlap between studies and the results are conflicting. The findings must be validated, by methods appliable in clinical practice, in order to identify promising candidate biomarkers.

Sammendrag

Kreft i prostata er en av de vanligste krefttypene blant menn over hele verden. Diagnostisering av prostatakreft kan være en tidskrevende og ubehagelig prosess som ofte fører til overdiagnostisering. Det er derfor behov for nye, mer presise biomarkører for prostatakreft. En av de potensielle biomarkørene er korte, ikke-kodende mikroRNA, kjent for å regulere genuttrykk. mikroRNA er blitt knyttet opp mot en rekke biologiske prosesser, som blant annet cellulær differensiering og apoptose. Forskning viser at endret uttrykk av enkelte mikroRNA kan brukes til å skille kreft fra kontroll, og at uttrykk av enkelte mikroRNA kan være informative når det gjelder kreftstatus.

Målet med denne masteroppgaven var å validere endringen i uttrykk hos en gruppe mikroRNA som gjennom et tidligere sekvenseringsprosjekt er vist å ha en signifikant endring i uttrykk som følge av behandling. Som en del av det teoretiske arbeidet med denne studien ble et litteratursøk gjennomført med hensikt å identifisere hvilke mikroRNA som er best egnet som biomarkører for prostatakreft. Ved å bruke den tilegnede informasjonen fra litteratursøket i kombinasjon med resultatet fra sekvenseringen ble seks mikroRNA valgt ut for validering.

I den eksperimentelle delen av studiet ble isolert RNA fra serumprøver fra prostatakreftpasienter revers transkribert til cDNA og analysert ved hjelp av RT-qPCR. Enkelte prøver ga ikke resultater og ble derfor utelatt fra dataanalysen. Analyse av resultater fra de resterende prøvene viste en signifikant endring i uttrykk som følge av behandling for to av de valgte mikroRNAene. Gjentakelse av de eksperimentelle prosedyrene med nytt RNA bør gjennomføres for å avgjøre om degradert RNA var årsak til at noen av prøvene ikke ga resultat. mikroRNA med en signifikant endring i uttrykk bør ytterligere valideres og den regulerende rollen til disse mikroRNAene bør kartlegges.

Litteratursøket viste at på tross av at det er forsket mye på mikroRNA som biomarkører, er det lite overlapp mellom studier og flere motstridende resultater. Funnene må valideres, gjennom metoder som kan brukes i klinisk sammenheng, for å identifisere aktuelle biomarkørkandidater.

Acknowledgements

Abbreviations

BPH	Benign prostate hyperplasia
cDNA	Complementary DNA
CLL	Chronic lymphatic leukaemia
CTC	Circulating tumour cell
CZ	Central zone
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
FDA	Food and Drug Administration
fPSA	Free PSA
iPSA	Intact PSA
KLK3	Kallikrein-3
LUTS	Lower urinary tract symptoms
miRNA	MicroRNA
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NCI	National Cancer Institute
NGS	Next generation sequencing
NIH	National Institute of Health
PCA3	Progensa prostate cancer antigen 3
PHI	Prostate health index
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PSA	Prostate-specific antigen
PZ	Peripheral zone
REK	Regional Ethical Committee
RISC	RNA-inducing silencing complex
RT-qPCR	Quantitative real-time polymerase chain reaction
TNM classification	Tumour, Node, Metastasis classification
TPM	Transcripts per kilobase million
TZ	Transition zone
UMI	Unique molecular identifier

Contents

List of Figures

List of Tables

1. Introduction

Cancer, a broad group of diseases characterized by uncontrolled cell division, is one of the world's largest health problems. In 2018, the Global Cancer Observatory estimated the number of new cancer cases to be approximately 18.1 million and the number of cancer-related deaths approximately 9.6 million (Bray et al., 2018). After cardiovascular disease, this makes cancer the second leading cause of deaths worldwide. The incidence rate is predicted to increase in the years to come due to higher life expectancy and environmental factors like air pollution, diet and tobacco smoking (You and Henneberg, 2017). Because the cancer burden will continue to exert physical, emotional, and financial strain on individuals, families and health system, the need for solutions that help lighten this burden will increase in the years to come. Advancements in medicine and healthcare such as development and improvement of methods used for detecting cancer are a part of these solutions.

1.1   Prostate cancer

1.1.1 Epidemiology

Prostate cancer, or *cancer prostatae*, is cancer that arises in the prostate, a gland in the male reproductive system. Prostate cancer is the most common type of cancer among men in Norway and the second most common cancer among men worldwide (Stewart and Wild, 2014). The incidence rates have been stable the last years, but the rates differ between countries (Larsen et al., 2019). Europe and North-America represented 60 % of all newly registered prostate cancer cases and 41 % of all prostate cancer related deaths in 2012 (Stewart and Wild, 2014). The five-year survival rate for men diagnosed with prostate cancer is relatively high (94.5 % in Norway) compared to other types of cancers. However, there are cases of lethal, metastatic prostate cancer resistant to treatment (Sartor and de Bono, 2018). The most frequent sites for prostate cancer cells to metastasize are the local lymph nodes and the skeleton, especially the spine, hip, or pelvic bones. Often, patients diagnosed with prostate cancer at young age have poor outcome, especially when compared to those diagnosed at high age (Gupta et al., 2017).
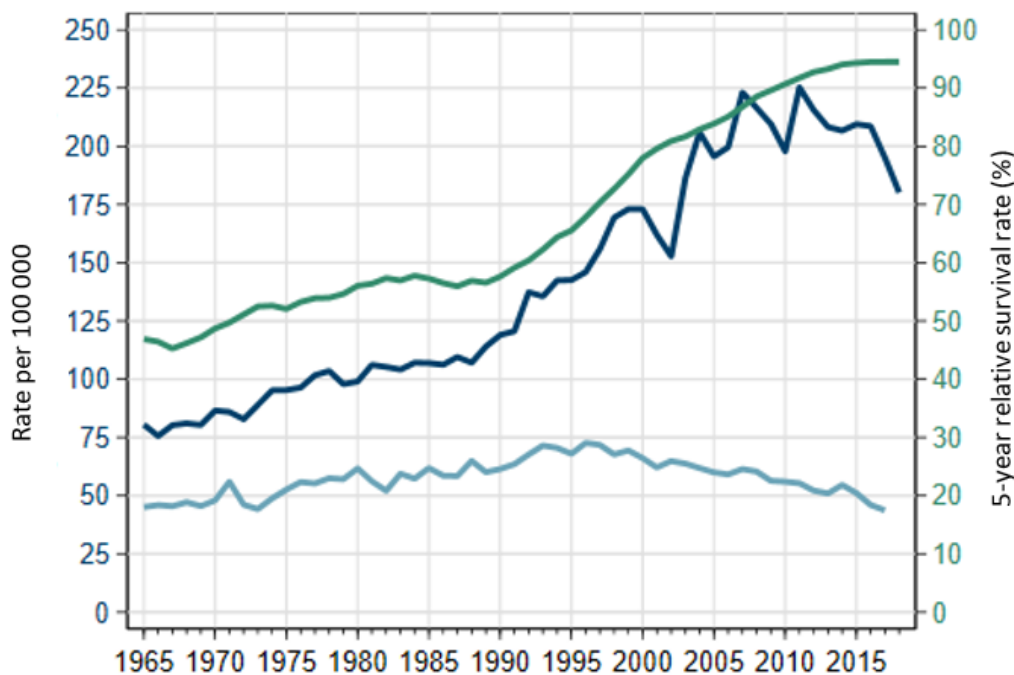
Figure 1.1: The figure shows trends in prostate cancer incidence rates (dark blue), five-year survival rate (green) and mortality (light blue) in Norway in the time period 1965 – 2015 (Larsen et al., 2019).

There are three established risk factors for developing prostate cancer: age, ethnicity and family history (Leitzmann and Rohrmann, 2012). In 2018, 4850 patients were diagnosed with prostate cancer in Norway and the median age of these patients was 70 years (Kreftregisteret, 2019). In most cases, prostate cancer is relatively slowly growing and it may take up to 20 years from the first cellular changes until the tumour is large enough for it to give symptoms and to be detectable (Roberts et al., 2000). The incidence rate and mortality is higher in African American men than in white men (Rawla, 2019). The reason for this is unclear, but genetic factors as well as social and environmental differences are conceivable causes. Men with a family history of prostate cancer and especially those with relatives diagnosed with prostate cancer at young age have high risk of developing prostate cancer (Johns and Houlston, 2003). Having more than one relative with a prostate cancer diagnosis further increases the risk (Stewart and Wild, 2014). Less established risk factors for prostate cancer are hormones, environment and lifestyle (Gann, 2002). Physical activity together with intake of vegetables and soy are mentioned as preventive factors, while smoking, obesity and frequent consumption of meat and dairy products are factors that may enhance the risk of prostate cancer.

1.1.2 Anatomy and pathology

The prostate gland is located inferiorly to the bladder, encircling the urethra, as illustrated by Figure 1.2 (Marieb and Hoehn, 2016). The gland is about the size of a walnut and is enclosed by a thick connective tissue capsule. Embedded in a mass of smooth muscle and dense connective tissue are 20 to 30 tuboalveolar glands. These glands compose the prostate parenchyma. The functional role of the prostate is to produce a weak alkaline solution containing proteins that play an important role in the activation of the sperm cells, citrate and prostate-specific antigen (PSA) (McKay and Sharma, 2019). PSA is a glycoprotein enzyme also known as kallikrein-3 (KLK3) that is encoded by the human *KLK3* gene. The prostatic secretion is released into the prostatic urethra as prostatic smooth muscle contracts during ejaculation and it represents approximately one third of the total semen volume. The remaining part, a yellowish fluid rich containing fructose and other substances, is produced by *vesicula seminalis*.
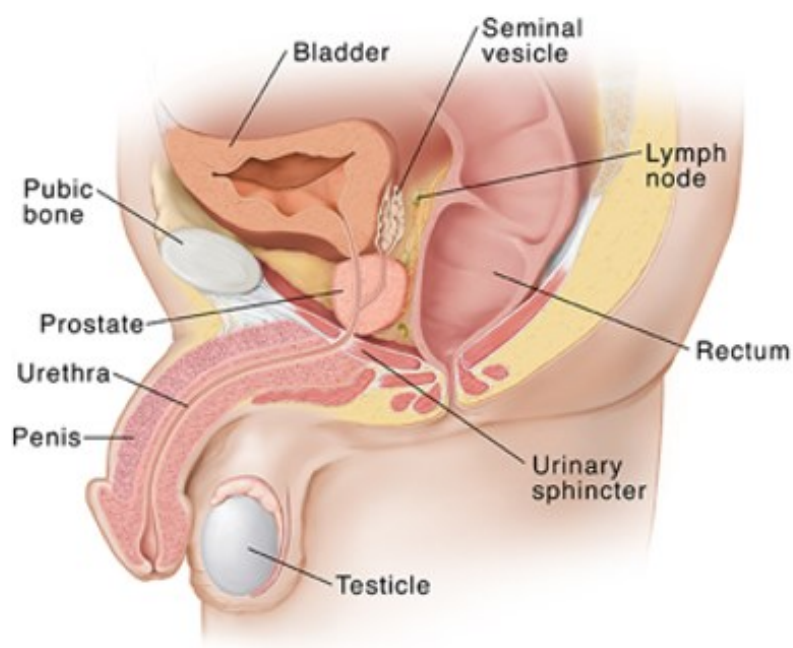
Figure 1.2: Anatomy of the male pelvis. The prostate is located inferior to the bladder, encircling the urethra (Gee).

The prostate gland can be subdivided in lobes or in zones. A subdivision into five lobes, the anterior and posterior lobe, two lateral lobes and a median lobe, is mainly used in anatomy whereas zones are more frequently used in pathology. The zonal classification is based on the four distinct glandular regions of the prostate gland, illustrated in Figure 1.3 and described in Table 1.1 (Kovacs, 2001).
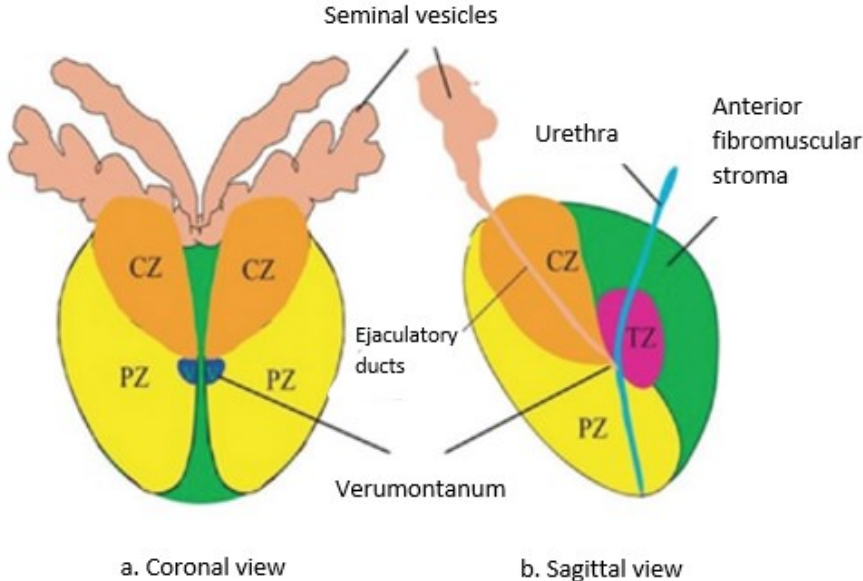
Figure 1.3: The zonal anatomy of the prostate consists of the peripheral zone (PZ), the central zone (CZ), the transition zone (TZ) and the anterior fibromuscular zone (Lahoti et al., 2018).

Table 1.1: Description of prostatic zones (McKay and Sharma, 2019).

Name	Fraction of gland (%)	Description
Peripheral zone	70	The peripheral zone represents the largest contribution of the prostate. It surrounds the distal part of the urethra and is where most of the cancers develop.
Central zone	25	The central zone composes the base of the gland that surrounds the ejaculating tubes. It is located posterior to the transition zone and the urethra. Only a small part (2.5 %) of prostate cancers arise here, but they tend to be more aggressive than others.
Transition zone	5	The transition zone surrounds the proximal urethra. Approximately 20 % of the prostate cancers arise in this zone. The transition zone grows throughout life and it is here the disease benign prostate hyperplasia may develop.
Anterior fibro-muscular zone	Not applicable	The anterior fibro-muscular zone is as the name implies composed of muscle and fibrous tissue and is therefore not always considered a zone. It is devoid of glandular elements.

The three most prevalent prostatic diseases are prostatitis, benign hyperplasia, and cancer. Prostatitis, or inflammation of the prostate, is a disease that affects men at any age (Lawrentschuk and Perera, 2000). Prostatitis can be caused by an acute, bacterial infection or most often, it can be chronic and keep recurring. Bacterial prostatitis is treated with antibiotics and chronic prostatitis is treated with α-blocking agents targeting the smooth muscle of the prostate (Sharp et al., 2010). Benign Prostatic Hyperplasia (BPH) is a non-cancerous enlargement of the prostate, which develop in the transition zone. It is a common condition, and by the age of 70 years, 50 % of all men will have an enlarged prostate (Tveter, 2019). The prostate can be severely enlarged, weighing up to 200 grams, which is ten times the normal weight and will then compress the urethra. The standard treatment for BPH is surgery whereby the hyperplastic tissue is removed.

Typically, this is accomplished by transurethral resection of the prostate where the hyperplastic tissue that affects the urethra is removed with the help of a cystoscope. Diseases of the prostate have different causation but share several symptoms. Lower urinary tract symptoms (LUTS) constitute a group of clinical symptoms that can be caused by one of the diseases of the prostate or diseases of the bladder or urethra (Lepor, 2005). Typically, these symptoms include prolonged micturition, weakened and/or unsteady stream, the feeling of deficient bladder emptying, discomfort during urination, incontinence and increased urgency and frequency of urination.

1.1.3 Diagnostics and classification of prostate cancer

Symptoms and Prostate-specific antigen (PSA) testing
Roughly 95 – 99 % of all prostate cancers are adenocarcinomas (cancer that develops in glandular epithelium). Prostate cancer at an early stage is asymptomatic because the tumour is too small to interfere with adjacent tissues (McLaughlin et al., 2005). In the majority of cases the tumour never reaches the symptomatic size, and the patient dies of other causes without being aware of his prostate cancer. But in the cases where the cancer grows and occupies more volume, symptoms like back pain, blood in the urine, difficulties with and/or frequent urination may be experienced (Dasgupta and Kirby, 2011). If prostate cancer is suspected, the first step towards a diagnosis is to measure the level of prostate-specific antigen (PSA) in the blood. Normally, only a small quantity of PSA leaks into the circulatory system and increased levels of PSA can indicate prostate cancer or other disorders of the prostate. The level of PSA is elevated if it is higher than 4.0 ng/ml.

Digital rectal examination

Based on symptoms and/or a suspicious PSA test, the next step towards a diagnosis is a digital rectal examination (DRE) where the doctor palpates the size and shape of the prostate using a gloved finger. Most tumours are located in the peripheral zone of the gland, making it possible to discover abnormalities through a DRE if the tumour is large enough (Helsedirektoratet, 2015). If the doctor discovers lumps or abnormal areas under the examination, a prostate biopsy is the next step to confirm a prostate cancer diagnosis.

Prostate biopsy and Gleason grading

The Norwegian Directorate of Health recommends an ultrasound guided biopsy when prostate cancer is suspected (Helsedirektoratet, 2015). By this procedure transrectal ultrasound is used to guide a special needle to obtain 5 – 6 needle biopsies from each side of the gland in a grid-like pattern (Litwin and Tan, 2017). The biopsies are examined by a pathologist who evaluates whether a cancer is present and, eventually, evaluates its histological grade. Grading is done according to the system developed by the pathologist Donald F. Gleason in 1966 (Moch et al., 2016). Cancerous tissue is graded from 1 (most differentiated) to 5 (least differentiated) based on the growth pattern and the degree of glandular differentiation. Examples of Gleason grades are presented in Figure 1.4. The two most prevalent growth patterns (grade 1-5) in the tumour are added, and the result is defined as the Gleason score for the tumour. For a growth pattern, or grade, to be included, it needs to extend over more than 5 % of the tissue sample. Grade 1 and 2 are rarely or never used. Gleason scores from needle biopsies are usually between grade 6 - 10. As a rule, cancer with low Gleason score is less aggressive than one with a high Gleason score (Dasgupta and Kirby, 2011).
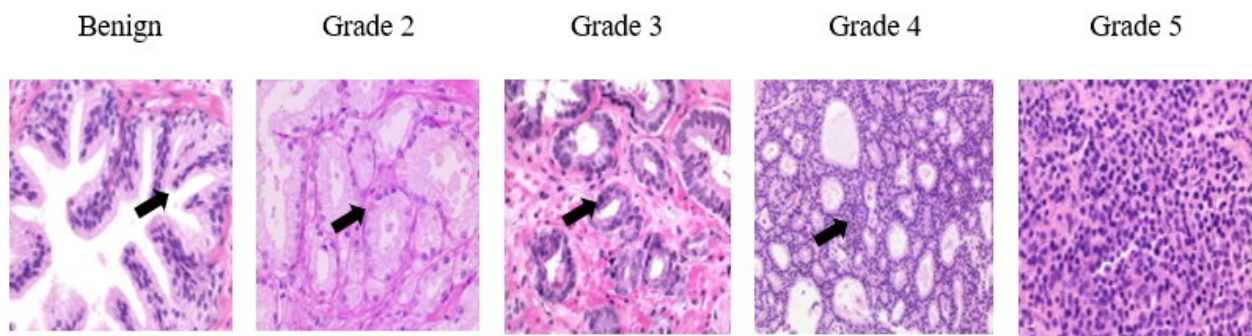
Figure 1.4: Histological appearance of prostate cancer, illustrating features associated with different Gleason grades (Ramnani, 2020). In a benign prostate, large glands with infoldings (arrow) are composed of a two-layered epithelium with small basal cells and secretory cells with a round nucleus and pale cytoplasm. Grade 1 resembles normal prostate tissue and is therefore not included in this figure. Grade 2 has larger glands with the infoldings not as easily observed. At grade 3, the glands tend to be round and smaller than normal and are seen to infiltrate among the normal glands. At grade 4 the glands have more irregular shapes and tend to coalesce into glandular complexes. Grade 5 is recognised by files or sheets of highly irregular cells, with no or few recognisable glands.

Tumour, Node, Metastasis (TNM) classification

To describe the extent of the prostate cancer, the Tumour, Node, Metastasis (TNM) classification system is used. As the name indicates, the system consists of T stage (local stage determination), N stage (lymph node status) and M stage (skeleton metastases) (Helsedirektoratet, 2015). The T stage, or primary tumour stage, is determined by DRE and ranges the primary tumour from T1 to T4 based on the extension of the tumour. At stage T1 and T2, the tumour is confined within the prostate, whereas at stage T3 and T4 the tumour extends through the capsule (T3) and invades nearby structures (T4). The N stage describes the lymph node status (whether the cancer has spread to lymph nodes). N0 means no spread to the lymph nodes, while the higher number after the letter N, the higher number of lymph nodes with cancer. The M stage describes whether metastasis is observed, in non-regional lymph nodes (M1a), bone (M1b) or other sites (M1c). M0 refers to no observed metastasis.

The examinations used to determine the status of these stages are supervised by a urologist and involve skeleton scintigraphy, magnetic resonance picture diagnostic (MRI) and radiography. Based on Gleason score, PSA level and T stage, the patient is diagnosed with low-risk or high-risk prostate cancer. In order for a patient to be diagnosed with low-risk prostate cancer, both Gleason score, PSA level and T stage must be within a defined area, as described in Table 1.2 (Helsedirektoratet, 2015).

Table 1.2: Classification of prostate cancer status

Risk	PSA	Gleason score	Clinical stage
Low	< 10 mg/dL	≤ 6	< T2a
Intermediate	> 10 < 20 mg/dL	= 7	T2b – T2c
High	> 20	8 – 10	> T3a

1.1.4 Treatment

Active surveillance

The standard treatment for patients diagnosed with low-risk prostate cancer is active surveillance. Active surveillance involves regular examinations where PSA-levels and tissue biopsy samples are closely monitored. The Norwegian Directorate of Health has a set of recommendations regarding the shift from active surveillance to active treatment. The recommendations state that if the PSA doubling time is less than 3 years, if a re-biopsy gives a Gleason score higher than 7 or if more than two biopsies are positive then active treatment should be considered (Helsedirektoratet, 2015). The patient's opinion needs to be addressed when considering the shift from active surveillance to active treatment. Often, when a patient is diagnosed with cancer, he wants treatment. In many cases an elderly patient will die of other causes than the prostate cancer, and active surveillance is often a far better alternative than invasive diagnosis and treatment.

Curative treatment

The curative treatment for localized prostate cancer includes radical prostatectomy (surgical removal of the prostate) and high-dose radiation therapy. Radiation therapy is often applied in combination with hormone therapy. The aim of the hormone therapy is to reduce the testosterone level in the blood. At an early stage of the disease, the cancerous cells depend on testosterone to grow, and the growth rate of the cancer can be reduced by reducing the level of testosterone. The risk profile of the cancer, together with the patient's age and general health condition, are the primary factors evaluated when deciding which treatment to use. Of the men diagnosed with prostate cancer in Norway in 2017, the patient group treated with radical prostatectomy had a median age of 66 years and an average PSA-value of 11 ng/mL (Kreftregisteret, 2019). The patient group treated with radiation therapy had a median age of 73 years and an average PSA-level of 18 ng/mL. In other words, younger men with low-risk prostate cancer are more frequently treated with radical prostatectomy, while high-dose radiation therapy is more often used to treat older men with high-risk prostate cancer.

Radical prostatectomy and radiation are curative treatments but are often associated with urine leak and sexual dysfunction. In some cases the side effects of prostate cancer treatment are so severe that they lead to loss of life quality (Stensvold et al., 2013).

1.2 Biomarkers

Biological markers, or biomarkers, have been used in medical practice for a long time. The general definition of a biomarker was established by the U.S Food and Drug Administration (FDA) and the National Institutes of Health (NIH) to be:

"A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention (FDA-NIH, 2016)."

Biomarkers can be used for cancer diagnosis and for evaluation of prognosis. A cancer biomarker is by The National Cancer Institute (NCI) Dictionary of cancer terms defined as:

"A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease." (NCI, 2020).

1.2.1 Prostate-Specific Antigen (PSA)

In 1987, PSA was introduced as a biomarker for prostate cancer in the USA (Collin et al., 2008). Since that time, PSA has been central in the process of diagnosing prostate cancer. PSA is an established biomarker for prostate cancer, but there are deficiencies associated with its use as a diagnostic test. The level of PSA may also be increased as a result of other diseases of the prostate, such as benign prostate hyperplasia (BHP) (Mettlin et al., 1993). Moreover, there are cases where patients with advanced prostate cancer have low PSA levels (Balk et al., 2003). Based on these insecurities there is a need for more precise ways to separate not only cancer from non-cancer, but also low-risk prostate cancer from the high-risk prostate cancer (Thompson et al., 2004). Overdiagnosis, that is when a patient is diagnosed with cancer not destined to cause harm, has been and still is a central topic associated with prostate cancer (Moynihan et al., 2012). For these patients, early detection and treatment will be unnecessary and cause more harm than good. A study by Rosario et al. showed that approximately one third of the men having prostate biopsies experienced issues that required clinical follow up, such as fever, pain, transient urinary difficulties, bleeding or infection (Rosario et al., 2012).

1.2.2 Alternative biomarkers for prostate cancer

The necessity of only treating patients who need treatment has led to a rapidly developing research field aiming to find new and improved biomarkers for prostate cancer. A part of the diagnostic process involves invasive biopsies so any new biomarker should be informative about the aggressivity of the cancer. This may help limit further examinations and treatment to the cases where it is required. Several biomarkers have been proposed, but only a few of these have so far been approved by the FDA.

Among the alternative biomarkers for prostate cancer, PSA derivatives such as Prostate health index (PHI), four-kallikrein panel and PSA glycoforms have been explored (Filella and Gimenez, 2012). The PHI blood test combines three isoforms of PSA in the mathematical formula (p2PSA/fPSA)*√totaltPSA to detect prostate cancer (Lepor et al., 2016). The majority of circulating PSA in the blood is bound to protease inhibitors. The unbound fraction, called free PSA (fPSA) exists in several isoforms, such as benign PSA, intact PSA (iPSA) and proPSA. The most stable form of proPSA, [-2]proPSA, can be detected using automated immunoassays, called p2PSA. The PHI test was approved by

FDA in 2012. There are few published studies about the PHI test in use, but the studies that are published show that when including the PHI test in the assessment, fewer men are biopsied (White et al., 2018).

Another FDA-approved molecular biomarker is the Progensa Prostate Cancer Antigen 3 (PCA3) test that detects PCA3 messengerRNA (mRNA) in urine (Merola et al., 2015). Normally, only a small amount of this mRNA is present in urine, but in prostate cancer it is increased (60-100 times). Studies have shown promising results when it comes to applying the PCA3 test in the diagnostic process, but there is still a need to establish optimal cut-off values and to prove a correlation between PCA3 and tumour aggressiveness before the test can be used in a clinical setting. Like the other suggested biomarkers, also the PCA3 test seem to be best suited as a secondary test used to determine whether or not a biopsy should be performed, and not as a diagnostic test itself (Kretschmer and Tilki, 2017).

The enumeration of circulating tumour cells (CTCs) is another alternative biomarker. As the name implies, CTCs are cancer cells that are malignant in origin. These cells circulate in the blood stream and through extravasation they may serve as seeds for metastases in distant organs. Studies have shown that the number of CTCs can be used as a prognosticator of overall survival in patients with metastatic castration-resistant prostate cancer (cancer that continues to grow despite that the testosterone level has been reduced to low levels). It remains to determine the relationship between CTCs and PSA and their prognostic utility in the diagnostic process (de Bono et al., 2008). The methods used to analyse CTCs in the blood can be divided into two types. Biological methods that use highly specific antigen binding for positive selection of these cells and filter-based physical methods that capture the cells based on their size. The challenge with using the number of CTCs in the blood as a prognostic marker is that they are relatively infrequent and that the detection techniques are expensive and time consuming (Saini, 2016).

1.3   MicroRNA as biomarker for prostate cancer

1.3.1 MicroRNA

MicroRNAs (miRNAs) are frequently mentioned as alternative biomarkers for prostate cancer. These short (~22 nucleotides) non-coding double stranded RNA molecules are thought to regulate at least one third of the protein-coding genes in the human genome (Gregory and Shiekhattar, 2005). They inhibit protein synthesis by blocking the translation of mRNA. The miRNA biosynthesis, illustrated by Figure 1.5 starts with primary miRNA (pri-miRNA) transcribed from chromosomal genes (Clark and Pazdernik, 2013). The double stranded stem-region of the pri-miRNA is cut by the enzyme Drosha, resulting in a hairpin-formed precursor miRNA (pre-miRNA). Pre-miRNA is translocated from the nucleus to the cytoplasm where it is trimmed by the nuclease Dicer, forming mature miRNA. Together with a set of proteins one of the strands of the miRNA is assembled into an RNA-induced silencing complex (RISC). The RISC searches for target mRNA with complementary nucleotide sequences to the miRNA strand. If the degree of complementarity between the miRNA and the mRNA is high enough the mRNA is cut and degraded. A partial sequence match between the miRNA strand and a target mRNA can lead to translational repression of the mRNA. Based on which mRNA they act upon, the regulatory role of miRNAs has been connected to biological processes like cellular differentiation, cellular division and apoptosis (Wach et al., 2012).
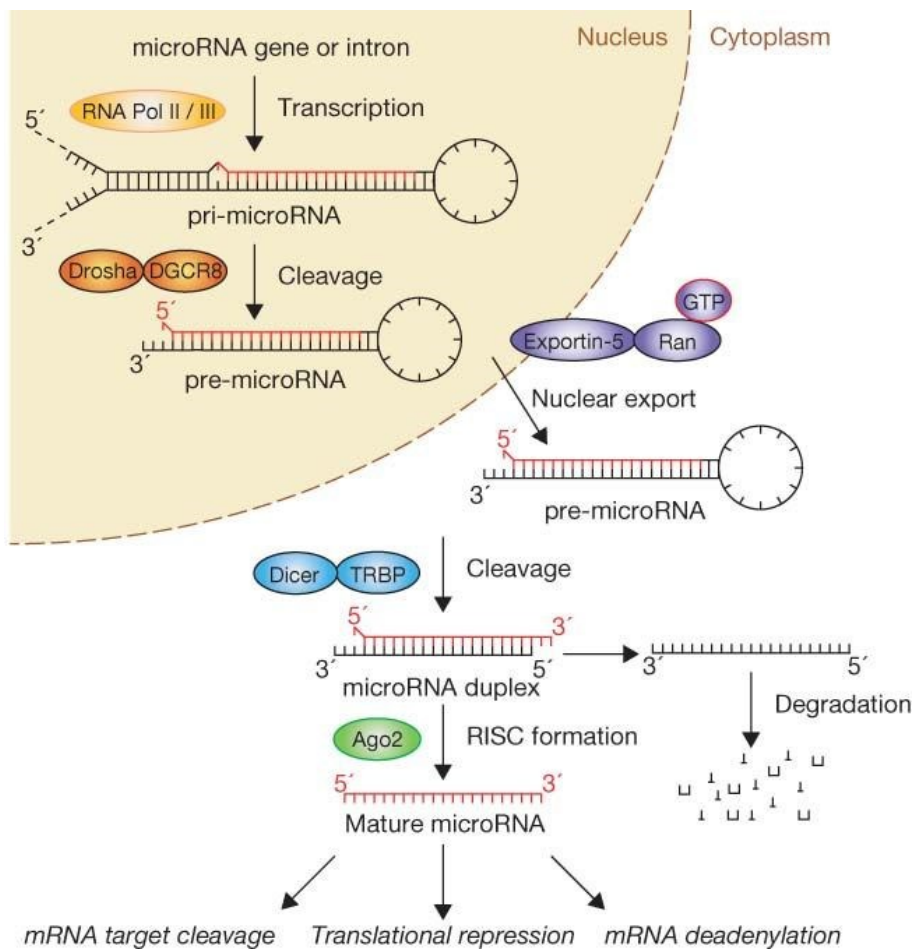
Figure 1.5: The biogenesis and function of miRNA (Winter et al., 2009). The synthesis of miRNA consists of several steps. First, the miRNA is transcribed by polymerase II in the nucleus as pri-miRNA. The pri-miRNA is processed by Drosha, a RNase III endonuclease, resulting in a hairpin-formed pre-miRNA. This pre-miRNA is translocated to the cytoplasm where it is further processed, resulting in a premature, double-stranded miRNA-miRNA complex (Winter and Diederichs, 2011). The mature miRNA strand in this complex becomes a part of the RNA-inducing silencing complex (RISC), where it is responsible for recognizing and binding complementary mRNA. Whether the mRNA is degraded or repressed depends on the degree of complementarity between the miRNA and mRNA.

1.3.2 MicroRNA and cancer

The role of miRNAs in cancer is connected to their regulation of two central types of genes: proto-oncogenes and tumour suppressor genes. Proto-oncogenes are genes that normally contribute to cellular growth and division. If mutations transform a proto-oncogene to its overactive form, an oncogene, the cell will start to divide in an uncontrolled fashion, a classical feature of a cancer cell. Tumour suppressor genes are genes that normally regulate cell growth and proliferation. If one or several mutations reduce the function of a tumour suppressor gene, it may result in uncontrolled growth and cancer. One of the best known tumour suppressor genes, p53, is mutated in more than 50 % of all known cancer types (Mello and Attardi, 2018). Because of their central role in controlling gene expression, it should come as no surprise that miRNAs often are found to be dysregulated in cancer. A study by Calin et al. was among the first studies pointing at the connection between miRNAs and cancer. The study demonstrated that in 68 % of chronic lymphatic leukaemia (CLL) cases, miR15 and miR16 were located in a 30 kb deletion (Calin et al., 2002). Several studies show that an abnormal expression of various miRNAs can be connected to several types of cancers, including prostate cancer (Filella and Foj, 2017). An example is the study by Cochetti et al. that found miR-25-3p to be downregulated in prostate cancer, suggesting that it has a tumour suppressor role (Cochetti et al., 2016a). A second miRNA connected to a cancerous pathway is let-7b-5p. It is downregulated in prostate cancer and is proposed to act as a tumour suppressor targeting the regulatory factors RAS and MYC, which are involved in tumorigenesis (Moustafa et al., 2017).

1.3.3 MicroRNA as a biomarker

There are several reasons why miRNAs may be suitable biomarkers for cancer. They are present in a variety of biological samples and the methods used for detection are rapid, and inexpensive. Research shows that miRNA profiles can be informative regarding the type of cancer, differentiation stage and the cancer status (Lu et al., 2005). miRNAs are relatively stable in clinical samples like serum and urine and they are easily detectable with polymerase chain reaction (PCR)-based methods (Filella et al., 2018). In addition, studies have demonstrated that miRNA profiles may be different in cancerous tissues compared with healthy tissues. The difference in expression of several circulating miRNAs, like for example miR-324-5p, has been shown to separate prostate cancer from other diseases of the prostate, like BPH (Jin et al., 2019). Two of the most promising possible biomarkers for prostate cancer are miR-375 and miR-141-3p. Several studies have shown miR-375 to be upregulated in prostate cancer compared to healthy controls and patients diagnosed with BPH (Gao et al., 2016). Also, a study by Wach et al. showed that the expression of miR-375 was higher in high-risk prostate cancer (T3-T4) than in low-risk prostate cancer (T1-T2) (Wach et al., 2015a). A study by Porzycki et al included a population of 20 men diagnosed with prostate cancer and found the expression of miR-141-3p higher in these patients than in the control group (Porzycki et al., 2018).

2. Aim of the study

Due to the deficiencies associated with the use of PSA as a diagnostic test for prostate cancer, there is a pressing need for more precise biomarkers. There are many steps before a potential new biomarker can be used in the diagnostic process, such as determining its ability to discriminate between cancer and healthy controls, its reproducibility and proving that it can be easily detected and analysed by methods applicable in clinical practice.

The background for this project is a previous project that by Next Generation Sequencing (NGS) found a significant change in expression of certain miRNA in pre- compared to post-operative serum samples from prostate cancer patients. Before one of these miRNAs can be considered as a new biomarker for detecting prostate cancer, there is a need to validate these results by a faster and cheaper method like RT-qPCR.

The main aim of this project is to validate the change in miRNA expression following treatment and to generate knowledge about some of the miRNAs that may serve as alternative biomarkers for prostate cancer. The research goals to fulfil this aim will be reflected in the presented work and organisation of the thesis paper. These are as follows:

- Perform a literature search in order to explore miRNAs suggested as biomarkers.
- Set up a study design based on NGS results and the literature search.
- Perform a RT-qPCR validation of the differential expression of circulating miRNAs in pre- compared to post-operative serum samples from prostate cancer patients.

3. Materials and methods

3.1 Workflow

This master thesis is a part of a larger project with its workflow presented in Figure 3.1. The work described in this thesis began in step 6 with receiving results from Next Generation Sequencing (NGS). Together with the results from a literature study, the NGS data were used to select six putatively relevant miRNA targets for RT-qPCR verification. The study design process (step 6) also included the selection of suitable reference miRNA and RT-qPCR plate design.

Figure 3.1: Workflow for the whole project. This master thesis started with receiving results from the NGS project.

The master thesis work can be divided into two parts as follows:

1) a theoretical part consisting of setting up a study design based on the results from NGS and a literature study.

2) an experimental part consisting of the validation of the change in expression for six chosen miRNAs in pre- compared to post-operative serum samples by RT-qPCR and analysis of these results.

3.2 Clinical samples

This thesis was a part of a larger project approved by the Regional Committees for Medical and Health Research Ethics (REK 2014/1487). A written informed consent was acquired from all patients.

A total of 50 serum samples from 25 prostate cancer patients were provided by Biobank1. Each patient donated one pre-operative serum sample and one post-operative serum sample taken three months after the patient had a radical prostatectomy. As presented in Table 3.1, the age of the patients was within the range 51 – 78 years, with a mean age of 67 years. All patients were diagnosed with a Gleason score 7 tumour. The PSA levels before surgery varied from 4.55 – 45.9 µg/L.

Table 3.1: Clinical information of the cohort. The cohort consisted of 25 patients, each donating one pre-operative sample and one post-operative sample. The age of the patients was between 51 – 78 years and the mean age was 67 years. All patients were diagnosed with a Gleason score 7 tumour. PSA before surgery varied from 4.55 µg/L to 45.9 µg/L.

Patient sample number	Age	Gleason score	PSA before surgery (µg/L)	PSA after surgery (µg/L)
1	64	7 (3+4)	4.55	< 0.06
2	65	7 (3+4)	11.2	< 0.06
3	67	7 (3+4)	9.43	< 0.06
4	51	7 (4+3)	43	< 0.06
5	73	7 (3+4)	6.6	< 0.06
6	68	7 (3+4)	45.9	< 0.06
7	56	7 (3+4)	7.8	< 0.06
8	65	7 (4+3)	15.8	<0.6
9	69	7 (4+3)	23.8	0.0
10	76	7 (4+3)	16.7	< 0.06
11	72	7 (3+4)	9.2	< 0.06
12	66	7 (4+3)	10.9	0.08
13	67	7 (4+3)	7.3	< 0.06
14	68	7 (3+4)	5.6	< 0.06
15	74	7 (3+4)	7.8	< 0.06
16	69	7 (3+4)	5.1	0.0
17	70	7 (3+4)	10.6	< 0.06
18	62	7 (3+4)	8.2	< 0.06
19	78	7 (4+3)	9.1	< 0.06
20	74	7 (4+3)	34.8	< 0.1
21	73	7 (3+4)	26.5	< 0.06
22	52	7 (4+3)	40.9	< 0.06
23	69	7 (3+4)	5.1	< 0.06
24	66	7 (3+4)	11.7	< 0.06
25	65	7 (3+4)	6.7	< 0.1

3.3 Blood sample processing

Blood samples from the patients were processed within 2 hours after collection. The blood was collected in serum gel tubes (5 mL). After blood collection the serum tube was turned five times and placed on the bench for 30 minutes before centrifugation (2200g, 10 minutes). Following centrifugation, the serum was aliquoted in 0.5 ml cryotubes. Samples were stored until further use at - 80 °C in a freezer with temperature monitoring.
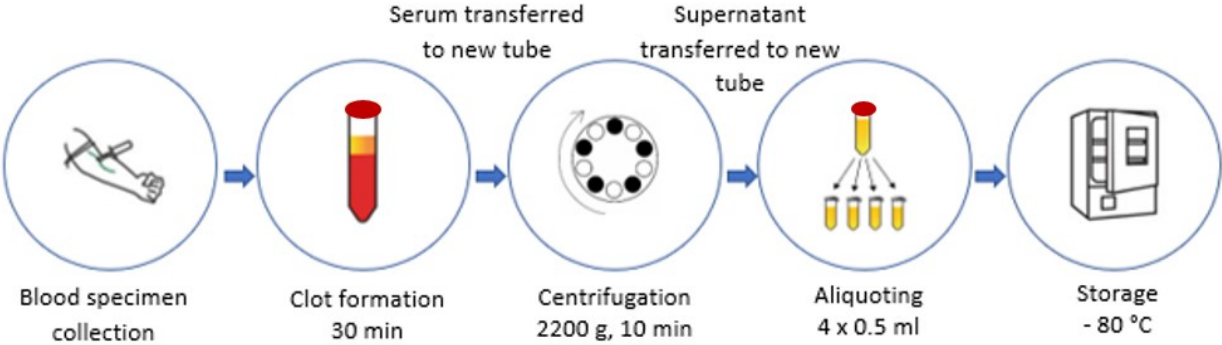
Figure 3.2: Blood sample processing

3.4 RNA isolation and Next Generation Sequencing

Serum samples were transported on dry ice overnight to Qiagen Genomic Services in Denmark where RNA isolation and NGS were conducted. The RNA was isolated using miRNeasy Serum/Plasma Kit from QIAGEN according to manufacturer's instructions. The isolation protocol resulted in 14 µl RNA.

3.4.1 Next generation sequencing

NGS enables the sequencing of large quantities of DNA, RNA or small RNAs like miRNA. NGS can be divided into three steps: library preparation, sequencing, and data analysis. The library preparation for NGS was done using QIAseq miRNA Library Kit from Qiagen. 5 µl total RNA was used to create miRNA NGS libraries. Adapters containing unique molecular identifiers (UMIs) were ligated to the RNA before the RNA was reverse transcribed to cDNA. PCR (22 cycles) was used to amplify the cDNA. Quality control of the library preparation was performed using Bioanalyzer 2100 (Agilent). The libraries were pooled in equimolar ratios and qPCR was used for quantification of the library pools. The sequencing was done on a NextSeq500 sequencing instrument according to the manufacturer's instruction. Raw data was de-multiplexed (connecting samples to sequences by using the barcode information) before bcl2fastq software (Illumina inc.) was used to generate FASTQ files (text files with sequence data) for each sample.

3.5 Literature study and selection of miRNA

3.5.1 Literature study

A literature study was conducted in order to explore miRNAs suggested as biomarker for prostate cancer and for choosing relevant targets for the validation of NGS by RT-qPCR. The literature collection databases Pubmed and Google Scholar were used in search of primary and secondary literature. The online database search terms used were *(((("prostatic neoplasms" OR "prostate cancer" OR "prostate cancers")) OR "Prostatic Neoplasms"[Mesh])) AND (microrna OR micrornas OR mirna OR mirnas OR micrornas[Mesh]).*
The search included the following areas of research: medical biochemistry, biotechnology, molecular biology.

3.5.2 Selection of miRNA

The strategy for selecting relevant targets for the validation of NGS data by RT-qPCR was to: 1) use the literature search to find miRNAs suggested as biomarkers for prostate cancer and 2) compare these results with the results from NGS.

For a miRNA to be considered as a target, the potential miRNA had to fulfil the following criteria:
- The miRNA was represented in the literature.
- The miRNA showed a significant change in expression from pre-operative to post-operative sample.
- The statistics supported the selection:
    - Trimmed mean of M-values (TMM) should be similar within each group.
    - The miRNA should have a relatively high expression.

Following this, a secondary literature search was conducted for each possible miRNA candidate, using, with miR-25-3p as an example, the search string: ""*prostate cancer" AND miRNA AND biomarker AND mir-25-3p".*

3.6 Quantitative Reverse Transcriptase PCR

Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) enables the quantification of miRNA by combining the conversion of RNA to cDNA by reverse transcriptase with amplification of the newly synthesized cDNA by PCR methodology. The raw data generated from RT-qPCR is Ct (threshold cycle) values. Ct is the intersection between an amplification curve and a threshold line and serves as a relative measure of the concentration of the target in the PCR reaction.

The PCR step consists of repetitive cycles with each cycle consisting of three steps:
Denaturation: a high-temperature step resulting in the separation of the double-stranded cDNA molecule. Denaturation is necessary to facilitate the annealing of the primers to the single strands in the next step.
Annealing: By lowering the temperature the primers are able to bind to their complementary strands. The primers provide the 3' OH group necessary for extension of the primer by DNA polymerase in the next step.
Extension: DNA polymerases extend the primers that are covalently attached to the single strands of the denatured cDNA molecule (Farrell, 2010).

Isolated RNA was exposed to reverse transcriptase in order to yield cDNA using the miRCURY LNA RT kit according to the manufacturer's instructions (Qiagen). RT-qPCR was performed using the miRCURY LNA miRNA Custom PCR Panel protocol according to the manufacturer's instructions (Qiagen). Reaction volumes of 10 µl were added to PCR plates pre-coated with commercial pre-validated primer assays and run in a StepOnePlus instrument according to the PCR cycling conditions in Table 3.3. The project set up was 2 cDNA reactions per sample and 2 PCR replicates per cDNA.

Table 3.3: PCR cycling conditions

Step	Time	Temperature
PCR initial heat activation	2 min	95 °C
2-step cycling:		
Denaturation	10 s	95 °C
Combined annealing/extension	60 s	56 °C
Number of cycles	40	
Melting curve analysis		60 – 95 °C

3.7 Quality control

Spike ins

Working with RNA may be challenging because the samples can contain inhibitors of cDNA synthesis or PCR amplification, despite using best standard procedures when isolating the RNA. These inhibitors can lead to variable efficiencies of cDNA synthesis or PCR amplification between samples. To check for differences in RNA isolation, cDNA synthesis and PCR amplification, known amounts of RNA transcripts with known sequence, called RNA spike-ins, are added to the sample prior to RNA isolation and cDNA synthesis. This makes it possible to calibrate the measurements obtained from the experimental procedures by comparing the wells containing the RNA spike-ins. The RNA isolation was accomplished by Qiagen, hence also the addition of the spike-ins used to control the RNA isolation (UniSp2, UniSp4, UniSp5). UniSP6 RNA spike-in was added during the reverse transcription reaction to give the possibility to evaluate the cDNA synthesis and UniSP3 was used to control the qPCR.

Reference miRNA

The purpose of including a reference gene, or housekeeping gene, in a PCR reaction is to be able to normalize the expression of the target gene to the expression of one or more stably expressed genes simultaneously measured in the same sample. The reference gene should show minimal change in expression levels despite different samples or experimental conditions (Schaefer et al., 2010). Based on the NGS results, four of the most stably expressed miRNAs across all samples were selected as reference miRNAs for normalization.

Non-template control

RNase-free water was used as non-template control with the purpose of detecting contamination. No signal indicates good laboratory practices and no contamination. The non-template control plate was the last plate analysed.

Melting curve analysis and flags

After PCR, melting curve analysis was performed. This method works by warming the samples containing many copies of the cDNA from 60 – 95 °C. As the sample is heated, the strands in the double-stranded cDNA copies will start to separate. The point where 50 % of the cDNA is separated, is called the melting temperature. By using a fluorescence dye, this melting process can be monitored. The fluorescent dye binds to double-stranded DNA molecules, and when bound it fluoresces brightly. As the sample is heated up, more and more double-stranded molecules separate and the degree of fluorescence decreases. The degree of fluorescence is plotted against the temperature, which results in characteristic melting plots for each sample. A melting plot with more than one peak indicates that several products have been amplified. Reactions with amplification efficiency below 1.6 and samples with more than one peak were removed.

3.8 Statistical analyses

Ct values and melting points were exported from the StepOnePlus instrument and pre-processed by using Excel. The online analysis tool GeneGlobe was planned to use for the data analysis. Because the experimental work was delayed due to Covid-19 and because the plate design made it challenging to upload the raw data to GeneGlobe, the main analysis was performed by Qiagen services. The Ct values of the technical replicates were averaged. The statistics were generated using R by Qiagen services. The plots presented in this paper were made by the author using Excel.

4.   Results

This master thesis includes the study of the change in expression of circulating miRNA isolated from pre- and post-operative serum samples from 25 prostate cancer patients. This chapter will present individual results for each miRNA target, in addition to results for the cohort.

4.1 Literature study

The extensive research on miRNA as potential biomarkers for prostate cancer has resulted in a variety of miRNAs found to be dysregulated in prostate cancer. The literature search resulted in the candidates presented in Table 4.1. These are among the miRNAs most frequently mentioned as potential biomarkers for prostate cancer. Of these miRNAs, six were selected for validation based on their appearance in the literature in combination with the NGS results. The literature found on these six miRNAs, miR-25-3p, let-7b-5p, miR-375, miR-18a-3p, miR-324-5p and miR-141-3p is described below.

Table 4.1: Some of the most frequently explored circulating miRNAs related to prostate cancer (left column), their implication in prostate cancer (middle column) and the references (right column).

miRNA	Dysregulation found in relation to prostate cancer	Reference
let-7a	Downregulated	(Kelly et al., 2015)
let-7b	Downregulated	(Zedan et al., 2018)
let-7c	Downregulated	(Cochetti et al., 2016b)
miR-18a-3p	Upregulated	(Ibrahim et al., 2019)
miR-21	Upregulated	(Ibrahim et al., 2019) (Gao et al., 2016)
miR-25-3p	Downregulated	(Cochetti et al., 2016b) (Srivastava et al., 2014)
miR-26	Downregulated	(Cochetti et al., 2016b)
miR-27a-3p	Upregulated	(Lyu et al., 2019)
miR-34a	Downregulated	(Zedan et al., 2018)
miR-93	Upregulated	(Zedan et al., 2018)
miR-103a	Upregulated	(Mello-Grand et al., 2019)
miR-125b	Upregulated	(Zedan et al., 2018)
miR-141	Upregulated	(Ibrahim et al., 2019) (Kelly et al., 2015)
miR-145	Upregulated	(Kelly et al., 2015)
miR-148a	Upregulated	(Paunescu et al., 2019) (Dybos et al., 2018)
miR-150-5p	Downregulated	(Paunescu et al., 2019)
miR-155	Upregulated	(Kelly et al., 2015)
miR-221	Upregulated	(Ibrahim et al., 2019)
miR-324	Upregulated	(Jin et al., 2019)
miR-375	Upregulated	(Gao et al., 2016) (Wach et al., 2015b) (Haldrup et al., 2014)
miR-424	Downregulated	(Suer et al., 2019)
miR-572	Upregulated	(Suer et al., 2019)
miR-628-5p	Downregulated	(Srivastava et al., 2014)

4.1.1 let-7b-5p

The let-7 family consists of thirteen miRNAs encoded in a highly conserved region of the genome. The five publications presented in Table 4.2 reported downregulation of circulating let-7 (let-7b, let-7a, let-7c and let-7e) in blood samples from prostate cancer patients when compared to healthy controls or patients with BPH.

Table 4.2: Summary of literature search results related to circulating members of the let-7 family and prostate cancer

Type of sample	Method	Regulation of let-7	Reference
Plasma, tissue	RT-qPCR	The study showed let-7b in plasma to be downregulated in metastatic prostate cancer patients (n=21) compared to healthy controls (n=25).	(Zedan et al., 2018)
Plasma	GeneChip microarray	The study showed let-7b and let-7c to be downregulated in prostate cancer patients (n=152) compared to BPH patients (n=40)	(Knyazev et al., 2016)
Plasma	GeneChip microarray and RT-qPCR	The study showed let-7c and let-7e to be downregulated in plasma from prostate cancer patients (n=80) compared to healthy controls (n=54).	(Chen et al., 2012)

4.1.2 miR-18a-3p

Dysregulation of miR-18a-3p has been associated with prostate cancer as well as other types of cancers (Wang et al., 2013). A study by Ibrahim et al. found miR-18a-3p to be upregulated in prostate cancer patients compared to healthy controls and BPH patients.

Table 4.3: Summary of literature search results on circulating miR-18a-3p and prostate cancer

Type of sample	Method	Regulation of miR-18a-3p	Reference
Plasma	RT-qPCR	The study showed a significant difference in miR-18a-3p expression when plasma from prostate cancer patients was compared with plasma from healthy controls and BPH patients.	(Ibrahim et al., 2019)

4.1.3 miR-25-3p

Dysregulation of miR-25-3p has been connected to prostate cancer. The two publications presented in Table 4.4 found miR-25-3p to be downregulated in serum from prostate cancer patients compared to healthy controls.

Table 4.4: Summary of literature search results on circulating miR-25 and prostate cancer

Type of sample	Method	Regulation of miR-25	Reference
Serum	RT-qPCR	The study showed miR-25-3p to be downregulated in prostate cancer patients (n=64) compared to BPH patients (n=60). It was also found that decreased expression of miR-25-3p was associated with an increase in malignancy.	(Cochetti et al., 2016a)
Serum	RT-qPCR	The study showed miR-25-3p to be downregulated in serum from prostate cancer patients (n=40) compared to healthy controls (n=32).	(Srivastava et al., 2014)

4.1.4 miR-141

The five studies presented in Table 4.5 confirm the upregulation of miR-141 in prostate cancer compared to BPH patients and healthy control group. High expression of miR-141 was also associated with metastatic prostate cancer.

Table 4.5: Summary of literature search results on circulating miR-141 and prostate cancer

Type of sample	Method	Regulation of miR-141	Reference
Whole blood	RT-qPCR	The study showed miR-141 to be upregulated in prostate cancer patients (n=75) compared to BPH-diagnosed patients (n=27).	(Kelly et al., 2015)
Plasma-derived circulating microvesicles	RT-qPCR	The study showed miR-141 to be upregulated in prostate cancer patients (n=78) compared to healthy controls (n=28).	(Bryant et al., 2012)
Serum, tissue	RT-qPCR	The study showed miR-141 to be upregulated in tumour tissue (n=36) compared to benign tissue (n=36) and upregulated in serum samples from patients with high-grade tumours compared to intermediate or low-grade tumours.	(Brase et al., 2011)
Serum, tissue	RT-qPCR	The study showed miR-141 to be upregulated in serum from prostate cancer patients (n=25) compared to healthy controls (n=25).	(Cheng et al., 2013)
Plasma	RT-qPCR	The study showed miR-141 to be upregulated in metastatic prostate cancer (n=30) compared to localized prostate cancer (n=50).	(Ibrahim et al., 2019)

4.1.5 miR-324

Dysregulation of miR-324 in relation to cancer is frequently reported in the literature. One paper is included in Table 4.6. Several publications have connected miR-324 to various types of cancer, but this was the only one connecting it to prostate cancer. The study found miR-324 to be upregulated in prostate cancer patients compared to healthy controls.

Table 4.6: Summary of literature search results on circulating miR-324 and prostate cancer

Type of sample	Method	Regulation of miR-324	Reference
Serum	RT-qPCR	The study showed the expression of miR-324 to be significantly higher in serum from prostate cancer patients than in serum from healthy controls and patients diagnosed with BPH. The study also showed that the level of circulating miR-324 was associated with Gleason score and tumour stage.	(Jin et al., 2019)

4.1.6 miR-375

miR-375 in relation to prostate cancer is one of the most frequently reported miRNAs in the literature. Increased levels of circulating miR-375 is well-documented in prostate cancer patients, as referred to in the six presented publications in Table 4.7.

Table 4.7: Summary of literature search results on circulating miR-375 and prostate cancer

Type of sample	Method	Regulation of miR-375	Reference
Plasma	RT-qPCR	The study showed miR-375 to be significantly higher in the prostate cancer group (n=57) than in the BPH group (n=28).	(Gao et al., 2016)
Serum	RT-qPCR	The study showed miR-375 to be upregulated in prostate cancer patients compared to BPH patients and healthy controls.	(Wach et al., 2015a)
Serum	RT-qPCR	The study showed miR-375 to be upregulated in prostate cancer patients (n=31) compared to patients diagnosed with BPH (n=13).	(Haldrup et al., 2014)
Plasma	RT-qPCR	The study showed miR-375 to be upregulated in plasma from prostate cancer patients (n=50) compared to patients diagnosed with BPH (n=22).	(Endzeliņš et al., 2017)
Serum, tissue	RT-qPCR	This study showed mir-375 to be upregulated in patients with metastatic prostate cancer (n=7) compared to localized prostate cancer (n=14). miR-375 was also found upregulated in tumour tissue compared to benign tissue	(Brase et al., 2011)
Serum, tissue	RT-qPCR	miR-375 was found upregulated in prostate cancer patients (n=25) compared to healthy controls (n=25).	(Cheng et al., 2013)

4.2 Study design

When planning a validation project like this, there are several important issues that need to be addressed. Selecting relevant targets, selecting stably expressed reference miRNAs for normalization, the plate setup and number of replicates are some of these. The choices made regarding these issues will be discussed in Chapter 5.

The cDNA reaction is probably the most sensitive step in the experiment. The cDNA reaction was run in two replicates for each pre- and post-operative sample. The cDNA reactions for all samples from all patients were run in the same day. The PCR setup was organized in two replicates per cDNA. A total of four PCR-datapoints per pre- and post-operative sample were obtained for all of the 25 patients. Custom Pick&Mix PCR plates pre-coated with primer assays were chosen to minimalize technical errors. The instrument used was a StepOnePlus capable of running 96 well plates. By including four reference miRNAs, six targets, UniSP6 for cDNA synthesis control and UniSP3 as qPCR control, all samples from the same patient were fitted into the same PCR plate with two cDNA reactions per pre- and post-operative sample and two PCR replicates per cDNA. The resulting plate design is presented in Appendix D.

Targets

The six selected targets were chosen based on their occurrence in the literature in addition to the results of our NGS-study, as presented in Table 4.8.

Table 4.8: Log fold change (LogFC) describing the change in expression between pre- and post-operative samples, the normalization method average Trimmed mean of M (TMM) values, p-values and Benjamini-Hochberg false discovery rate (FDR) corrected p-values for the six selected miRNA targets. The criteria used for selection were: TMM values > 15, FDR < 0.05 and minimal variance within each group (pre and post).

miRNA	LogFC	Pre TMM	Post TMM	p-value	FDR
let-7b-5p	0.50	41431	58767	< 0.0001	0.0022
mir-18a-3p	0.69	39	65	0.0005	0.0207
miR-25-3p	0.73	13844	22978	< 0.0001	0.0004
miR-141-3p	-0.34	17	15	0.1483	0.4964
miR-324-5p	0.66	242	382	0.0001	0.0054
mir-375	-0.57	265	201	0.0083	0.1119

References

Because the project was based on data from a previous NGS project, reference miRNAs were selected from the ones that by NGS were found to be most stably expressed in the samples. Several reference miRNAs are ideally included in a validation project to make sure that at least one can be used for normalization in case some references fail to give results, or if some show too much variation in expression across samples. The targets selected were miR-30e-5p, miR-320a, let-7i-5p and miR-425-5p. Table 4.9 shows the statistical values obtained from NGS data for the four selected reference miRNAs. Low stability values indicate good stability across samples.

Table 4.9: Stability/TPM_AVG ratio, Stability, the stability measure as calculated by Normfinder and TPM average, an estimate of the abundance of the miRNA across the two groups for the four selected reference miRNAs. Lower values of (Stability/TPM_AVG) ratio indicates good stability across all samples.

miRNA	Stability/TPM_AVG	Stability	TPM average
let-7i-5p	0.23	1424	6154
miR-30e-5p	0.13	274	2131
miR-320a	0.22	437	1979
miR-425-5p	0.24	748	3105

4.3 Quality control

4.3.1 Melting curve analysis

A melting curve analysis was performed in order to evaluate the specificity of the amplification products. A single peak in the melting curve indicates that a single product was amplified during the PCR. For the PCR reactions that gave rise to multiple peaks, these were flagged and removed from the dataset.
Nine plates did not give result for both pre- and post-operative samples and were omitted from the data analysis. The remaining plates had single peaks in the melting curves and few flags, indicating good sample quality.

4.3.2 Sample quality control using spike-ins

Two types of spike-ins were used, UniSp6 for cDNA synthesis control and UniSp3 for qPCR control. Both spike-ins indicated that the RT-reaction and qPCR were successful. Figure 4.1 show the raw Ct-values obtained for the control assay UniSp6 and Figure 4.2 the raw Ct-values obtained for the control assay UniSp3. Raw Ct-values for UniSp6 were in the range 18.35 to 19.93 and raw Ct-values for UniSp3 were in the range 18.28 to 20.97.
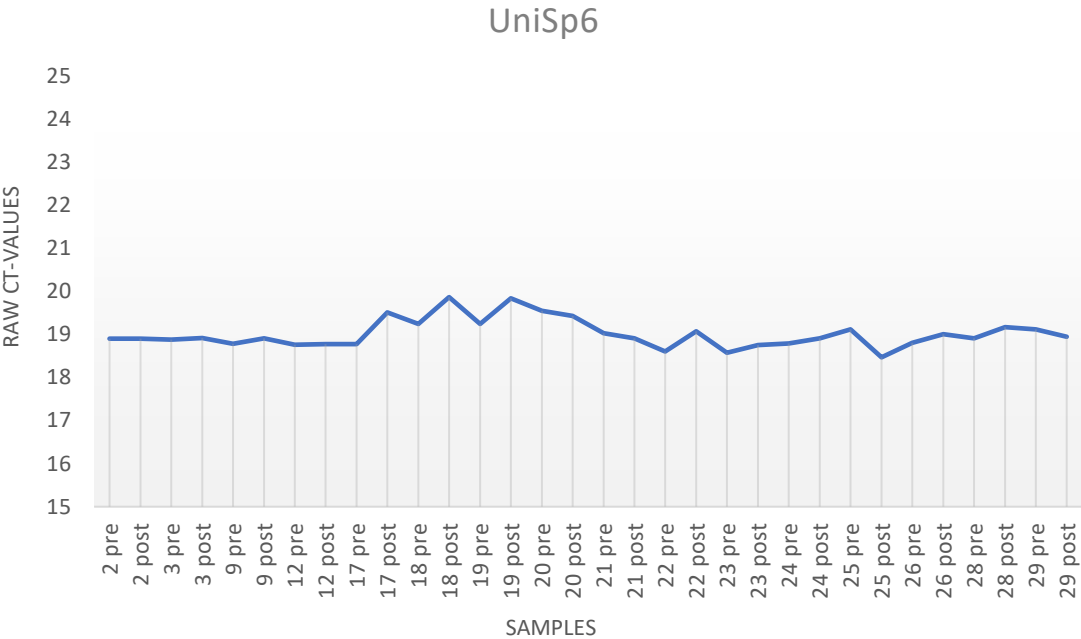
Figure 4.1: Raw Ct-values obtained for the control assay UniSp6
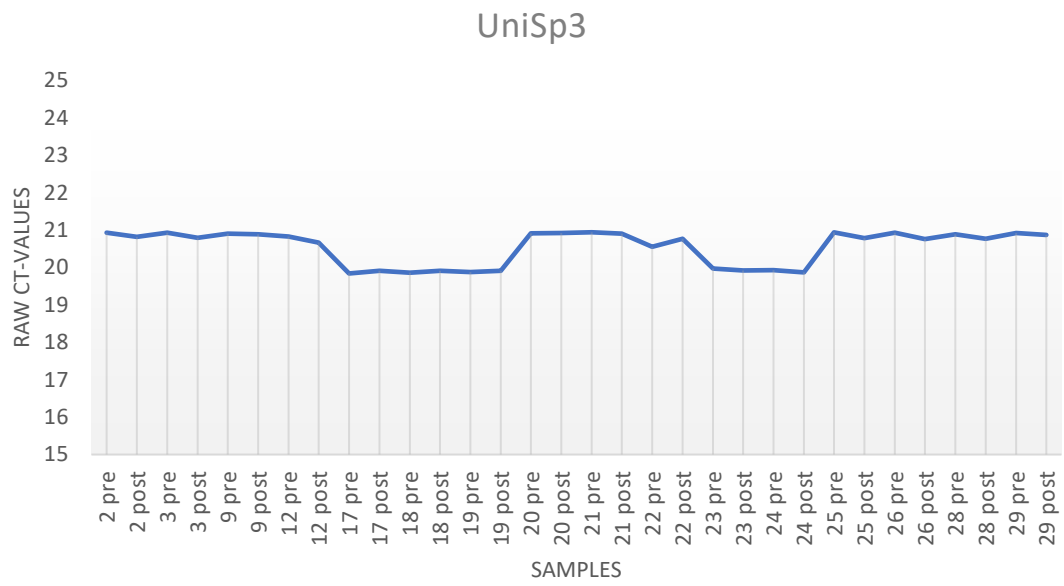
Figure 4.2: Raw Ct-values obtained for the control assay UniSp3

4.3.3 Signal from negative controls

In the last plate all wells contained "no-template" samples. The purpose of this plate was to detect any RNA contamination in the RT step. Assays detected 5 Ct lower than the "no-template" control are included in the analysis. There was no signal in any of the wells, except in the wells pre-coated with the control UniSp3.
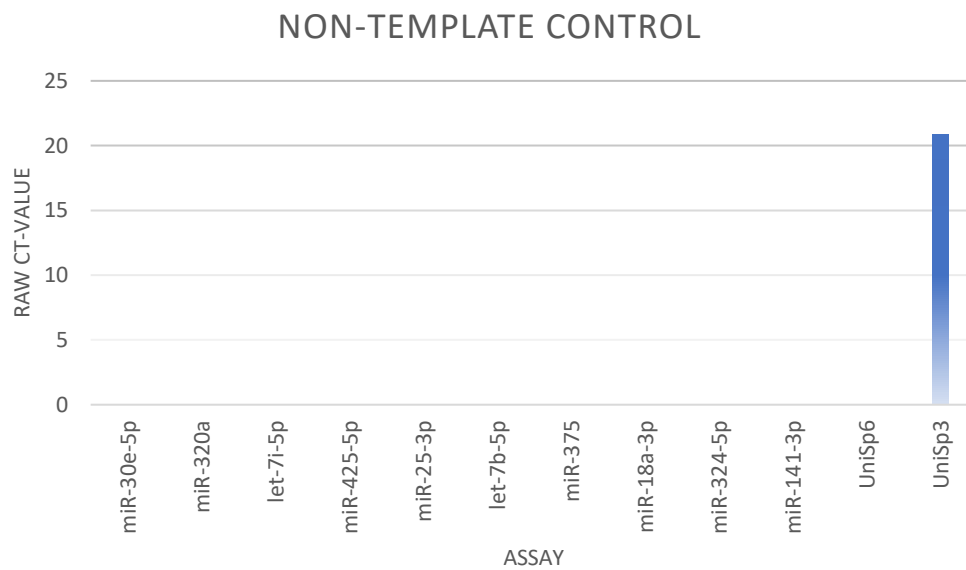
Figure 4.3: Raw Ct-values for the negative control sample

4.3.4 Normalization

Of the four normalizer assays, all were detected in all samples. The range of the raw Ct-values are presented in Table 4.10. By using Normfinder, the best normalizer was found to be the average of miR-425-5p, let-7i-5p and one of the targets; miR-18a-3p.
The formula used to calculate the normalized Ct values are:

Normalized Ct = normalizers assay mean Ct (sample 1) – assay Ct (miRNA of interest sample 1),

Normalized Ct = normalizers assay mean Ct (sample 2) – assay Ct (miRNA of interest sample 2)…

A higher value indicates that the miRNA is more abundant in the particular sample.

Table 10:The variation in raw Ct-values for the four reference miRNAs.

	Raw Ct-values		
	Pre-operative group	**Post-operative group**	**Total range**
miR-30e-5p	28.89 – 34.21	25.65 – 35.93	25.65 – 35-93
miR-320a	26.16 – 30.94	24.55 – 30.13	24.55 – 30.94
let-7i-5p	29.32 – 34.25	27.84 – 34.20	27.84 – 34.20
miR-425-5p	28.13 – 35.27	22.46 – 31.88	22.46 – 35.27

4.4 RT-qPCR

Isolated RNA from pre- and post-operative serum samples donated from 25 prostate cancer patients was reverse transcribed to cDNA and analysed by RT-qPCR. The PCR results from nine of these patients were omitted from the data analysis because they failed to give sufficient data for both pre- and post-operative samples. In the data from the remaining patients, five of the six targets had signal in all samples. All the reference miRNA had signal in all samples.

When comparing the group of pre-operative samples with post-operative, two miRNAs were found to be differentially expressed using a cut-off of p-value < 0.05. The two most differentially expressed miRNAs, and the only two found to be significantly differentially expressed, were miR-25-3p and let-7b-5p. Table 4.11 below shows the individual results for the six miRNA targets included in this study.

Table 4.11: Differentially expressed miRNAs, with average normalized Ct-values for each group, the standard deviation (SD) across the groups, fold change and p-value from the t-test. Averaged Ct-values from 16 patients were included in the pre-operative and in the post-operative group.

miRNA	PRE average normalized Ct-values	POST average normalized Ct-values	Standard deviation (SD)	Fold change (FC)	p-value
miR-25-3p	3,32	3,53	0.30	1.1512	0.016
let-7b-5p	1,46	1,73	0.49	1.2105	0.0414
miR-324-5p	-2,90	-2,50	0.7439	1.3130	0.0518
miR-141-3p	-3,67	-4,72	1.6945	-1.7871	0.087
miR-375	-1,63	-2,17	1.3783	-1.4601	0.1339
miR-18a-3p	-2,74	-2,77	0.54482	-1.0182	0.8507

4.4.1 miR-25-3p

miR-25-3p was one of the miRNAs found to be significantly differentially expressed in pre- compared to post-operative samples. Findings from the literature reported miR-25-3p to be downregulated in prostate cancer. This is consistent with the normalized Ct-values presented in Appendix H, table A.8. Normalized Ct-values for miR-25-3p were lower in the pre-operative group than in the post-operative group. The average normalized Ct-value for the pre-operative group was 3.32, while the average normalized Ct-value for the post-operative group was 3.53.

4.4.2 hsa-let-7b-5p

Let-7b-5p was one of the miRNAs found to be significant differentially expressed in pre- compared to post-operative samples. Findings from the literature reported let-7b-5p to be downregulated in prostate cancer. This is consistent with the normalized Ct-values presented in Appendix H, table A.8. Normalized Ct-values for let-7b-5p were lower in the pre-operative group than in the post-operative group. The average normalized Ct-value for the pre-operative group was 1.40, while the average normalized Ct-value for the post-operative group was 1.79.

4.4.3 miR-375

The literature search reviled that miR-375 was one of the best candidates as biomarkers for prostate cancer. Published research reported miR-375 to be upregulated in prostate cancer patients. Comparing normalized Ct-values from pre- and post-operative samples did not give a significant change using a cut-off of p-value < 0.05. The normalized Ct-values from pre-operative samples tended to be higher than from the post-operative samples. The average normalized Ct-value for the pre-operative group was -1.63, while the average normalized Ct-value for the post-operative group was -2.17.

4.4.4 miR-18a-3p

Findings from the literature search reported miR-18a-3p to be upregulated in prostate cancer patients compared to healthy controls. The difference between pre- and post-operative samples in this study were so small that this target was included as one of the normalizers. The average normalized Ct-value for the pre-operative group was -2.75, while the average normalized Ct-value for the post-operative group was -2.77.

4.4.5 miR-324-5p

Another miRNA reported to be upregulated in prostate cancer patients was miR-324-5p. The normalized Ct values for miR-324-5p obtained from this experiment tended to be higher in pre- compared to post-operative samples. With a p-value of 0.0518, there was just not a significant change in expression between the two groups. The average normalized Ct-value for the pre-operative group was -2.90, while the average normalized Ct-value for the post-operative group was -3.37.

4.4.6 miR-141-3p

In addition to miR-375, miR-141-3p is reported as one of the candidates as biomarkers for prostate cancer. Findings from the literature reported miR-141-3p to be upregulated in prostate cancer. The paired t-test showed no significant change in expression between the two groups. Normalized Ct values tended to be higher in pre- compared to post-operative samples. The average normalized Ct-value for the pre-operative group was -3.66, while the average normalized Ct-value for the post-operative group was -4.72.

5. Discussion

According to various research studies, approximately 20-40% of the newly registered prostate cancer cases in the USA and Europe could be due to overdiagnosis through extensive PSA testing. This is supported by the fact that an increase in prostate cancer incidence was observed at the same time as the introduction of the PSA test in the diagnostic process in 1990 (Lu-Yao and Greenberg, 1994). As can be observed from Table 3.1, even though the Gleason scores for the patients in this cohort were the same, the PSA levels before surgery varied from 4.55 – 45.9 µg/L.

The potential of circulating miRNAs as alternative biomarkers for prostate cancer has been explored by a large number of studies over the recent years. Emerging evidence suggest that the level of some miRNAs can be used to separate cancer from non-cancer, and that the expression of some miRNAs differ depending on the aggressivity of the cancer. Several studies have aimed to find miRNAs that could serve as potential biomarkers for prostate cancer, based on differences in expression in cancer patients compared to healthy controls, or compared to patients diagnosed with BPH. So far, there are few published reports that have addressed what happens with the expression of these potential biomarkers after the patients have undergone radical prostatectomy, and the cancerous tissue is removed. This study aimed to validate the change in expression for a number of circulating miRNAs in serum samples collected from prostate cancer patients that by NGS showed a significant change in expression following surgery. This thesis was a part of a larger project that sent pre- and post-operative serum samples from twenty-five men diagnosed with prostate cancer to Qiagen, Denmark for NGS. The resulting NGS data was used to assembly a list of miRNA candidates for validation, based on their significant change in expression following removal of cancerous tissue. NGS is a method that generates a large quantity of information. However, the method is time-consuming and expensive, and it can be challenging to sort out relevant information. RT-qPCR is a faster and cheaper method more appliable in clinical practice. If some of the miRNAs with a significant change in expression following surgery were to be further explored as biomarkers for prostate cancer, one of the first steps is to prove that they can be easily detected in a clinical setting.

The survival rate for prostate cancer is high (95 %) compared to other types of cancers. Yet, there is a small patient group that is diagnosed with far more severe cancer, representing 5.09 % of deaths caused by cancer in the US in 2017 (US Cancer Statistics Working Group, 2020). The investigation of miRNA as an alternative biomarker to the

PSA test is motivated of the fact that it would be beneficial for both the patient and the health care system to be able to separate these groups as early as possible.

5.1 The literature study

A literature search was performed before the onset of the laboratory work, to evaluate which miRNAs held the greatest potential as biomarkers for prostate cancer. The findings from this literature search were used when working on the details of the study design, such as the selection of miRNA targets. The literature study reviled a large research field that, since one of the first published studies by Calin et al. in 2002, has generated a high number of potential biomarkers for prostate cancer. The high number of suggested miRNAs for this purpose prove that it is a subject of relevance.

The aim of the literature search was to find relevant miRNAs that could be used for the validation of NGS data. The aim was fulfilled, as a list of miRNAs was produced, and a few miRNAs found in this list were found to have a significant change in expression following surgery. One could argue that one of the limitations with the literature search performed in this thesis was that given the large amount of research performed on miRNAs and cancer, some relevant miRNAs may have been left out of the resulting miRNAs found in Table 4.1. The challenge with a literature study like this, and literature studies in general, is to cover the enormous amount of research in the given research field. In the work of this thesis, it soon became clear that the amount of information related to this subject was enormous, and that limitations needed to be set in order to keep it within the work and time frame of a master thesis. Including all the miRNAs that has been explored in relation to prostate cancer would have been time consuming and inefficient. The strategy applied was therefore to include the miRNAs that by methods similar to this study were found relevant.

Common for the studies that the miRNA candidates in Table 4.1 were suggested by was that the they were small, with cohorts between 10 – 50 patients. In addition to small study sizes, contradicting results and the use of various methods makes interpretation and comparison of results from previous publications challenging. In some cases, small cohorts may give unreliable results, such as suggesting a specific miRNA as a biomarker, even though it may not be suited. This would be visible if another study were to validate the results and failed to do so. Another issue that appeared during the work with the literature study was that often, the protocols used for the project were different. This makes it hard to compare the results between different studies performed at different

laboratories. There are a lot of techniques being applied, but reference methods are missing. These challenges highlight the importance of validation projects.

A large-scale validation project would normally include a large number of targets for validation, thereby making sure that the ones that are relevant are included. These validation projects are carried out in lab facilities with equipment capable of large-scale analyses. Bearing in mind that this was a master project, it was, in agreement with advisors from Qiagen, decided that the literature search and this project design, with six targets selected based on significant difference between groups and also for their appearance in existing literature, would be suitable.

The literature search resulted in twenty-one miRNAs suggested as circulating biomarker candidates for prostate cancer. Strikingly, only a few of these miRNAs were found to be associated with prostate cancer in more than one study. The reason for the limited overlap between studies are complex. As previously mentioned, small cohorts and differences in methods are likely factors contributing to the limited overlap. Whether it is serum, plasma or purified micro-vesicles that are used as starting materials may also contribute. In addition, differences in isolation methods, miRNA detection methods (RT-qPCR or array-based methods) and statistical methods are also likely sources to limited overlap between miRNA studies. The relevance of the miRNAs that have been found to be associated with prostate cancer in more than one study, can be considered higher as it proves that they remain associated with prostate cancer despite varying experimental conditions.

5.2 Experimental procedures

5.2.1 RT-qPCR validation of NGS data

Samples and replicates from the same patient were analysed on the same PCR plate, as a step to minimize technical variations. Of the twenty-five PCR plates, nine failed to give results for both pre- and post-operative samples. Resultingly, sixteen plates were included in the data analysis. Due to challenges with the software intended to use for the data analysis, and because the time simply ran out because of corona-closed labs, the data analysis was performed by Qiagen. One of the benefits with doing the data analysis at Qiagen is that they are experienced with these types of project, the software they use is capable of analysing advanced set-ups and they were already familiar with the project.

The two miRNAs that by RT-qPCR showed a significant change in expression following surgery was miR-25-3p and let-7b-5p. These two miRNAs, together with miR-18a-3p and miR-324-5p were among the miRNAs that the NGS project reviled as the most differentially expressed between the two groups. The successful validation of the change in expression for miR-25-3p and let-7b-5p should be further explored. miR-324-5p had a p-value of 0.0518, which is just below the limit for significance. Knowing that some samples were left out of the data analysis, and that the NGS project reviled a significant change in expression for miR-324-5p, it is recommended to further explore this miRNA. For miR-18a-3p, the difference between pre- and post-operative samples was small enough for the miRNA to be suitable as a reference miRNA. The existing literature on miR-18a-3p and prostate cancer was based on small study size, which may have led to false positive results.

The two miRNAs that based on existing literature holds the greatest potential as biomarkers for prostate cancer are miR-375 and miR-141-3p. These miRNAs were not selected for this validation study based on their statistical results from NGS, but rather for their appearance in the literature. miR-141-3p came up late in the PCR run (after 35 – 37 cycles) and for many replicates miR-141-3p was not detectable. miR-375 on the other hand showed more stability and would therefore be to prefer by these two miRNAs. Further verification should be performed to conclude whether the expression of miR-375 is altered by prostate cancer and if it returns to normal levels after treatment. Even though this project did not reveal a significant change in expression, it might be because the samples were taken to soon after surgery. It is possible that the levels are returning to normal, but that it takes longer time than three months.

The results from this experiment are conflicting. Looking away from the plates that failed to give results from both pre- and post-operative samples, this experiment successfully validated the change in expression following treatment for two, and nearly three, of the six targets that from NGS showed a significant change in expression from pre- to post-operative samples. For these targets to be considered as biomarkers for prostate cancer, they need to be easily detected in clinical practice, something that cannot be said about all the samples in this cohort. In order to conclude about the potential of these miRNAs as biomarkers for prostate cancer, the experiment should be repeated with newly isolated RNA in hope of getting results from all patient samples included in this study. Increasing the number of samples included in the data analysis would have increased the strength of the statistical methods and the results obtained from them. As mentioned in Chapter 4.4, the normalized Ct-values from all targets except miR-18a-3p tended to be consistent with the literature, even though miR-25-3p and let-7b-5p were the only two targets that had a significant change in expression using a cut-off of p-value < 0.05. For example, miR-375, which is likely the miRNA with the greatest potential as a biomarker for prostate cancer according to existing literature, had an average normalized Ct-value of -1.63 before surgery and -2.17 three months after surgical removal of the cancerous tissue. Previous studies have reported miR-375 to be upregulated in prostate cancer patients. By assuming that the level of circulating miRNA returns to normal after the cancer is removed, the average normalized Ct-values support this. If all samples had provided results, and these where all included in the data analysis, then, based on trends and existing literature, miR-375 and the other targets might also have showed a significant change in expression when comparing the two groups.

5.2.2 Quality control

An underlying contribution to the aim of the study, to generate knowledge about potential biomarkers, is to produce high-quality, reliable results. The quality control consisting of melt curve analysis, non-template control and spike-ins overall indicated good quality control.

The reason for why some samples gave results, while other did not, is unclear. For two of the patient samples, one of the cDNA replicates for the post-operative sample had increased Ct-values for UniSp6, indicating that inhibition or other errors had occurred during the RT-reaction. The results from the replicates with increased UniSp6 Ct-values were omitted from the data analysis. The resulting Ct-value for these samples were therefore based on one cDNA replicate, not two as for the others.

For the patient samples that failed to give results, the Ct-values for both UniSp6 and UniSp3 were fine, suggesting that both the cDNA synthesis and the PCR reaction worked as planned. Repeating the experiment with newly isolated RNA would have given an indication whether it was the quality of the returned RNA or technical errors that caused that some samples failed to give results.

5.3 Evaluation of study design

An initial high-throughput sequencing of miRNA expression in pre- and post-operative serum samples from prostate cancer patients was performed to identify miRNAs that showed a change in expression following surgery. Because this project builds and depends on the work of the previous sequencing project, many factors regarding study design was already set, such as patient group and cohort size.

At the present, looking back, it seems like a better choice to isolate new RNA instead of using the left-over RNA from the NGS project. The argument that the technical variations should be minimized is highly valued, but due to the insecurities regarding why some samples gave results while other did not, a fresh RNA isolation would have been preferred. However, at the time, the idea was that a new RNA isolation could be done if the first samples failed to give result and it was suspected to be due to degraded RNA. Because of Covid-19 and closed labs, repeating the RNA isolation unfortunately was not possible.

The number of patients in the cohort was also a subject of discussion. For statistical reasons, a large cohort is desired. However, in this project it was determined that a validation of the NGS results should be performed at the same set, the 25 patients, as was analysed by NGS. As observed from the literature search, free standing project aiming to investigate miRNAs that could function as biomarkers, should be as large as possible. Small study sizes increase both false positive results and false negative results (Kok et al., 2017).

5.3.1 Selection of targets

Ideally, a target would be a miRNA that is well documented in the literature as a potential biomarker for prostate cancer by being able to separate between cancer and non-cancer and also be among the miRNAs reviled by NGS to have a significant change in expression in post- compared to pre-operative samples. In a large-scale validation project, six targets are far from enough. But because of time, budged and instrumental capacity (96 wells instead of 365 wells), six relevant targets were included. Comparing the results from the literature search with the results from NGS showed that some of the miRNAs with the largest variation in expression between the two groups, could not be found in previously published literature. Some of the miRNAs most frequently mentioned in the literature showed little, or none, change in expression in the two groups. Hence, selecting these targets has been a challenging process. Some of the targets, like miR-375 is well documented in the literature and is known as one of the most promising alternative biomarkers for prostate cancer. In the NGS data though, is was not among the best candidates. The selection of miR-375 is therefore an example of a target that was based more on literature than that the actual NGS data. miR-141-3p is also well-documented in the literature with the potential of separate cancer from non-cancer. There are examples on opposite thinking as well. Some of the targets were hard to find literature on, specifically with relation to prostate cancer. But because the NGS data showed a significant change in expression, some of these miRNAs were included as targets anyway. As expected, the miRNAs with the largest change in expression were also the ones that showed a significant change by a paired t-test of the RT-qPCR data.

5.3.2 Selection of references

To improve the accuracy with RT-qPCR, the identification of stably expressed housekeeping genes for normalization of miRNA expression is a principal step. These reference miRNAs used for normalization are internal controls that make it possible to normalize the gene expression of distinct miRNAs. Housekeeping genes, in this case reference miRNAs, needs to show minimal variability in expression between different experimental conditions and physiological states of the patient (Causin et al., 2019). Ideally, multiple references should be included in a validation project like this to ensure that if some of the selected reference miRNAs fail to give results or show too much variation in expression, there are still references to use for normalization. Because of plate design, four reference miRNAs were included in this study. The reference miRNAs were based on NGS data as among the miRNAs that showed greatest stability across of

samples.

The software Normfinder was used, by Qiagen services, to calculate the stability value of the miRNAs found in all samples and to propose a normalizer. By using Normfinder, the best normalizer was found to be the average of three miRNAs. Out of the four included reference miRNA in the study, two were included in the average used for normalization; miR-425-5p and let-7i-5p. Surprisingly, one of the targets was also among the three miRNAs proposed as a normalizer.

5.3.3 Selection of spike-ins

Following advisement from Qiagen's advisors, UniSp6 were selected for cDNA synthesis control and UniSp3 for qPCR and interplate calibrator. Including these two controls enabled the possibility to omit samples where the spike-ins indicated errors in one of the reactions. As mentioned, this was done in two of the samples, where the Ct-value for UniSp6 indicated errors in the RT reaction for one of the cDNA replicates.
Qiagen added spike-ins when performing the RNA isolation and preparations for NGS. Because these spike-ins indicated good quality of the samples, it was decided not to include more controls than UniSP6 and UniSp3 in this RT-qPCR verification study. Including more quality controls would have occupied more wells on the PCR plate, which would have decreased the number of included targets and references. If, as proposed, a new RNA isolation was to be performed, followed by a repetition of this experiment, controls for RNA isolation should be included. Including controls for RNA isolation is also argued by the issue that some of the plates gave results only for pre- or post-samples. One reason for that some of these samples failed to give results for any of the included miRNAs could be that the quality of the isolated RNA was low. Whether this was the case would have been determined if an additional spike-in were included. The spike in controls UniSp2, UniSp4 and UniSp5 are suitable RNA isolation controls.

5.3.4 Custom PCR Panels

miRCURY LNA miRNA Custom PCR Panels are filled with dried down primer sets by the customer's choice. These PCR plates pre-coated with primer assays were chosen as a step to minimize technical errors. Having the primers already in the PCR plate means that they do not need to be added to the master mix. Hence, the risk of pipetting errors related to the addition of primers are excluded. The challenge with using custom PCR plates is that because they are pre-coated with primers, it needs to be planned in detail how the samples should be organized into the plate before the plates can be ordered.

Because the aim was to compare pre-operative samples with post-operative samples, it was desired to gather all samples from the same patient into the same plate. This was accomplished by using the following numbers of assays: six targets, four references and two spike-ins. Using this plate-setup, 25 PCR plates, plus one extra plate with no-template controls to test for contamination were included in the experimental phase. One of the limitations with using these plates was that the plate design process and the delivery time were time consuming, resulting in a delay in the project time plan. These are valuable lessons for the future. Delivery time and time to consult with sales advisors from the companies the supplies are ordered from should not be overseen. Unexpected events may occur, such as in this case where 20 plates were delayed and were not received until May 13th.

5.3.5 Evaluation of PCR procedures

A high-quality template is essential in RT-qPCR and a fundamental principle is to use high quality RNA for the reverse transcription (RT) step where the template is generated. Errors during RNA isolation and cDNA synthesis are likely to compromise the accuracy and efficiency of qPCR. This may give inaccurate and erroneous results. Because the RNA isolation was performed by Qiagen Services in Denmark, quality control of the isolated RNA was also performed by them. Intracellular nucleases and/or various compounds of blood or bile can compromise the RNA quality. A common method to ensure that the RNA-purity is high enough, is Nanodrop. Clean RNA is indicated by an A260/A280 ratio above 1.8. One of the limitations with using nanodrop as a quality method is that it is insensible to degraded RNA. Qiagen reported that the samples were of high quality and well suited for further analyses. In this case, where degraded RNA is one possible cause to why some samples failed to give results, this would not have been detected by a method like Nanodrop. In addition to quality controls like Nanodrop, the use of internal controls like spike-ins are necessary to ensure high quality in every step of the experimental procedures.

In a RT-qPCR experiment, the RT reaction step is considered the most fragile step in the experimental procedures. Error induced at this stage will likely be amplified in the following PCR steps. The RT reactions for all samples were therefore performed at the same day. Because the success of qPCR, that is the specificity, efficiency and accuracy, depends on the primers used, they should be specific for the gene of interest. When using gene-specific primers, one amplification product at the end of each PCR reaction is expected. These amplification products can be verified through dissociation curve

analysis based on properties like melting point, GC content and size. The melting curve analysis revealed that the amplification products in each PCR assay peaked at the same melting point. This indicated the presence of a single PCR product, as desired.

Number of replicates

One of the limitations of the study design was the low amount of returned RNA. The RNA was isolated using the miRNeasy Serum/Plasma Kit, which resulted in 14 μl isolated RNA. 5 μl was used for NGS and some were used for quality measurements. Based on this knowledge, and by assuming that some amounts get lost in pipetting, it was determined to run two cDNA reactions per sample. For each cDNA reaction 2 μl template RNA were added. As mentioned, the cDNA synthesis is the most critical step and it would have been desired to include 3 cDNA reactions per sample, making it possible to exclude one if it differed from the other two. Because this was not an option due to low quantity of isolated RNA, the solution was to do two cDNA reactions per sample and two PCR replicates per cDNA. Looking back, this was a good solution at the given time. For some samples, only one of the cDNA reactions gave results, and instead of omitting the entire patient sample, the results from the other cDNA reaction were used.

5.4 Further studies

Time was a limiting factor at the end of this master thesis, partly due to the delayed delivery of the custom PCR plates and partly due to Covid-19 and closed labs. This resulted in a more theoretical thesis than planned, where the data analysis unfortunately needed to be performed by Qiagen and not the author. Ideally, it would have been interesting to repeat the project with newly isolated RNA to look at the reproducibility and also to see if using newly isolated RNA would prevent that some of the samples fail to give results. The amount of returned RNA from the NGS project was low. By performing a new RNA isolation, it would have been possible to run several small pilot studies prior to the main experiment. In these pilots one could have tested several possible reference miRNAs in addition to testing various concentrations and input RNA to the cDNA synthesis. Increased concentration would have reduced the Ct-values and perhaps contributed to successful validation of more miRNAs.

A possible reason for why the change in expression is small in some of the miRNAs is that taking the post-operative sample three months after surgery might be too soon. For further studies it would be useful to explore the expression in the selected miRNAs in samples taken longer time after surgery. If this project were to be repeated, it would also have been useful to include a group of healthy controls. In that way, it would have been easier to say something about the change observed between the pre- and post-operative samples.

6. Conclusion

Evidence show that miRNAs could serve as alternative biomarkers for the detection of prostate cancer. A literature search reviled that a large number of miRNAs has been proposed as biomarkers. The aim of this study was to, by RT-qPCR, validate the change in expression of a set of miRNAs that by NGS were shown to have a significant change in expression following radical prostatectomy. The miRNAs were selected both for their appearance in the literature and based on the NGS results. Of the six selected miRNA targets, two of these showed a significant change in expression following surgery. The reason for why the other targets did not show a significant change might be due to the fact that several samples were omitted from the data analysis because they failed to give sufficient results. By repeating the experiment and include results from the same set of patients that were included in the NGS project, the results might have been consisting. As confirmed by the literature search, small study size is a central issue related to miRNAs and prostate cancer.

Further studies are recommended to validate these findings. Following experiments should repeat the RNA isolation and in that way used newly isolated RNA instead of stored RNA to see if this increase the number of samples that give results. It would be useful in following studies to include healthy controls and samples taken later than three months after surgery, to see if the expression of the miRNA returns to normal levels.

Literature

Balk, S. P., Ko, Y. J. & Bubley, G. J. 2003. Biology of prostate-specific antigen. *J Clin Oncol,* 21**,** 383-91.

Brase, J. C., Johannes, M., Schlomm, T., Fälth, M., Haese, A., Steuber, T., Beissbarth, T., Kuner, R. & Sültmann, H. 2011. Circulating miRNAs are correlated with tumor progression in prostate cancer. *Int J Cancer,* 128**,** 608-16.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A. & Jemal, A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin,* 68**,** 394-424.

Bryant, R. J., Pawlowski, T., Catto, J. W., Marsden, G., Vessella, R. L., Rhees, B., Kuslich, C., Visakorpi, T. & Hamdy, F. C. 2012. Changes in circulating microRNA levels associated with prostate cancer. *Br J Cancer,* 106**,** 768-74.

Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F. & Croce, C. M. 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A,* 99**,** 15524-9.

Causin, R. L., Pessôa-Pereira, D., Souza, K. C. B., Evangelista, A. F., Reis, R. M. V., Fregnani, J. H. T. G. & Marques, M. M. C. 2019. Identification and performance evaluation of housekeeping genes for microRNA expression normalization by reverse transcription-quantitative PCR using liquid-based cervical cytology samples. *Oncology letters,* 18**,** 4753-4761.

Chen, Z. H., Zhang, G. L., Li, H. R., Luo, J. D., Li, Z. X., Chen, G. M. & Yang, J. 2012. A panel of five circulating microRNAs as potential biomarkers for prostate cancer. *Prostate,* 72**,** 1443-52.

Cheng, H. H., Mitchell, P. S., Kroh, E. M., Dowell, A. E., Chéry, L., Siddiqui, J., Nelson, P. S., Vessella, R. L., Knudsen, B. S., Chinnaiyan, A. M., Pienta, K. J., Morrissey, C. & Tewari, M. 2013. Circulating microRNA profiling identifies a subset of metastatic prostate cancer patients with evidence of cancer-associated hypoxia. *PLoS One,* 8**,** e69239.

Clark, D. P. & Pazdernik, N. J. 2013. *Molecular Biology*, Elsevier Science.

Cochetti, G., Poli, G., Guelfi, G., Boni, A., Egidi, M. & Mearini, E. 2016a. Different levels of serum microRNAs in prostate cancer and benign prostatic hyperplasia: Evaluation of potential diagnostic and prognostic role. *OncoTargets and therapy,* 9**,** 7545-7553.

Cochetti, G., Poli, G., Guelfi, G., Boni, A., Egidi, M. G. & Mearini, E. 2016b. Different levels of serum microRNAs in prostate cancer and benign prostatic hyperplasia: evaluation of potential diagnostic and prognostic role. *Onco Targets Ther,* 9**,** 7545-7553.

Collin, S. M., Martin, R. M., Metcalfe, C., Gunnell, D., Albertsen, P. C., Neal, D., Hamdy, F., Stephens, P., Lane, J. A., Moore, R. & Donovan, J. 2008. Prostate-cancer mortality in the USA and UK in 1975-2004: an ecological study. *Lancet Oncol,* 9**,** 445-52.

Dasgupta, P. & Kirby, R. S. 2011. *ABC of Prostate Cancer*, Wiley.

De Bono, J. S., Scher, H. I., Montgomery, R. B., Parker, C., Miller, M. C., Tissing, H., Doyle, G. V., Terstappen, L. W., Pienta, K. J. & Raghavan, D. 2008. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res,* 14**,** 6302-9.

Dybos, S. A., Flatberg, A., Halgunset, J., Viset, T., Rolfseng, T., Kvam, S. & Skogseth, H. 2018. Increased levels of serum miR-148a-3p are associated with prostate cancer. *Apmis,* 126**,** 722-731.

Endzeliņš, E., Berger, A., Melne, V., Bajo-Santos, C., Soboļevska, K., Ābols, A., Rodriguez, M., Šantare, D., Rudņickiha, A., Lietuvietis, V., Llorente, A. & Linē, A. 2017. Detection of circulating miRNAs: comparative analysis of extracellular vesicle-incorporated miRNAs and cell-free miRNAs in whole plasma of prostate cancer patients. *BMC Cancer,* 17**,** 730.

Farrell, R. E. 2010. Chapter 18 - RT-PCR: A Science and an Art Form. *In:* Farrell, R. E. (ed.) *RNA Methodologies (Fourth Edition).* San Diego: Academic Press.

Fda-Nih, B. W. G. 2016. *BEST (Biomarkers, EndpointS, and Other Tools) Resource*, Food and Drug Administration (US).

Filella, X., Fernández-Galan, E., Fernández Bonifacio, R. & Foj, L. 2018. Emerging biomarkers in the diagnosis of prostate cancer. *Pharmacogenomics and personalized medicine,* 11**,** 83-94.

Filella, X. & Foj, L. 2017. miRNAs as novel biomarkers in the management of prostate cancer. *Clin Chem Lab Med,* 55**,** 715-736.

Filella, X. & Gimenez, N. 2012. Evaluation of [−2] proPSA and Prostate Health Index (phi) for the detection of prostate cancer: a systematic review and meta-analysis. *Clinical chemistry and laboratory medicine : CCLM / FESCC,* 51**,** 1-11.

Gann, P. H. 2002. Risk factors for prostate cancer. *Reviews in urology,* 4 Suppl 5**,** S3-S10.

Gao, Y., Guo, Y., Wang, Z., Dai, Z., Xu, Y., Zhang, W., Liu, Z. & Li, S. 2016. Analysis of circulating miRNAs 21 and 375 as potential biomarkers for early diagnosis of prostate cancer. *Neoplasma,* 63**,** 623-8.

Gee, W. H. Fairview Health Services: Krames StayWell.

Gregory, R. I. & Shiekhattar, R. 2005. MicroRNA biogenesis and cancer. *Cancer Res,* 65**,** 3509-12.

Gupta, S., Gupta, A., Saini, A. K., Majumder, K., Sinha, K. & Chahal, A. 2017. Prostate Cancer: How Young is too Young? *Current urology,* 9**,** 212-215.

Haldrup, C., Kosaka, N., Ochiya, T., Borre, M., Høyer, S., Orntoft, T. F. & Sorensen, K. D. 2014. Profiling of circulating microRNAs for prostate cancer biomarker discovery. *Drug Deliv Transl Res,* 4**,** 19-30.

Helsedirektoratet. 2015. *Nasjonalt handlingsprogram med retningslinjer for diagnostikk, behandling og oppfølging av prostatakreft* [Online]. Oslo: Helsedirektoratet. Available: https://www.helsedirektoratet.no/retningslinjer/prostatakreft-handlingsprogram/Prostatakreft%20%E2%80%93%20Nasjonalt%20handlingsprogram%20med%20retningslinjer%20for%20diagnostikk,%20behandling%20og%20oppf%C3%B8lging.pdf/_/attachment/inline/a0bb1696-5a09-4cd6-93da-05907e829974:b994ab8e47204ecbfa4560030b1f9c64cb7c598c/Prostatakreft%20%E2%80%93%20Nasjonalt%20handlingsprogram%20med%20retningslinjer%20for%20diagnostikk,%20behandling%20og%20oppf%C3%B8lging.pdf [Accessed 05.11 2019].

Ibrahim, N. H., Abdellateif, M. S., Kassem, S. H., Abd El Salam, M. A. & El Gammal, M. M. 2019. Diagnostic significance of miR-21, miR-141, miR-18a and miR-221 as novel biomarkers in prostate cancer among Egyptian patients. *Andrologia,* 51**,** e13384.

Jin, Y. Y., Tong, S. Q. & Tong, M. 2019. Diagnostic Value of Circulating miR-324 for Prostate Cancer. *Clin Lab,* 65.

Johns, L. E. & Houlston, R. S. 2003. A systematic review and meta-analysis of familial prostate cancer risk. *BJU Int,* 91**,** 789-94.

Kelly, B. D., Miller, N., Sweeney, K. J., Durkan, G. C., Rogers, E., Walsh, K. & Kerin, M. J. 2015. A Circulating MicroRNA Signature as a Biomarker for Prostate Cancer in a High Risk Group. *J Clin Med,* 4**,** 1369-79.

Knyazev, E. N., Fomicheva, K. A., Mikhailenko, D. S., Nyushko, K. M., Samatov, T. R., Alekseev, B. Y. & Shkurnikov, M. Y. 2016. Plasma Levels of hsa-miR-619-5p and hsa-miR-1184 Differ in Prostatic Benign Hyperplasia and Cancer. *Bull Exp Biol Med,* 161**,** 108-11.

Kok, M. G. M., De Ronde, M. W. J., Moerland, P. D., Ruijter, J. M., Creemers, E. E. & Pinto-Sietsma, S. J. 2017. Small sample sizes in high-throughput miRNA screens:

A common pitfall for the identification of miRNA biomarkers. *Biomolecular detection and quantification,* 15**,** 1-5.

Kovacs, E. J. 2001. Wheater's Functional Histology: A Text and Colour Atlas. *Archives of Pathology & Laboratory Medicine,* 125**,** 708-708.

Kreftregisteret 2019. Årsrapport 2018 med resultater og forbedringstiltak fra Nasjonalt kvalitetsregister for prostatakreft. Oslo.

Kretschmer, A. & Tilki, D. 2017. Biomarkers in prostate cancer – Current clinical utility and future perspectives. *Critical Reviews in Oncology/Hematology,* 120**,** 180-193.

Lahoti, A., Lakhotiya, A., Ingole, S., Dhok, A. & Mudaliar, P. 2018. Role and application of diffusion-weighted imaging in evaluation of prostate cancer. *Indian Journal of Medical and Paediatric Oncology,* 39**,** 349-354.

Larsen, I., Møller, B., Johannesen, T., Larønningen, S., Robsahm, T., Grimsrud, T. & Ursin, G. 2019. *Cancer in Norway 2018 - Cancer incidence, mortality, survival and prevalence in Norway,* Kreftregisteret.

Lawrentschuk, N. & Perera, M. 2000. *Benign Prostate Disorders*, MDText.com, Inc., South Dartmouth (MA).

Leitzmann, M. F. & Rohrmann, S. 2012. Risk factors for the onset of prostatic cancer: age, location, and behavioral correlates. *Clin Epidemiol,* 4**,** 1-11.

Lepor, A., Catalona, W. J. & Loeb, S. 2016. The Prostate Health Index: Its Utility in Prostate Cancer Detection. *Urol Clin North Am,* 43**,** 1-6.

Lepor, H. 2005. Pathophysiology of lower urinary tract symptoms in the aging male population. *Reviews in urology,* 7 Suppl 7**,** S3-S11.

Litwin, M. S. & Tan, H. J. 2017. The Diagnosis and Treatment of Prostate Cancer: A Review. *Jama,* 317**,** 2532-2542.

Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B. L., Mak, R. H., Ferrando, A. A., Downing, J. R., Jacks, T., Horvitz, H. R. & Golub, T. R. 2005. MicroRNA expression profiles classify human cancers. *Nature,* 435**,** 834-8.

Lyu, J., Zhao, L., Wang, F., Ji, J., Cao, Z., Xu, H., Shi, X., Zhu, Y., Zhang, C., Guo, F., Yang, B. & Sun, Y. 2019. Discovery and Validation of Serum MicroRNAs as Early Diagnostic Biomarkers for Prostate Cancer in Chinese Population. *BioMed Research International,* 2019**,** 9306803.

Marieb, E. N. & Hoehn, K. 2016. *Human anatomy & physiology*.

Mckay, A. C. & Sharma, S. 2019. Anatomy, Abdomen and Pelvis, Seminal Vesicle. *StatPearls [Internet].* StatPearls Publishing.

Mclaughlin, P. W., Troyer, S., Berri, S., Narayana, V., Meirowitz, A., Roberson, P. L. & Montie, J. 2005. Functional anatomy of the prostate: implications for treatment planning. *Int J Radiat Oncol Biol Phys,* 63**,** 479-91.

Mello-Grand, M., Gregnanin, I., Sacchetto, L., Ostano, P., Zitella, A., Bottoni, G., Oderda, M., Marra, G., Munegato, S., Pardini, B., Naccarati, A., Gasparini, M., Gontero, P. & Chiorino, G. 2019. Circulating microRNAs combined with PSA for accurate and non-invasive prostate cancer detection. *Carcinogenesis,* 40**,** 246-253.

Mello, S. S. & Attardi, L. D. 2018. Deciphering p53 signaling in tumor suppression. *Curr Opin Cell Biol,* 51**,** 65-72.

Merola, R., Tomao, L., Antenucci, A., Sperduti, I., Sentinelli, S., Masi, S., Mandoj, C., Orlandi, G., Papalia, R., Guaglianone, S., Costantini, M., Cusumano, G., Cigliana, G., Ascenzi, P., Gallucci, M. & Conti, L. 2015. PCA3 in prostate cancer and tumor aggressiveness detection on 407 high-risk patients: a National Cancer Institute experience. *Journal of experimental & clinical cancer research : CR,* 34**,** 15-15.

Mettlin, C., Jones, G., Averette, H., Gusberg, S. B. & Murphy, G. P. 1993. Defining and updating the American Cancer Society guidelines for the cancer-related checkup: prostate and endometrial cancers. *CA Cancer J Clin,* 43**,** 42-6.

Moch, H., Cubilla, A. L., Humphrey, P. A., Reuter, V. E. & Ulbright, T. M. 2016. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part A: Renal, Penile, and Testicular Tumours. *Eur Urol,* 70**,** 93-105.

Moustafa, A. A., Ziada, M., Elshaikh, A., Datta, A., Kim, H., Moroz, K., Srivastav, S., Thomas, R., Silberstein, J. L., Moparty, K., Salem, F. E., El-Habit, O. H. & Abdel-

Mageed, A. B. 2017. Identification of microRNA signature and potential pathway targets in prostate cancer. *Exp Biol Med (Maywood),* 242**,** 536-546.

Moynihan, R., Doust, J. & Henry, D. 2012. Preventing overdiagnosis: how to stop harming the healthy. *BMJ : British Medical Journal,* 344**,** e3502.

Nci 2020. NCI Dictionary of Cancer Terms. https://www.cancer.gov/publications/dictionaries/cancer-terms: National Cancer Institute.

Paunescu, I. A., Bardan, R., Marcu, A., Nitusca, D., Dema, A., Negru, S., Balacescu, O., Balacescu, L., Cumpanas, A., Sirbu, I. O., Petrut, B., Seclaman, E. & Marian, C. 2019. Biomarker Potential of Plasma MicroRNA-150-5p in Prostate Cancer. *Medicina (Kaunas, Lithuania),* 55**,** 564.

Porzycki, P., Ciszkowicz, E., Semik, M. & Tyrka, M. 2018. Combination of three miRNA (miR-141, miR-21, and miR-375) as potential diagnostic tool for prostate cancer recognition. *Int Urol Nephrol,* 50**,** 1619-1626.

Ramnani, D. 2020. *Adenocarcinoma of Prostate* [Online]. Available: https://www.webpathology.com/case.asp?case=20 [Accessed 28.02.2020].

Rawla, P. 2019. Epidemiology of Prostate Cancer. *World journal of oncology,* 10**,** 63-89.

Roberts, M. J., Teloken, P., Chambers, S. K., Williams, S. G., Yaxley, J., Samaratunga, H., Frydenberg, M. & Gardiner, R. A. 2000. Prostate Cancer Detection. *In:* Feingold, K. R., Anawalt, B., Boyce, A., Chrousos, G., Dungan, K., Grossman, A., Hershman, J. M., Kaltsas, G., Koch, C., Kopp, P., Korbonits, M., Mclachlan, R., Morley, J. E., New, M., Perreault, L., Purnell, J., Rebar, R., Singer, F., Trence, D. L., Vinik, A. & Wilson, D. P. (eds.) *Endotext.* South Dartmouth (MA): MDText.com, Inc.

Rosario, D. J., Lane, J. A., Metcalfe, C., Donovan, J. L., Doble, A., Goodwin, L., Davis, M., Catto, J. W., Avery, K., Neal, D. E. & Hamdy, F. C. 2012. Short term outcomes of prostate biopsy in men tested for cancer by prostate specific antigen: prospective evaluation within ProtecT study. *Bmj,* 344**,** d7894.

Saini, S. 2016. PSA and beyond: alternative prostate cancer biomarkers. *Cellular oncology (Dordrecht),* 39**,** 97-106.

Sartor, O. & De Bono, J. S. 2018. Metastatic Prostate Cancer. *New England Journal of Medicine,* 378**,** 645-657.

Schaefer, A., Jung, M., Miller, K., Lein, M., Kristiansen, G., Erbersdobler, A. & Jung, K. 2010. Suitable reference genes for relative quantification of miRNA expression in prostate cancer. *Experimental & molecular medicine,* 42**,** 749-758.

Sharp, V. J., Takacs, E. B. & Powell, C. R. 2010. Prostatitis: diagnosis and treatment. *Am Fam Physician,* 82**,** 397-406.

Srivastava, A., Goldberger, H., Dimtchev, A., Marian, C., Soldin, O., Li, X., Collins, S. P., Suy, S. & Kumar, D. 2014. Circulatory miR-628-5p is downregulated in prostate cancer patients. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine,* 35**,** 4867-4873.

Stensvold, A., Dahl, A. A., Brennhovd, B., Smastuen, M. C., Fossa, S. D., Lilleby, W., Steinsvik, A., Axcrona, K. & Smeland, S. 2013. Bother problems in prostate cancer patients after curative treatment. *Urol Oncol,* 31**,** 1067-78.

Stewart, B. W. & Wild, C. P. 2014. World cancer report 2014.

Suer, I., Guzel, E., Karatas, O. F., Creighton, C. J., Ittmann, M. & Ozen, M. 2019. MicroRNAs as prognostic markers in prostate cancer. *Prostate,* 79**,** 265-271.

Thompson, I. M., Pauler, D. K., Goodman, P. J., Tangen, C. M., Lucia, M. S., Parnes, H. L., Minasian, L. M., Ford, L. G., Lippman, S. M., Crawford, E. D., Crowley, J. J. & Coltman, C. A., Jr. 2004. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *N Engl J Med,* 350**,** 2239-46.

Tveter, K., Wahlqvist, R. 2019. Prostatahyperplasi. *Store medisinske leksikon*.

Us Cancer Statistics Working Group, N. 2020. U.S. Cancer Statistics Data Visualizations Tool, based on 2019 submission data (1999-2017). Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute.

Wach, S., Al-Janabi, O., Weigelt, K., Fischer, K., Greither, T., Marcou, M., Theil, G., Nolte, E., Holzhausen, H. J., Stohr, R., Huppert, V., Hartmann, A., Fornara, P., Wullich, B. & Taubert, H. 2015a. The combined serum levels of miR-375 and urokinase plasminogen activator receptor are suggested as diagnostic and prognostic biomarkers in prostate cancer. *Int J Cancer,* 137**,** 1406-16.

Wach, S., Al-Janabi, O., Weigelt, K., Fischer, K., Greither, T., Marcou, M., Theil, G., Nolte, E., Holzhausen, H. J., Stöhr, R., Huppert, V., Hartmann, A., Fornara, P., Wullich, B. & Taubert, H. 2015b. The combined serum levels of miR-375 and urokinase plasminogen activator receptor are suggested as diagnostic and prognostic biomarkers in prostate cancer. *Int J Cancer,* 137**,** 1406-16.

Wach, S., Nolte, E., Szczyrba, J., Stohr, R., Hartmann, A., Orntoft, T., Dyrskjot, L., Eltze, E., Wieland, W., Keck, B., Ekici, A. B., Grasser, F. & Wullich, B. 2012. MicroRNA profiles of prostate carcinoma detected by multiplatform microRNA screening. *Int J Cancer,* 130**,** 611-21.

Wang, J. L., Hu, Y., Kong, X., Wang, Z. H., Chen, H. Y., Xu, J. & Fang, J. Y. 2013. Candidate microRNA biomarkers in human gastric cancer: a systematic review and validation study. *PLoS One,* 8**,** e73683.

White, J., Shenoy, B. V., Tutrone, R. F., Karsh, L. I., Saltzstein, D. R., Harmon, W. J., Broyles, D. L., Roddy, T. E., Lofaro, L. R., Paoli, C. J., Denham, D. & Reynolds, M. A. 2018. Clinical utility of the Prostate Health Index (phi) for biopsy decision management in a large group urology practice setting. *Prostate Cancer and Prostatic Diseases,* 21**,** 78-84.

Winter, J. & Diederichs, S. 2011. MicroRNA biogenesis and cancer. *Methods Mol Biol,* 676**,** 3-22.

Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nature Cell Biology,* 11**,** 228-234.

You, W. & Henneberg, M. 2017. Cancer incidence increasing globally: The role of relaxed natural selection. *Evolutionary applications,* 11**,** 140-152.

Zedan, A. H., Hansen, T. F., Assenholt, J., Pleckaitis, M., Madsen, J. S. & Osther, P. J. S. 2018. microRNA expression in tumour tissue and plasma in patients with newly diagnosed metastatic prostate cancer. *Tumour Biol,* 40**,** 1010428318775864.

Appendices

A. Protocol: miRNeasy Serum/Plasma Kit (Qiagen, Germany)

Procedure

1. Prepare serum or plasma or thaw frozen samples.

2. Add 5 volumes QIAzol Lysis Reagent (see Table 2 for guidelines). Mix by vortexing.

3. Place the tube containing the lysate on the benchtop at room temperature (15–25°C, 5 min).

4. Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 108 copies/µl working solution) and mix thoroughly.

5. Add chloroform of an equal volume to the starting sample to the tube containing the lysate (see Table 2 for guidelines). Vortex or shake vigorously for 15 s.

6. Place the tube containing the lysate on the benchtop at room temperature for 2–3 min.

7. Centrifuge (12,000 x g, 4°C, 15 min).

8. Transfer the upper aqueous phase to a new collection tube. Add 1.5 volumes of 100% ethanol and mix thoroughly. Do not centrifuge.

9. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into a RNeasy MinElute spin column in a 2 ml collection tube. Centrifuge (≥8000 x g, 15 s). Discard the flow-through.

10. Repeat step 9. Discard flow-through.

11. Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Centrifuge (≥8000 x g, 15s). Discard the flow-through.

12. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Centrifuge (≥8000 x g, 15s). Discard flow-through.

miRNeasy Serum/Plasma Procedure

Serum/plasma

Add QIAzol Lysis Reagent

Add chloroform and shake

Separate phases

Add ethanol to aqueous phase

Bind total RNA including small RNAs

Wash

Elute

Total RNA including small RNAs
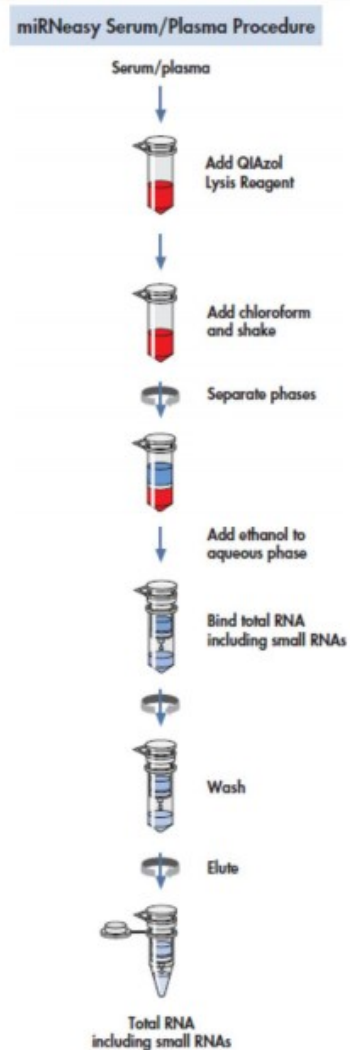
13. Pipet 500 µl of 80% ethanol onto the RNeasy MinElute spin column. Centrifuge (≥8000 x g, 2 min). Discard the collection tube with the flow-through.

14. Place the RNeasy MinElute spin column into a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min to dry the membrane.

15. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14 µl RNase-free water directly to the center of the spin column membrane. Centrifuge for 1 min at full speed to elute the RNA.

Table A.1: QIAzol Lysis Reagent volumens for various serum/plasma volumes

Serum/plasma (µl)	Protocol step 2: QIAzol Lysis Reagent (µl)	Protocol step 5: chloroform (µl)	Protocol step 7: opprox. Volume of upper aqueous phase (µl)	Protocol step 8: 100 % ethanol (µl)
≤ 50	250	50	150	225
100	500	100	300	450
200	1000	200	600	900

B.   Protocol: miRCURY LNA RT Kit (Qiagen, Germany)

Reverse transcription reactions were prepared on ice according to table A.2. A PCR cycler were used for the incubation steps in table A.3.

Table A.2: Reverse transcription reaction setup per sample.

Component	Amount (µl)
5x miRCURY SYBR® Green RT Reaction Buffer	2
RNase-free water	4,5
10x miRCURY RT Enzyme Mix	1
UniSp6 RNA spike-in	0,5
Template RNA	2
Total reaction volume	10

Table A.3: Reverse transcription reaction temperature cycling protocol

Step	Time	Temperature
Reverse transcription step	60 min	42 °C
Inactivation of reaction	5 min	95 °C
Storage	∞	4 °C

C. Protocol: miRCURY LNA miRNA Custom PCR Panel (Qiagen, Germany)

cDNA dilutions were prepared by using 3 µl cDNA + 117 µl nuclease-free water. A reaction mix were prepared according to table A.4. 10 µl per well was dispersed into the PCR plates before the plates were sealed, carefully vortexed and briefly centrifuged. After waiting 5 minutes for the primers to dissolve, the plates were placed into the real-time cycler and run according to the PCR cycling conditions in table A.5.

Table A.4: Reaction set-up per sample for miRCURY LNA miRNA Custom PCR Panels

Component	Volume per reaction (µl)
2x miRCURY SYBR Green Master Mix	5
ROX Reference Dye	0,5
cDNA template (diluted 1:40)	4
RNase-free water	0,5
Total reaction volume	10

Table A.5: PCR cycling conditions

Step	Time	Temperature	Ramp rate
PCR initial heat activation	2 min	95 °C	Maximal/fast mode
2-step cycling:			
Denaturation	10 s	95 °C	Maximal/fast mode
Combined annealing/extension	60 s	56 °C	Maximal/fast mode
Number of cycles	40		
Melting curve analysis		60 – 95 °C	

D. Plate setup

			1	2	3	4	5	6	7	8	9	10	11	12
A		pre	cDNA 1	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3
B			cDNA 1	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3
C		pre	cDNA 2	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3
D			cDNA 2	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3
E		post	cDNA 1	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3
F			cDNA 1	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3
G		post	cDNA 2	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3
H			cDNA 2	hsa-miR-30e-5p	hsa-miR-320a	hsa-let-7i-5p	hsa-miR-425-5p	hsa-miR-25-3p	hsa-let-7b-5p	hsa-miR-375	hsa-miR-18a-3p	hsa-miR-324-5p	hsa-miR-141-3p	UniSp6	UniSp3

E. Sequence of miRNAs assayed

The expression of ten miRNAs, four references and six targets, were investigated in pre- and post-operative serum samples from prostate cancer patients. Sequence, together with genomic location for these miRNAs is shown in table A.6.

Table A.6: Sequence and genomic locations for the selected miRNAs.

| miRNA name	Sequence	Genomic location
hsa-miR-30e-5p	UGUAAACAUCCUUGACUGGAAG	Chromosome 1
hsa-miR-320a	AAAAGCUGGGUUGAGAGGGCGA	Chromosome 8
hsa-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU	Chromosome 12
hsa-miR-425-5p	AAUGACACGAUCACUCCCGUUGA	Chromosome 3
hsa-miR-25-3p	AGGCGGAGACUUGGGCAAUUG	Chromosome 7
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU	Chromosome 22
hsa-miR-375	GCGACGAGCCCCUCGCACAAACC	Chromosome 2
hsa-miR-18a-3p	UAAGGUGCAUCUAGUGCAGAUAG	Chromosome 13
hsa-miR-324-5p	CGCAUCCCCUAGGGCAUUGGUG	Chromosome 17
hsa-miR-141-3p	CAUCUUCCAGUACAGUGUUGGA	Chromosome 12

F.   Amplification plots

Figure A.2: Amplification plots for the 25 samples. The numbers indicate patient number and the amplification plots marked with a red X were omitted from the data analysis because they failed to give results for both pre- and post-operative samples.
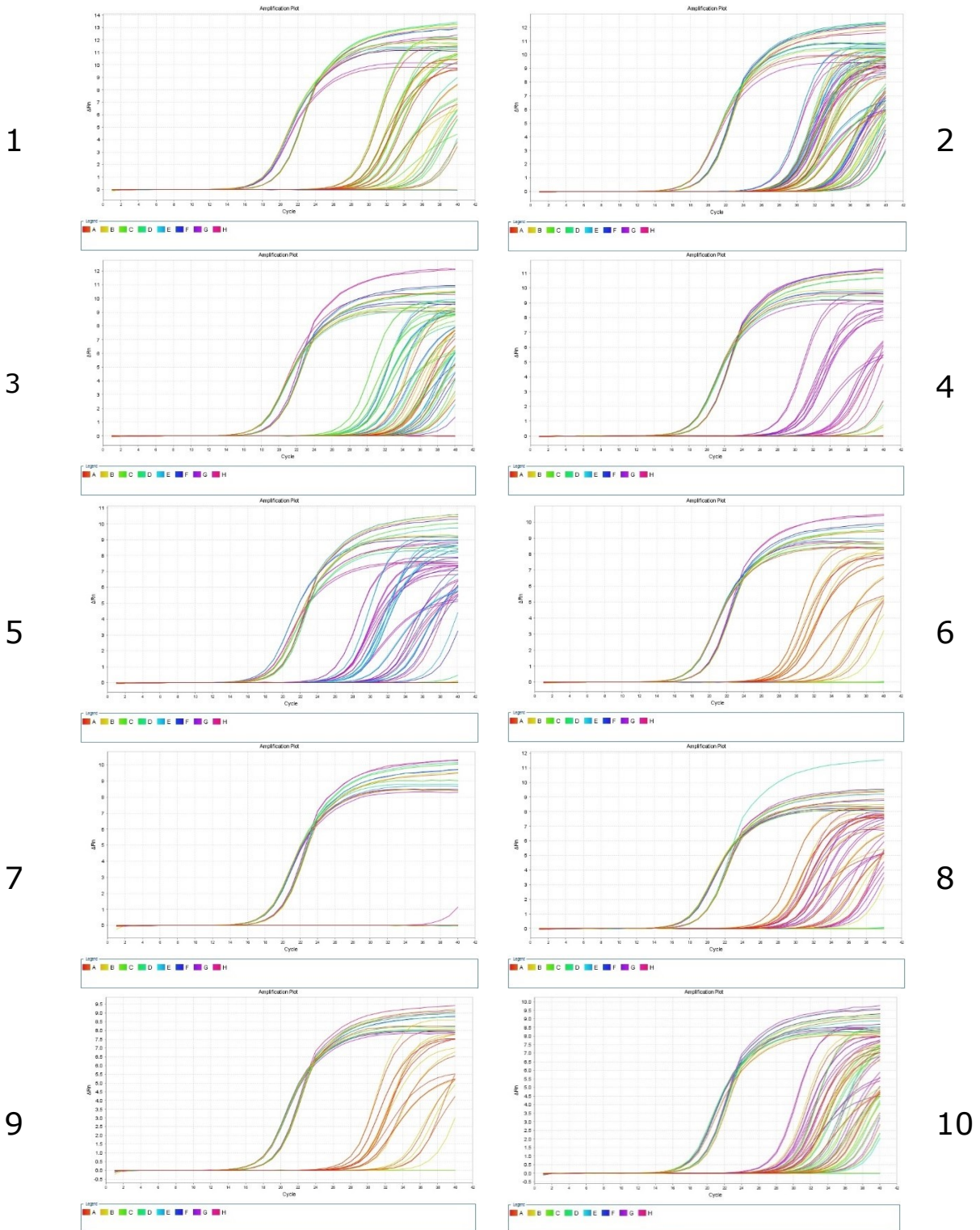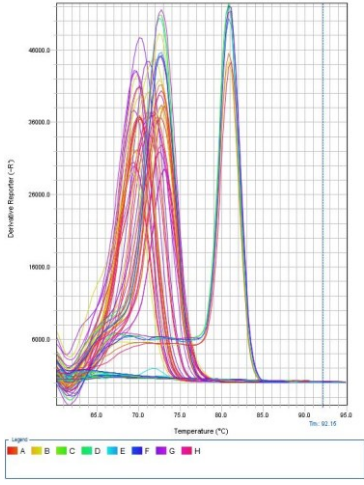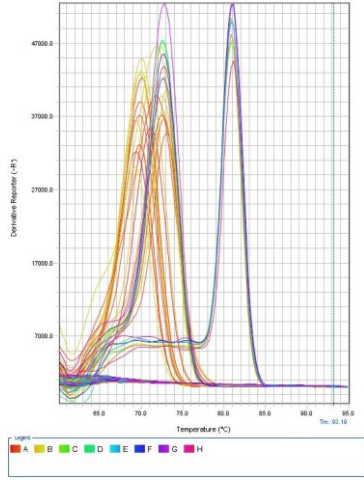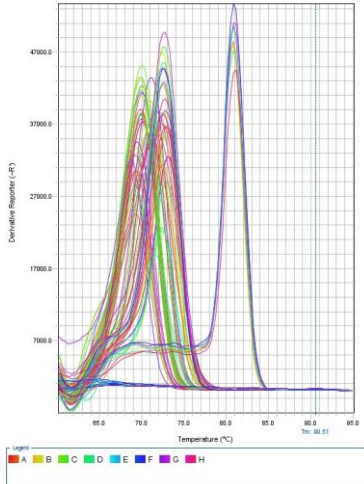
11
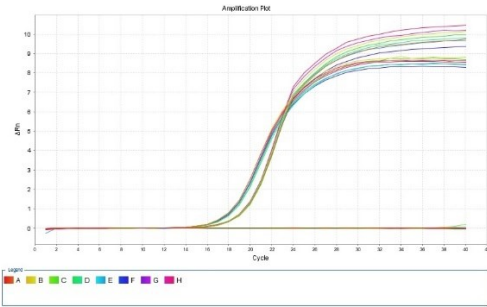
12
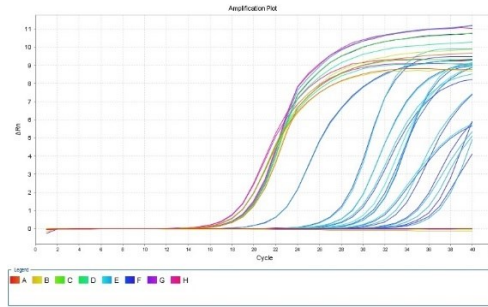
13
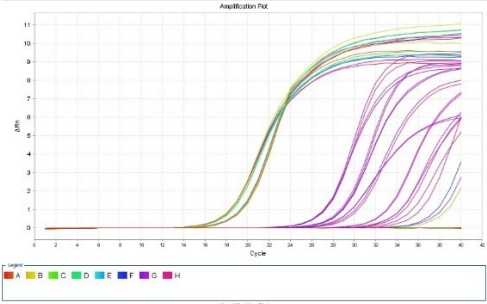
14
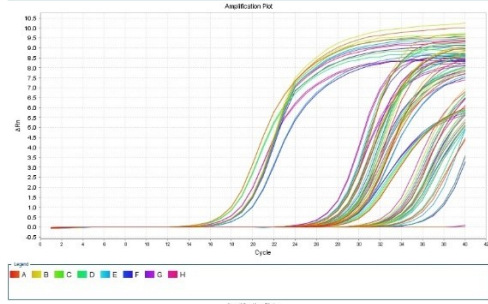
15
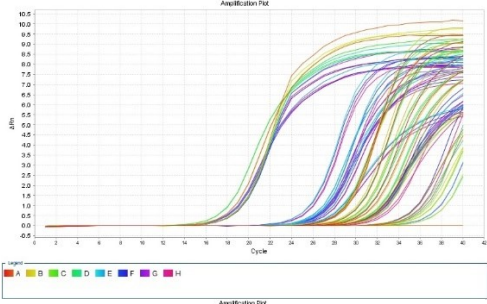
16
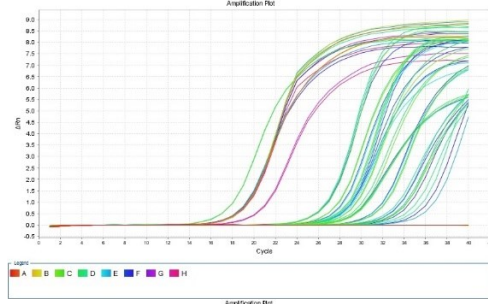
17
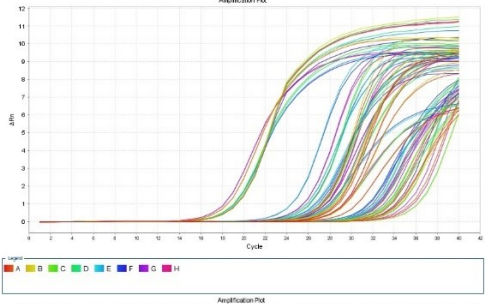
18
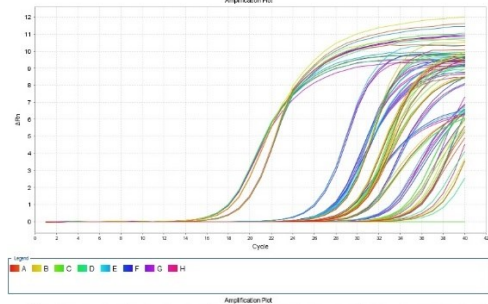
19
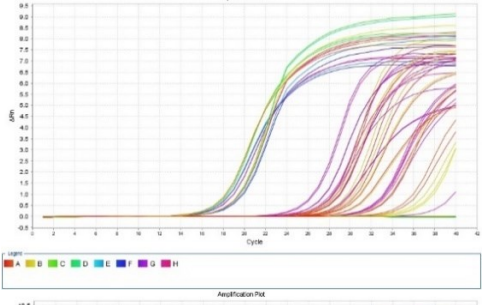
20
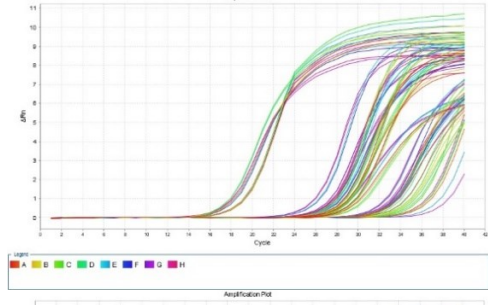
21
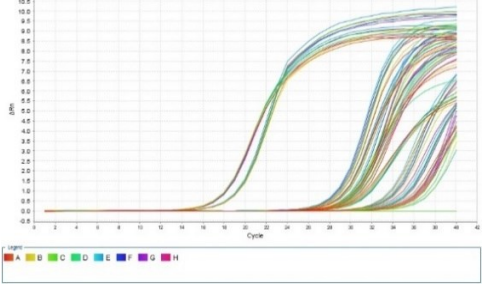
22
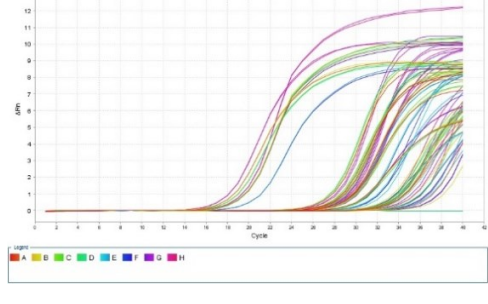
23
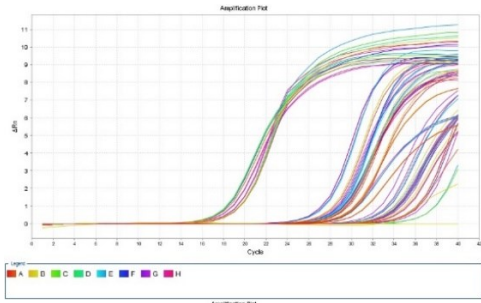
24
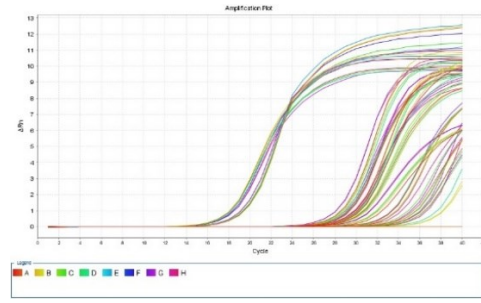
25
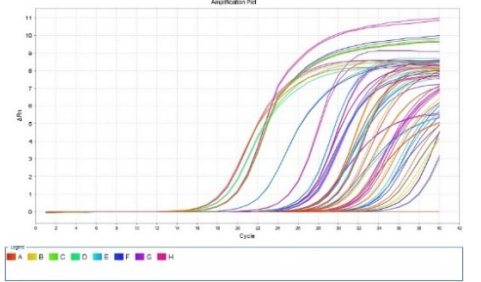
G. Melting Plots

Figure A.3: Melting plots for the 25 samples. The numbers indicate patient number and the melting plots marked with a red X were omitted from the data analysis because they failed to give results for both pre- and post-operative samples.
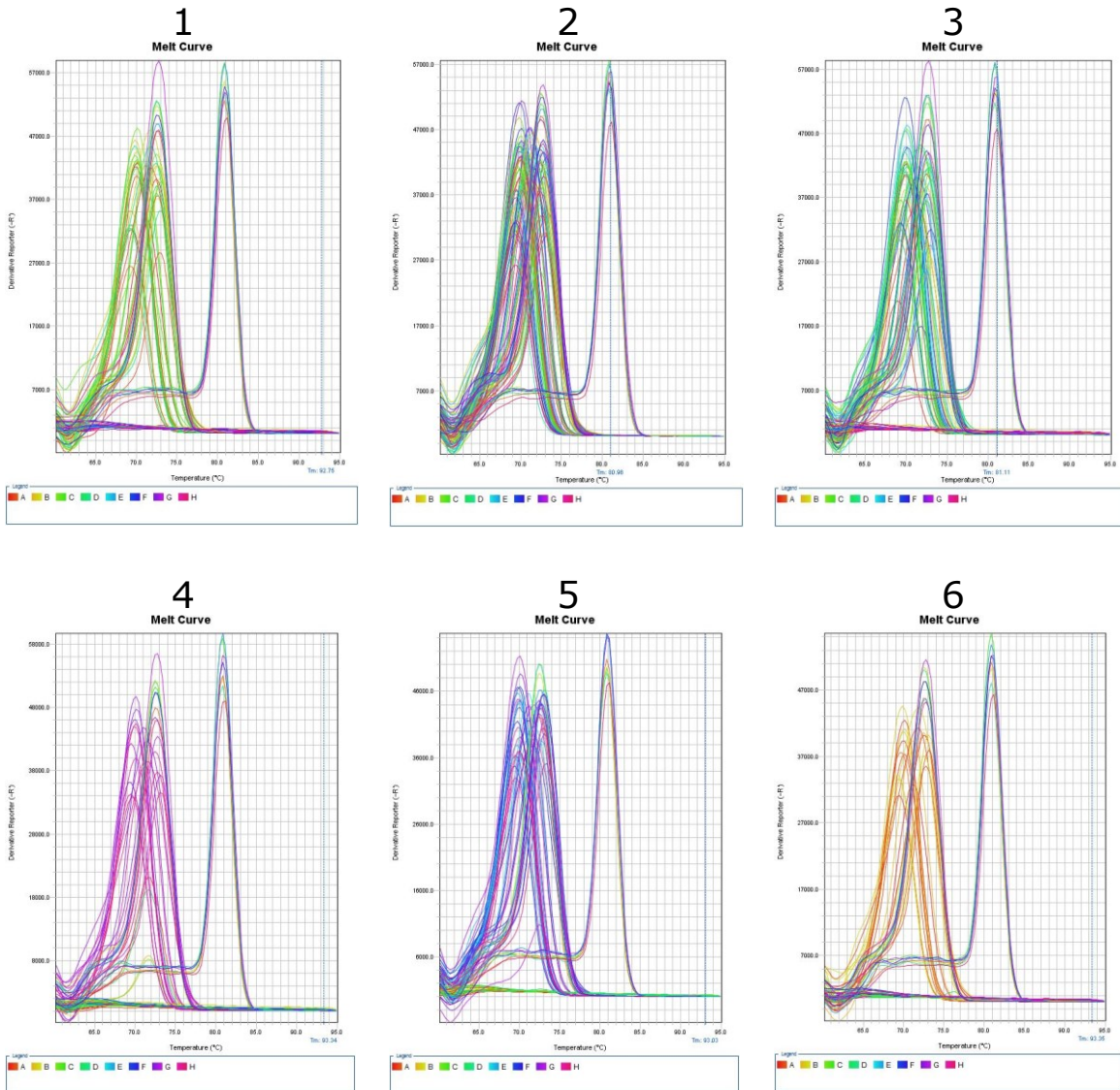
7
Melt Curve
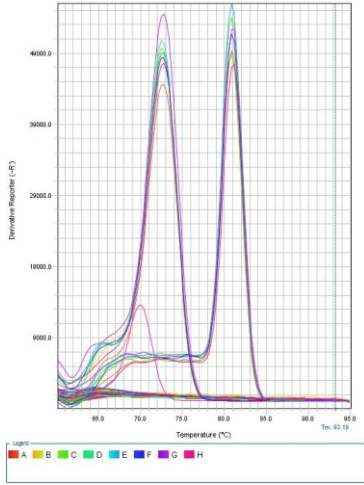
8
Melt Curve

9
Melt Curve

10
Melt Curve

11
Melt Curve
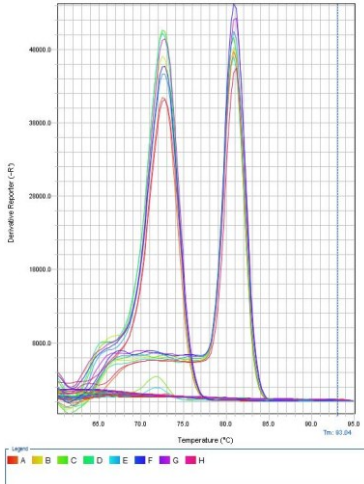
12
Melt Curve
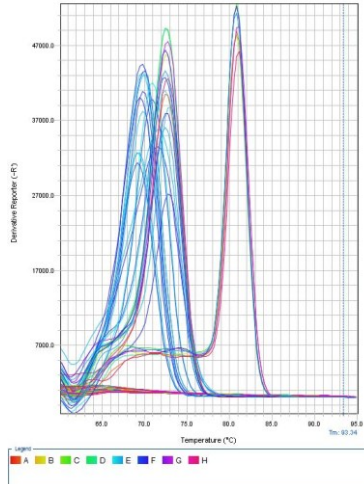
13
Melt Curve
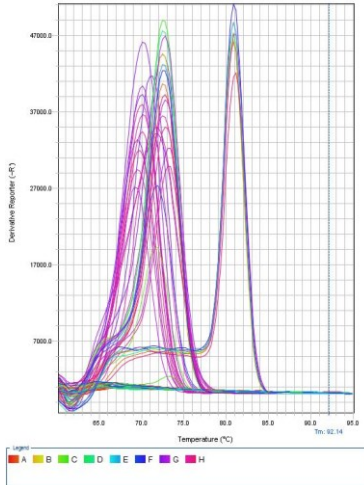
14
Melt Curve
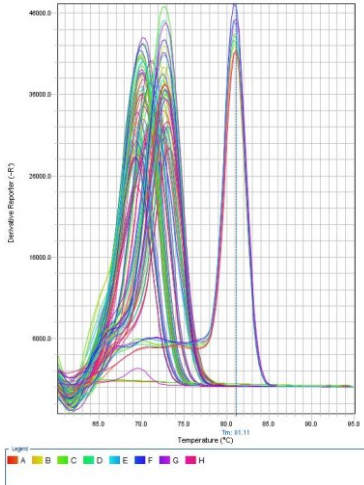
15
Melt Curve
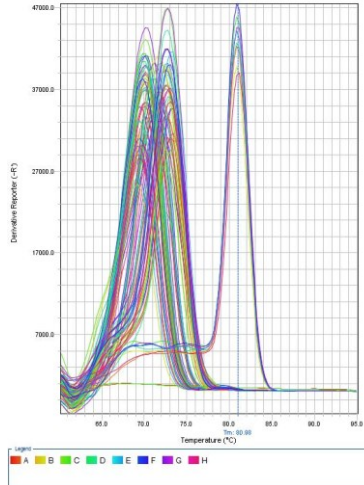
16

Melt Curve
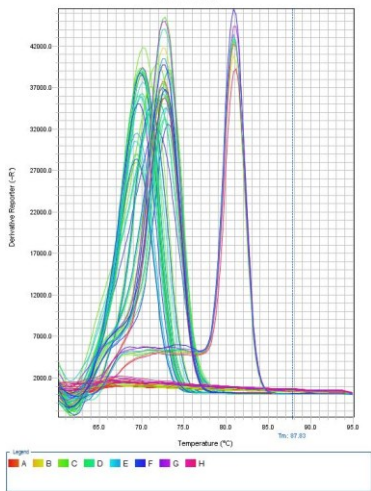
17

Melt Curve
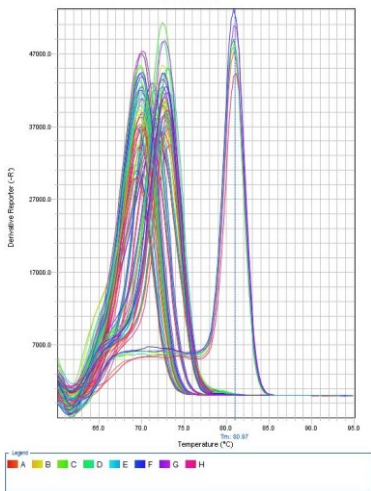
18

Melt Curve
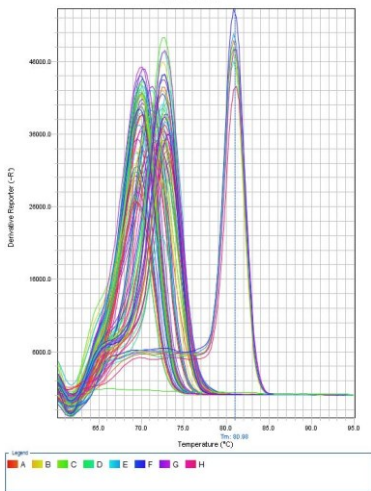
19

Melt Curve
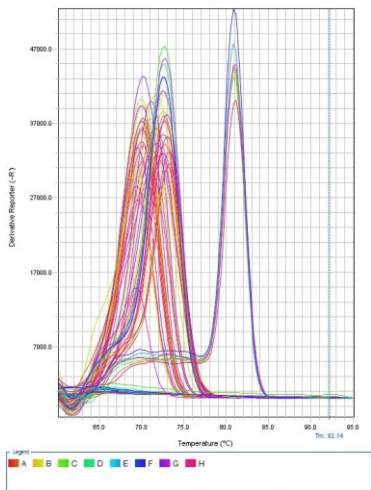
20

Melt Curve
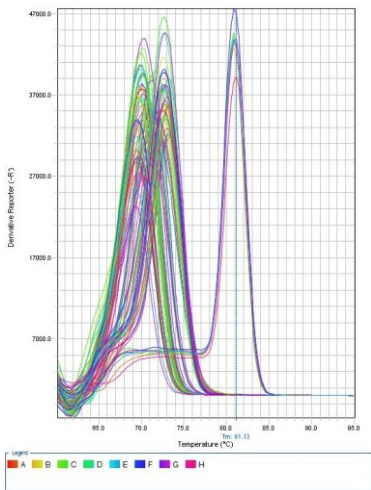
21

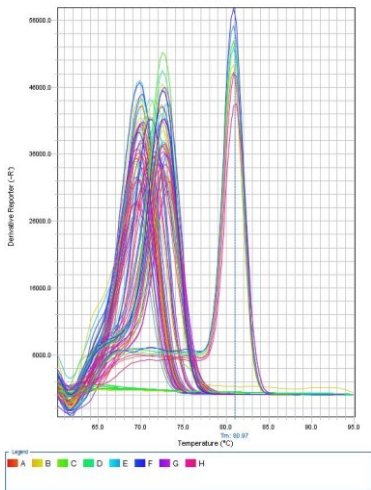**Melt Curve**
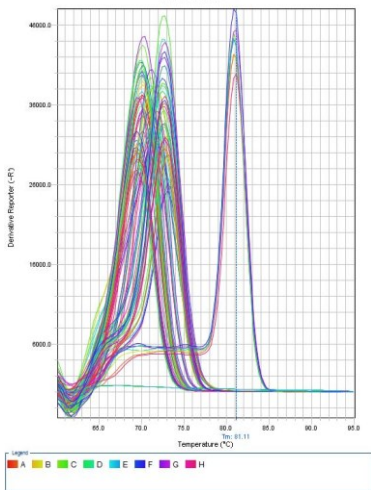
22

Melt Curve
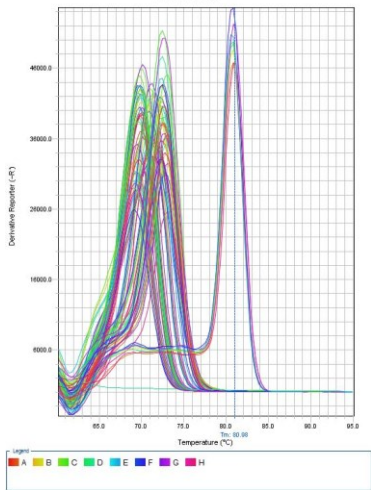
23

Melt Curve

24

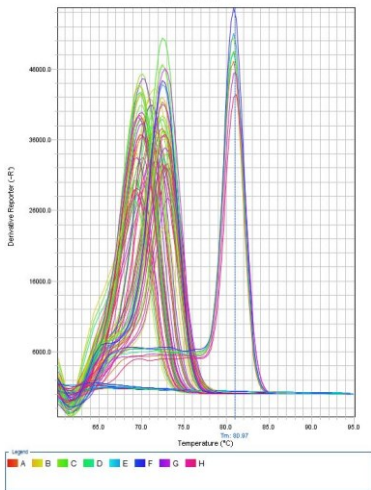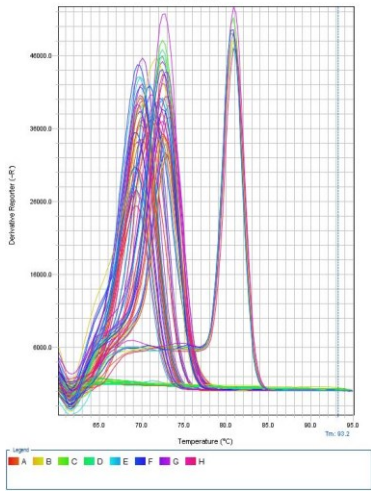**Melt Curve**

25

Melt Curve

H. Raw and normalized Ct values

In table A.7 it is shown the raw Ct-values for each miRNA from pre-and post-operative samples from the 25 patients.

Table A.7: Raw Ct-values for each miRNA target

Patient	miR-25-3p		let-7b-5p		miR-375		miR-18a-3p		miR-324-5p		miR-141-3p	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	29,9	-	30,8	-	32,4	-	35,3	-	35,8	-	36,9	-
2	28,8	28,4	31,4	29,8	34,4	34,7	36,0	35,0	35,6	33,2	35,1	36,3
3	30,8	30,4	32,7	32,6	34,4	35,4	35,1	36,4	34,9	36,0	36,4	-
4	-	28,9	-	30,7	-	34,8	-	35,0	-	35,3	-	36,6
5	-	22,7	-	28,4	-	32,8	-	33,7	-	33,3	-	35,5
6	28,9	-	29,9	-	33,9	-	34,8	-	35,4	-	36,4	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	27,7	29,7	29,5	31,9	32,4	34,5	34,0	36,1	34,5	36,4	36,1	35,5
9	28,9	-	30,8	-	31,9	-	35,2	-	35,3	-	36,0	-
10	30,3	28,5	31,5	29,7	35,8	32,9	35,5	35,0	36,2	33,1	37,0	36,8
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	28,9	-	30,9	-	33,7	-	35,7	-	35,4	-	36,6
13	-	28,0	-	30,8	-	33,9	-	35,4	-	30,4	-	36,1
14	29,0	28,6	30,3	30,5	34,3	34,5	35,1	34,9	35,4	34,6	36,9	36,7
15	30,0	26,7	31,8	27,8	32,9	33,4	35,8	32,5	36,0	31,5	36,2	35,6
16	27,8	27,9	30,8	29,7	32,9	32,6	34,1	34,1	35,0	34,4	35,9	36,7
17	28,1	26,4	29,5	27,7	33,5	33,2	34,3	32,3	34,2	32,6	36,1	35,3
18	29,4	30,0	30,8	28,5	35,2	31,4	35,6	33,4	35,5	32,4	37,0	37,0
19	29,1	27,0	31,5	28,8	34,0	33,4	36,3	33,3	36,1	32,6	35,2	37,1
20	28,8	27,0	29,8	28,7	34,0	33,1	35,1	33,6	35,0	33,8	35,0	35,9
21	29,8	29,5	30,9	31,3	34,6	34,5	35,8	36,2	36,1	35,4	36,7	36,1

22	28,5	29,0	30,8	30,6	34,6	34,5	34,7	36,0	33,9	34,8	35,8	35,8
23	27,5	26,5	30,9	29,9	34,6	33,6	35,0	33,4	35,3	34,7	35,3	36,0
24	29,7	29,1	31,8	30,9	34,1	34,2	35,3	34,2	35,8	35,7	36,8	35,2
25	29,8	25,9	31,9	27,9	33,7	32,8	35,6	31,8	36,1	32,8	-	36,5

In table A.8 it is shown the normalized Ct-values for each miRNA from pre-and post-operative samples from the 25 patients.

Table A.8: Normalized Ct-values for each miRNA target for the 16 patient samples that were included in the data analysis.

Patient	miR-25-3p		let-7b-5p		miR-375		miR-18a-3p		miR-324-5p		miR-141-3p	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
2	3,35	3,79	1,74	2,37	-1,24	-2,56	-2,83	-2,83	-2,42	-1,02	-1,97	-4,14
3	2,74	3,58	0,82	1,34	-0,90	-1,39	-1,64	-2,40	-1,38	-1,98	-2,91	-
8	3,31	3,22	1,47	1,07	-1,44	-1,59	-2,95	-3,21	-3,46	-3,42	-5,11	-2,53
10	3,43	3,61	2,25	2,43	-2,02	-0,75	-1,67	-2,87	-2,40	-0,97	-3,23	-4,67
14	3,09	3,03	1,75	1,13	-2,20	-2,90	-3,00	-3,27	-3,33	-3,06	-4,79	-5,09
15	3,10	3,14	1,22	1,20	0,18	-3,64	-2,77	-2,74	-2,96	-1,71	-3,16	-5,81
16	3,71	3,72	0,76	1,91	-1,34	-1,01	-2,50	-2,50	-3,40	-2,78	-4,39	-5,07
17	2,91	3,15	1,53	1,86	-2,55	-3,66	-3,26	-2,82	-3,19	-3,11	-5,07	-5,82
18	3,34	3,74	1,88	2,22	-2,50	-0,73	-2,88	-2,73	-2,79	-1,73	-4,28	-4,63
19	3,83	3,72	1,39	1,90	-1,11	-2,65	-3,42	-2,55	-3,24	-1,93	-2,27	-6,39
20	2,98	3,52	1,98	3,52	-2,17	-2,55	-3,33	-3,05	-3,22	-3,23	-3,23	-5,31
21	3,14	3,53	2,13	1,72	-1,57	-1,48	-2,77	-3,13	-3,15	-2,30	-3,67	-3,01
22	3,22	3,42	0,10	1,76	-2,91	-2,08	-2,95	-3,64	-2,20	-2,46	-4,03	-3,43
23	4,95	4,89	1,51	1,54	-2,11	-2,22	-2,57	-2,02	-2,88	-3,30	-2,84	-4,54
24	3,11	2,87	1,06	1,11	-1,24	-2,20	-2,49	-2,26	-2,97	-3,71	-3,94	-3,28
25	2,98	3,52	0,88	1,54	-0,94	-3,38	-2,89	-2,32	-3,35	-3,37	-	-7,01

Biobank1®