

Expression Profiling of circulating micro-RNAs in Prostate Cancer

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by

Brian Daniel Kelly

MB BCh BAO BMedSci MSc MRCSI



Under the supervision of

Dr. Nicola Miller

and the direction of

Professor Michael Kerin

Discipline of Surgery

National University of Ireland, Galway

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I would like to dedicate this thesis to Veronica and Tadhg.

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ABBREVIATIONS

A	adenine
ADPC	androgen dependent prostate cancer
ADT	androgen deprivation therapy
AIPC	androgen independent prostate cancer
AJCC	american joint committee on cancer
AR	androgen receptor
AS	active surveillance
AUC	area under the curve
bp	base pair(s)
BPH	benign prostatic hyperplasia
C	cytosine
Ca ⁺⁺	calcium ions
cDNA	complementary DNA
CIS	carcinoma <i>in-situ</i>
CRP	C-reactive protein
CRPC	castration-resistant prostate cancer
CSAP	cryosurgical ablation of the prostate
CSC	cancer stem cells
Ct	cycle threshold
ddH ₂ O	ultra-pure water
ddNTP	dideoxyribonucleotide(s)
DHT	5- α -dihydrotestosterone
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide(s)
DTT	dithiothreitol
DRE	digital rectal exam
EBRT	external beam radiotherapy
EC	endogenous control
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-mesenchymal transition
EN2	Engrailed-2
ERSPC	European randomized study of screening for prostate cancer
ESR	erythrocyte sedimentation rate
FSH	follicle-stimulating hormone
g	gram
G	guanine
G	gauge
GP	general practitioner
HGPIN	high grade prostatic intraepithelial neoplasm
HPC1	hereditary prostate cancer gene 1
IPSS	international prostate symptom score
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LH	luteinising hormone
LHRH	luteinising hormone-releasing hormone
M	molar
M	million
mg	milligram
miRNA	microRNA
mL	millilitre
mRNA	messenger RNA
mM	millimolar
NCCP	National Cancer Control Program
NIH	National Institutes of Health
NPV	negative predictive value
NaOH	sodium hydroxide
nm	nanometers
PIN	prostatic intraepithelial neoplasm
PCa	prostate cancer
PCA3	prostate cancer antigen 3
PCR	polymerase chain reaction
PLCO	prostate lung colorectal and ovarian cancer screening trial
PPV	positive predictive value
PSA	prostate specific antigen
RAPAC	rapid access prostate assessment clinic
RT PCR	reverse transcriptase PCR
RQ-PCR	relative quantification PCR
RNA	ribonucleic acid
ROC	receiver operating characteristic curve
RRP	radical retropubic prostatectomy
T	thymine
TAN	tumour associated normal
TE	tris-EDTA buffer
TNM	Tumour, Node, Metastasis staging system
TRUS	transrectal ultrasound guided biopsy of the prostate
TURP	transurethral resection of the prostate
U	unit(s)
USA	united states of america
°C	degrees centigrade
µg	microgram
µL	microlitre
µM	micromolar
WW	watchful-waiting
ZEB1	zinc-finger E-box binding homebox 1
ZEB2	zinc-finger E-box binding homebox 2

COMMUNICATIONS ORIGINATING FROM THIS WORK

Peer Reviewed Published Manuscripts

A review of expression profiling of circulating microRNAs in men with prostate cancer.

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Kelly BD, Miller N, Durkan GC, Sweeney KJ, Rogers E, Walsh K, Kerin MJ.

Submitted to Urology Gold. Under Review.

Published Abstracts

Circulating miRNAs as blood based markers for prostate cancer.

B.D. Kelly, N Miller, G.C. Durkan, K.J. Sweeney, E. Rogers, K. Walsh, M.J. Kerin.

Ir J Med Sci. 2011 Mar;180 Suppl 3:S72-115

Circulating microRNA signatures: a novel biomarker in prostate cancer.

Kelly BD, Miller N, Durkan G, Rogers E, Walsh K, Kerin MJ.

Int J Surg. 2011 Sep; 9(7):493-582

Circulating microRNA signatures: a novel biomarker in prostate cancer.

Kelly BD, Miller N, Durkan G, Rogers E, Walsh K, Kerin MJ.

British Journal of Surgery 2011;98(S3):1-80

Circulating microRNAs: Novel biomarkers for prostate cancer in the screening setting.

Kelly BD, Miller N, Durkan GC, Rogers E, Kerin MJ, Walsh K.

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Circulating microRNA signatures: a novel minimally invasive biomarker for prostate cancer

Kelly BD, Miller N, Durkan GC, Sweeney KJ, Rogers E, Walsh K, Kerin MJ

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Circulating microRNA signatures: A novel minimally invasive biomarker for prostate cancer.

Kelly BD, Miller N, Durkan GC, Sweeney KJ, Rogers E, Walsh K, Kerin MJ. *Journal of Urology* 2012; 187 (S4) E1-936

Circulating *miR-141* as a diagnostic biomarker for prostate cancer and for monitoring response to treatment.

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Presentations to Learned Societies

“Circulating miRNAs as blood based markers for prostate cancer”.

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“Circulating microRNA signatures: a novel biomarker in prostate cancer”.

SARS/ASiT Plenary Prize Session (Prize Winner)

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“Circulating microRNA signatures: a novel biomarker in prostate cancer”.

International Surgical Congress of the Association of Surgeons of Great Britain and Ireland (ASGBI). Bournemouth, May 2011.

“Circulating microRNA signatures: a novel minimally invasive biomarker for prostate cancer”.

Plenary Prize Session.

Sir Peter Freyer Meeting, Galway. September 2011.

“Circulating microRNAs: A novel minimally invasive biomarker for prostate cancer”.

Irish Society of Urology. September 2011.

“Circulating microRNA signatures: A novel minimally invasive biomarker for prostate cancer”.

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“Circulating microRNAs: Novel biomarkers for prostate cancer in the screening setting”.

British Association of Urological Surgeons (BAUS), Annual Meeting, Liverpool, June 2011.

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ABSTRACT

Mi(cro)RNAs are small non-coding RNAs whose differential expression in tissue has been implicated in the development and progression of many malignancies, including prostate cancer. The discovery of miRNAs in the blood of patients with a variety of malignancies makes them an ideal, novel biomarker for prostate cancer diagnosis.

The aim of this study was to identify a unique expression profile of circulating miRNAs in patients with prostate cancer attending a rapid access prostate assessment clinic.

To conduct this study blood and tissue samples were collected from 102 patients (75 with biopsy confirmed cancer and 27 benign samples) following ethical approval and informed consent. These patients were attending a prostate assessment clinic. Samples were reverse-transcribed using stem-loop primers and expression levels of each of 12 candidate miRNAs were determined using real-time quantitative PCR. MiRNA expression levels were then correlated with clinicopathological data and subsequently analysed using qBasePlus software and Minitab.

Circulating miRNAs were detected and quantified in all subjects. The analysis of miRNA mean expression levels revealed that 4 miRNAs were significantly dysregulated, including let-7a ($p=0.005$) which has known tumour suppressor characteristics, along with miR-141 ($p=0.01$) which has oncogenic characteristics.

In 20 patients undergoing a radical retropubic-prostatectomy, the expression levels of miR-141 returned to normal at day 10 post-operatively. A panel of 4 miRNAs could be used in combination to detect prostate cancer with an AUC of 0.783 and a PPV of 80%.

These findings identify a unique expression profile of miRNA detectable in the blood of prostate cancer patients. This confirms their use as a novel, diagnostic biomarker for prostate cancer.

Chapter 1

Introduction

1.1 Prostate Cancer

1.1.1 Disease Burden

In the western world, prostate cancer (PCa) is the second most frequently diagnosed cancer in men, and the third leading cause of cancer death in men^{1,2}. However, in the USA, PCa is the most common cancer in men and is the second leading cause of death in men³. In 2008, it was estimated that there were 382,000 new cases of PCa diagnosed in the European Union⁴. PCa is rare in men younger than 50 years of age but the risk of developing PCa rises significantly with age beyond this age. Autopsy studies of men without clinical evidence of malignancy reveal a high detection rate of clinically undetected PCa, which increases from 40% at the age of 50 to 80% by the age of 80⁵. Given that the population of the western world has risen dramatically as a result of the baby boom following the Second World War, PCa is likely to be an increasing public health problem over the decades to come.

The incidence of PCa is higher in African Americans (100 per 100,000) than in Caucasians (70 per 100,000) and the incidence is quite low in Native American Indians, Hispanics and in Orientals. However the prevalence of latent PCa is similar in all ethnic groups despite the wide variation in the clinical incidence of PCa.

Currently, PCa diagnosis is based on measurement of serum prostatic specific antigen (PSA), digital rectal examination (DRE) and the histological analysis of prostatic biopsies. A variety of management options are available to patients, ranging from active surveillance to surgery (radical prostatectomy) and radiotherapy. Hormonal and chemotherapy regimens exist for metastatic disease².

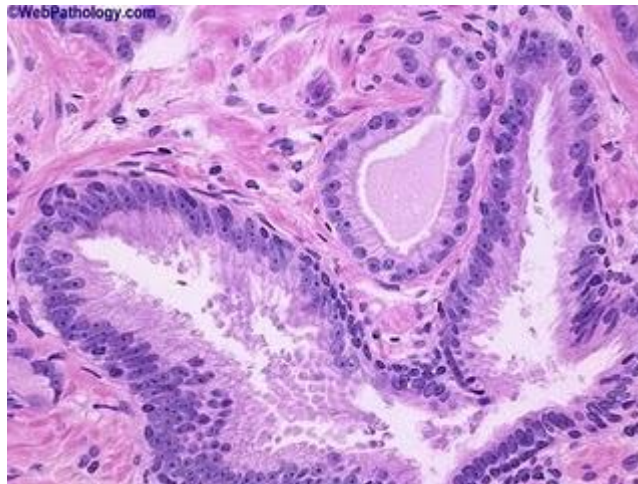
However, PCa can vary from being a cancer that is asymptomatic and does not metastasise to an incredibly aggressive cancer that can metastasise causing significant pain, morbidity and ultimately death. Recent improvements in management techniques have resulted in a decrease in morbidity and mortality. The issue is that many men die with PCa rather than from it, giving rise to the theory that many men have clinically insignificant cancers. A sensitive, specific biomarker is needed to clearly identify those that have a cancer that needs radical treatment from those that have a clinically insignificant tumour.

1.1.2 Prostatic Intraepithelial Neoplasm

Prostate intraepithelial neoplasm (PIN) represents the precancerous end of the morphological change of cellular proliferations within prostatic ducts, ductules and the acini⁶. PIN is considered to be carcinoma *in-situ* (CIS), or a premalignant lesion of the prostatic ducts. There are two grades of PIN identifiable (low and high grade). High grade PIN (HGPIN) is considered to be the pre-invasive stage of invasive prostate adenocarcinoma.

PIN is generally asymptomatic and has no effect on PSA levels. HGPIN is common in men with PCa. HGPIN is found in up to 80% of radical prostatectomy specimens⁷. Patients with an elevated PSA or an abnormal DRE who undergo a transrectal ultrasound guided (TRUS) biopsy and are diagnosed with HGPIN usually undergo a second TRUS biopsy with 6-12 months as the detection of invasive prostate adenocarcinoma at repeat biopsy is approximately 30%⁸.

Figure 1.1 High grade PIN⁹



1.1.3 Gleason Score

The Gleason grading system is used as the histological grading system for PCa. Based on a cancer's microscopic appearance it is assigned a Gleason Score. The score is applied to TRUS needle biopsies, tissue chips from transurethral resection of the prostate (TURP) and radical retropubic prostatectomy specimens (RRP).

The scores range from 1 – 5. With 1 being low grade, well differentiated, while 5 is high grade, poorly differentiated prostate carcinoma. The uro-pathologist assigns a

score to the most common tumour pattern and then assigns a score to the second most common tumour pattern identified¹⁰. For example, if the most common tumour pattern is grade 4, and the second most common tumour pattern is grade 3, the Gleason Score would be $4+3 = 7$.

The Gleason Score is important for the prognosis of the disease and plays a pivotal role in selecting the most appropriate treatment for men with PCa^{11,12}. Interestingly, in RRP specimens, uro-pathologists also assign a score to the third most common pattern, the tertiary component. Again, this has important prognostic implications.

However, inter-observer and intra-observer variability has been reported. The subjective nature of the scoring system precludes an absolute precise diagnosis^{13,14}.

Figure 1.2 Gleason Score for PCa

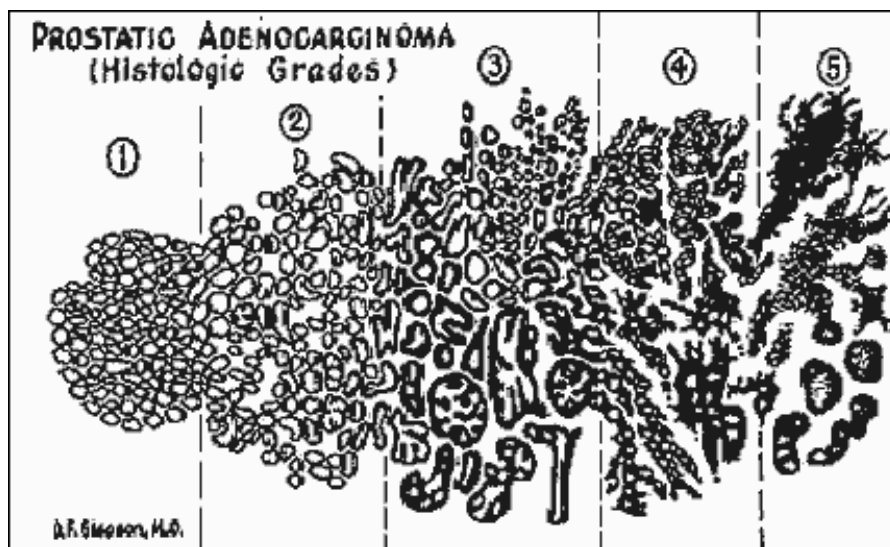


Table 1.1 Features of Gleason Score

Gleason Score	
Gleason Score 2 – 4	<p>Well differentiated</p> <p>Small glands, closely packed</p> <p>Cancer cells behave in a predictable manner</p> <p>Least aggressive, least likely to metastasis</p>
Gleason Score 5 – 6	<p>Moderately well differentiated</p> <p>Variable sized glands with little stroma</p> <p>May also see a cribriform pattern of several cells fused together</p> <p>Cancer cells behave in a predictable manner</p> <p>Most common grade</p>
Gleason Score 7	<p>Can be considered a moderately well differentiated or a poorly differentiated cancer</p> <p>Glands are incompletely formed</p>
Gleason Score 8 – 10	<p>Poorly differentiated</p> <p>Single cells have broken away and may be found within vascular lumen</p> <p>Cancer cells can behave in a unpredictable manner</p> <p>Aggressive cancer</p>

1.1.4 Histological variants

The vast majority of PCa is adenocarcinoma but there are some histological variants of prostate carcinoma, however these account for less than 10%. The clinical behaviour of these morphological variants varies, with some having a worse or better prognosis.

1.1.5 Genetics

Race, family history and specific gene variants attribute specific risk to the development of PCa. Men with a first-degree relative with PCa have double the risk of developing PCa, and those with two first-degree relatives with PCa have a fivefold greater risk as compared with men with no family history¹⁵. In the USA, African American men are more commonly affected with PCa than Caucasian or Hispanic men and they develop a higher grade of disease earlier^{16,17}. Investigation of twin studies in Scandinavia identified that forty percent of PCa risk is attributable to inherited factors¹⁸.

Mutations in BRCA1 and BRCA2 genes are known to be risk factors for ovarian cancer and breast cancer in women. These genes have also been implicated in the pathogenesis of PCa¹⁹. Another gene which is more recently described in PCa is the Hereditary PCa gene 1 (HPC1)¹⁷. The loss of cancer suppressor genes occurs early in the pathogenesis of PCa. The loss of these suppressor genes have been localized to chromosomes 8p, 10q, 13q, and 16q. P53 mutations are relatively uncommon in localised PCa but are seen more frequently in metastatic PCa²⁰.

1.1.6 TNM Staging

The tumour, node, metastasis (TNM) staging, as described by the American Joint Committee on Cancer (AJCC), is most commonly used in clinical practice along with the Gleason Score²¹. This staging system evaluates the size of the tumour, the extent of involved lymph nodes, and metastasis.

Table 1.2 TNM classification of PCa

TNM	
T (Tumour)	TX: tumour cannot be assessed T0: no evidence of primary tumour
	T1: tumour not clinically apparent T1a: tumour found in resected specimen (<5%) T1b: tumour found in resected specimen (>5%) T1c: tumour found at biopsy for elevated PSA
	T2: tumour confined to prostate T2a: tumour involves one lobe of prostate T2b: tumour involves both lobes of prostate T3: tumour palpable, extends beyond capsule T3a: tumour extends beyond capsule T3b: tumour invades seminal vesicles T4: tumour is fixed or invades adjacent anatomy
N (Node)	NX: regional lymph nodes cannot be assessed N0: no regional lymph node metastasis N1: metastasis to regional lymph node(s)
M (Metastasis)	Mx: presence of distant metastasis cannot be assessed M1: distant metastasis M1a: metastasis to non-regional lymph nodes M1b: metastasis to bone M1c: metastasis to other distant sites

1.1.7 Signs and Symptoms

As PCa traditionally develops in the peripheral zone of the prostate, initially it can be asymptomatic. However, as the tumour grows it may cause urinary symptoms as the tumour surrounds the prostatic urethra causing obstruction. Symptoms include frequency, urgency, nocturia, weak stream, poor flow, intermittency, tenesmus and the sensation of incomplete voiding. Patients may also develop difficulty in achieving an erection and painful ejaculation, although these symptoms are rare.

Patients can present with bone or back pain, particularly in patients with metastatic disease. On DRE, patients may have a palpable nodule in the prostate.

1.1.8 PSA

The two main prognostic factors for treatment efficacy and patient survival are the Gleason grade and also the PSA level at the time of diagnosis²². PSA is a kallikrein-like serine protease produced almost exclusively by the epithelial cells of the prostate gland. PSA liquefies the semen in the seminal coagulum and allows sperm to swim freely²³. PSA is also involved in dissolving the cervical mucus, thus allowing the entry of sperm.

Currently PSA is used as the gold standard, along with DRE, for assessing patients at risk from PCa. However, PSA can be elevated for a variety of reasons including; age, prostatic volume, inflammation, infection, recent ejaculation and also post DRE. Pooled data from a meta-analysis reveals that DRE has a sensitivity of 53% and specificity of 83% and PSA has a sensitivity of 72% with a specificity of 93% with a positive predictive value of 25%²⁴. PSA can result in the over-diagnosis and subsequently over treatment of PCa that is perhaps clinically insignificant^{25,26}.

The term prostatic specific antigen is in fact a misnomer. Although it is an antigen it is not specific to the prostate. It is detectable in prostate tissue, semen and other bodily fluids. In women, it is detectable in the female ejaculate, breast milk and amniotic fluid²⁷. In tissue samples, PSA stains are used to identify if metastases are of prostatic origin²⁸.

1.1.9 PCA3

Prostate cancer antigen 3 (PCA3) is a prostatic gene that expresses a piece of non-coding RNA. PCA3 is exclusively expressed in human prostatic tissue. The PCA3 gene is highly upregulated in PCa^{29,30}. Given that this gene is specific to PCa, it has the potential to be a useful as a tumour marker in clinical practice³¹.

PCA3 levels can be obtained from first pass urine in men after a prostatic massage³². It has been shown to be less sensitive but more specific than PSA for the diagnosis of PCa, and with superior positive (PPV) and negative predictive values (NPV)³³. PCA3 levels do not increase with prostatic volume, unlike PSA³⁴. In clinical practice, PCA3 has been shown to be useful in the management of men undergoing repeat TRUS biopsy^{32,35,36}.

1.1.10 EN2

The HOX genes are a family of homeodomain-containing transcription factors that determine the early identity of cells and tissues during embryonic development³⁷. Engrailed-2 (EN2) is another member of this group. EN2 is a transcriptional repressor, but it also has a role in translational regulation³⁸. In addition to its developmental role, EN2 has recently been shown to be a potential oncogene in both breast cancer and PCa^{39,40}.

EN2 is detectable in the urine, however, unlike PCA3, no prostatic massage is required prior to collection of the first pass urinary specimen. The presence of EN2 in urine was highly predictive of PCa, with a sensitivity of 66% and a specificity of 88.2%. This highlights the potential that this molecule has as a urinary biomarker for the diagnosis of PCa⁴⁰. No correlation between EN2 and PSA has been identified.

1.1.11 TRUS Biopsy

In men with an elevated PSA or an abnormal DRE, they are offered a TRUS biopsy so as to ascertain if PCa is present within the prostate. This involves inserting an ultrasound probe into the rectum through which 12 biopsies are taken in a standard sextant method; with 2 biopsies taken from the base, mid-gland and apex of both lobes of the prostate.

Figure 1.3 Schematic representation of 12 Core TRUS Biopsy

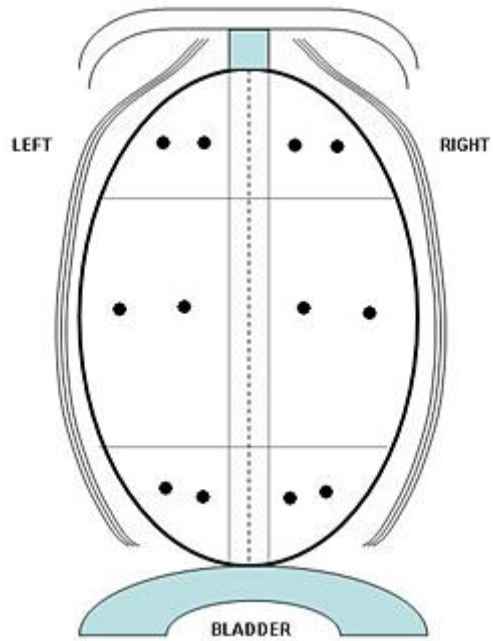


Figure 1.4 Image of TRUS Biopsy

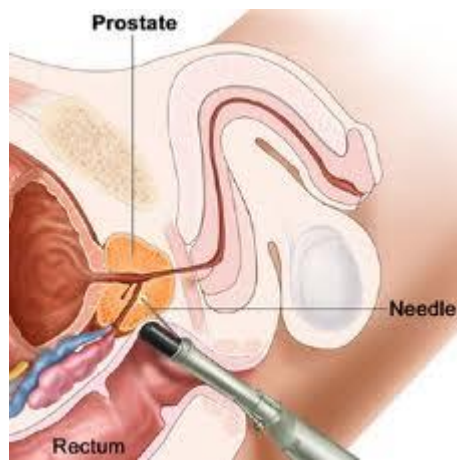
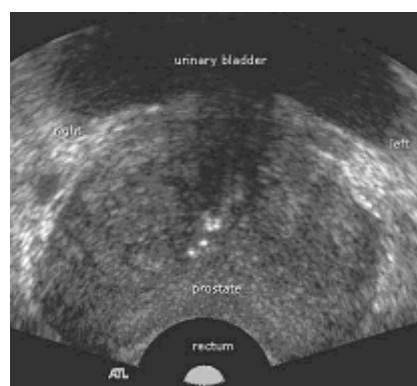


Figure 1.5 TRUS image of prostate



1.1.12 D'Amico Risk Classification

Risk stratification schemes have been developed based on the PSA level, biopsy Gleason score, and clinical T-stage. This risk stratification scheme is associated with the risk of PSA failure and prostate cancer-specific mortality following radical treatment⁴¹.

Table 1.3 D'Amico Risk Stratification

D'Amico Risk	
Low	Diagnostic PSA <10ng/ml AND Highest Biopsy Gleason Score ≤6 AND Clinical Stage T1c or T2a
Intermediate	Diagnostic PSA >10 but <20ng/ml OR Highest Biopsy Gleason Score =7 OR Clinical Stage T2b
High	Diagnostic PSA >20ng/ml OR Highest Biopsy Gleason Score ≥8 OR Clinical Stage T2c/T3

1.1.13 Treatment options

The therapeutic management of PCa has become increasingly complex due to the various therapeutic options available to the clinician and patient, which have equal oncological efficacy but significantly different, treatment-related side-effects. Treatment decisions for each clinical stage and risk group of PCa should be based on national, American or European guidelines. Furthermore, a multidisciplinary approach is advised from the beginning in patients with high-risk PCa because it is very likely that adjuvant treatment will be necessary for locally advanced disease.

1.1.14 Active surveillance

Active Surveillance (AS) is the term for the conservative management of PCa. Introduced in the past decade, it includes an active decision not to treat the patient immediately and to follow him with close surveillance and treat at pre-defined thresholds that classify progression (i.e. short PSA doubling time and deteriorating

histopathological factors on repeat biopsy). In these cases, the treatment options are intended to be curative⁴².

1.1.15 Watchful waiting

Watchful Waiting (WW) also known as ‘deferred treatment’ or ‘symptom-guided treatment’, this term was coined in the pre-PSA screening era (before 1990) and referred to the conservative management of PCa until the development of local or systemic progression, at which point the patient would be treated palliatively with transurethral resection of the prostate (TURP) or other procedures for urinary tract obstruction and hormonal therapy or radiotherapy for the palliation of metastatic lesions⁴².

1.1.16 Radical prostatectomy

The surgical treatment of PCa consists of a RRP, which involves the removal of the entire prostate gland between the urethra and the bladder, and resection of both seminal vesicles along with sufficient surrounding tissue to obtain a negative margin. Often, this procedure is accompanied by a bilateral pelvic lymph node dissection. This procedure can be performed as an open procedure, laparoscopic or robot-assisted laparoscopic operation.

In men with localised PCa and a life expectancy > 10 years, the goal of a RRP by any approach must be eradication of disease, while preserving continence and whenever possible, potency⁴³. There is no age threshold for RRP and a patient should not be denied this procedure on the grounds of age alone⁴⁴. Increased co-morbidity greatly increases the risk of dying from non PCa related causes^{45,46}. An estimation of life expectancy is paramount in counselling a patient about surgery⁴².

1.1.17 Radiotherapy

To date there are no randomised studies comparing radical prostatectomy (RP) with either external beam radiation therapy (EBRT) or brachytherapy for localised PCa. However, the National Institutes of Health (NIH) consensus set up in 1988 is that external irradiation offers the same long-term survival results as surgery and that EBRT provides a quality of life at least as good as that provided by surgery^{42,47,48}.

1.1.18 Brachytherapy

Brachytherapy is a form of radiotherapy where radiation seeds are inserted into the prostate. Transperineal brachytherapy is a safe and effective technique that generally requires fewer than 2 days of hospitalisation. Low-dose rate brachytherapy can be considered for low risk PCa patients with a prostate volume < 50 mL and an IPSS<12.

1.1.19 Cryotherapy

Cryosurgical ablation of the prostate (CSAP) uses freezing techniques to induce cell death by; dehydration, resulting in protein denaturation, direct rupture of cellular membranes by ice crystals, vascular stasis and microthrombi, resulting in stagnation of the microcirculation with consecutive ischaemia and apoptosis⁴⁹⁻⁵².

Freezing of the prostate is ensured by placement of 12 to 15 17G-cryoneedles under TRUS guidance, placement of thermosensors at the level of the external sphincter and the bladder neck, and the insertion of a urethral warmer. Two freeze-thaw cycles are used under TRUS guidance, resulting in a temperature of -40°C in the mid-gland and at the neurovascular bundle. The ideal candidates for CSAP are those who have organ-confined PCa and those identified as having minimal tumour extension beyond the prostate⁴⁹⁻⁵¹. The prostate should be less than 40 mL in size. Prostate glands greater than 40 mL should be hormonally downsized in order to avoid any technical difficulty in placing cryoprobes under the pubic arch. PSA levels should be less than 20 ng/mL, and the biopsy Gleason score should be < 7⁴².

1.1.20 Hormonal treatment

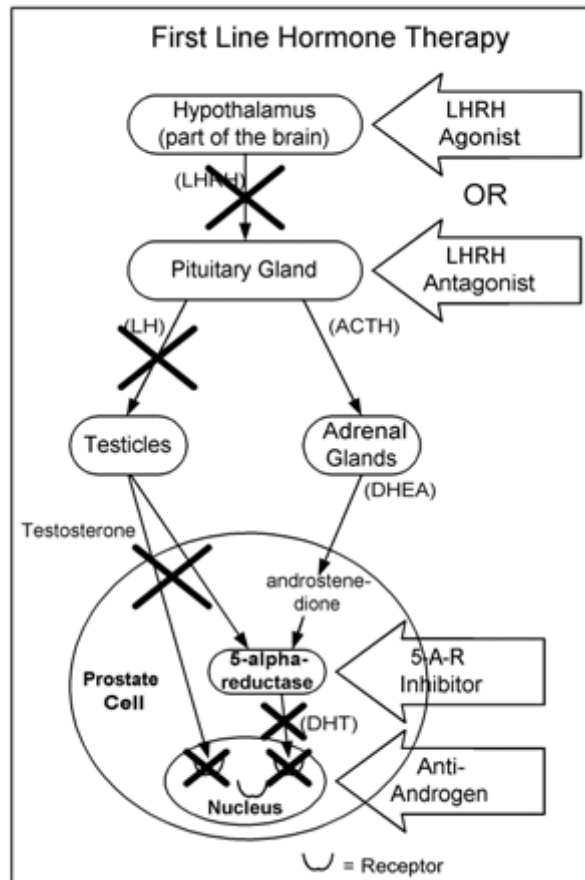
Prostate cells are physiologically dependent on androgens to stimulate growth, function and proliferation. Testosterone, although not tumorigenic, is essential for the growth and perpetuation of tumour cells⁵³. The testes are the source of most of the androgens, with only 5-10% (androstenedione, dihydroepiandrosterone and dihydroepiandrosterone sulphate) being derived from adrenal biosynthesis.

Testosterone secretion is regulated by the hypothalamic-pituitary-gonadal axis. The hypothalamic luteinising hormone-releasing hormone (LHRH) stimulates the anterior pituitary gland to release luteinising hormone (LH) and follicle-stimulating hormone (FSH). Luteinising hormone stimulates the Leydig cells of the testes to secrete testosterone⁴².

Within the prostate cells, testosterone is converted by the enzyme 5- α -reductase into 5- α -dihydrotestosterone (DHT), which is an androgenic stimulant about 10 times more powerful than the parent molecule⁵⁴. Circulating testosterone is peripherally aromatised and converted into oestrogens, which together with circulating androgens, exert a negative feedback control on hypothalamic LH secretion⁴².

Long-acting LHRH agonists (busereline, gosereline, leuproreline and triptoreline) have been used in advanced PCa for more than 15 years and are currently the main forms of androgen deprivation therapy (ADT)^{55,56}. They are synthetic analogues of LHRH, generally delivered as depot injections on a 1-, 2-, 3-, or 6-monthly basis by initially stimulating pituitary LHRH receptors, inducing a transient rise in LH and FSH release. This then elevates testosterone production (known as the 'testosterone surge' or 'flare up' phenomenon), which begins within approximately 2-3 days of the first injection and lasts through approximately the first week of therapy^{42,57}.

Figure 1.6 Mechanism of action of LHRH Agonists/Antagon



1.1.21 Prostate Cancer Screening

There are 3 recent PCa trials which investigate the role of screening. The European Randomized Study of Screening for Prostate Cancer (ERSPC) was initiated in the early 1990s to evaluate the effect of screening with PSA testing on death rates from PCa. The ERSPC trial revealed that there is a huge economic cost for screening and this only gave a 20% early survival benefit. Men who had clinically insignificant cancers were exposed to the complications associated with TRUS Biopsy and repeated biopsies for those on active surveillance. This European trial showed that 1410 men would need to be screened and 48 additional cases of PCa would need to be treated to prevent a single death from PCa²⁶.

The Prostate, Lung, Colorectal, and Ovarian (PLCO) cancer screening trial investigated the effect of screening with PSA testing and DRE on death rates from

PCa. The PLCO trial from the US revealed no statistically significant difference on mortality rates for men undergoing a screening program and those not in a screening program. This is an interesting result given that it comes from a country that has high rates of PSA and DRE testing of patients with their primary care physicians or urologist²⁵.

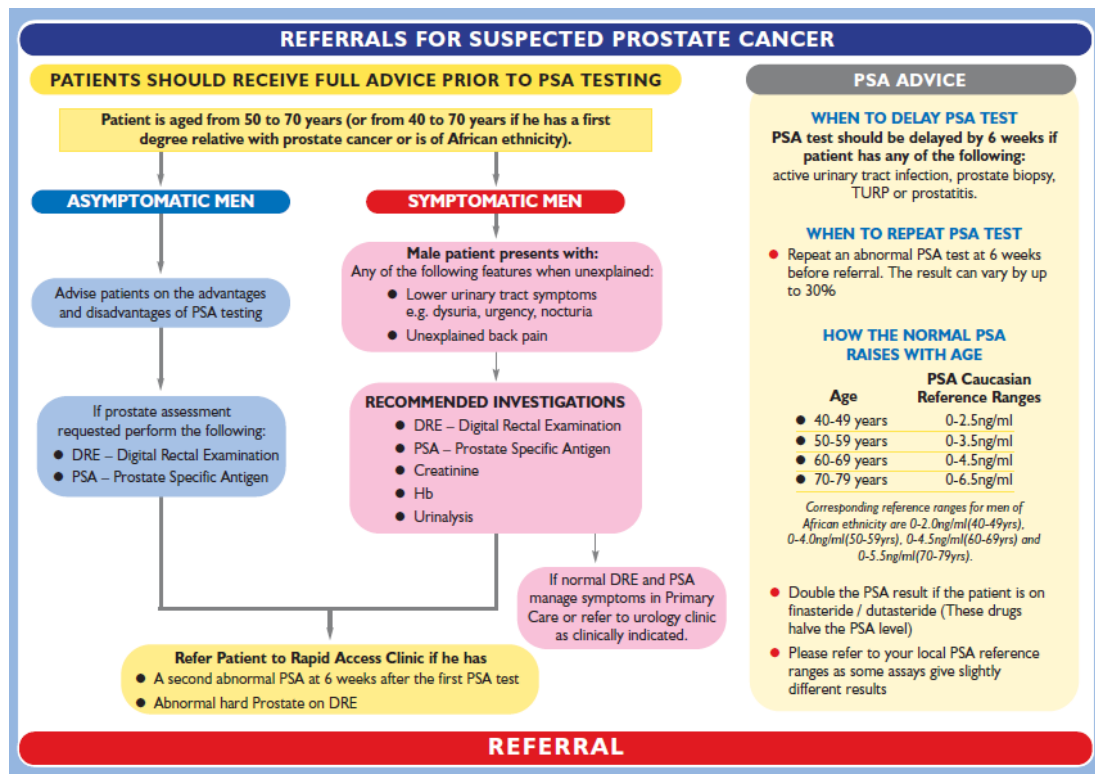
A more recent European study with longer follow up, a lower reference range for a “normal” PSA value and also screening in a younger age group revealed that the number needed to screen to diagnose a cancer and also the number needed to treat were actually considerably lower than the previous studies mentioned above. Deaths from PCa was reduced by over 50% over a 14 year screening period⁵⁸.

Clearly much controversy exists on the topic of screening for PCa. However, given that many studies show that cancer incidence, rates of diagnosis in a screening setting and the mortality rates of cancers are very similar, nearly identical for both prostate and breast cancer, then surely there is a case for arguing for the role out of a national screening program for men in this country, just as there are breast check centres through-out the country. Yet, there are many people that believe that the current roll out of PCa screening is not an example of evidence based medicine, something which clinicians strive to use in everyday practice.

1.1.22 Rapid Access Prostate Assessment Clinic

In Ireland the National Cancer Control Program (NCCP) has established rapid access prostate assessment clinics (RAPAC) for the evaluation of men with an elevated PSA-for –age, an abnormal DRE or a positive family history of PCa. Although screening for PCa is not performed in Ireland, RAPACs have been established to assess men who have made a decision to have their prostate assessed after discussion with their general practitioner (GP).

Figure 1.7 NCCP RAPAC referral guidelines



1.2 miRNAs

1.2.1 Introduction to miRNAs

MiRNAs are small non-coding endogenous RNA molecules that vary in length from 18-25 nucleotides. There are numerous dysregulated miRNAs that are implicated in the pathogenesis of cancer. MiRNAs play a role in the expression of over 60% of human genes⁵⁹. MiRNAs can be up or down-regulated. The up-regulation of oncogenic miRNAs (also known as oncomirs) and the down-regulation of tumour suppressor miRNAs have been demonstrated in many malignancies⁶⁰⁻⁶³.

Overexpression or up-regulation of the oncogenic miRNAs essentially silences the apoptotic factors within the cell, giving rise to malignancy and ultimately metastasis.

1.2.2 miRNA Biochemistry

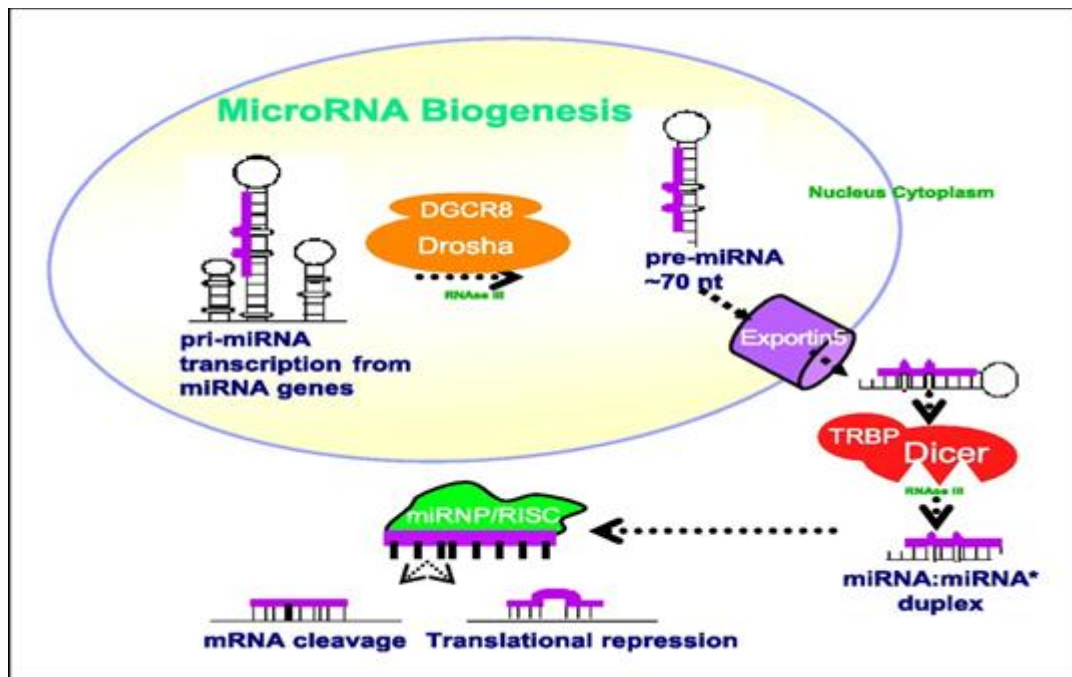
MiRNAs are located within the introns and exons of protein coding genes within DNA and they are also located in the intergenic region⁶⁴. Transcription of the intronic and exonic miRNA appears to occur in parallel with transcription of a host gene, however, transcription of miRNA located at the intergenic regions appear to be transcribed independently⁶⁴.

1.2.3 miRNA Regulation

MiRNAs regulate gene expression at the transcriptional and post-transcriptional level. It is hypothesised that a single miRNA can have as many as 200 target mRNA sites and that a single mRNA may have the ability for different miRNAs to bind at its sites⁶⁵. Dysregulation of miRNA has been associated with the development of cancer and approximately up to 50% of miRNA genes are located in cancer-related genomic locations⁶⁶.

The biosynthesis of miRNA is complex. The transcription of a miRNA gene begins with initiation of transcription by RNA Polymerase II. This results in the formation of a large molecule, pri-miRNA⁶⁷. The enzyme Drosha and the nuclear protein DGCR8 then cleaves a miRNA precursor (pre-miRNA) which is exported from the nucleus to the cytoplasm by Exportin5^{68,69}. Drosha and DGCR8 form a large complex which is known as the microprocessor complex⁷⁰. In the cytoplasm the pre-miRNA is then cleaved by Dicer and an RNA transactivator binding protein into a small dsRNA duplex (miRNA:miRNA*). This duplex molecule is composed of a mature miRNA and a complementary strand (miRNA*)⁷¹. The mature miRNA is then incorporated in a further complex, named RNA-induced silencing complex (RISC). The miRNA* strand is then usually eliminated by cleavage. The mature miRNA molecule is then free to interact with a variety of mRNA targets, resulting in either cleavage or inhibiting protein synthesis⁷¹.

Figure 1.8 A simplified representation of miRNA biogenesis⁷²



1.2.4 MiRNAs and cancer

MiRNA have been initially identified to play an important role in cellular processes such as differentiation, cell growth and death. As the dysregulation of these processes are integral to the development of cancer, it was not surprising that these molecules were soon implicated in carcinogenesis. This was first proven by Calin *et al* who revealed that *miR-15a* and *miR-16-1* were deleted from chromosome 13q14.3. This resulted in an increased expression of the anti-apoptotic gene *BCL2* in the development of chronic lymphocytic leukemia⁶¹. The theory of miRNAs existing as either oncogenic or tumour suppressor miRNA was initially put forward by Lu *et al* in 2005. They observed that miRNA expression had either been upregulated or downregulated when comparing tumour tissue with a normal control⁶³.

Abnormal miRNA expression in PCa tissue had been identified for a number of miRNAs. These miRNAs correspond with cancer progression, invasion and ultimately metastasis of PCa⁷³⁻⁷⁵. If particular miRNA expression results in genetic dysregulation and ultimately leads to cancer progression then miRNA may have the potential to be biomarkers for correlation with PCa diagnosis, histological grade,

stage, classification and also prognosis. With time this could lead to the development of novel therapeutic strategies for PCa management.

The majority of papers in the literature that investigates the role and expression of miRNA in PCa do so by analysing the expression profiles of miRNA in PCa tissue specimens and comparing them to either tumour associated normal tissue (TAN) or normal prostate tissue controls. Expression profile analysis of miRNA in human malignancy has revealed that miRNAs are both expressed and are strongly specific for tissue and also for tumour. MiRNA expression levels have been shown to correlate with tumour stage. Lu *et al* also revealed that miRNAs are superior to mRNA for profile expression patterns. The analysis of miRNA could accurately distinguish 12 from 17 carcinomas, whereas mRNA could only distinguish 1 out of the 17 carcinomas⁶³.

1.2.5 MiRNA expression profiling in prostate cancer tissue

Over the years, a number of studies have investigated dysregulation of miRNA in PCa specimens. MiRNA expression profiles were investigated using micro-arrays and RQ-PCR. Lu *et al* analysed 217 mammalian miRNA in a variety of cancers and normal tissue (334 samples), including 6 PCa specimens and 8 normal tissue controls. MiRNA had been identified to be downregulated in PCa in comparison between the 2 tissue groups⁶³. Volinia *et al* in 2006 analysed 56 PCa tumour tissue and 7 non-cancerous controls. This revealed 39 miRNAs that were upregulated and 6 miRNA that were downregulated in PCa samples⁷⁶. This early work revealed differences in miRNA expression in tumours in comparison to non-cancerous tissue samples.

Later, in 2007, Porkka *et al* identified a unique miRNA signature for PCa. They studied 4 benign prostatic hyperplasia (BPH) samples and 9 PCa specimens along with 6 PCa cell lines and 9 PCa xenografts samples. The PCa xenografts investigated were LuCaP41, LuCaP49, LuCaP69, LuCaP70, LuCaP73, LuCaP86.2, LuCaP92.1, LuCaP93 and LuCaP115. Of the 9 PCa specimens, 5 were untreated and 4 were hormone refractory. Differential expression of 51 miRNAs was identified. 37 were downregulated and 14 upregulated in comparison of the cancer and noncancerous

tissue. Through hierarchical clustering of these tumour samples by their miRNA it was possible to accurately separate the BPH from the PCa specimens. It was also possible to classify these tumours according to their androgen dependence (AD)⁷³.

Ozen *et al* had a similar number in their cohort, 16 PCa tissues and 10 benign peripheral zones. The tissues were extracted from radical prostatectomy specimens. There was widespread downregulation of a variety of miRNAs using micro-array based profiling. Using quantitative polymerase chain reaction (RQ-PCR) to confirm the downregulation, *miR-125b*, *miR-145* and *let-7c* were downregulated in PCa⁷⁷.

Ambs *et al* in 2008 analysed miRNA and mRNA expression in 60 PCa tumours and 16 non-cancerous prostate tissues. The results of this microarray were validated with RQ-PCR. This revealed significant upregulation in prostate tumours of the key components of miRNA processing and also of several genes carrying miRNA coding sequences in their introns through mRNA analysis⁷⁸. The same group later published a paper on miRNA expression and perineural invasion. There was a total of 19 miRNAs which were found to be differentially expressed in tumours with perineural invasion. *MiR-224* was found to have the highest level of expression by the perineural invasive tumours⁷⁴.

Comparing 76 tissue pairs (Tumour and TAN) Schaefer *et al* identified 5 miRNAs that correlated with either Gleason Grade score (*miR-31*, *-96* and *-205*) or the tumour staging (*miR-125b*, *-205* and *-222*). *MiR-205* classified 72% of the PCa specimens correctly, and combining it with *miR-183* improved the correct classification of the samples to 84%⁷⁹. A study by Tong *et al* compared miRNA expression from PCa specimens. 40 prostatectomy specimens were microdissected into PCa areas and uninvolved areas. 20 of these specimens were from patients who had early chemical relapses. 5 miRNAs were identified to be downregulated in PCa (*miR-23b*, *-100*, *-145*, *-221* and *-222*). Of the 16 patients with biological recurrence of PCa within 2 years of surgery, *miR-135b* and *miR-194* were found to be overexpressed⁸⁰. This would suggest that particular miRNA play a pivotal role in the progression of carcinoma.

MiR-221 was found by Spahn *et al* to be downregulated in samples from 92 patients. Upon follow up it was noted that *miR-221* was associated with Gleason scoring and clinical recurrence. PCa specimens with downregulation of *miR-221* were found to

have high Gleason scores, more advanced staged tumours and clinical recurrence. The 10 year survival rates of patients with high and low *miR-221* expression were 87.2% and 53.5%. This is suggestive that *miR-221* can be used as a novel prognostic indicator in patients with high-risk PCa⁸¹.

However, *miR-221* and *miR-222* have been shown in a variety of cancers to be upregulated, including PCa^{80,82-84}. Both of these miRNAs are overexpressed in the PC3 cell model of aggressive PCa when compared with LNCaP and 22Rv1 cell line models of slowly growing carcinomas⁸². Another study comparing androgen dependent (AD) and androgen independent (AI) LNCaP cells reveals that both of these 2 miRNAs are upregulated in castration resistant cells⁸³. *MiR-221* and *miR-222* binds to a target mRNA, the cyclin-dependent kinase inhibitor p27^{Kip1}, which results in tumour growth through p27^{Kip1} downregulation^{82,83,85}.

Particular miRNAs are associated with androgen dependence and independence. Ribas *et al* identified 16 androgen receptor (AR) responsive miRNAs from their analysis of 2 AR positive PCa cell lines. The androgen induced AR binds to a *miR-21* promoter and this results in an over expression of *miR-21*. This overexpression is believed to be associated with AD cell growth and also in the development of castration resistant PCa. Inhibition of *miR-21* reduced the androgen induced cell proliferation of PCa and the overexpression of *miR-21* promoted tumour growth and also castration resistance *in vivo*⁸⁶.

Table 1.4 Dysregulated miRNAs in Prostate Cancer tissue

Upregulated	Downregulated	Study Design	Technique	Validation	Reference
92, 101, 146a, 16, 20a, 21, 32, 34a, let7d, 148, 181, 184, 195, 196, 198, 199a	Let7a, 128, 218-2	540 Samples including 6 malignancies	Microarray	Yes, Northern Blotting	Volinia <i>et al</i> ⁷⁶
184, 198, 210, 296, 345	92, 100, 125a, 125b, 141, 143, 145, 16, 195, 205, 221, 222, 23b, let7a	6 PCa Cell Lines, 9 PCa Xenografts, 4 BPH, 9 PCa	miRNA array hybridization	Yes, RQPCR	Porkka <i>et al</i> ⁷³
106b, 32, let7i, 125a, 181, 188, 196, 370, 99b, 425	145, 205, 221, 34a, let7b, 128, 345	60 PCa Vs 16 TAN	Microarray	Yes, RQPCR	Ambs <i>et al</i> ⁷⁸
-	99b, 100, 125b, 145, 16, 205, 21, 221, 222, 23b, 30a, 34a, let7c	16 PCa Vs 10 TAN	Microarray	Yes, RQPCR	Ozen <i>et al</i> ⁷⁷
20a, 25, let7a, 122a, 129, 141, 302	24, 100, 125b, 143, 145, 205, 221, 222, 23b	40 PCa Vs 40 TAN	Microarray	Yes, RQPCR	Tong <i>et al</i> ⁸⁰
375, 182, 96, 183, 182*	16, 31, 125b, 145, 149, 181b, 184, 205, 221, 222	76 Tissue Pairs (Tumour and TAN)	Microarray	Yes, RQPCR	Schaeffer <i>et al</i> ⁷⁹
-	16, 29a, 125b, 221	4 PCa Vs 4 BPH	Microarray	Yes, RQPCR	Spahn <i>et al</i> ⁸¹

1.2.6 MiRNA and Prostate Cancer stem cells

Cancer stem cells (CSCs) are a particular group of cells that play an important role in the pathogenesis of cancer and metastasis⁸⁷. Prostate CSC have increased clonogenic potential, tumour initiating ability and metastatic capabilities⁸⁸⁻⁹⁰. MiRNAs appear to function as regulators of CSC characteristics⁹¹⁻⁹³. The dysregulation of miRNAs have been identified in stem cells in comparison with normal tissue^{94,95}. *MiR-34a* is one such miRNA, *miR-34a* has strong anti-tumour and anti-metastatic effects. *MiR-34a* is downregulation of in CD44⁺ PCa cells. The upregulation of *miR-34a* in CD44⁺ PCa cells results in the inhibition of clonogenic growth, inhibition of metastasis and tumour regeneration^{96,97}.

1.2.7 The role of specific miRNAs in prostate cancer

As mentioned previously, miRNAs play an integral role in the pathogenesis of cancer. While the upregulation of known tumour suppressors can be viewed as a good prognostic indicator, the upregulation of known oncomirs are signs of more advanced disease. However, these upregulated oncomirs are attractive targets for therapy in the future.

1.2.7.1 The role of *miR-21*

MiR-21 has been shown to be upregulated in a variety of cancers including; prostate, colorectal, hepatocellular and lung. This miRNA appears to direct cell growth by inhibiting cellular apoptosis. In *In-vitro* studies, inhibiting *miR-21* resulted in increased apoptosis in DU145 and PC-3 cell lines, while no effect was seen on LNCaP cells. This highlights that *miR-21* appears to have a role in the pathogenesis of AR negative PCa^{98,99}. Gene targets of *miR-21* have a potential therapeutic role in advanced androgen independent PCa in the future¹⁰⁰.

1.2.7.2 The role of *miR-125b*

MiR-125b is another well-known oncomir in PCa. The downregulation of this miRNA is associated with the regulation of cellular proliferation^{101,102}. Transfection of cells (LNCaP) with this miRNA resulted in significant PCa growth through the development of castration resistance by reducing BAK1 gene expression¹⁰³.

1.2.7.3 The roles of *miR-143* and *miR-145*

MiR-143 is downregulated in PCa and is further decreased during PCa progression. The gene V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) is a potential target of *miR-143* and lower levels of this miRNA is associated with pathogenesis of PCa due to the lack of its inhibitory effect on KRAS^{104,105}. Both *miR-143* and *miR-145* are downregulated in metastatic tumour samples. These miRNAs are correlated with bone metastasis, increasing Gleason Score and levels of free PSA. The upregulation of these miRNAs in PC-3 cell lines resulted in a less invasive morphological pattern, however, it did not have a similar effect on LNCaP cell lines, which are derived from lymph node metastases. Given that these 2 miRNAs are associated with bone metastases, this highlights their potential as biomarkers of disease progression⁵⁹.

1.2.7.4 The role of the *miR-200* family

The *miR-200* family appears to play an important role in regulating epithelial-mesenchymal transition (EMT) by targeting the zinc-finger E-box binding homebox 1 (ZEB1) and ZEB2^{106,107}. The downregulation of the *miR-200* family resulted in the upregulation of ZEB1 and ZEB2. The transfection of PC-3 cells with the *miR-200* family considerably reduced the expression of ZEB1 and ZEB2, which inhibited cell migration and invasion¹⁰⁶.

1.2.7.5 The roles of *miR-221* and *miR-222*

MiR-221 and *miR-222* are both oncomirs which are implicated in metastatic PCa¹⁰¹. One of their target mRNAs is p27kip1, binding to and suppressing this mRNA results in tumour growth⁸². They also play an integral role in the development of castration resistant PCa (CRPC). *MiR-221* is upregulated in both androgen dependent prostate cancer (ADPC) and androgen independent prostate cancer (AIPC)⁸³. *MiR-221* stimulated the growth of both LNCaP and LNCaP-AI¹⁰⁸.

1.2.8 Circulating miRNAs and prostate cancer

MiRNAs are ideal molecules for blood based biomarkers for the detection of cancer as they are dysregulated in carcinogenesis and are highly stable in both tissue and in blood samples^{63,109-111}. MiRNAs have the potential to be used as far superior biomarkers than PSA for the diagnosis and for monitoring response to treatment of PCa. A variety of studies have documented the differential expression of miRNAs in the circulation of patients with cancer when compared to healthy controls, making miRNA an ideal non-invasive biomarkers¹¹²⁻¹¹⁴. Mitchell *et al* also confirmed that tumour derived miRNAs are present in the circulation (present in both serum and plasma) at sufficient levels in order to be used as a suitable biomarker. MiRNAs are resistant to endogenous ribonuclease (RNase) activity as well as variations in temperature and pH^{109,115}.

The exact mechanisms whereby miRNAs are released into the circulation have been debated within the scientific literature. One potential theory is that miRNAs simply leak into the circulation via tissue degradation in a passive, energy independent process. However, there is increasing evidence that miRNAs are actively secreted into the circulation within exosomes and microvesicles and perhaps may even be selected to be transferred to distant cells^{114,116,117}. MiRNAs are also present within Ago complexes in the circulation. MiRNAs in the circulation contained within microvesicles or within Ago complexes may originate from different cell types and may actually reflect a tissue specific miRNA expression profile¹¹⁸.

There has been wide variability in results when miRNAs from patients' serum has been used to differentiate between those with or without PCa. Mitchell *et al* analysed serum samples from a cohort of 50 individuals, 25 patients with metastatic PCa and 25 male, age matched controls. This revealed increased expression of *miR-100*, *-125b*, *-141*, *-143* and *-296* in the serum of the metastatic PCa group. *MiR-141* had the greatest differential expression in the PCa group in comparison with the control group. In fact it had a 46 fold overexpression. Serum levels of *miR-141* could detect individuals with PCa with a high accuracy, with 100% specificity and 60% sensitivity with an area under the curve (AUC) of 0.907¹⁰⁹. This identified a blood based PCR analytical tool for the detection of PCa. Brase *et al* later identified *miR-141* to be upregulated and could be used to differentiate between metastatic and

localised PCa¹¹⁹. However, Mahn *et al* encountered difficulties with detecting *miR-141* in the circulation, this is surprising given that Mitchell and Brase found it to be expressed early in PCa sera^{109,119,120}

Lodes *et al* identified a unique miRNA signature for PCa based on an extraction technique from serum samples. In an analysis comprising of 13 samples (5 from patients with PCa, 8 age matched controls) 15 upregulated miRNAs were identified¹²¹. Brase *et al* investigated the expression profile of miRNAs in the progression of PCa from organ confined disease to metastatic disease. Following an initial screening study of 21 patients (14 with localised PCa, 7 with metastatic PCa) and subsequent validation studies were performed with serum samples to investigate the expression profile of 5 dysregulated miRNAs. *MiR-375* was useful to identify those patients with metastatic disease but also a positive lymph node status. *MiR-141* was also identified as upregulated in the serum of patients with higher grade tumours¹¹⁹.

MiR-21 has previously been identified as an oncomir, being upregulated in a number of cancers. A study by Zhang *et al* into the expression levels of *miR-21* and disease progression reveals many interesting results. The serum of 56 patients were included in this study; 20 with localised PCa, 20 with androgen dependent prostate cancer (ADPC), 10 hormone refractory prostate cancer (HRPC) and 6 patients with benign prostatic hyperplasia (BPH). Patients with HRPC expressed higher levels of *miR-21*. Of the patients with HRPC who subsequently received chemotherapy, those who were resistant to chemotherapy had higher levels of *miR-21* in comparison with patients who were sensitive to chemotherapy, but there was no validation of these results in a separate group. Although numbers in this study were small, this identifies the potential of miRNAs as not only a biomarker for diagnosis, but also for disease progression and for response to treatment¹²².

Yaman *et al* investigated the expression profile of 3 miRNAs; *miR-21*, *-141* and *-221* in plasma. 71 patients were included in this study, 51 with PCa and 20 healthy controls. A significant upregulation of *miR-21* and *miR-221* was observed. Levels of all 3 miRNAs were upregulated in the patients with metastatic disease as compared with those with localised PCa, again confirming that miRNAs have the potential to be used as biomarkers for diagnosis and disease progression¹²³. Zheng *et al* also

identified that *miR-221* was upregulated in patients with PCa. In addition, *miR-221* was also found to be significantly elevated in patients with ADPC¹⁰⁸.

Moltzahn *et al* also identified a miRNA signature that could be used to diagnose PCa and also correlate with disease progression in the serum of 48 patients, 36 with PCa and 12 controls. 5 miRNAs were identified to be upregulated and 4 down-regulated. Three miRNAs, *miR-93*, *-106a* and *-24* were significantly dysregulated in patients with metastatic disease again identifying that there is a miRNA expression profile unique to both localised PCa and also to metastatic PCa¹²⁴.

Heneghan *et al* analysed the expression profile of patients with a variety of malignancies including prostate, colon, breast, renal and melanoma. This study revealed a significant downregulation of the tumour suppressors *miR-145* and *-155* in PCa. Whilst the downregulation of these 2 miRNAs was also observed in a number of the malignancies included in this study, a significant upregulation of *let-7a* was found in prostate and breast cancer and an upregulation of *miR-195* exclusive to breast cancer samples. Using 3 miRNAs in combination; *miR-195*, *-155* and *let-7a* could be used a biomarker for breast cancer with a sensitivity of 94%¹²⁵. This process of using a miRNA signature to identify a particular cancer has the potential to be used as a blood based biomarker across other malignancies.

The receiver operating characteristic (ROC) curve is a standard analytical tool for the evaluation of diagnostic tests. The area under the curve (AUC) is the average sensitivity over a range of specificities. A biomarker with no predictive value will have a AUC of 0.5 or less, whereas a biomarker with a perfect ability to detect disease would have a value of 1.

Mahn *et al* analysed the expression of 4 miRNAs in patients with localised PCa (n=37), metastatic PCa (n=8), BPH (n=18) and healthy controls (n=20). Three of these 4 miRNAs were significantly upregulated in patients with PCa in comparison to patients with BPH, *miR-26a*, *-195* and *let-7i*. MiR-26a could differentiate PCa from BPH with an AUC of 0.703 yet PSA could differentiate between the 2 clinical conditions with a superior AUC of 0.834. However, in patients with an elevated PSA level, *miR-26b* could differentiate those with cancer from those with BPH with an AUC of 0.918, this included 37 PCa patients and 7 BPH patients. These 3 miRNAs along with the 4th miRNA, *miR-32*, could be used in combination to further enhance

their diagnostic potential with an AUC of 0.758. Of 10 patients who underwent a radical prostatectomy, the expression levels of *miR-26a* and *miR-195* were significantly reduced at post-operative day 7, returning to normal expression levels. This further highlights the diagnostic potential of the overall expression profile of many oncomirs and tumour suppressor miRNAs ¹²⁰. This is not the first time that expression levels of miRNAs have returned to normal for patients undergoing oncological surgery. This has previously been described in breast cancer and colorectal cancer patients ^{113,126}.

Table 1.5 Studies investigating the expression profiles of circulation miRNAs in prostate cancer

Upregulated	Downregulated	Study Numbers	Study Design	Validation	Blood Type	References
100, 125b, 141, 143, 296	-	50	25 Metastatic PCa Vs 25 Controls	Yes	Serum	Mitchell <i>et al</i> ¹⁰⁹
16, 92a, 103, 107, 197, 34b, 328, 485-3p, 486-5p, 92b, 574-3p, 636, 640, 766, 885-5p	-	13	5 PCa Vs 8 Controls	Yes	Serum	Lodes <i>et al</i> ¹²¹
9*, 141, 200b, 375, 516a	-	21	7 Metastatic PCa Vs 14 PCa	Yes	Serum	Brase <i>et al</i> ¹¹⁹
Let7a	145, 155	83	20 PCa Vs 63 Controls	No	Whole-blood	Heneghan <i>et al</i> ¹²⁵
21	-	56	50 PCa Vs 6 BPH	No	Serum	Zhang <i>et al</i> ¹²²
21, 221	-	71	51 PCa Vs 20 Controls	No	Plasma	Yaman <i>et al</i> ¹²³
93, 106a, 874, 1207-5p, 1274a	24, 26b, 30c, 223	48	36 PCa Vs 12 Controls	No	Serum	Moltzahn <i>et al</i> ¹²⁴
221	-	43	28 PCa Vs 20 Controls	No	Plasma	Zheng <i>et al</i> ¹⁰⁸
26a, 195, let7i	-	83	45 PCa Vs 38 Controls	No	Serum	Mahn <i>et al</i> ¹²⁰
141, 298, 346, 375	-	50	25 Metastatic PCa Vs 25 Controls	Yes	Serum	Selth <i>et al</i> ¹²⁷

Whilst miRNAs have enormous potential as biomarkers for PCa, work to elucidate their role as systemic markers of this disease remains in its infancy. To date there are a limited number of studies that investigate circulating levels of miRNAs in men with PCa, with study numbers varying from a total of 13 up to 83 patients. Studies range from comparing localised PCa to controls, and some comparing metastatic PCa patients with controls, with only four studies having a validation cohort.

1.2.9 Urinary miRNAs

Urinary miRNAs are a promising tumour marker in urothelial cancer and miRNAs have also been identified in other bodily fluids such as peritoneal fluid and saliva. This highlights the potential that these molecules have as potential biomarkers across a variety of media¹²⁸⁻¹³⁰. However, this is a relatively novel discovery and many further studies are required in this area to elucidate the true potential of urinary miRNAs to act as biomarkers for PCa.

1.2.10 MiRNA Profiling methods

There are several methods for the detection and quantification of miRNAs such as Northern blotting and in situ hybridization techniques, hybridisation based microarray platforms (which allow high throughput miRNA profiling), and single miRNA approaches such as quantitative RT-PCR (reverse transcriptase/polymerase chain reaction)¹³¹⁻¹³³.

1.2.11 MiRNA extraction

The isolation of miRNAs from tissue specimens requires modification of existing RNA extraction protocols due to the small size and unique structure of these molecules. Column based approaches have been adapted to either selectively capture and retain both large and small RNA fractions (e.g. using Qiagen RNeasy kits) and co-purification methods have also been developed to isolate the total RNA which is inclusive of the small RNA fraction¹³⁴.

Currently RNA is extracted from a variety of circulating media, including whole-blood, serum and plasma using a variety of different extraction techniques. There is no established consensus on what is the optimum media from which to isolate RNA. Pritchard *et al* recently identified that a variety of circulating miRNAs are highly expressed in one or more blood cell types. They suggested that acceptable ranges for blood cell counts should be established for miRNAs that are vulnerable to blood cell effects¹³⁵. While most articles to date have focused on free or exosomal miRNAs extracted from either serum or plasma, Heneghan *et al* have highlighted an RNA extraction technique from whole blood using a trizol-based extraction technique which results in a higher yield of miRNAs in comparison to serum or plasma samples. The validity of this process was demonstrated by the fact that there was no significant difference in the white cell count, haemoglobin or haematocrit levels between the cancer and control groups of patients in this article^{126,136}. With this lack of consistency regarding starting material and methodology across results must be viewed with caution.

1.2.12 MicroArray

A microarray is a tool utilised for the analysis of gene expression which allows high throughput analysis. Specific sequences are immobilized to a surface and reacted with labelled complementary DNA (cDNA) targets. A signal resulting from hybridization of the labelled target with the specific, immobilized probe identifies which RNAs are present in the unknown target sample. Initially, microarrays were unable to distinguish between signals from pre-miRNA, pri-miRNA and mature miRNA sequences, however labelling and probe design have been improved to address these issues.

As many miRNAs have similar structures and have very different physiological roles, accurate distinction between them is important. More recently, miRNA microarray platform have been developed using locked nucleic acid modified capture probes which improve the probe thermostability and increases the specificity^{137,138}. These improvements allow for the discrimination between miRNAs with single nucleotide differences. Alternative high-throughput miRNA profiling techniques have been developed which are a bead-based flow cytometric approach which offers high specificity for closely related miRNAs as hybridisation occurs in solution⁶³.

1.2.13 Northern blotting

Northern blotting is a technique whereby RNA is fractionated on a polyacrylamide gels using electrophoresis and hybridization. Traditionally this technique has been used for the analysis of mRNA expression. However, this is a low throughput technique which has limited ability to distinguish between individual miRNAs that have only 1-2 nucleotide differences in their sequences. To date, the most common use of this technique in miRNA analysis is for the validation of candidate miRNAs identified by array-based expression profiles.

1.2.14 Real time quantitative PCR

Real time quantitative polymerase chain reaction (RT-PCR) uses a reverse transcription reaction to convert extracted RNA into cDNA. cDNA is then amplified using PCR and quantification of the amplicons in real time. Gene-specific, stem-loop RT primers can be used to discrimination between slightly differing miRNA sequences and between miRNAs and their corresponding precursors¹³⁹⁻¹⁴².

RT-PCR depends on the correct normalization of the genes of interest to stably expressed endogenous controls. RT-PCR is a relatively low cost method which allows for efficient analysis of miRNA. to date, most centres are using RT-PCR to validate miRNA data obtained from miRNA microarray expression profiling.

1.3 Study Rationale

The aim of this study was to determine if a panel of circulating miRNAs could distinguish patients with PCa from those with benign disease attending a rapid access prostate assessment clinic. MiRNAs dysregulated in the circulation of men with PCa are a potential biomarker for the diagnosis, prognosis and for monitoring response to therapy for PCa.

1.4 Study Aims

The specific aims of the study were;

- To identify a suitable endogenous miRNA control(s) for valid and accurate RQ-PCR.
- To determine if circulating miRNAs were dysregulated in the circulation of patients with PCa from a panel of 12 candidate miRNAs.
- To determine if a panel of circulating miRNAs could identify patients with PCa in a high risk group.
- To determine if circulating miRNAs had the potential to act as biomarkers for PCa in clinical practice.
- To determine if the miRNA expression profile returned to normal after a radical retropubic prostatectomy.

Chapter 2

Materials and Methods

2.1 Biobank

2.1.1 Department of Surgery Biobank

The Department of Surgery BioBank at NUI Galway was established in 1992. With ethics approval from the Galway University Hospitals Clinical Research Ethics Committee, breast specimens including malignant, benign and normal breast tissues retrieved during surgery were initially stored for breast cancer research projects. Following approval from the Galway University Hospitals Clinical Research Ethics Committee, the Biobank was expanded to include patients with PCa. Blood, tumour and tumour-associated normal (macroscopically normal appearing tissue adjacent to the tumour site) tissue specimens have since been routinely collected from patients with PCa. Clinical and pathological data have been anonymised and stored in a dedicated computer database system, Shire.

2.1.2 Sample collection from PCa patients

Following a detailed discussion of the programme, patients were given a written information leaflet (see appendix) further describing the BioBank process and its implications, and then asked for informed consent if they wished to participate. Consenting patients agreed to blood and tissue specimens being taken prior to and during their treatment. Blood samples (whole blood, serum and plasma) were typically collected preoperatively and tissue specimens retrieved from patients at the time of surgical resection of their tumour or at the time of their initial TRUS biopsy.

2.1.3 Blood collection

Whole blood was collected pre-operatively in two Vacurette EDTA K3E blood bottles (Grenier Bio-one) and one Vacutainer Serum Separator Tubes II (Becton Dickinson). One EDTA bottle was processed for plasma and the other unprocessed.

Samples for serum collection were left to clot at room temperature for 30 minutes and then all samples destined for serum and plasma collection were centrifuged at 2000rpm at 4°C for 10 minutes. The plasma and serum were then removed (by pipette), aliquoted and stored at -20°C until required. The unprocessed whole blood sample was stored at 4°C until required.

2.1.4 Tissue collection

Resected specimens were collected immediately following surgical excision and transferred to the pathology laboratory in their resected state (i.e. prior to adding formalin or any other preservative). The specimens were then examined by a pathologist prior to removal of pairs of tissue samples from the macroscopically visible tumour and from the adjacent macroscopically normal tissue at the tumour edge (tumour-associated normal samples). Tissue samples were immediately snap-frozen in liquid nitrogen and then transferred to long-term storage at -80°C.

2.1.5 Anonymisation of patient data

Data (including demographic and limited clinicopathological data) pertaining to all samples used was recorded in the 'Shire' laboratory information management system. This generates unique alphanumeric codes and also assigns a specific storage location for each sample. All samples were relabelled with these anonymised codes prior to storage. Clinical case notes were reviewed to obtain patient clinical and pathological details.

2.2 Study groups

Prior written informed consent was obtained from each participant in these cohorts and ethical approval obtained from the ethics review board of Galway University Hospital.

2.2.1 Prostate cancer group

A cohort of patients was identified from whom blood samples were available or could be obtained. This group was representative of a typical group of PCa patients, and served as the cohort for testing the hypothesis that circulating miRNAs had potential as PCa biomarkers. Their clinical and demographic details are summarized below.

Table 2.1 Patient demographics

	Prostate Cancer Group
Number	75
Age:	
Median	64 years
Mean	65.3 years
Range	48 – 85 years
PSA: (µg/L)	
Median	7.4
Mean	11.55
Range	(1.42 – 52.24)

Table 2.2 Gleason Score

Gleason Score	Number
3+3	28
3+4	20
4+3	14
4+4	6
4+5	7

Table 2.3 D'Amico Risk Stratification

Risk Stratification	Numbers (75)
Low	28
Intermediate	11
High	36

Table 2.4 Metastasis

Metastases	Numbers (75)
No metastases	65
Metastases	10

2.2.2 Benign Group

The benign group were healthy individuals with no known malignancy or active inflammatory condition who provided whole blood samples. These were patients who had an elevated PSA or an abnormal DRE who presented to the RAPAC for a TRUS Biopsy to outrule PCa. Following TRUS Bx, these patients were found to have a benign histological finding. These patients were of a similar age to the PCa group. This group's characteristics are summarized below.

Table 2.5 Patient demographics

	Benign Group
Number	27
Age:	
Median	65 years
Mean	65.1
Range	(48 – 80 years)
PSA: (µg/L)	7.45
Median	11.6
Mean	(1 – 77)
Range	

2.3 RNA extraction from whole blood samples

Total RNA was extracted from whole blood, serum and plasma samples using a modification of the Tri Reagent® BD (Molecular Research Centre) co-purification protocol, as follows: Using 1 mL of whole blood, or its derivatives, phase separation was performed by the addition of 3mL of Trizol and 200µL of 1-bromo-4-methoxybenzene to augment the RNA phase separation process. The homogenous lysate was divided between two 2 mL collection tubes and then centrifuged at 12000g for 15 minutes at 4°C. The clear aqueous phase (approximately 1 mL) from each tube was then removed, transferred to fresh collection tubes respectively and RNA precipitated by the addition of 1 mL of isopropanol and centrifugation of the solution at 12000g for 8 minutes at 18°C. Following the removal of the supernatant, the RNA pellet was then washed with 1 mL of 75% ethanol. An additional ethanol wash was performed to improve the purity of RNA isolated, which was reflected in an improved 260/280 nm spectrophotometry ratio. Each RNA pellet was briefly air-dried and then solubilised using 30µL of nuclease free water. Hence each 1mL of whole blood yielded 60µL of total RNA when the two matched RNA pellets were solubilised, combined, and finally transferred to storage tubes prior to storage at -80°C.

2.4 RNA analysis

2.4.1 Analysis of RNA concentration

Concentration and purity of miRNA was assessed using a Nanodrop spectrophotometer. With the sampling arm of the instrument open, a 1 µL aliquot of RNA was pipetted onto the lower measurement pedestal. Closure of the sampling arm caused the liquid to bridge the gap between the fibre optic ends of the instrument. Spectral measurement was initiated with a tightly controlled path-length of 0.1 cm. RNA concentration was automatically calculated by the operating software using the following formula:

$$\text{RNA concentration (ng / } \mu\text{l)} = (\text{A}_{260} \times \text{e})/\text{b}$$

A₂₆₀=Absorbance at 260nm, e=extinction coefficient(ng-cm/mL),
b=pathlength (cm)

‘Other’ was selected as the sample type on the operating software and an extinction coefficient of 33 was used. RNA with an absorbance ratio at 260 and 280 nm (A_{260}/A_{280}) between 1.8 and 2.2 was deemed indicative of pure RNA. Lower ratio was indicative of the presence of protein or phenol which resulted in high absorption at 280 nm. A ratio at 260 and 230 nm (A_{260}/A_{230}) between 1.8 and 2.2 was considered acceptable. The presence of contaminants could result in lower ratios.

Figure 2.1 The concentration and purity of (a) miRNA assessed using the NanoDrop1000® spectrophotometer with a constant value of 33 and (b) large RNA assessed using the NanoDrop1000® spectrophotometer with a constant value of 40.

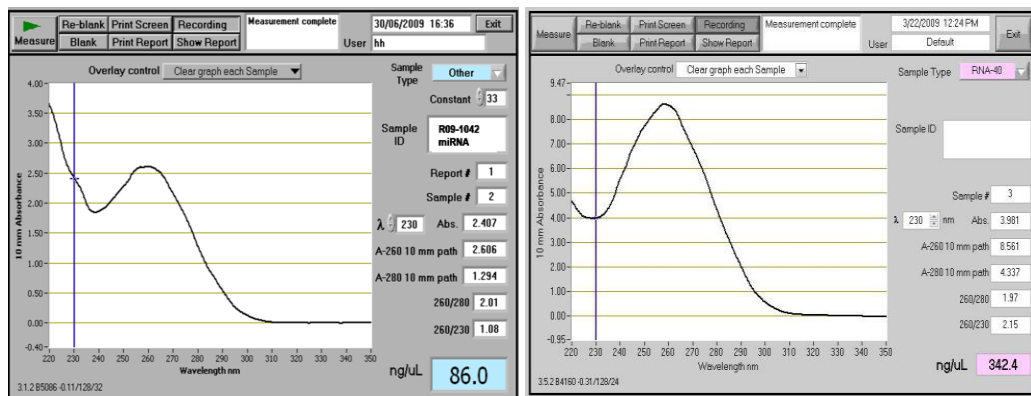


Figure 2.1 (a)

Figure 2.1 (a)

The concentration and purity of the large (>200nt) and total RNA were also assessed using the NanoDrop1000® spectrophotometer (NanoDrop Technologies). The sample type ‘RNA-40’ was selected (figure 2.1b). Spectrophotometry was performed as above with A_{260}/A_{280} ratios of 1.6 - 1.9 indicating RNA that is essentially free of DNA and proteins. The higher the A_{260}/A_{280} ratio, the better the purity of the RNA extracted.

2.4.2 Candidate miRNA selection

Selection of specific candidate miRNAs for expression analysis in this study was based on information gleaned from previous studies within the Discipline of Surgery at NUI Galway and a review of the internationally published literature on both tissue and circulating miRNAs in men with PCa.

Table 2.6 Candidate miRNAs selected which were dysregulated in either blood or PCa tissue

Dysregulated miRNA	Source	Reference
<i>let7a</i>	Blood, Tissue	73,76,80,125
<i>miR-21</i>	Blood, Tissue	76,77,122,123
<i>miR-34a</i>	Tissue	77,78
<i>miR-125b</i>	Blood, Tissue	73,77,79-81,109
<i>miR-141</i>	Blood, Tissue	73,109,119
<i>miR-143</i>	Blood, Tissue	73,80,109
<i>miR-145</i>	Blood, Tissue	73,77-80,125
<i>miR-155</i>	Blood	125
<i>miR-221</i>	Blood, Tissue	73,77-81,108,123
<i>miR-375</i>	Blood, Tissue	79,119
<i>miR-16</i>	Blood, Tissue	125,143-146
<i>miR-425</i>	Tissue	147

2.4.3 Reverse transcription of miRNA to cDNA

Reverse transcription involves the conversion of single-stranded RNA into double-stranded cDNA, which can subsequently be amplified using the polymerase chain reaction (PCR). Primers were obtained from MWG Biotech if sequences were available. Otherwise assays containing stem-looped primer were purchased from Applied Biosystems. Each reaction was primed using a gene-specific stem-loop primer. All reagents were included in the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RNA was diluted to 1 ng / μ L in a final volume of 5 μ L. MiRNA was reverse-transcribed as follows:

Small RNA (1 ng / μL) 5.0 μL

dNTPs (100 mM) 0.17 μL

10X RT buffer 1.65 μL

Nuclease-free water 4.57 μL

RNase inhibitor (20 U / μL) 0.21 μL

Multiscribe™ RT (50 U / μL) 1.1 μL

Stem-loop primer (50 nM) 3.1 μL

Samples were incubated at 16°C for 30 minutes, 42°C for 30 minutes and finally 85°C for 5 minutes to denature double-stranded duplexes. The reaction was performed using an AB9700 GeneAmp thermal cycler (Applied Biosystems). An RT-negative control was included in each batch of reactions.

2.4.4 Real-time quantitative polymerase chain reaction (RQ-PCR)

RQ-PCR allows accumulating amplified DNA/cDNA to be detected and measured as the reaction progresses, i.e. in real time. It is possible to detect the amount of amplified product by incorporating a DNA-binding dye or fluorescently labelled gene-specific probe in the reaction. The RQ-PCR reaction consists of an exponential phase, in which the amount of amplified product approximately doubles during each cycle of denaturation, primer annealing and template extension; and a non-exponential or plateau phase in which reduced reagents limit the reaction. The point at which enough amplified product has accumulated to produce a detectable fluorescent signal is known as the threshold cycle or Ct and the greater the amount of starting template, the lower the Ct value.

2.4.5 PCR amplification efficiencies

Amplification efficiency of 100% indicates an efficient amplification of product for each cycle during the exponential phase in an optimal PCR reaction. To determine the amplification efficiency of a PCR assay, serial dilutions (neat to 10^{-6}) of cDNA templates were prepared and amplified using the same thermal cycling conditions used for subsequent gene analyses. By plotting Ct against the dilution factor, a dilution curve was constructed. Amplification efficiencies (E) were calculated for each PCR assay using the formula:

$$E = (10^{-1/\text{slope}} - 1) \times 100$$

(where slope = slope of dilution curve)

Amplification efficiencies between 90-110 % indicate a relatively robust and reproducible RT-qPCR assay. Low E could be indicative of pipetting error. High E could represent inefficient amplification. The R² value of the dilution curve represents the linearity of the data. R² should be ≥ 0.98 for each dilution curve.

2.4.6 Endogenous controls

For reliable RQ-PCR data, corrections need to be made for variations between reactions, which may have been introduced during any of the steps from the sample preparation through to the amplification of the target. The optimal means of correcting for technical and biological sources of variation (e.g. differences in sample procurement, RNA extraction, the amount of starting template, RNA quality, enzymatic efficiencies, or even sample-to-sample inconsistencies in cellular subpopulations) is to normalise RQ-PCR data to an endogenous control (EC) gene. The accuracy of RQ gene expression analysis is critically dependent on proper normalisation of the data as inappropriate normalisation of RQ-PCR data can lead to incorrect conclusions¹⁴⁷⁻¹⁴⁹.

A reliable EC gene is ideally stably expressed across a whole sample set, is expressed along with the target in the samples of interest, and demonstrates equivalent storage stability, extraction, and quantification efficiency as the target of interest. However, few EC genes fulfilling all of these criteria are known¹⁵⁰.

While ECs have been validated for quantification of mRNA expression in various experimental settings, similar reports of validated ECs for miRNA expression are limited¹⁴⁷⁻¹⁴⁹. As EC genes are tissue and organ specific, it would not necessarily be appropriate to infer that a normalising gene for one tissue type would be an equally reliable normaliser for another tissue type.

To date there is no consensus regarding an appropriate EC for miRNA expression in whole-blood. As such, the preliminary part of this study aimed to identify a miRNA or miRNAs sufficiently stably expressed in the blood of PCa patients and controls to be suitable for use as endogenous control genes.

2.4.7 RQ-PCR of miRNA

The PCR reactions were carried out in final volumes of 10 μ L using an Applied Biosystems 7900HT instrument. Reaction mix consisted of:

TaqMan Fast Master Mix (2X) 5.0 μ L

Nuclease-free water 1.68 μ L

TaqMan probe (0.2 μ M) 0.5 μ L

Forward primer (1.5 μ M) 1.5 μ L

Reverse primer (0.7 μ M) 0.7 μ L

MiRNA-specific cDNA 0.7 μ L

Once all samples had been added to the plate, it was sealed using MicroAmp optical adhesive film lids (Applied Biosystems). Plates were then centrifuged at 2000 rpm for 1 minute, and placed in the 7900 instrument. Standardised 'Fast' thermal cycling conditions were applied. This consisted of 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Inter-assay control (*miR-26b* cDNA synthesised from a colorectal tumour sample) and calibrator (i.e. nuclease-free water) were included in each 96-well plate. All reactions were performed in triplicate. The threshold standard deviation for intra- and inter-assay replicates was 0.3.

2.4.8 Relative quantification

In order to relatively quantify miRNA and gene expression between different blood samples, the Ct values generated by Applied Biosystems 7900HT Fast Real-Time PCR System were scaled to a calibrator sample (i.e. nuclease-free water). Data was exported to qBase plus software (Biogazelle). Relative quantities were generated using the formula:

$$\Delta Ct = \text{average Ct (test sample)} - \text{average Ct (calibrator sample)}$$

The ΔCt values were converted to a linear form using the formula:

$$E^{-\Delta Ct}$$

(where E = amplification efficiency)

Non-biological sample-to-sample variation that could be introduced by protocol-dependent inconsistencies was corrected for by using EC genes in order to achieve accurate, reproducible and biologically relevant miRNA and mRNA quantification. The comparative Ct ($\Delta\Delta Ct$) method was used to calculate the expression of a target gene relative to the EC gene using the formula¹⁵¹:

$$\Delta\Delta Ct = \boxed{\begin{array}{c} [Ct \text{ target gene} - Ct \text{ EC gene}] \\ \text{Test Sample} \end{array}} - \boxed{\begin{array}{c} [Ct \text{ target gene} - Ct \text{ EC gene}] \\ \text{Calibrator Sample} \end{array}}$$

The $\Delta\Delta Ct$ values were converted to a linear form using the formula: $E^{-\Delta\Delta Ct}$.

2.5 Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (Chicago, IL, USA) and Minitab (Version 16) statistical software. Univariate comparison of other variables was assessed using χ^2 test or Fisher's exact test for nominal or ordinal data.

Distribution of continuous data was determined using the Kolmogorov-Smirnov Z test. Student's t-test or ANOVA, and Mann-Whitney U or Kruskal-Wallis tests were used for parametric and non-parametric continuous data respectively. Paired-samples t-test or Wilcoxon test was used for paired parametric and non-parametric

continuous data respectively. A p value of less than 0.05 was considered statistically significant for all tests.

To determine sensitivity and specificity of miRNA expression levels in distinguishing cancer cases from controls, Receiver Operating Characteristics (ROC) were constructed and the area under the curve (AUC) was calculated. The AUC is a surrogate marker of the ability of each miRNA to differentiate between cancer cases and controls; it does so by computing sensitivity and specificity for each possible cut off point of the individual miRNAs. This was performed univariately for each individual miRNA, and multivariately for combinations of target miRNAs in our panel via logistics regression analysis.

Chapter 3

Identification of MiRNAs as Endogenous Controls for RQ-PCR

3.1 Introduction

A variety of methods have been utilised to study miRNA expression levels including; northern blotting, bead-based flow cytometry and microarray technology^{63,152,153}. However, RQ-PCR is remains the current gold standard for quantifying miRNA expression as this method has superior sensitivity and specificity, with a wide dynamic range and low template requirements in comparison with the semi-quantitative methods mentioned above. Stem loop primers have now been developed specifically for individual miRNAs to convert it to its complementary DNA (cDNA)¹⁴².

RQ-PCR is used to detect the change in expression of the target of interest relative to a control or a reference. However, a number of factors may influence the expression levels which may or may not be a direct consequence of the disease process in question. In order for the data produced to be reliable, corrections must be made for the variation between reactions which are introduced during the steps from the RNA extraction through to the PCR amplification step. This is also important to compensate for the differences in the quality and quantity of RNA used in the reactions^{154,155}.

An ideal EC gene, also known as a reference or a house-keeping gene, should be stably expressed across a whole sample set, is expressed along with the target in the samples of interest, and should demonstrate equivalent storage stability, extraction, and quantification efficiency as the target of interest. Although there are very few EC genes which fulfil all of these criteria¹⁵⁰.

An unreliable EC gene will result in the inappropriate normalisation of RQ-PCR data. This can lead to inaccurate results and conclusions. There is increasing evidence that EC genes appear to be tissue-specific and as such, the appropriate choice of an EC gene may be directly dependent on the tissue of interest^{147-149,155-157}.

The choice of an EC gene or genes is an important component of any study design. There are a number of ECs that have been identified in different cancer tissues^{156,158-}

¹⁶⁰. However, there is limited data on suitable EC for miRNA extracted from whole-blood, serum or plasma of PCa patients.

A number of approaches have been utilised to normalise miRNA for RQ-PCR for miRNA extracted from blood. Some authors add a synthetic miRNA to normalise the RQ-PCR data¹²⁰. Other authors investigating circulating miRNAs in men with PCa have used *miR-16*, U6, RNU1A and RNU6B^{109,122,123,125}.

Table 3.1 Endogenous Controls used for circulating miRNAs

Endogenous Control	Blood type	Reference
<i>miR-16</i>	Serum	109
<i>C. Elegans</i> spike in <i>cel-miR-39</i> , <i>cel-miR-54</i> , <i>cel-miR-238</i>	Serum	119
<i>miR-16</i>	Whole-blood	125
U6 snRNA	Serum	122
RNU1A	Plasma	123
RNU6B	Plasma	108
<i>Syn-miR-39</i>	Serum	120
<i>C. Elegans</i> spike <i>miR-39</i>	Serum	127

3.2 Aims

The aim of this study was to evaluate a panel of five candidate EC genes from which to identify the most stably expressed gene or genes to normalise RQ-PCR data derived from whole-blood samples obtained from PCa patients and men with benign prostate disease who attended the RAPAC.

3.3 Materials and methods

3.3.1 Study group

Following ethical approval and written informed consent, whole-blood samples were collected prospectively from 66 men attending the RAPAC. Following TRUS biopsy, 38 were diagnosed with PCa and 28 had a benign histological finding. The men with a benign histological finding were men who were referred to the RAPAC for investigation of an elevated PSA or an abnormal DRE. All cancer patients had histologically confirmed PCa and their relevant. These blood specimens were obtained from men attending the RAPAC at Galway University Hospital, Galway, Ireland.

Table 3.2 Patient demographics

	Benign	Cancer
Numbers	28	38
Age:		
Mean	68 years	69 years
Range	(52 – 84 years)	(49 – 83 years)
PSA: (µg/L)		
Mean	11.9	12.1
Range	(3.9 – 20.6)	(4.6 – 40.5)

Table 3.3 Histology of study groups

Histology	Numbers
Benign	28
3+3	13
3+4	10
4+3	9
4+4	4
4+5	2

3.3.2 Candidate endogenous control genes

The expression of five miRNAs (*miR-10b*, *miR-16*, *miR-425*, *miR-21* and *let-7a*) were determined in the 66 blood samples. These miRNAs were chosen as candidate ECs for this study based on their expression in both the circulation of patients with PCa and in PCa tissue, or previous use as an EC gene for miRNA RQ-PCR analysis^{109,125}.

Table 3.4 Candidate miRNA selected for investigation as suitable endogenous controls

miRNA	Source	Reference
<i>let7a</i>	Blood (prostate), Tissue (prostate, breast, colorectal)	^{73,76,80,125}
<i>miR-10b</i>	Tissue (breast)	¹⁶¹
<i>miR-21</i>	Blood (pros), Tissue (prostate, breast, colon)	^{76,77,122,123}
<i>miR-16</i>	Blood (breast), Tissue (lymphoma, oral cancer, colorectal)	^{125,143-146}
<i>miR-425</i>	Tissue (colorectal)	¹⁴⁷

3.3.3 Blood collection

10ml of whole blood was collected from each participant in Vacuette EDTA K3E blood bottles (Grenier Bio-one). These samples were stored at 4°C (unprocessed and in the original sample bottles) until required.

3.3.4 MiRNA extraction from blood

Total RNA was extracted from 1ml aliquots of whole blood using a modified trizol co-purification technique. This is described in detail in section 2.3. To summarise, for each 1ml of whole blood, phase separation was performed by the addition of 3ml of trizol. 200µl of 1-bromo-4-methoxybenzene was then added to augment the RNA phase separation process. Total RNA was precipitated using isopropanol and washed with 75% ethanol prior to solubilisation with 60µl of nuclease free water.

3.3.5 Analysis of miRNA concentrations

The RNA concentration was determined using a Nanodrop® Spectrophotometer (NanoDrop Technologies). The wavelength-dependent extinction coefficient '33' was taken to represent the micro-component of all RNA in solution. In general concentrations ranging between 22 and 595ng/µl of miRNA were obtained for each sample.

3.3.6 Reverse transcription and RQ-PCR

The RNA samples were then reverse transcribed using primers specific to each miRNA target and RQ-PCR was carried out using TaqMan® miRNA primers and probes (Applied Biosystems, USA), as described in detail in section 2.4.4.

For each sample, 100ng of small RNA was reverse transcribed into cDNA using MultiScribe™ reverse transcriptase and gene-specific stem-loop primers which target the mature miRNA/snoRNA sequence. RQ-PCR was performed using TaqMan® probes which bind to a complementary sequence in the target gene between the forward and reverse primers. The RQ-PCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using default thermal cycling conditions.

PCR amplification efficiencies were calculated for each candidate EC RQ-PCR assay by generating a cDNA dilution curve and using the formula $E = (10^{-1/\text{slope}} - 1) \times 100$, where E is the amplification efficiency.

Triplicate samples were used throughout and each plate included an interassay control and calibrator synthesised from pooled colorectal cancer tissue. The threshold standard deviation accepted for intra- and inter-assay replicates was 0.3.

3.3.7 Data analysis

The stability of EC expression was analysed using two freely available software programmes, namely geNorm and NormFinder. Prior to geNorm and NormFinder analysis, the cycle threshold (Ct) values for each EC were converted to relative quantities (Q.rel.) by scaling to a calibrator sample and corrected for efficiency of amplification (E) using the formula: $Q.\text{rel.} = E^{-\Delta C_t}$, with $\Delta C_t = \text{average Ct (test sample)} - \text{average Ct of the calibrator sample}$.

GeNorm (version 3.4) is a visual basic application for Microsoft Excel, however, it is also incorporated into the qBase plus software (Biogazelle) that is used for relative quantification. GeNorm calculates a gene-stability measure (M) for all candidate EC genes in a given set of samples and determines the most reliable pair of ECs, namely those showing greatest stability of expression ratio across samples. It is based on a pair-wise comparison model.

NormFinder is an Excel add-in and uses an ANOVA-based model to estimate intra- and inter-group variation. It combines these estimates to produce a stability value for each candidate. Thus NormFinder indicates the single most stable EC or EC pair where the stability of the latter is greater than that of the single EC.

Statistical analyses were performed using Minitab (version 16). The distribution of continuous data was determined using the Kolmogorov-Smirnov Z test and two-sample t-tests or non-parametric tests were used as appropriate to compare relative quantities of each candidate EC between the cancer group and the control group.

3.4 Results

3.4.1 RQ-PCR

3.4.1.1 PCR amplification efficiencies

PCR amplification efficiencies were calculated for each of the candidate EC RQ-PCR assay using the formula $E=(10^{-1/\text{slope}}-1) \times 100$, using the slope of the plot of Ct versus log input of cDNA concentration, as shown in table 3.4. The amplification efficiencies were within the recommended range of 90-100% for all RQ-PCR assays.

Table 3.5 Amplification efficiencies

EC Name	Efficiency (%)
<i>miR-10b</i>	94.8
<i>miR-16</i>	108.4
<i>miR-21</i>	108.2
<i>miR-425</i>	96.1
<i>Let7a</i>	96.7

Figure 3.1 PCR amplification efficiencies for candidate EC genes calculated by the formula $E=(10^{-1/\text{slope}}-1) \times 100$ using the slopes of the dilution curves above

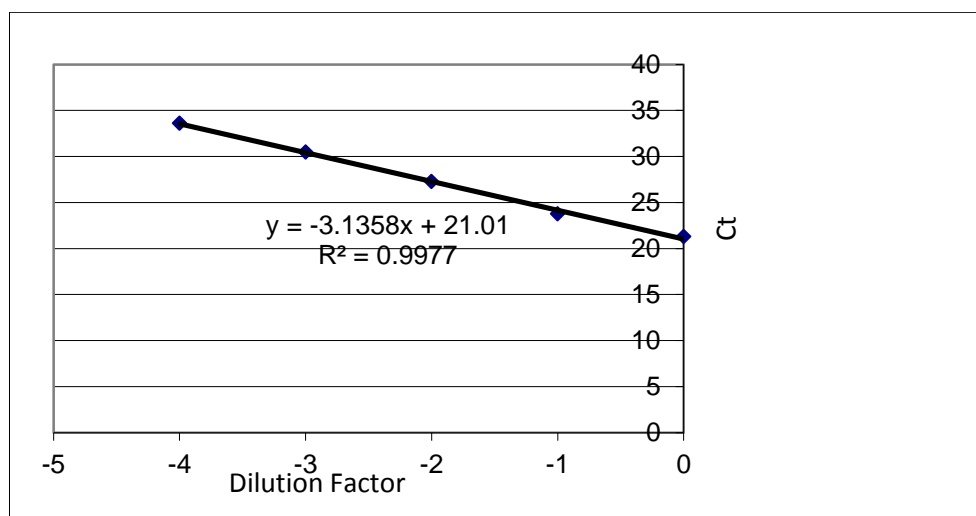


Figure 3.1 (a) *Mir-16* Dilution curves used to calculate RQ-PCR efficiencies

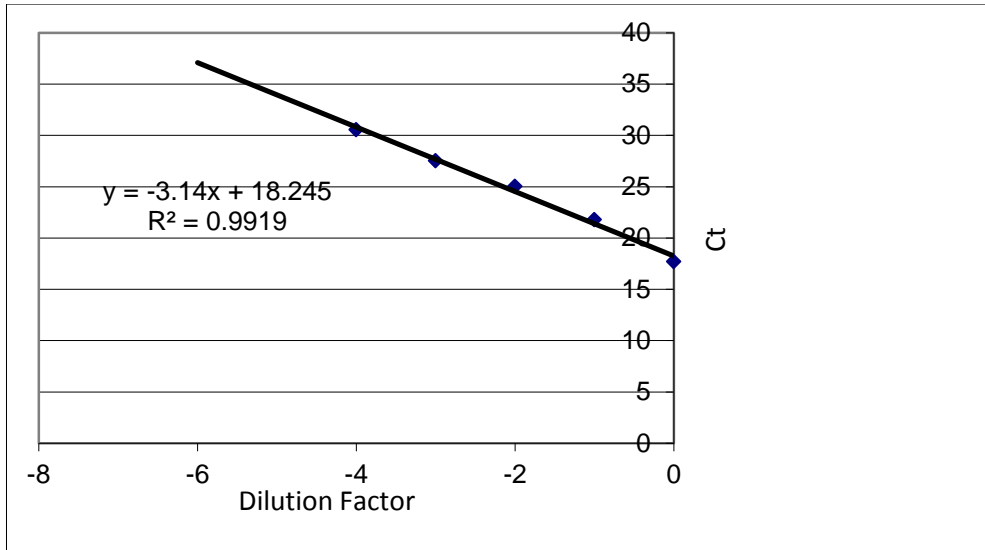


Figure 3.1 (b) *Mir-21* Dilution curves used to calculate RQ-PCR efficiencies

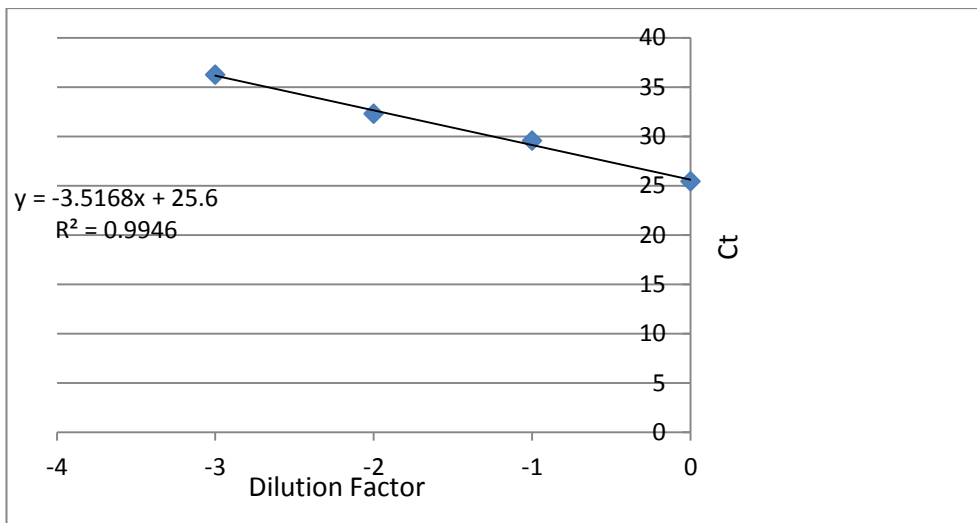


Figure 3.1 (c) *let7a* Dilution curves used to calculate RQ-PCR efficiencies

Table 3.6 Cycle threshold (Ct) values of candidate ECs

EC Gene	Ct Range	Ct Min	Ct Max
<i>miR-16</i>	13.87119	15.3491	29.22029
<i>miR-21</i>	13.63278	24.30479	37.93757
<i>miR-425</i>	15.23082	18.9982	34.22902
<i>Let7a</i>	14.86166	21.98975	36.85141

3.4.1.2 Relative quantities of candidate endogenous controls

The Ct value is the amplification cycle number at which the fluorescence generated within a reaction rises above a defined threshold fluorescence¹⁶². The five candidate ECs displayed a wide expression range, with Ct values between 15 and 39.

miR-16 and *miR-425* had a relatively high expression with median Ct values of 20 and 22 respectively. *Let7a* and *miR-21* had moderately abundant expression levels with median Ct values of 26 and 28 respectively. However, *miR-10b* had a low abundance with a median Ct value of 36.

Given that *miR-10b* had such a low expression, and that it was not determined in all samples the decision was made to exclude it from further analysis. Given that many samples for this particular miRNA had only reached the threshold level at or close to the final amplification cycle (equivalent to Ct 40), this essentially made *miR-10b* unsuitable as a potential endogenous control in RQ-PCR.

Ct values for each EC were converted to relative quantities (Q.rel.) using the formula: $Q.rel.=E^{-\Delta Ct}$, with ΔCt =average Ct (test sample Ct-average Ct of the calibrator sample).

3.4.2 Analysis of results

3.4.2.1 Statistical analysis

For analysis, the data was log transformed given the magnitude and the range of the miRNA expression levels (natural log, ln). The distribution of values were evaluated using the Kolmogorov-Smirnov Z-test. The Two-sample t-tests were used to compare relative expression between the cancer and benign groups for the four ECs.

P-values <0.05 were accepted as significant for all statistical tests. All four of these miRNAs were detectable in the circulation of the patients in this study group. There was no significant difference in the expression levels of *miR-16*, *-21* or *miR-425*.

Figure 3.2 Boxplots of miRNA expression

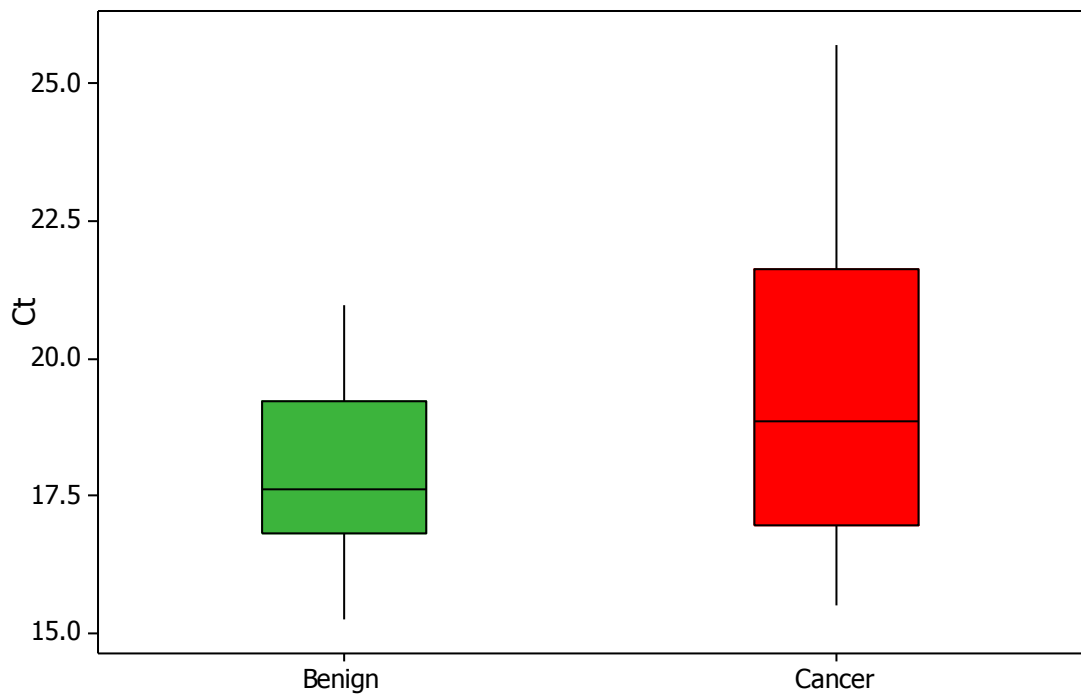


Figure 3.2 (a) *miR-16* expression

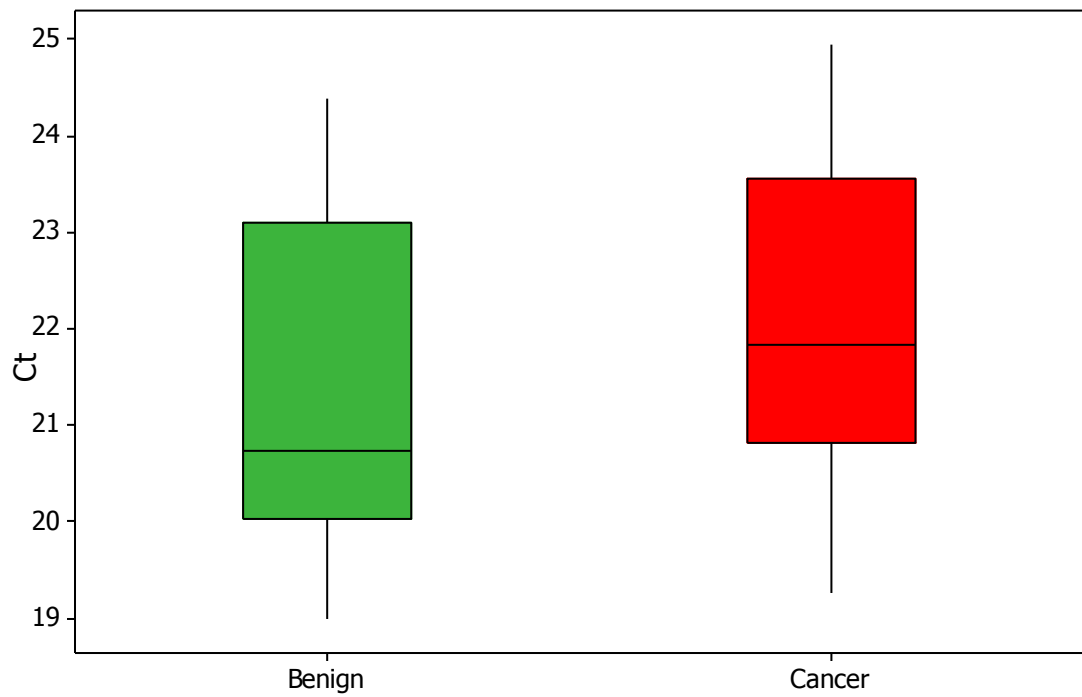


Figure 3.2 (b) *miR-425* expression

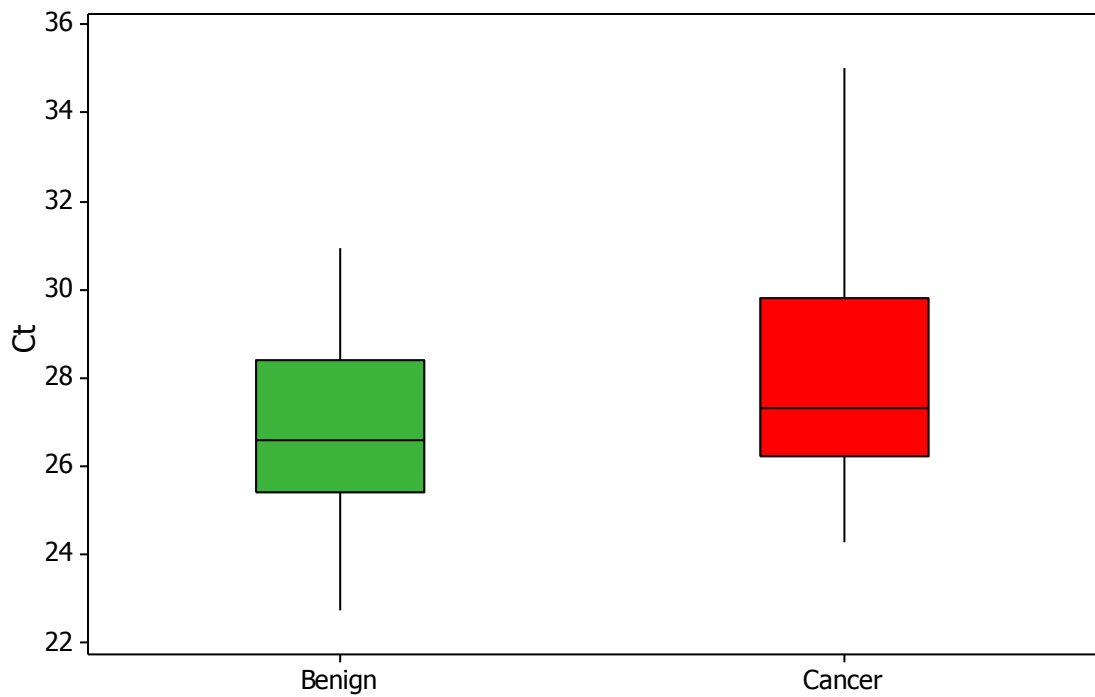


Figure 3.2 (c) *miR-21* expression

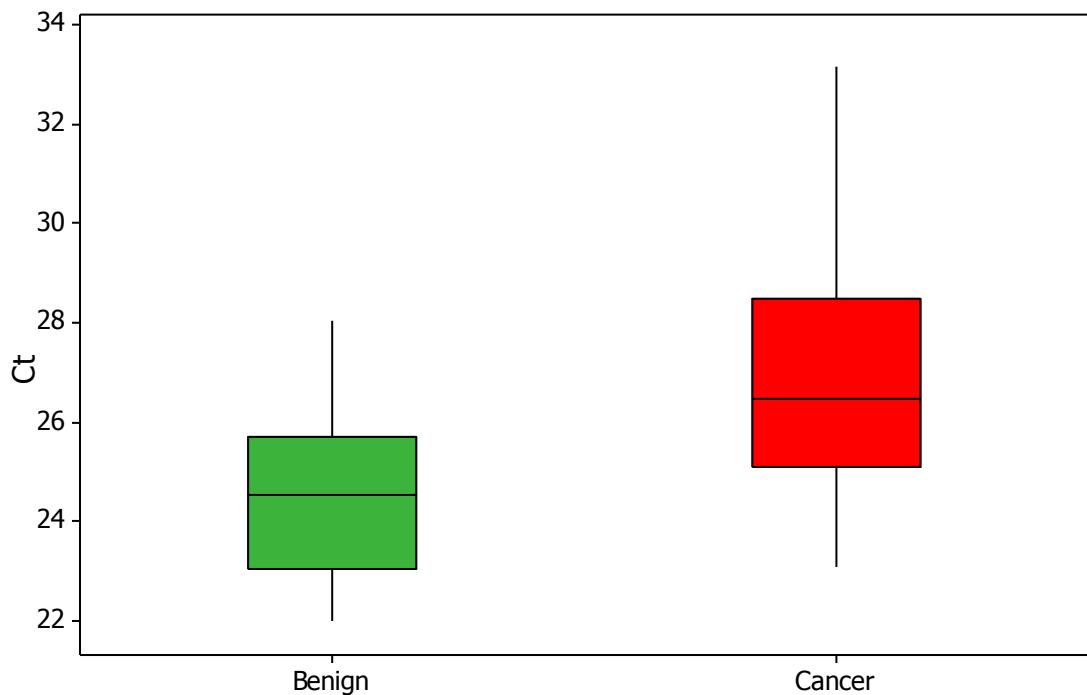


Figure 3.2 (d) *let7a* expression

Quantity of the candidate endogenous control genes *miR-16*, *miR-425*, *miR-21*, *let7a* relative to calibrator and corrected for amplification efficiency in the in the blood specimens of the Benign and Cancer groups. The boxes show the interquartile range and median and the whiskers indicate the range. There was no significant difference in the expression levels of *miR-16*, *-21* or *miR-425*, thus establishing the validity of EC comparison.

3.4.2.2 GeNorm and NormFinder analysis

The stability of the miRNA candidate ECs were examined using the geNorm and NormFinder software programmes. The NormFinder and geNorm candidate EC rankings are shown below. The lower the stability value, represent a greater gene expression stability. As such, the lower the stability value, the higher the gene stability.

It is not possible to directly compare the stability values of these two software packages. In general, NormFinder produces lower values than geNorm. However, it must be noted that both *miR-16* and *miR-425* are ranked by both programs as the

most stably expressed gene. Given this finding, it was decided that both of these miRNAs in combination would be used as the EC genes for this project.

Table 3.7 NormFinder and geNorm candidate EC rankings

Normfinder			geNorm		
Rank	Gene	Stability Value	Rank	Gene	Stability Value (M)
1	<i>miR-16</i>	0.211	1	<i>miR-16</i>	2.168
2	<i>miR-425</i>	0.225	2	<i>miR-425</i>	2.379
3	<i>miR-21</i>	0.261	3	<i>miR-21</i>	2.873
4	<i>Let7a</i>	0.294	4	<i>Let7a</i>	3.505
Best Combination	<i>miR-16 & miR-425</i>	0.206			

Greater expression stability is indicated by a lower stability value (M). Results for four EC candidates are given. The stability is calculated from the intra- and inter-group variation and the best combination of EC genes is also given. GeNorm stability is based on an estimate of the pairwise variation (M).

3.5 Discussion

If miRNA are to be used as a circulating biomarker PCa, then standardised, reliable and accurate methods of miRNA isolation and quantification is required. Given that RQ-PCR is highly sensitive, the use of an appropriate EC to normalise and correct for any non-biological variation.^{154,155} However, it has previously been demonstrated that the selection of an inappropriate choice of EC or ECs can produce misleading or inaccurate results^{147,149,155}.

For RQ-PCR, a gene-specific cDNA for each sample is synthesised using miRNA-specific primers. This introduces an additional non-biological variation which is avoided during the synthesis of cDNA from mRNA using random or oligo-dT primers. Given that miRNAs may regulate multiple gene targets within the same

pathway, small changes in the miRNA expression may be clinically and biologically significant¹⁶³.

Previously, other RNA species have been investigated as potential ECs including; rRNAs, tRNAs, snRNAs and snoRNAs. However, these molecules may be expressed at significantly higher levels than miRNA which may cause issues when quantifying both in the same dilution¹⁵⁰.

There is currently no consensus in the literature regarding suitable ECs for the normalisation of circulating miRNA RQ-PCR data from whole-blood, serum or plasma. Some authors have used a spike in synthetic miRNA or miRNAs as an EC^{109,164}. Whilst other authors have used ECs that were published in other studies. Given that the most stably expressed genes may be tissue specific, the most suitable EC or ECs may be uniquely different for different tissues¹⁵⁶.

Mir-16 and *miR-425* were found to be stably expressed in the circulation of all patients with little variability. As a result both were selected for use as endogenous controls, in combination. *Mir-16* has been used as a normaliser in a many studies investigating levels of miRNAs in whole blood, serum, plasma and tissue^{126,143-146}. *miR-425* has also been used as an endogenous control in our institution in both cancer tissue and in the circulation^{147,165}. The use of more than one EC in combination may increase the accuracy of quantitation compared to the use of a single EC^{150,166}. It should be noted that the use of both of these miRNAs in combination had a slightly lower stability value in comparison with the use of each miRNA individually as an EC.

The software programs geNorm and NormFinder were used to identify the most stably expressed genes for use as an EC. Both identified *miR-16* and *miR-425* as the most suitable EC. Given that NormFinder identified that using both in combination was the most stably expressed EC, the decision was made to use these miRNAs as the housekeeping genes for further studies investigating circulating miRNAs in men with PCa.

3.6 Conclusion

The investigation of circulating miRNAs as potential biomarkers for the diagnosis of men with PCa remains in its infancy. The use of RQ-PCR is a practical way to further investigate this potential. However, the accuracy of this method is dependent on normalisation to a stably expressed control gene or genes. This study identifies the use of *miR-16* and *miR-425* as the most stably expressed EC miRNAs (from a panel of five candidate miRNAs) in human whole-blood using an analysis of expression in men with PCa and those with a benign histological finding.

Chapter 4

The evaluation of circulating miRNAs in Prostate Cancer

4.1 Introduction

PCa is the most commonly diagnosed non-cutaneous malignancy in men and is the second leading cause of cancer death¹⁶⁷. It is estimated that up to 1 in 6 men will be diagnosed with PCa during their lifetime¹⁶⁸. Clinicians use a combination of a digital rectal examination (DRE) and a prostate specific antigen (PSA) and a transrectal ultrasound guided prostate biopsy (TRUS) to detect PCa. However, PCa screening trials, such as PLCO and ERSPC trials, have highlighted that despite an increase in the diagnosis of PCa using these tests, there is still no clear improvement in mortality^{25,26}.

However, PCa can vary from being a cancer that is asymptomatic and does not metastasise to an incredibly aggressive cancer that can metastasise causing significant pain, morbidity and ultimately death. Recent improvements in management techniques have resulted in a decrease in morbidity and mortality. The issue is that many men die with PCa rather than from it, giving rise to the theory that many men have clinically insignificant cancers. A sensitive, specific biomarker is needed to clearly identify those that have a cancer that needs radical treatment from those that have a clinically insignificant tumour.

In addition, PSA, a frequently used biomarker for the detection of PCa, is limited by its lack of sensitivity and specificity for PCa and therefore not considered an ideal biomarker. In clinical practice, PSA is used as the gold standard, along with DRE, for assessing patients at risk from PCa. However, PSA can be elevated for a variety of reasons including; age, prostatic volume, inflammation, infection, recent ejaculation and also post DRE. Pooled data from a meta-analysis reveals that DRE has a sensitivity of 53% and specificity of 83% and PSA has a sensitivity of 72% with a specificity of 93% with a positive predictive value of 25%²⁴. As a result, a search for a novel, minimally invasive, clinically relevant biomarkers for the detection of PCa is required.

Such a biomarker could potentially also be used to monitor response to treatment and for future disease recurrence. An ideal biomarker should be easily accessible (i.e. can be sampled in a relatively non-invasive fashion), sensitive enough to detect early presence of tumours in almost all patients and absent or minimal in healthy tumour free individuals¹⁶⁹.

4.2 Aims

The aims of this study were:

- To determine if circulating miRNAs were dysregulated in the circulation of patients with PCa from a panel of 12 candidate miRNAs.
- Analysis of any such miRNAs identified to establish if there was a relationship between the miRNA expression level and PSA, Gleason Score and risk stratification.
- To determine if circulating miRNAs had the potential to act as biomarkers for PCa in clinical practice.

4.3 Materials and methods

4.3.1 Study group

Following ethical approval and written informed consent, whole blood samples were collected prospectively from 102 patients attending the RAPAC in Galway University Hospital. These men were referred to the RAPAC as they had an elevated PSA-for-age or an abnormal DRE. None of the patient cohort had received chemotherapy or radiotherapy at the time of phlebotomy. Following a TRUS Biopsy 75 patients were diagnosed with PCa and the relevant demographic and clinicopathological details are summarised table 4.1. Age and stage of disease is representative of a typical cohort of PCa patients. 27 patients had a benign histological finding following TRUS Biopsy. These men had no current or previous malignancy. The PSA at diagnosis is also recorded for both groups. These blood specimens were obtained from the PCa patients prior to their TRUS Biopsy in the RAPAC in Galway University Hospital.

Table 4.1 Histology of study groups

Histology	Numbers
Benign	27
3+3	28
3+4	20
4+3	14
4+4	6
4+5	7

Table 4.2 D'Amico Risk Stratification

Risk Stratification	Numbers (75)
Low	28
Intermediate	11
High	36

Table 4.3 Metastasis

Metastasis	Numbers (75)
No metastasis	65
Metastasis	10

4.3.2 Blood collection

10ml of whole blood was collected from each participant in Vacuette EDTA K3E blood bottles (Grenier Bio-one). These samples were stored at 4°C (unprocessed and in the original sample bottles) until required.

4.3.3 Candidate miRNA targets

A panel of 12 individual miRNA targets were chosen for analysis in this study. These are listed in table 4.4 below. Selection of specific candidate miRNAs for expression analysis in this study was based on information gleaned from previous studies within the Discipline of Surgery at NUI Galway and a review of the

internationally published literature on both tissue and circulating miRNAs in men with PCa.

Table 4.4 Candidate miRNA selected for investigation

Dysregulated miRNA	Source	Reference
<i>let7a</i>	Blood (prostate), Tissue (prostate, breast, colorectal)	73,76,80,125
<i>miR-21</i>	Blood(prostate), Tissue (prostate, breast, colorectal)	76,77,122,123
<i>miR-34a</i>	Tissue (colorectal)	77,78
<i>miR-125b</i>	Blood (prostate), Tissue (prostate, breast)	73,77,79-81,109
<i>miR-141</i>	Blood (prostate), Tissue (prostate)	73,109,119
<i>miR-143</i>	Blood (prostate), Tissue (prostate)	73,80,109
<i>miR-145</i>	Blood (breast), Tissue (prostate, breast)	73,77-80,125
<i>miR-155</i>	Blood (breast)	125
<i>miR-221</i>	Blood (prostate), Tissue (prostate, breast)	73,77-81,108,123
<i>miR-375</i>	Blood (prostate), Tissue (prostate)	79,119

4.3.4 MiRNA extraction from blood

Total RNA was extracted from 1ml aliquots of whole blood using a modified trizol co-purification technique. This is described in detail in section 2.3. To summarise, for each 1ml of whole blood, phase separation was performed by the addition of 3ml of trizol. 200µl of 1-bromo-4-methoxybenzene was then added to augment the RNA phase separation process. Total RNA was precipitated using isopropanol and washed with 75% ethanol prior to solubilisation with 60µl of nuclease free water.

4.3.5 Analysis of miRNA concentrations

RNA concentration was determined using a Nanodrop® Spectrophotometer (NanoDrop Technologies).

4.3.6 Analysis of miRNA gene expression by RQ-PCR

RNA samples were reverse transcribed using primers specific to each miRNA target and real-time quantitative PCR (RQ-PCR) was carried out using TaqMan® miRNA primers and probes (Applied Biosystems, USA), as described in detail in section 2.4.4. PCR amplification efficiencies were calculated for each candidate EC RQ-PCR assay by generating a cDNA dilution curve and using the formula $E = (10^{-1/\text{slope}} - 1) \times 100$, where E is the amplification efficiency (as described in section 2.4.5 and 3.4.1.1). Amplification efficiencies falling within the desired range of 90-110% were achieved for all targets (table 4.5).

Table 4.5 Amplification efficiencies

EC Name	Efficiency (%)
<i>Let7a</i>	96.7
<i>miR-16</i>	108.4
<i>miR-21</i>	108.2
<i>miR-34a</i>	97.4
<i>miR-125b</i>	97.6
<i>miR-141</i>	106.4
<i>miR-143</i>	96.4
<i>miR-145</i>	108.7
<i>miR-155</i>	106.4
<i>miR-221</i>	98.7
<i>miR-375</i>	94.6
<i>miR-425</i>	96.1

Triplicate samples were used throughout and each plate included an interassay control and calibrator synthesised from pooled normal breast tissue. The threshold standard deviation accepted for intra- and inter-assay replicates was 0.3.

MiRNA expression levels were calculated using QbasePlus software with *miR-16* and *miR-425* as the endogenous controls (based on the endogenous control selection and validation process described in chapter 3).

4.3.7 Statistical analysis

Data were analysed using the software package SPSS (version 18) and Minitab (version 16). Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis (natural log). All datasets were normally distributed, as verified by the Kolmogorov-Smirnov Z-test, and therefore suitable for parametric testing. For all two-sample comparisons the two-sample t-test was used to assess differences in mean expression levels and the Mann-Whitney test to evaluate the differences in the median values. ANOVA, followed by Tukey HSD Post Hoc test, was used to compare the mean expression levels of

miRNAs across different stages of disease. Pearson's correlation was used to assess the relationship between miRNAs and the PSA levels. All tests were two tailed and results with a $p < 0.05$ were considered statistically significant. A receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) calculated to assess the ability of each miRNA with significant differential expression to differentiate between cancer cases and benign cases, by computing sensitivity and specificity for each possible cut-off point of the individual miRNAs.

4.4 Results

4.4.1 Detection of miRNAs in the circulation

All 12 of the chosen miRNA targets amplified satisfactorily during RQ-PCR and thus were readily detectable at the concentrations used in these assays. The Ct value is the amplification cycle number at which the fluorescence generated within a reaction rises above a defined threshold fluorescence¹⁶². The 12 miRNAs attained raw Ct values ranging between 16 and 34.

4.4.2 Relative quantities of miRNAs

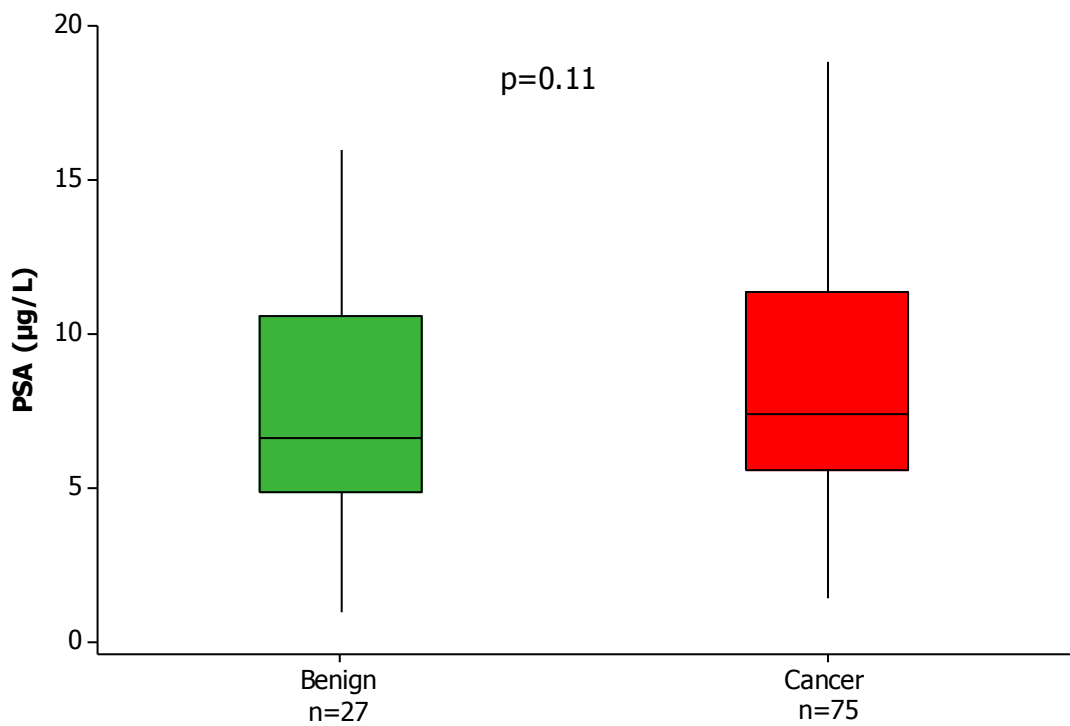
4.4.2.1 PSA

The majority of patients referred to the RAPAC were referred due to an elevated PSA-for-age, some had a normal PSA reading but an abnormal DRE. As such, it was not possible to identify those with PCa based solely on an elevated PSA. There was no significant difference in the PSA levels of both the PCa and the benign groups.

Table 4.6 Patient demographics

	Benign	Cancer
Number (102)	27	75
PSA: ($\mu\text{g/L}$)		
Median	7.45	7.4
Mean	11.6	11.55
Range	1 – 77	1.42 - 52.24

Figure 4.1 PSA of patients

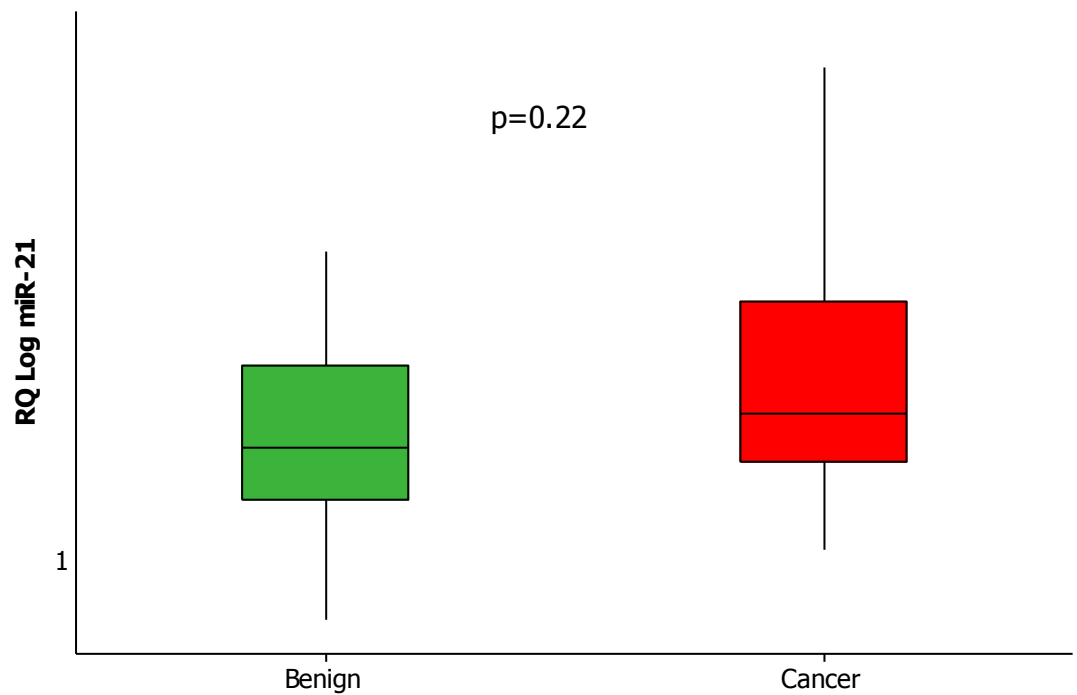


The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance ($p=0.11$).

4.4.2.1 *MiR-21*

There was no significant difference in expression levels of *miR-21* across the two groups ($p=0.22$). *miR-21* expression is illustrated in figure 4.2.

Figure 4.2 *MiR-21* expression levels in the benign and cancer groups

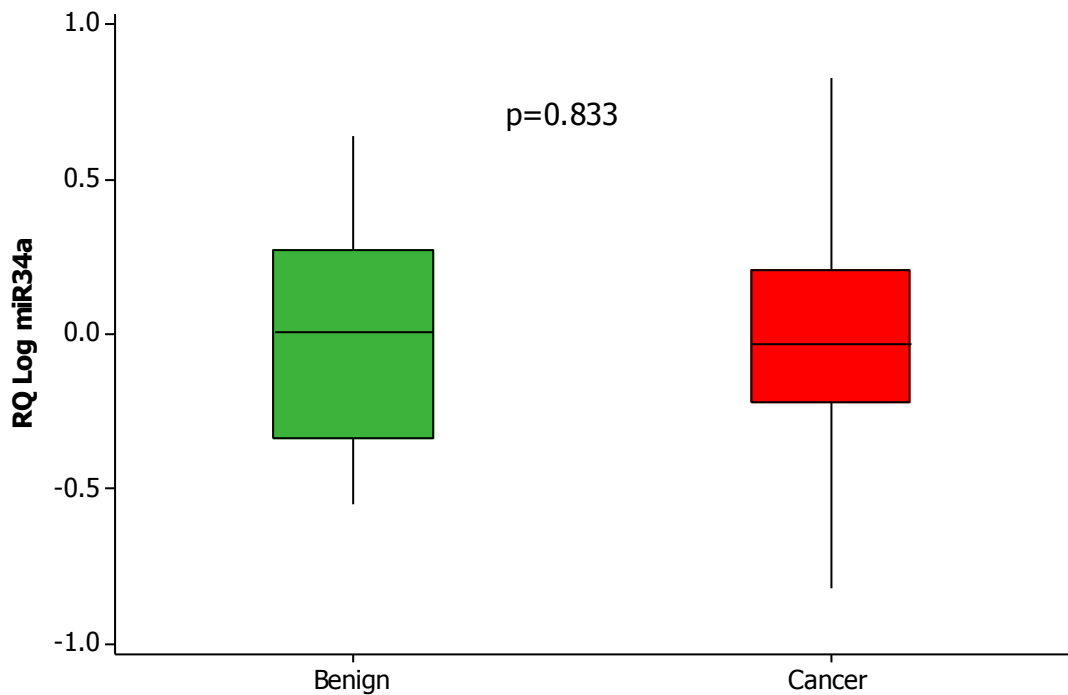


The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance ($p=0.22$).

4.4.2.2 *MiR-34a*

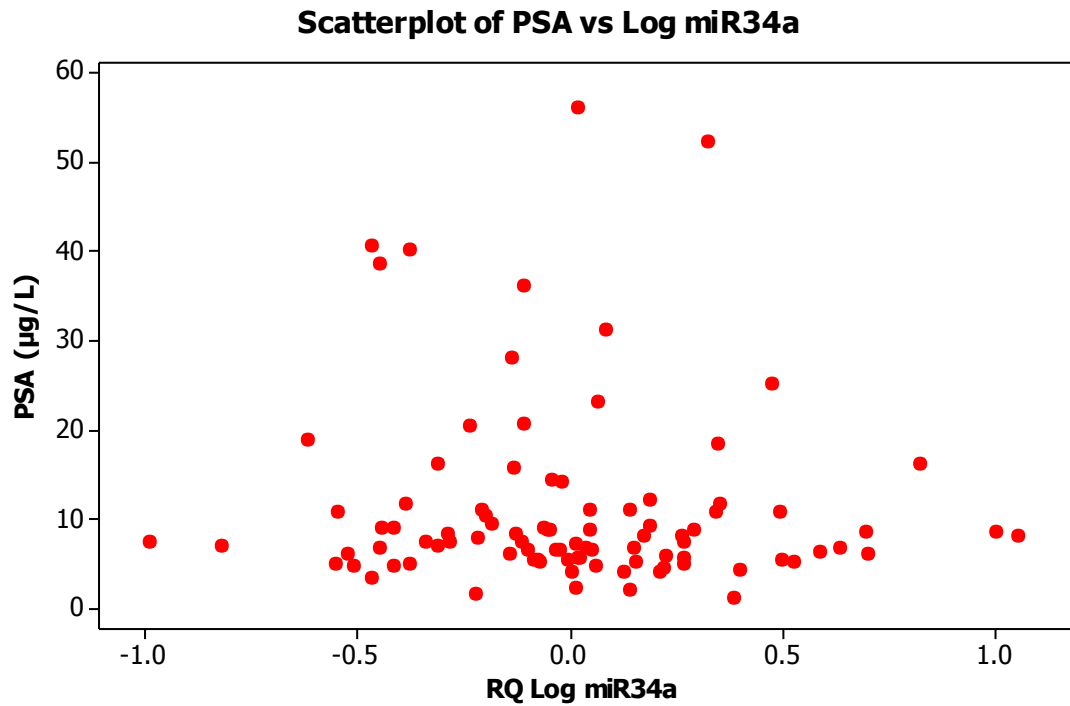
There was no significant difference in expression levels of *miR-34a* across the two groups ($p=0.833$). *miR-34a* expression is illustrated in figure 4.2. There was no significant correlation between *miR-34a* and PSA levels across both the cancer and benign groups (pearson correlation -0.075 , $p=0.474$).

Figure 4.3 *MiR-34a* expression levels in the benign and cancer groups



The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance ($p=0.833$).

Figure 4.4 Scatterplot of PSA Vs Log *miR-34a*



Scatterplot: No significant correlation between *miR-34a* and PSA levels across both the cancer and benign groups (pearson correlation -0.075, $p=0.474$).

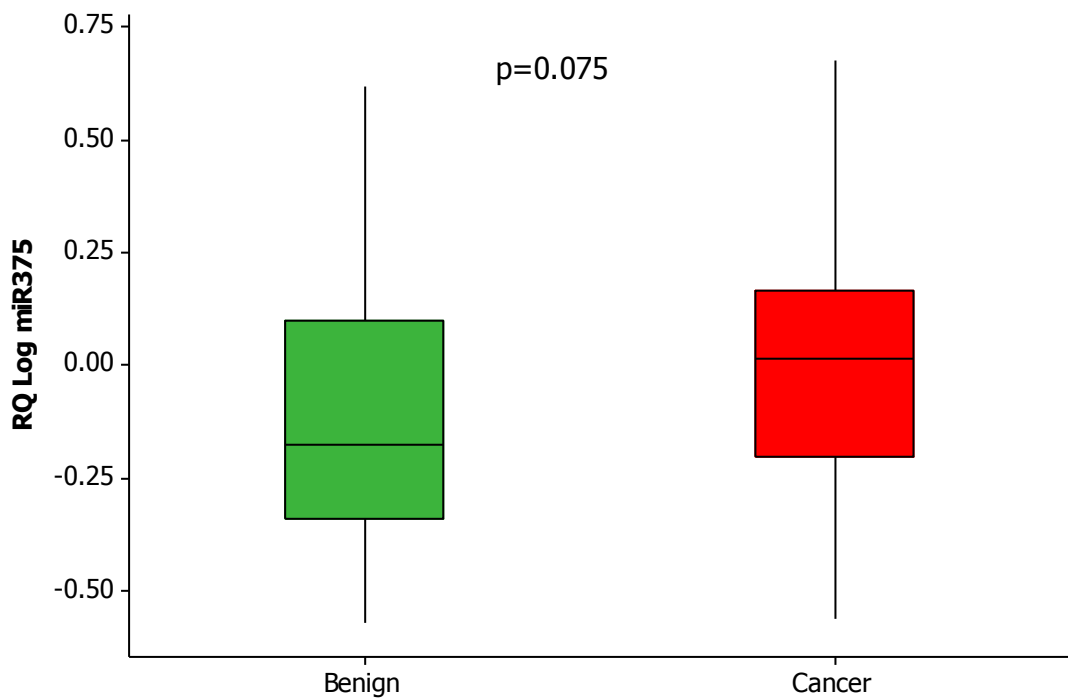
4.4.2.3 *MiR-143, -125b* and *-221*.

There was no significant difference in expression levels of *miR-143*, *-125b* or *-221* across the two groups ($p=0.821$, $p=0.721$ and $p=0.233$ respectively). There was no significant correlation between these miRNAs and PSA levels across both the cancer and benign groups.

4.4.2.4 *MiR-375*

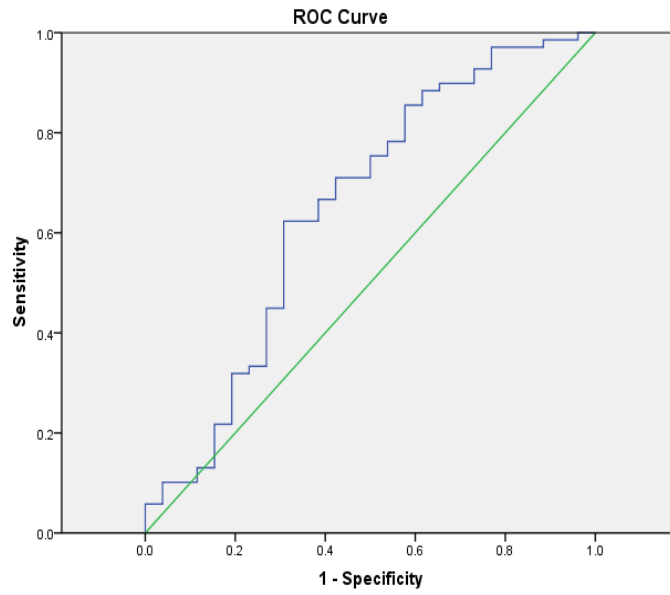
There was a trend towards upregulation of *miR-375* in the cancer group as illustrated in figure 4.5 the adjusted p-value after applying a Bonferroni correction was 0.075 for the two-sample t-test with an AUC of 0.651. There was no correlation between circulating *miR-375* and PSA levels ($p=0.829$) nor was there a correlation between increasing levels of *miR-375* and increasing D'Amico Risk stratification (anova analysis $p=0.151$). There was no significant increase in *miR-375* levels in patients with metastatic disease ($p=0.6$).

Figure 4.5 *MiR-375* expression levels in the benign and cancer groups



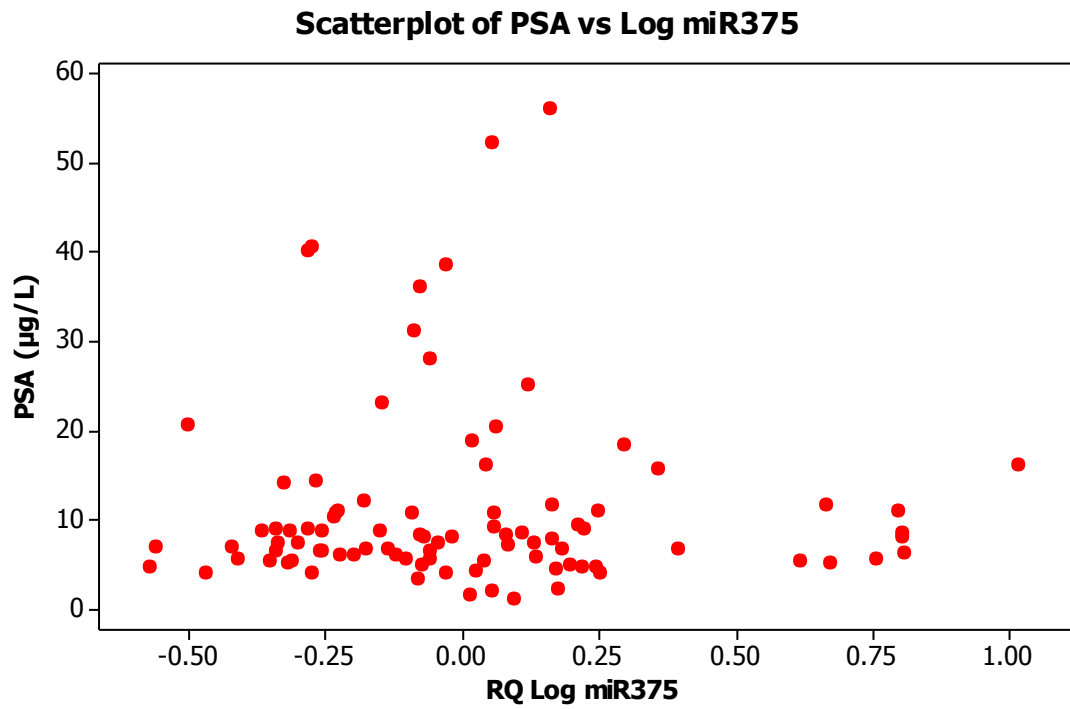
The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance ($p=0.075$).

Figure 4.6 Receiver operated characteristic (ROC) curve for miR-375 expression levels in the benign and cancer groups.



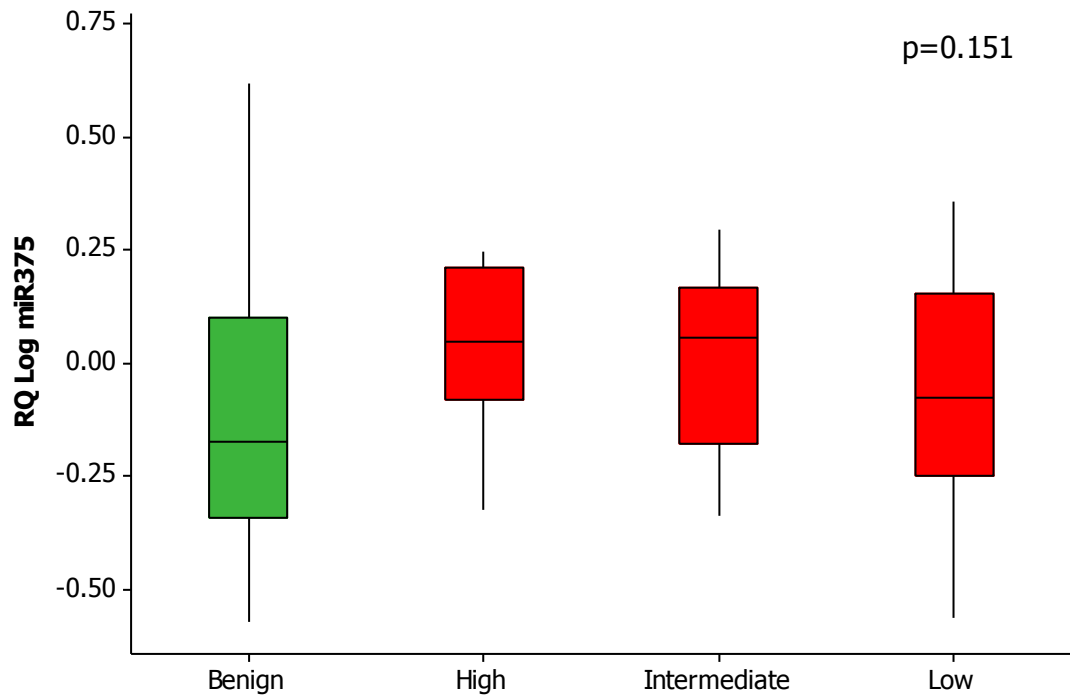
The area under the curve (AUC) was calculated as 0.651 with optimum cut-off yields for sensitivity of 76% and specificity of 61%.

Figure 4.7 Scatterplot of PSA Vs Log miR-375



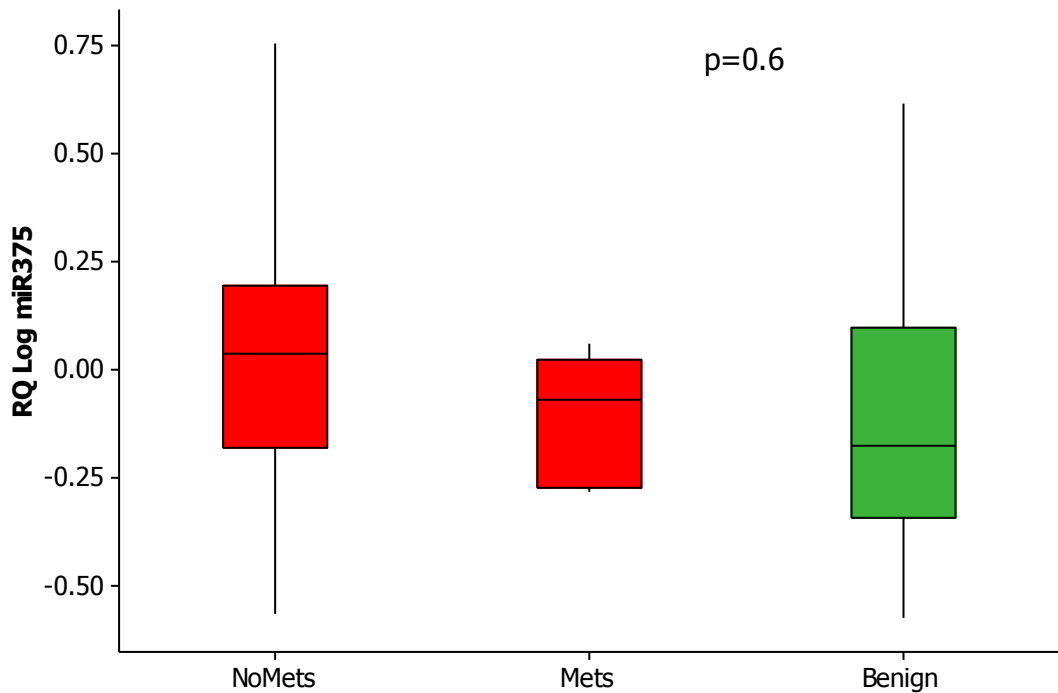
There was no correlation between circulating *miR-375* and PSA levels (p=0.829).

Figure 4.8 miR-375 expression in relation to D'Amico Risk Stratification.



As there was a trend towards significance of upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-375* in relation to D'Amico Risk Stratification. There was no significant difference found in this analysis ($p=0.151$).

Figure 4.9 miR-375 expression in relation to metastatic disease (p=0.6).

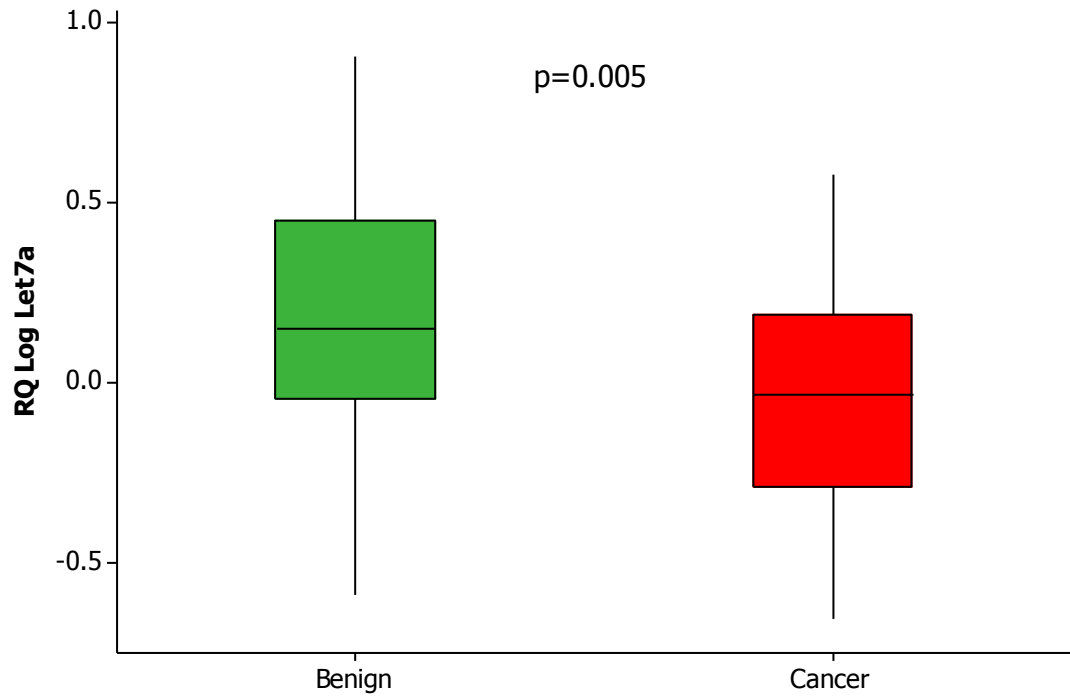


As there was a trend towards significance of upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-375* with the presence of metastases. There was no significant difference found in this analysis (p=0.6).

4.4.2.5 Let7a

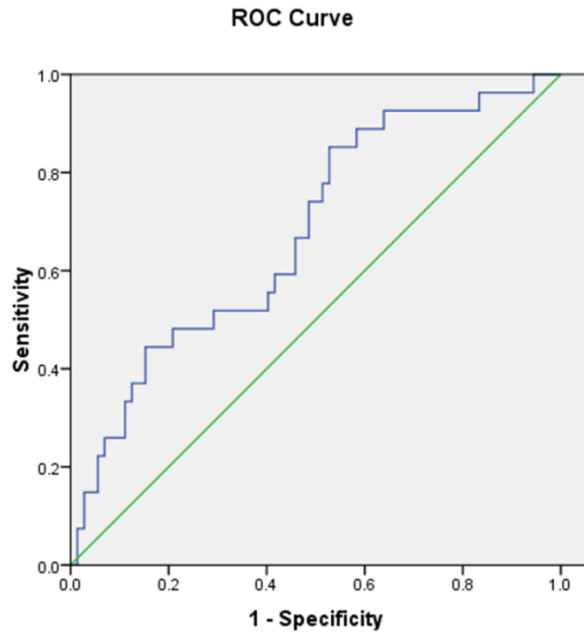
There was a significant downregulation of *let7a* in the cancer group as illustrated in figure 4.10. The adjusted p-value after applying a Bonferroni correction was 0.005 for the the two-sample t-test with an AUC of 0.678. There was no correlation between circulating *let7a* and PSA levels (p=0.826) however there was a correlation between decreasing levels of *let7a* and increasing D'Amico Risk stratification (anova analysis p=0.023). There was no significant decrease in *let7a* levels in patients with metastatic disease (p=0.1). The tumour-suppressor *let7a* had a sensitivity of 64% and a positive predictive value (PPV) of 70%.

Figure 4.10 *let7a* expression levels in the benign and cancer groups, $p=0.005$.



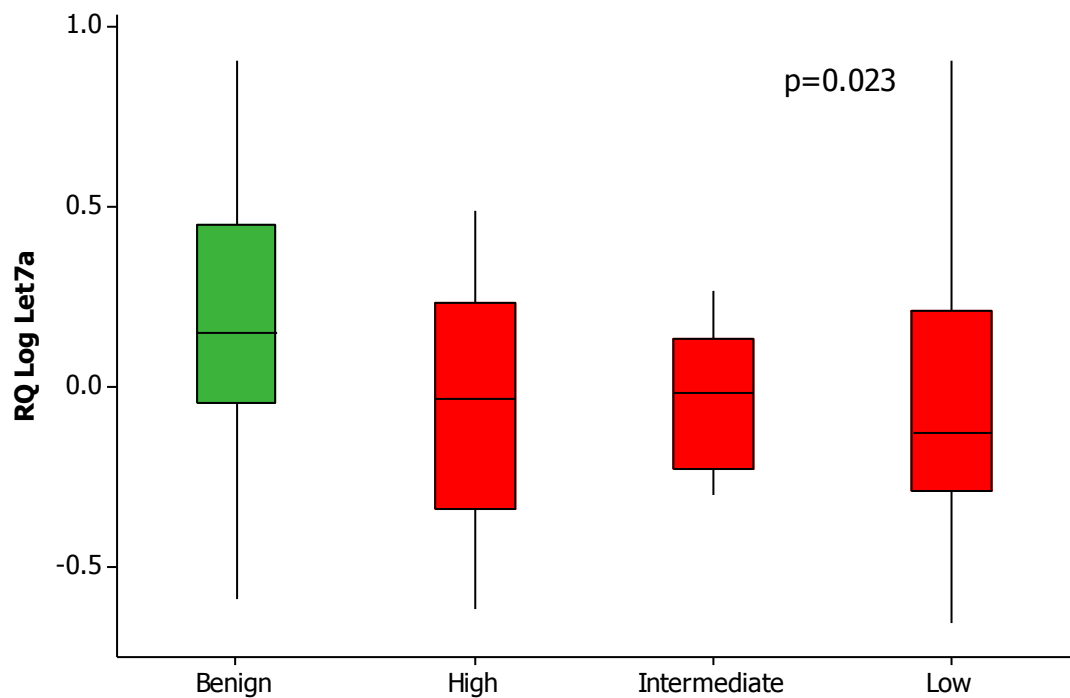
The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels reached statistical significance ($p=0.005$).

Figure 4.11 Receiver operated characteristic (ROC) curve for *let7a* expression levels in the benign and cancer groups.



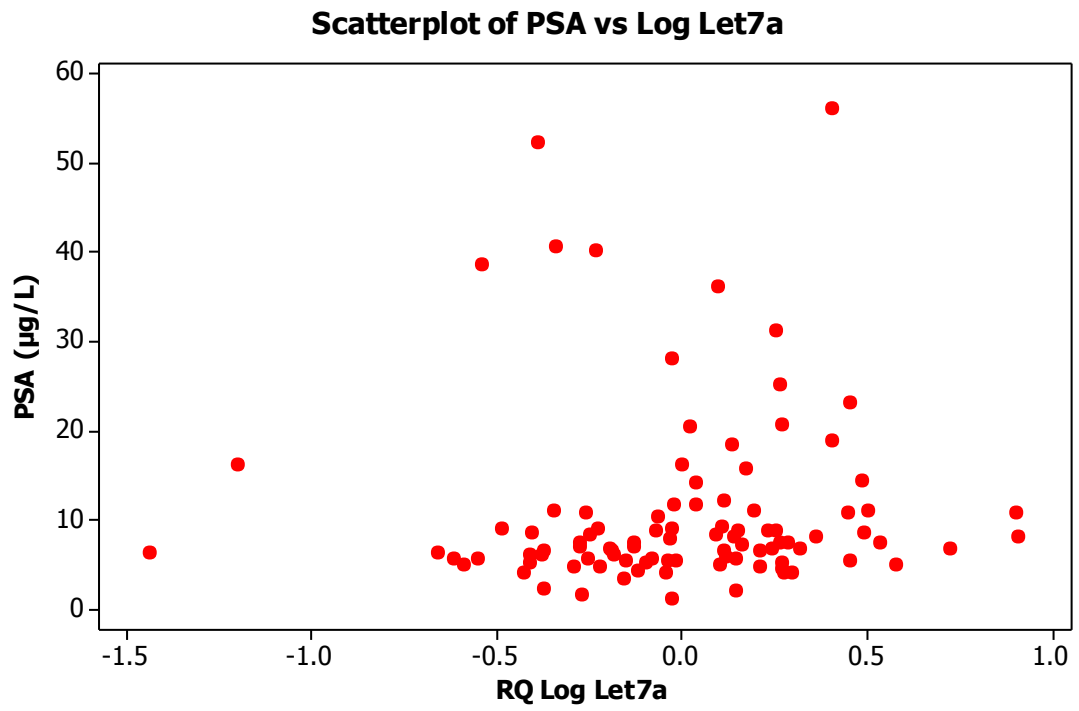
The area under the curve (AUC) was calculated as 0.678 with optimum cut-off yields for sensitivity of 64% and specificity of 62%.

Figure 4.12 *Let7a* expression in relation to D'Amico Risk Stratification (p=0.023)



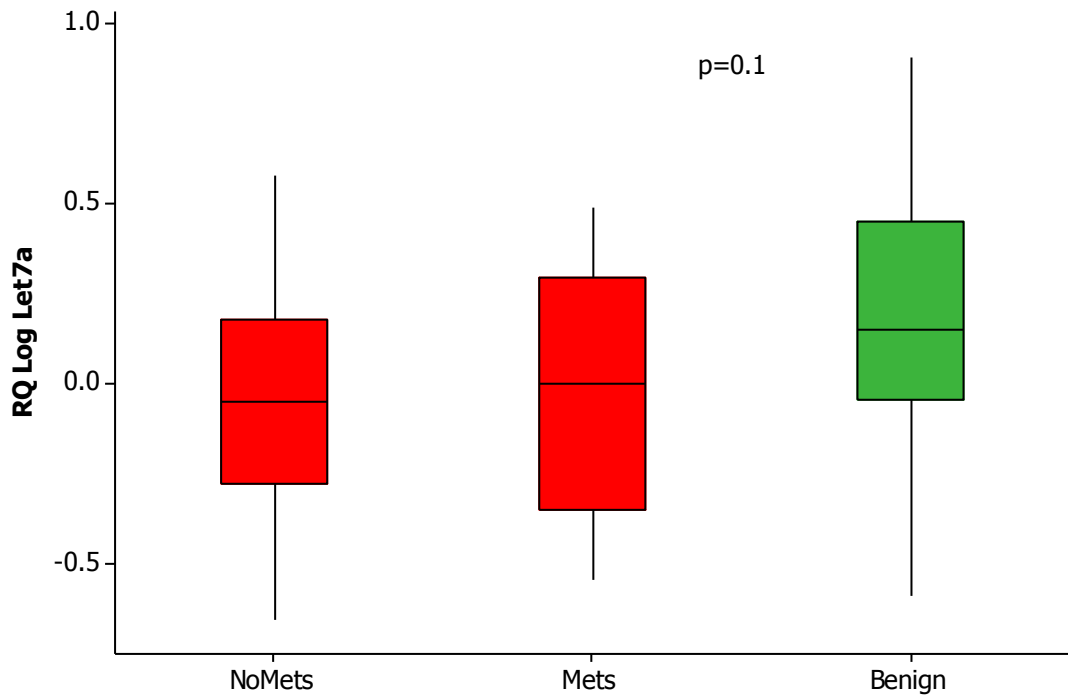
As there was a significant downregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *let7a* in relation to D'Amico Risk Stratification. There was a significant downregulation in the cancer groups (p=0.023).

Figure 4.13 4.7 Scatterplot of PSA Vs *let7a*



There was no correlation between circulating *let7a* and PSA levels ($p=0.826$).

Figure 4.14 *Let7a* expression in relation to metastatic disease.

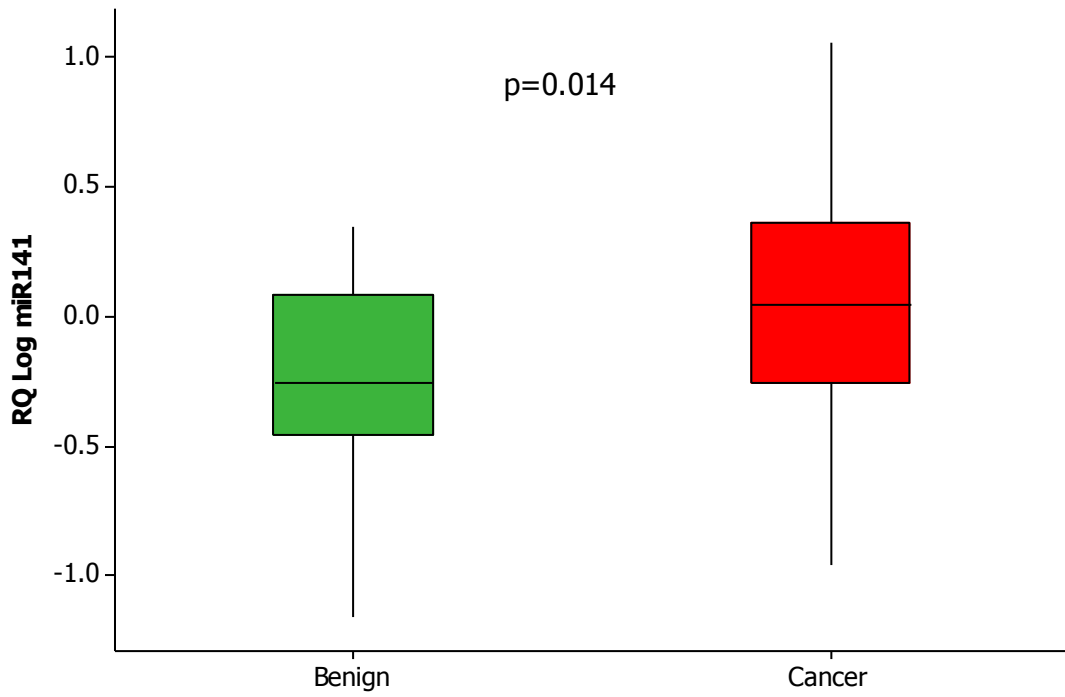


As there was a significant downregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *let7a* with the presence of metastases. There was no significant difference found in this analysis ($p=0.1$).

4.4.2.6 *MiR-141*

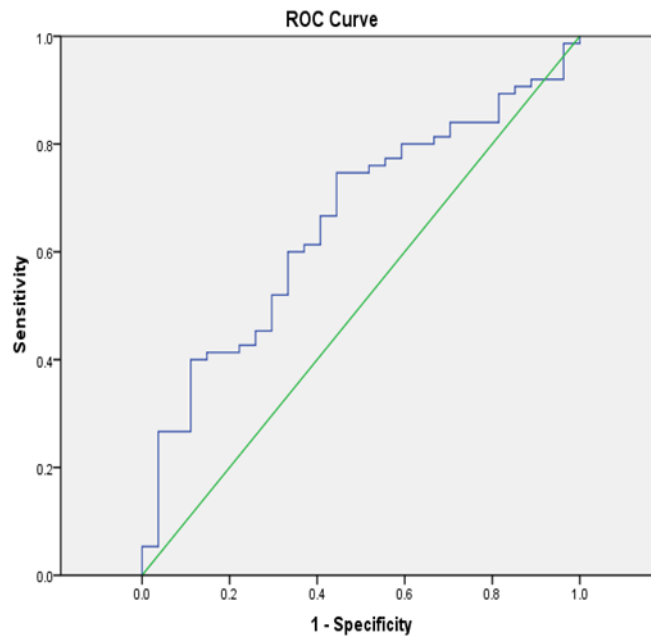
There was a significant upregulation of *miR-141* in the cancer group as illustrated in figure 4.15. The adjusted p-value after applying a Bonferroni correction was 0.014 for the two-sample t-test with an AUC of 0.655. There was no correlation between circulating *miR-141* and PSA levels ($p=0.276$) however there was a correlation between increasing levels of *miR-141* and increasing D'Amico Risk stratification (Anova analysis $p=0.028$). There was no significant increase in *miR-141* levels in patients with metastatic disease ($p=0.075$). The oncomir *miR-141* had a sensitivity of 76% and a positive predictive value (PPV) of 73%.

Figure 4.15 *miR-141* expression levels in the benign and cancer groups.



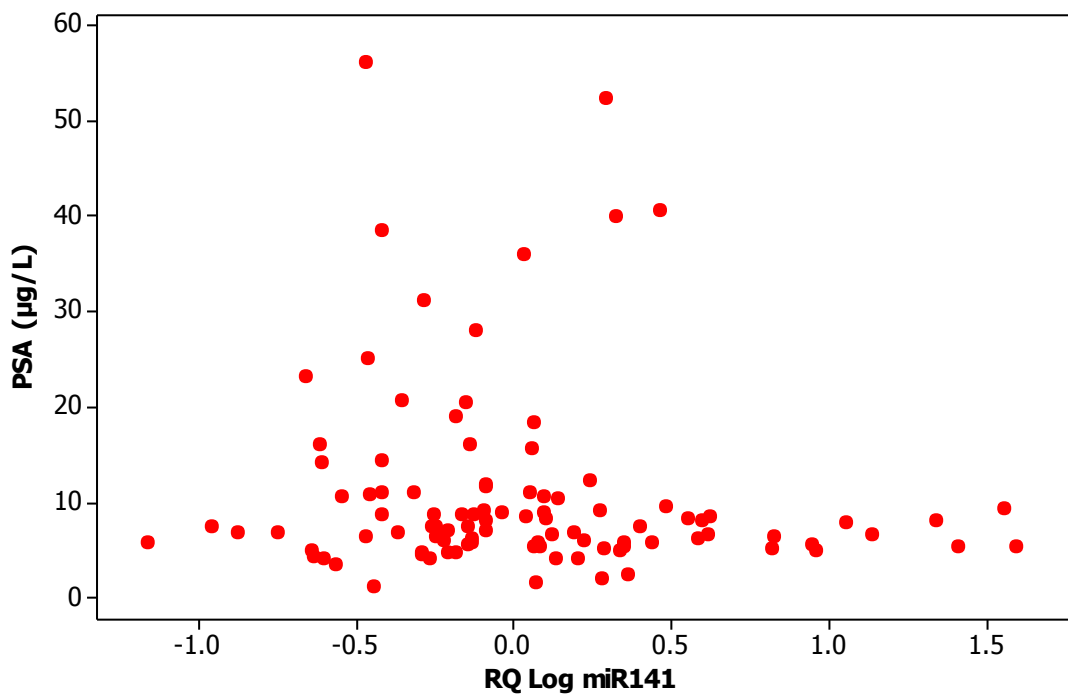
The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels reached statistical significance ($p=0.014$).

Figure 4.16 Receiver operated characteristic (ROC) curve for *miR-141* expression levels in the benign and cancer group.



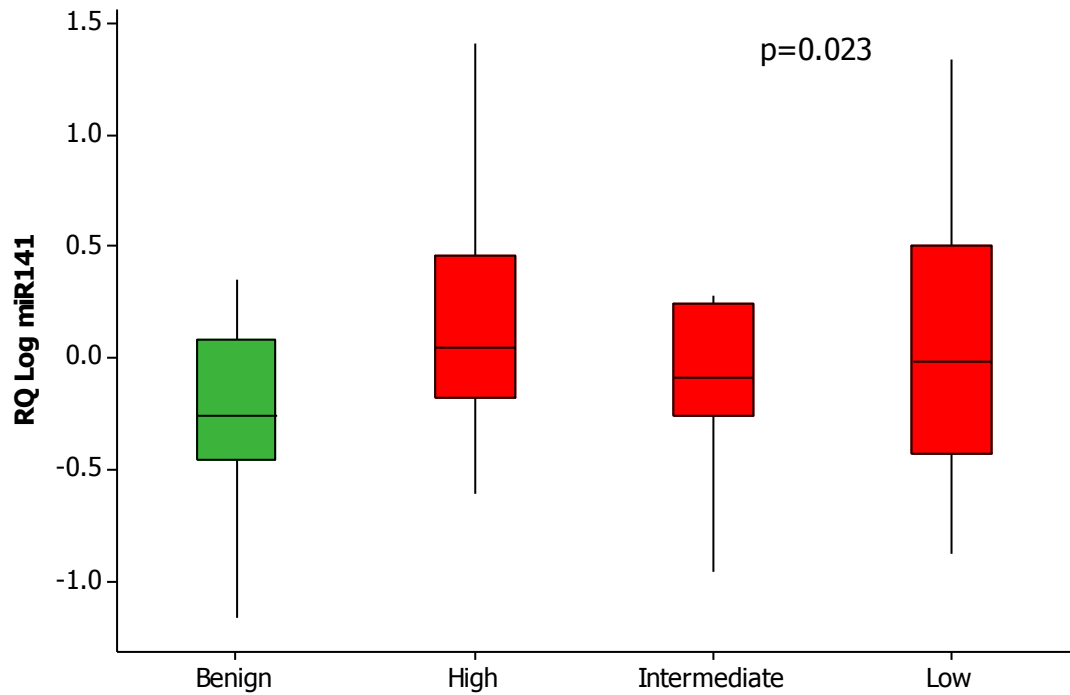
The area under the curve (AUC) was calculated as 0.655 with optimum cut-off yields for sensitivity of 76% and specificity of 59%.

Figure 4.17 Scatterplot of PSA Vs *miR-141*



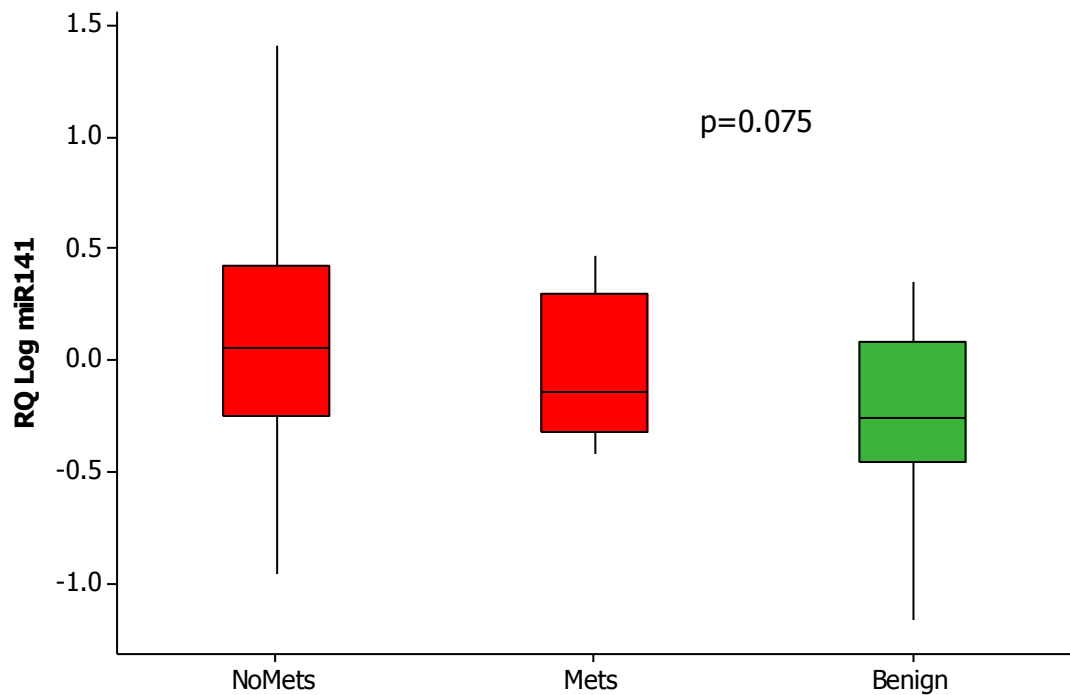
There was no correlation between circulating *miR-141* and PSA levels ($p=0.276$)

Figure 4.18 *miR-141* expression in relation to D'Amico Risk Stratification.



As there was a significant upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-141* in relation to D'Amico Risk Stratification. There was a significant upregulation in the cancer groups ($p=0.023$).

Figure 4.19 *miR-141* expression in relation to metastatic disease.

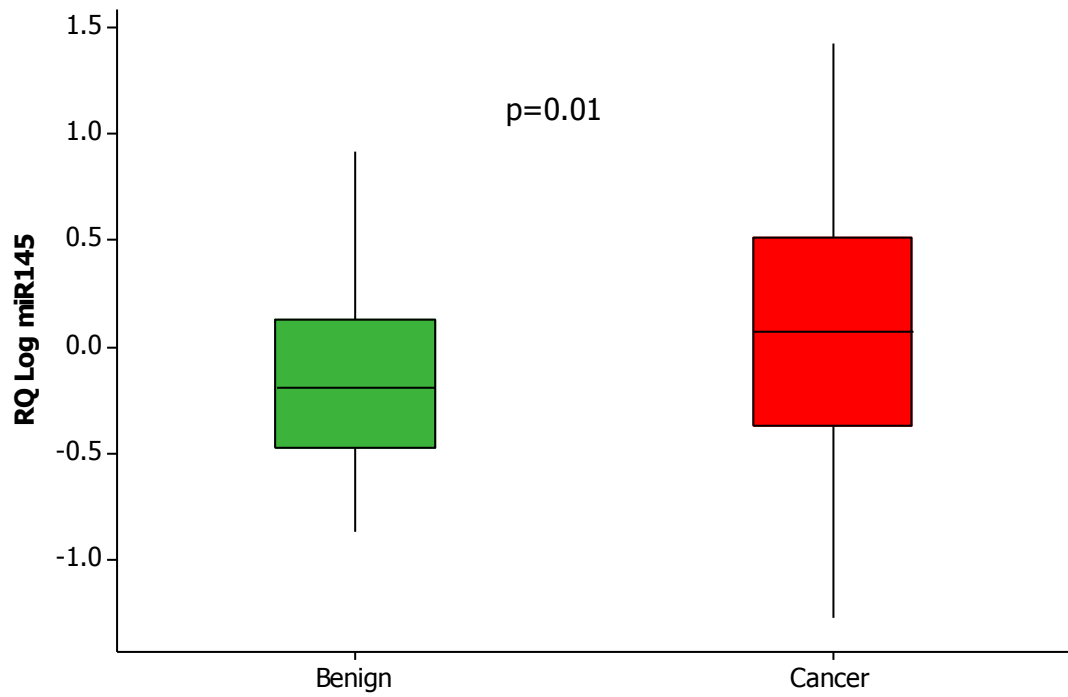


As there was a significant upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-141* with the presence of metastases. There was no significant difference found in this analysis ($p=0.075$).

4.4.2.7 *MiR-145*

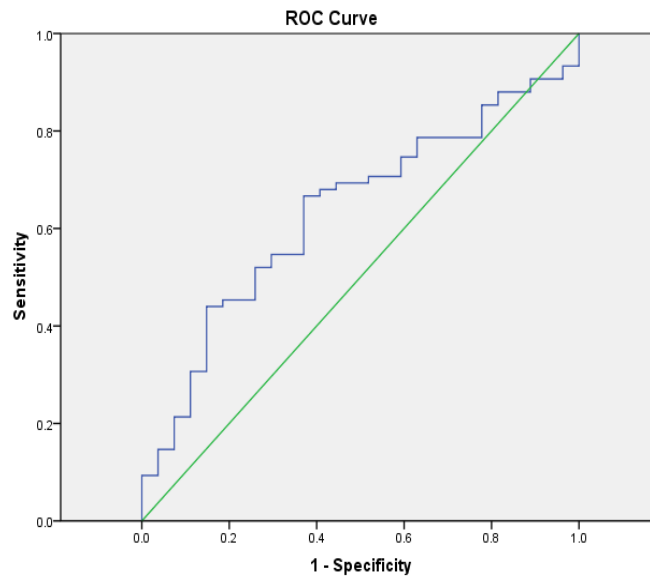
There was also a significant upregulation of *miR-145* in the cancer group as illustrated in figure 4.20. The adjusted p-value after applying a Bonferroni correction was 0.01 for the two-sample t-test with an AUC of 0.634. There was no correlation between circulating *miR-145* and PSA levels ($p=0.882$), there was no correlation between levels of *miR-145* and D'Amico Risk stratification (Anova analysis $p=0.277$). There was a trend towards significance with an increase in *miR-145* levels in patients with metastatic disease ($p=0.1$).

Figure 4.20 *miR-145* expression levels in the benign and cancer groups.



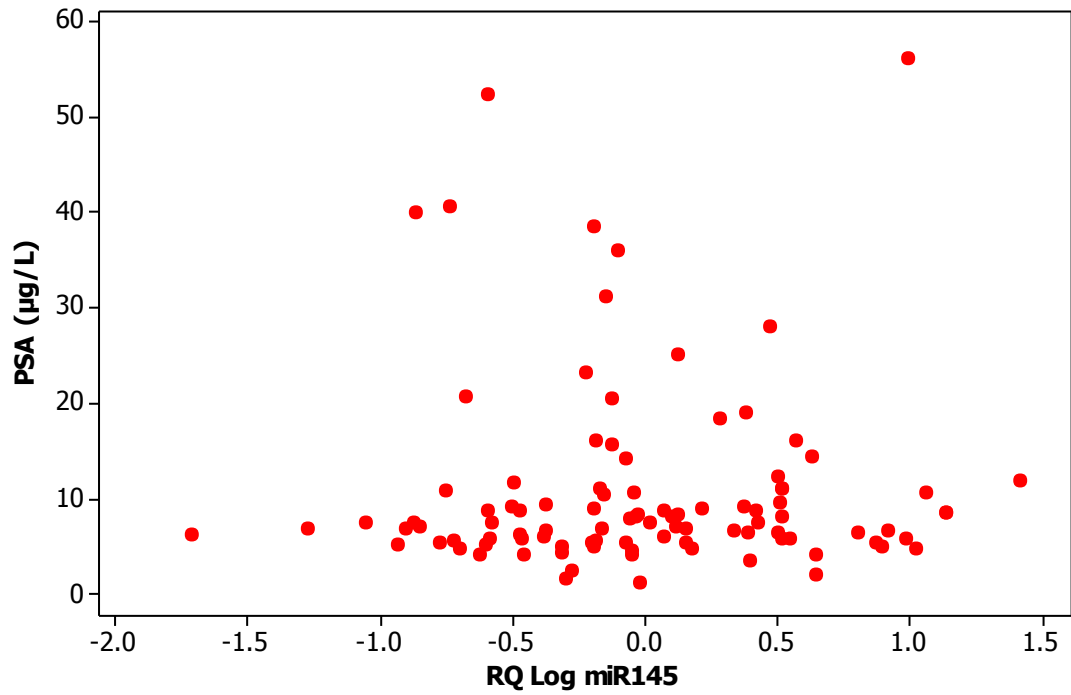
The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels reached statistical significance ($p=0.01$).

Figure 4.21 Receiver operated characteristic (ROC) curve for *miR-145* expression levels in the benign and cancer group.



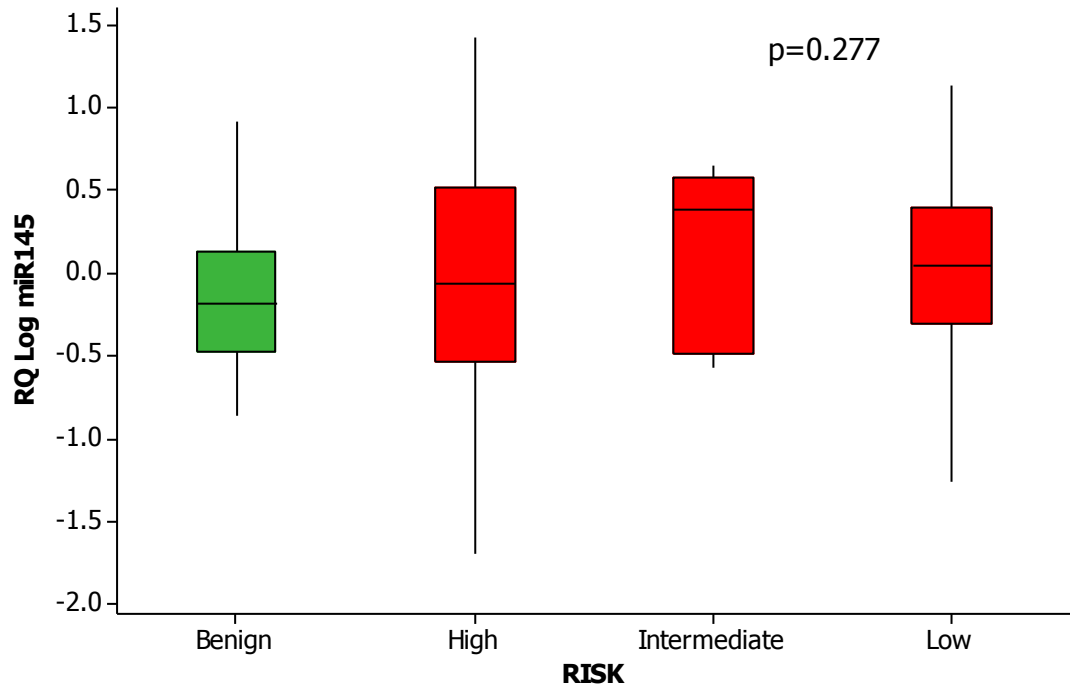
The area under the curve (AUC) was calculated as 0.634 with optimum cut-off yields for sensitivity of 64% and specificity of 60%.

Figure 4.22 Scatterplot of PSA Vs *miR-145*



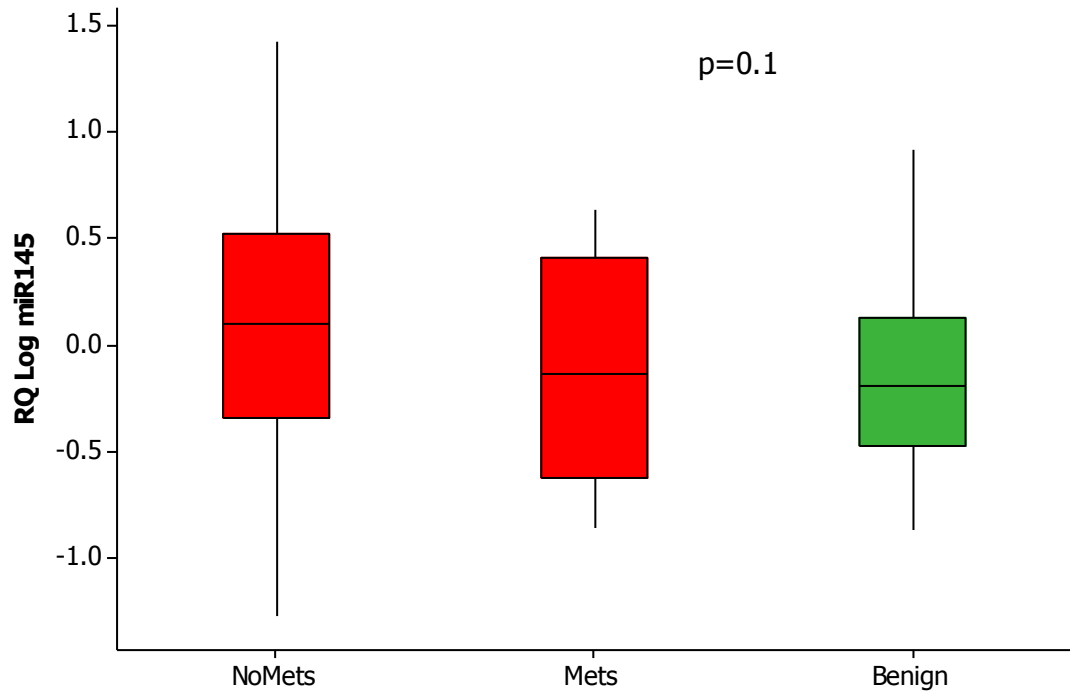
There was no correlation between circulating *miR-145* and PSA levels ($p=0.882$).

Figure 4.23 *miR-145* expression in relation to D'Amico Risk Stratification.



As there was a significant upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-145* in relation to D'Amico Risk Stratification. There was no significant upregulation in the cancer groups ($p=0.277$).

Figure 4.24 miR-145 expression in relation to metastatic disease.

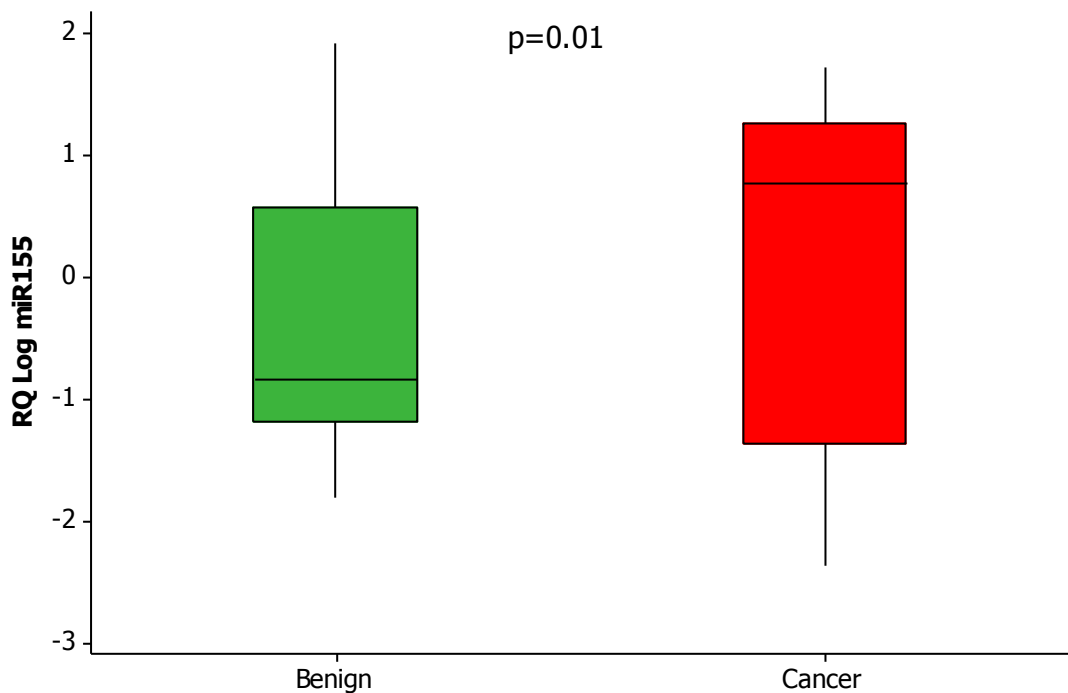


As there was a significant upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-145* with the presence of metastases. There was no significant difference found in this analysis ($p=0.1$).

4.4.2.8 *MiR-155*

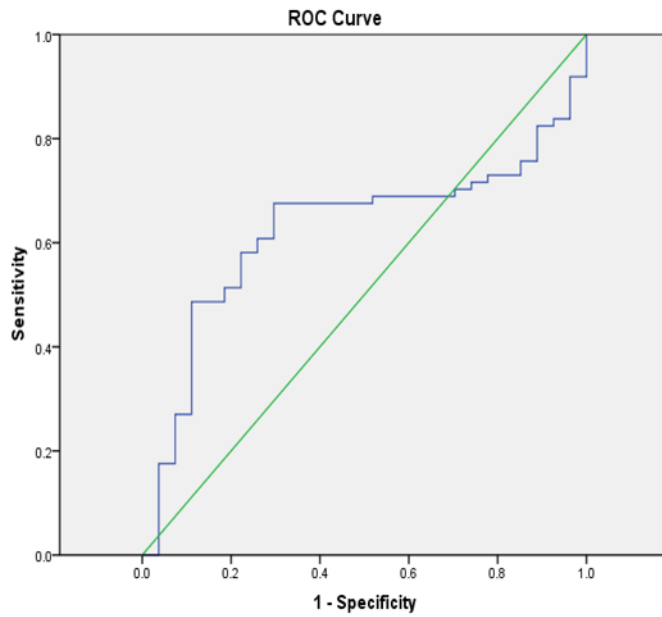
There was also a significant upregulation of *miR-155* in the cancer group as illustrated in figure 4.25. The adjusted p-value after applying a Bonferroni correction was 0.01 for the two-sample t-test with an AUC of 0.624. There was no correlation between circulating *miR-155* and PSA levels ($p=0.98$), there was no correlation between levels of *miR-155* and D'Amico Risk stratification (Anova analysis $p=0.115$). There was no significant increase in *miR-155* levels in patients with metastatic disease ($p=0.06$).

Figure 4.25 *miR-155* expression levels in the benign and cancer group, $p=0.01$



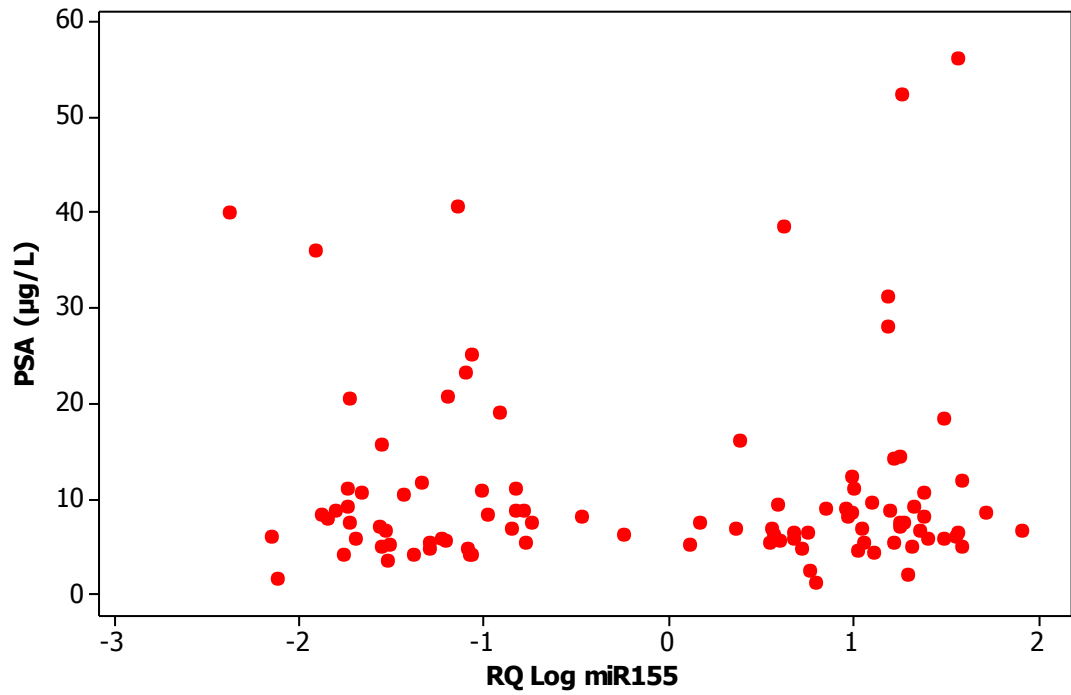
The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels reached statistical significance ($p=0.01$).

Figure 4.26 Receiver operated characteristic (ROC) curve for *miR-155* expression levels in the benign and cancer group.



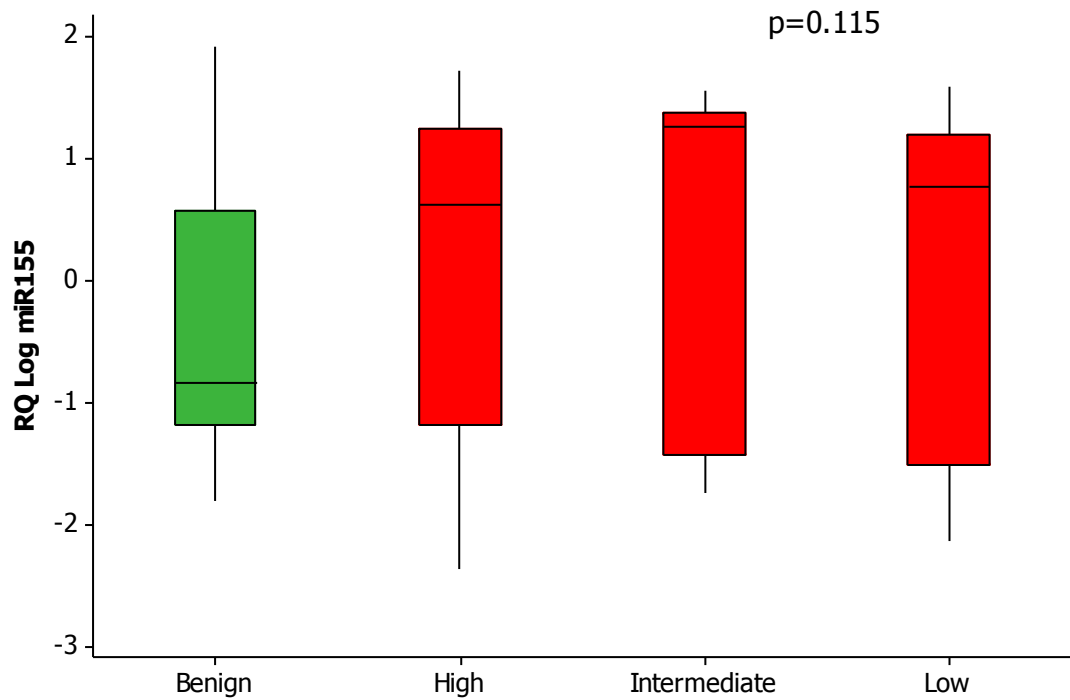
The area under the curve (AUC) was calculated as 0.624 with optimum cut-off yields for sensitivity of 62% and specificity of 65%.

Figure 4.27 Scatterplot of PSA Vs miR-155



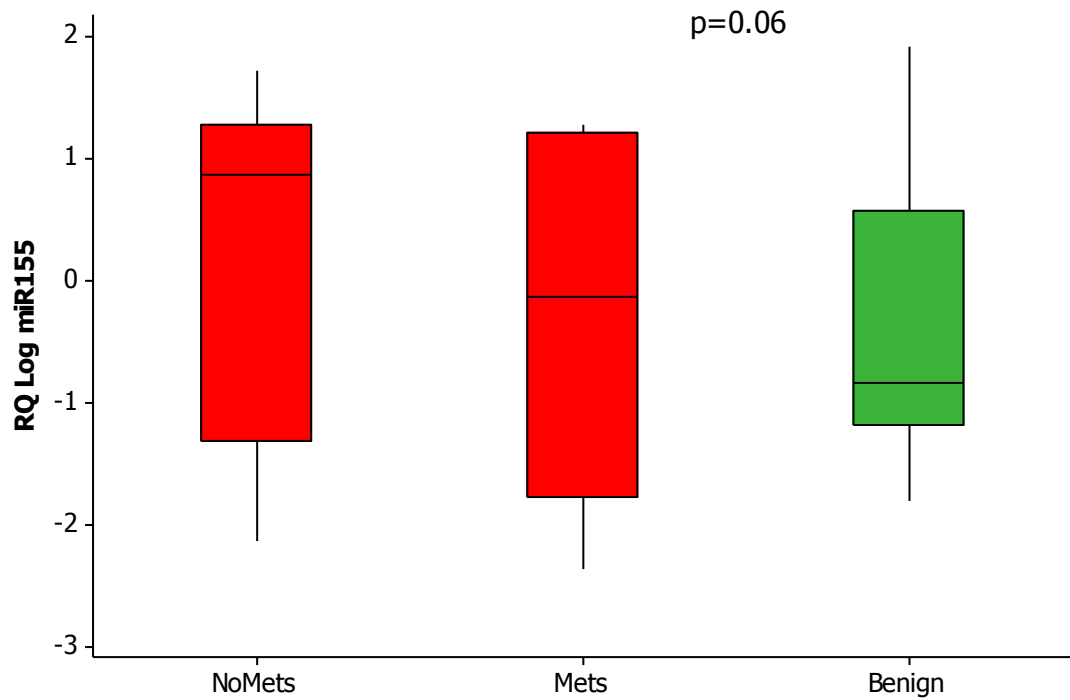
There was no correlation between circulating *miR-155* and PSA levels ($p=0.98$),

Figure 4.28 *miR-155* expression in relation to D'Amico Risk Stratification.



As there was a significant upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-155* in relation to D'Amico Risk Stratification. There was no significant upregulation in the cancer groups ($p=0.115$).

Figure 4.29 *miR-155* expression in relation to metastatic disease.



As there was a significant upregulation in the cancer group further analysis (using ANOVA) was performed to assess the relationship of *miR-145* with the presence of metastases. There was no significant difference found in this analysis ($p=0.06$).

Table 4.7 PSA and miRNA expression and clinicopathological correlations

Variable	PSA		Let7a		miR-141		miR-145		miR-155		Statistical test (parametric & non-parametric)
	Expression Level	p-value	Expression Level	p-value	Expression Level	p-value	Expression Level	p-value	Expression Level	p-value	
Histology		0.11		0.005*		0.014*		0.01*		0.01*	t-test / Mann-Whitney
Benign	8.72		0.167		-0.198		-0.178		-0.49		
Cancer	11.6		-0.063		0.071		0.064		0.18		
D'Amico Risk Stratification		0.02*		0.023*		0.023*		0.277		0.115	ANOVA / Kruskal-Wallis
Benign	8.724		0.1667		-0.1977		-0.1781		-0.491		
Low	6.461		-0.017		0.016		0.0269		0.131		
Intermediate	9.853		-0.1147		-0.1829		0.178		0.44		
High	15.712		-0.838		0.1917		0.0583		0.144		
Metastasis		0.001*		0.024*		0.075		0.01*		0.06	ANOVA / Kruskal-Wallis
Benign	8.724		0.331		-0.1977		-0.1781		-0.491		
No Metastasis	8.267		0.3869		0.089		0.093		0.246		
Metastasis	31.958		0.3475		-0.0448		-0.1234		-0.248		

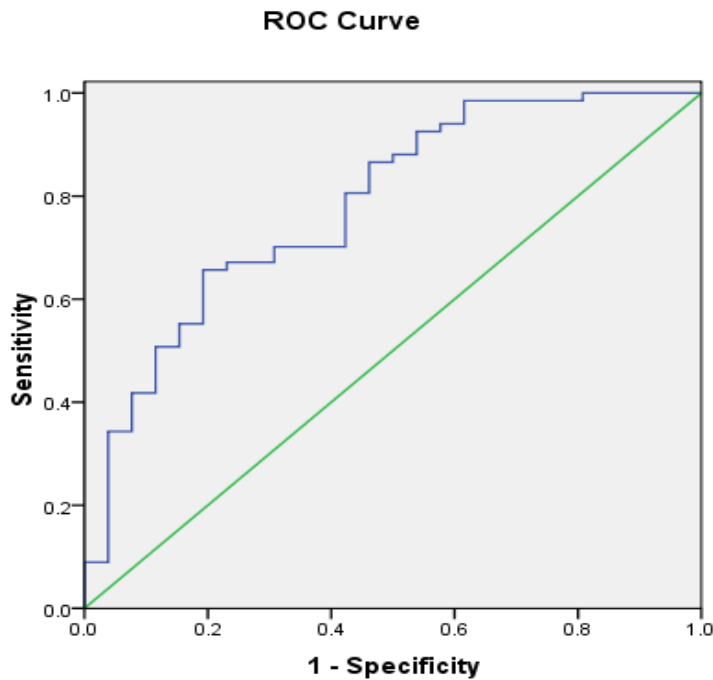
4.4.2.9 miRNAs in Combination

Using regression analysis, expression patterns were analysed in combination using the miRNAs as a diagnostic panel to improve upon the sensitivity. Using the 4 miRNAs mentioned above (*let-7a*, *miR-141*, *-145* and *miR-155*) the sensitivity improved to 97%, with a PPV of 80% and an AUC of 0.789.

Table 4.8 Dysregulated miRNAs, the p-value and AUC

miRNA	Up/Down Regulated	P Value	AUC
<i>Let7a</i>	↓	0.005	0.678
<i>miR-141</i>	↑	0.014	0.655
<i>miR-145</i>	↑	0.01	0.634
<i>miR-155</i>	↑	0.01	0.624
<i>miR-375</i>	↑	0.075	0.651

Figure 4.30 Receiver operated characteristic (ROC) curve expression levels in the benign and cancer group for the miRNAs in combination.



The area under the curve (AUC) was calculated as 0.789 with optimum cut-off yields for sensitivity of 80% and specificity of 64%.

4.5 Discussion

MiRNAs are ideal molecules for a blood-based biomarkers for the detection of cancer, as they are dysregulated in carcinogenesis and are highly stable in both tissue and in blood samples^{63,109-111}. Various studies have documented the differential expression of miRNAs in the circulation of patients with cancer when compared with healthy controls, making miRNA an ideal non-invasive biomarker¹¹²⁻¹¹⁴.

This study demonstrates that 4 miRNAs are significantly dysregulated in the circulation of patients with PCa. Combining these miRNAs to identify a unique PCa miRNA signature revealed a biomarker panel with an AUC of 0.783. A study investigating the expression profile of patients with gastric cancer using 5 circulating

miRNAs in combination had a sensitivity of 80%, a specificity of 81% and an AUC of 0.879¹⁷⁰.

To date there are a limited number of studies examining the expression profile of miRNAs in the circulation of PCa patients. Examining these papers reveals that there are a variety of different techniques used for RNA extraction and from different blood media such as whole blood, serum and plasma. Our institution has recently published on the variability of miRNA levels in whole blood, serum and plasma, and identified that whole blood contains a higher yield of miRNAs by RQ-PCR¹²⁶.

MiR-16 and *miR-425* were found to be stably expressed in the circulation of all patients with little variability. As a result both were selected for use as endogenous controls. *MiR-16* has been used as a normaliser in a many studies investigating levels of miRNAs in whole blood, serum, plasma and tissue^{126,143-146}. *miR-425* has previously been described as a suitable EC in tissue and recently as a suitable EC in the circulation of patients with colorectal cancer^{147,165}.

The oncomir *miR-141*, when quantified in circulation, has the ability to identify those men with PCa with an AUC of 0.655. This highlights a potential clinical use of miRNAs in the identification of patients with malignancy in a group deemed to be clinically high risk due to an elevated PSA. Mitchell *et al* have previously reported that *miR-141* could differentiate those with PCa with an AUC of 0.9, although all 25 of the PCa patients had metastatic disease¹⁰⁹. Levels of *miR-141* has been shown to increase as the stage of disease progresses from organ confined disease, to locally advanced disease and on to metastatic PCa^{119,123}. However, similar to Mitchell *et al* and using a RNA extraction technique from serum, Mahn *et al* encountered difficulties in the detection of *miR-141* in the sera¹²⁰. This further highlights the variability in results from different extraction methods, different blood products and the use of different endogenous controls. There is also evidence to support that RNase activity is increased in the serum of PCa patients however Mitchell *et al* identified that circulating miRNAs are stable against RNase activity^{109,171}.

In this study *let7a* was found to be significantly downregulated in PCa patients. *Let7a* has the ability to act as both an oncogene and tumour suppressor and is dysregulated in a number of malignancies¹²⁵. This is first reported downregulation of *let7a* in the circulation of patients with PCa, this also concurs with previous papers citing *let7a* as downregulated in PCa tumour tissue^{73,76}.

Not all miRNAs that are dysregulated in PCa tissue are released into the circulation. The exact mechanism by which miRNAs are released still remains unclear. MiRNAs could be passively leaked or actively secreted into the circulation. Passive leakage can occur by tissue degradation associated with malignancy, through this mechanism miRNAs could be released into the circulation in an energy free mechanism. miRNAs could also be actively secreted in an energy dependant mechanism similar to that of hormone or cytokine release in exosomes or microvesicles and to support this theory miRNAs have recently been identified within exosomes and microvesicles^{116,117}. MiRNAs are also present within Ago complexes in the circulation. MiRNAs in the circulation contained within microvesicles or within Ago complexes may originate from different cell types and may actually reflect a tissue-specific miRNA expression profile¹¹⁸

Using the 4 miRNAs (*miR-141*, *-145*, *-155* and *let7a*) in combination yielded a sensitive biomarker panel with an AUC of 0.789. Given a sensitivity of 80%, with few false negative results, illustrates that quantifying a panel of miRNAs in the circulation has the potential to reduce unnecessary TRUS biopsies from being performed, allows for risk stratification in active surveillance protocols and in the future may help with choices of therapeutic intervention for physicians, surgeons and patients. Internal and external validation with larger patient numbers will be necessary to investigate if this panel of miRNAs has the ability to accurately diagnose men with PCa.

4.6 Conclusion

This study has identified a panel of 4 miRNAs that have diagnostic potential superior to that of PSA and DRE for the detection of PCa. Three miRNAs were observed to be upregulated and one miRNA downregulated in association with PCa. MiRNAs, due to their detection, dysregulation and stability in blood, hold immense promise as future, novel, non-invasive biomarkers for PCa.

Chapter 5

The evaluation of miRNAs post radical prostatectomy

5.1 Introduction

PSA, a frequently used biomarker for the detection of PCa, is limited by its lack of sensitivity and specificity for PCa and therefore not considered an ideal diagnostic biomarker. In clinical practice, PSA is also used monitor response to treatment and is an excellent biomarker for detecting biochemical recurrence. After a radical prostatectomy the PSA level becomes undetectable ($<0.01 \mu\text{g/L}$) and a reading above $0.2 \mu\text{g/L}$ is considered to be a biochemical occurrence. Following radiotherapy or brachytherapy the PSA levels fall to a nadir reading, nadir being the lowest level reached. Again, a significant increase above this nadir (usually a rise $>2 \mu\text{g/L}$) is considered a biochemical recurrence.

5.2 Aims

The aim of this study was to evaluate the expression of five miRNA, four of which were significantly dysregulated in the circulation of men undergoing a radical prostatectomy (RRP) and to compare the pre-operative and post-operative levels to assess if they return to normal or a level similar to the group of men with benign prostatic disease.

5.3 Materials and methods

5.3.1 Study groups

5.3.1.1 Prostate cancer

Whole blood samples were collected prospectively from 20 patients prior to a RRP (either open or laparoscopic). All 20 patients had histologically confirmed PCa and none had received any neoadjuvant chemotherapy or radiotherapy prior to collection of the specimens or the surgery. Whole blood samples were collected from these patients again at mean day 10 post-operatively (range 5-21 days). Details of this cohort are summarised in tables 5.1, 5.2 and 5.3.

Table 5.1 PSA levels of the prostate cancer group

PSA	µg/L
Median	7.9
Mean	10.75
Range	2 - 14.2

Table 5.2 Histology of prostate cancer group

Gleason Score	Number (20)
3+3	8
3+4	8
4+3	3
4+4	1

Table 5.3 D'Amico Risk Stratification

D'Amico Risk Stratification	Number (20)
Low	8
Intermediate	9
High	3

5.3.1.2 Benign Group

This is the group as described previously in Chapter 4. 27 patients had a benign histological finding following a TRUS Biopsy. These men had no current or previous malignancy. The PSA at diagnosis is also recorded for both groups. These blood specimens were obtained from the PCa patients prior to their TRUS Biopsy in the RAPAC in Galway University Hospital.

Table 5.4 PSA levels of the benign group

	Benign
Number	27
PSA: µg/L	
Median	7.45
Mean	11.6
Range	1 - 77

5.3.2 Candidate miRNA targets

A panel of five individual miRNA targets were chosen for analysis in this study. These are listed in table 5.5 below. Four of these miRNAs were significantly dysregulated in the circulation of men with PCa as described in the previous chapter (*let7a*, *miR-141*, *-145* and *-155*) along with *miR-375*, which had a trend towards significance.

Table 5.5 Candidate miRNA selected for investigation

Candidate miRNA
<i>Let7a</i>
<i>miR-141</i>
<i>miR-145</i>
<i>miR-155</i>
<i>miR-375</i>

5.3.3 Blood collection

10ml of venous whole blood was collected from each participant in Vacuette EDTA K3E blood bottles (Grenier Bio-one). These samples were stored at 4°C (unprocessed and in the original sample bottles) until required.

5.3.4 MiRNA extraction from blood

As described in detail in chapter 2, Total RNA was extracted from whole blood using a modified trizol co-purification technique. For each 1ml of whole blood, phase separation was performed by the addition of 3ml of trizol. 200µl of 1-bromo-4-methoxybenzene was then added to augment the RNA phase separation process. The total RNA was precipitated using isopropanol and washed with 75% ethanol prior to solubilisation with 60µl of nuclease free water.

5.3.5 Analysis of miRNA concentration

RNA concentration was determined using a Nanodrop® Spectrophotometer (NanoDrop Technologies). In general concentrations ranging between 22 and 595ng/µl of miRNA were obtained for each sample.

5.3.6 Analysis of miRNA expression by RQ-PCR

RNA samples were reverse transcribed using primers specific to each miRNA target and real-time quantitative PCR (RQ-PCR) was carried out using TaqMan® miRNA primers and probes (Applied Biosystems, USA), as described in detail in chapter 2. The RQ-PCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using default thermal cycling conditions. PCR amplification efficiencies were calculated for each candidate EC RQ-PCR assay by generating a cDNA dilution curve and using the formula $E = (10^{-1/\text{slope}} - 1) \times 100$, where E is the amplification efficiency. Triplicate samples were used throughout and each plate included an interassay control and calibrator synthesised from pooled normal breast tissue. The threshold standard deviation accepted for intra- and inter-assay replicates was 0.3. MiRNA expression levels were calculated using QbasePlus software with *miR-16* and *miR-425* as an endogenous control (based on the endogenous control selection and validation process described in chapter 3).

5.3.7 Statistical analysis

Data were analysed using the software package SPSS (version 18) and Minitab (version 16). Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis (natural log). All datasets were normally distributed, as verified by the Kolmogorov-Smirnov Z-test, and

therefore suitable for parametric testing. For all two-sample comparisons the two-sample t-test was used to assess differences in mean expression levels and the Mann-Whitney test to evaluate the differences in the median values. ANOVA, followed by Tukey HSD Post Hoc test, was used to compare the mean expression levels of miRNAs across different groups. The paired sample t test was used to compare the differences in miRNA expression between pre- and post-operation. Unpaired sample t test was used to compare the differences in miRNA expression between PCa patients and the benign patients. A probability P value <0.05 was considered statistically significant.

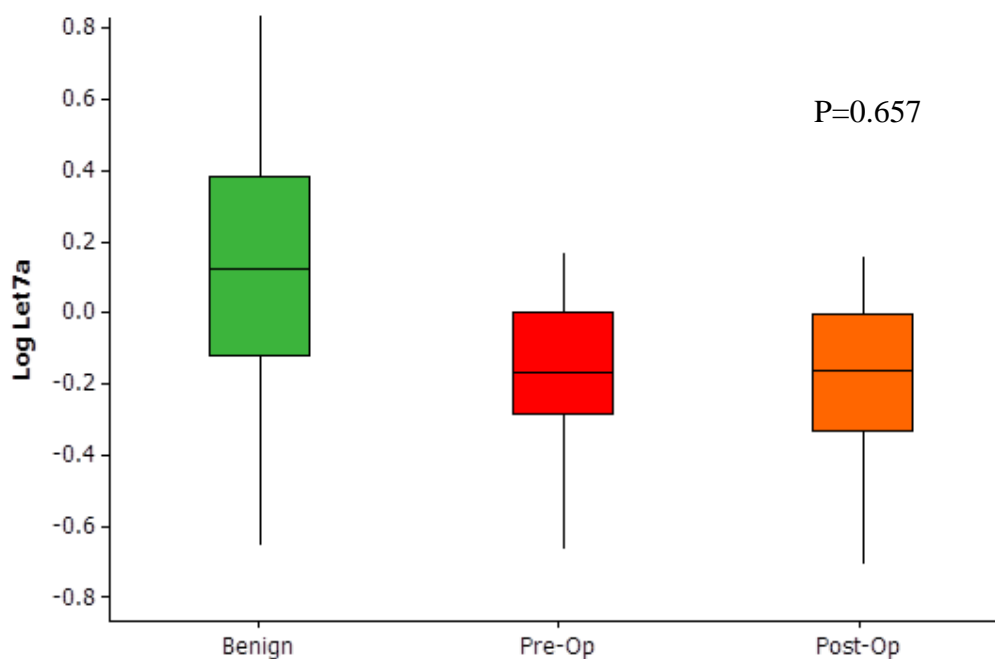
5.4 Results

The expression of all five miRNAs were detectable at variable levels in the circulation of all 67 samples. *MiR-16* and *miR-425* expression was stable and reproducible across all 67 samples and used to normalise RQ-PCR data.

5.4.1 *Let7a*

There was a significant downregulation of *let7a* in the cancer group as illustrated in figure 1. The adjusted p-value after applying a Bonferroni correction was 0.045 for the two-sample t-test. The expression levels of *let7a* did not return to a level similar to that of the benign group at day 10 post-operative (p=0.657).

Figure 5.1 *let7a* expression levels in the benign, pre-op and post-op cancer groups

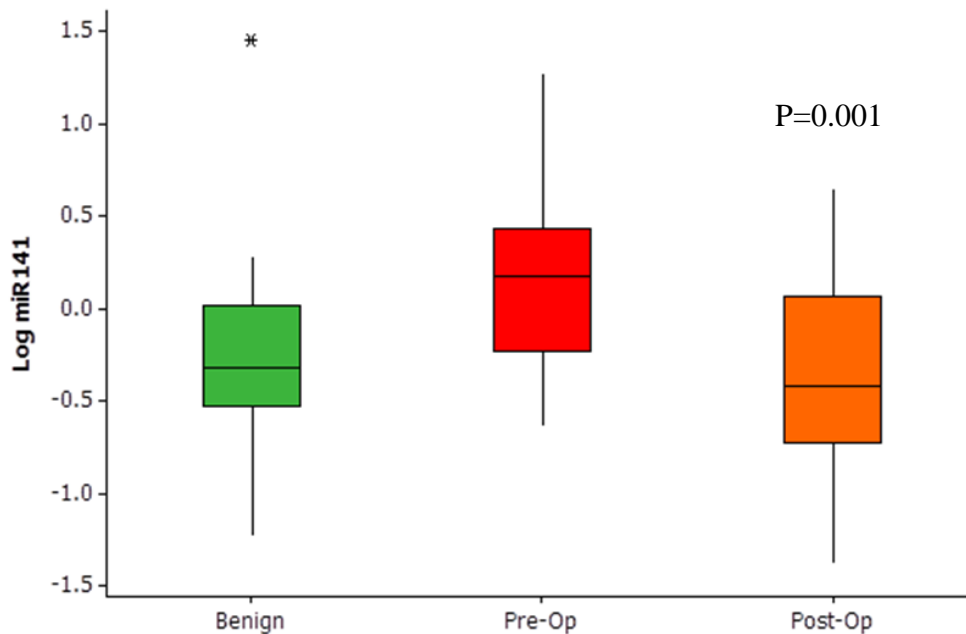


The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance in the post op setting (p=0.657).

5.4.2 *MiR-141*

There was a significant upregulation of *miR-141* in the cancer group as illustrated in figure 5.2. The adjusted p-value after applying a Bonferroni correction was 0.05 for the two-sample t-test. The expression levels of *miR-141* did return to a level similar to that of the benign group at day 10 post-operative (p=0.001).

Figure 5.2 *miR-141* expression levels in the benign, pre-op and post-op cancer groups

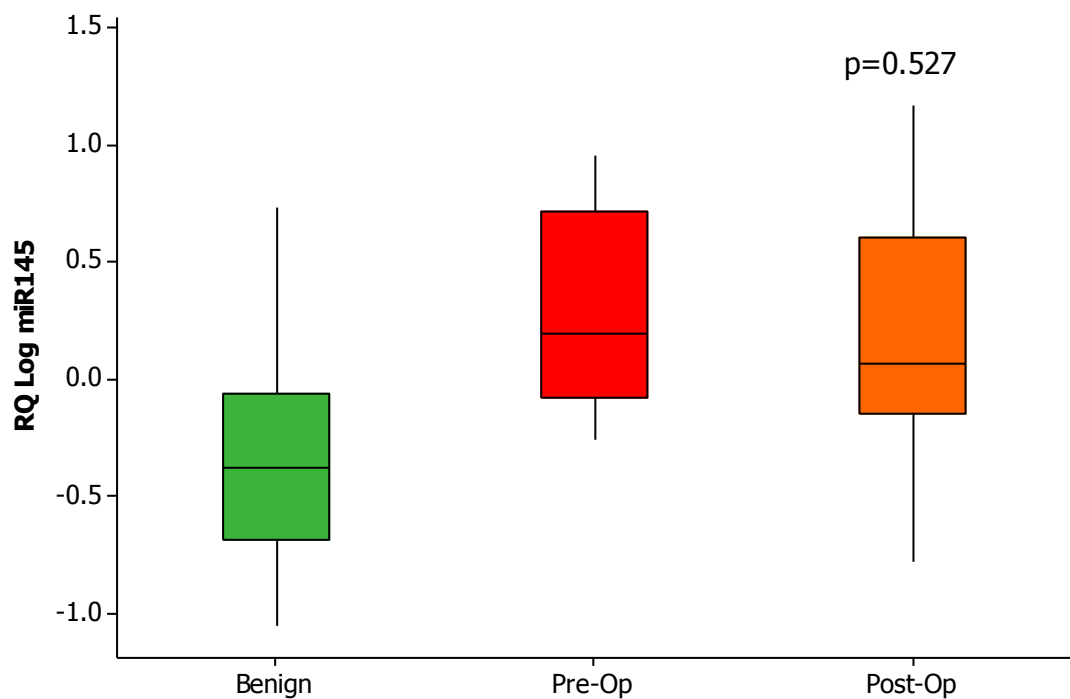


The boxes show the interquartile range and median, the whiskers indicate the range and outliers are depicted with the symbol (*). The difference in mean expression levels reached statistical significance in the post op setting returning to a level similar to the pre op values (p=0.657).

5.4.3 *MiR-145*

There was a significant upregulation of *miR-145* in the cancer group as illustrated in figure 5.3. The adjusted p-value after applying a Bonferroni correction was 0.005 for the two-sample t-test. The expression levels of *miR-145* did not return to a level similar to that of the benign group at day 10 post-operative ($p=0.527$).

Figure 5.3 *miR-145* expression levels in the benign, pre-op and post-op cancer groups

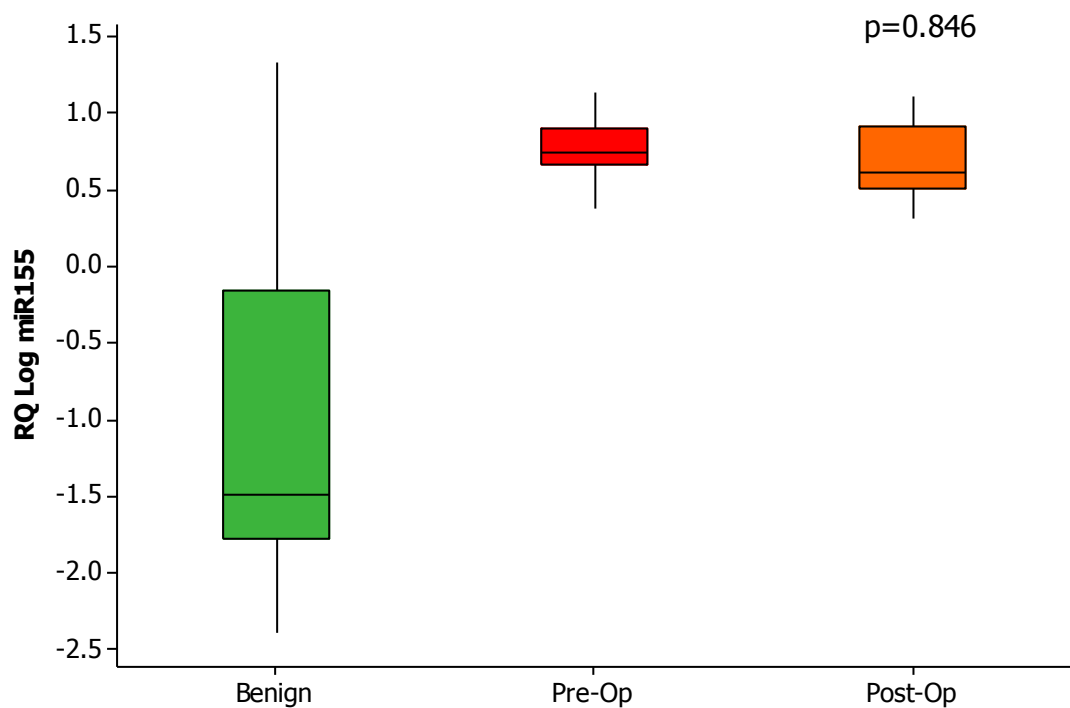


The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance in the post op setting ($p=0.527$).

5.4.4 *MiR-155*

There was a significant upregulation of *miR-155* in the cancer group as illustrated in figure 5.4. The adjusted p-value after applying a Bonferroni correction was 0.005 for the two-sample t-test. The expression levels of *miR-155* did not return to a level similar to that of the benign group at day 10 post-operative (p=0.846).

Figure 5.4 *miR-155* expression levels in the benign, pre-op and post-op cancer groups

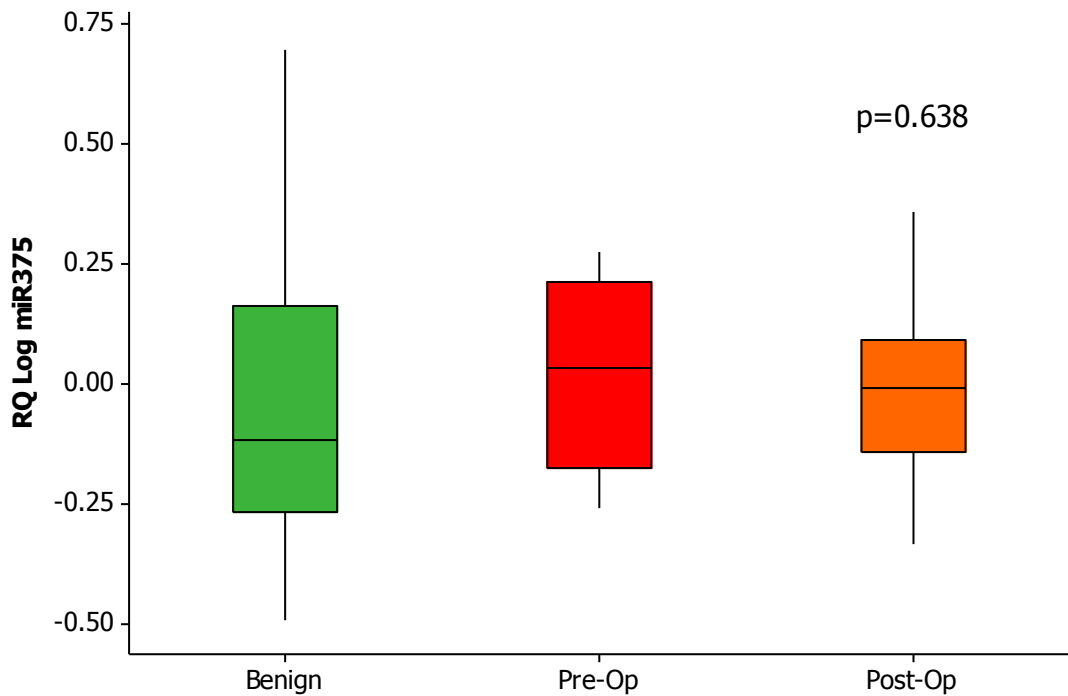


The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance in the post op setting (p=0.846).

5.4.5 *MiR-375*

There was no significant upregulation of *miR-375* in the cancer group as illustrated in figure 5.5. The adjusted p-value after applying a Bonferroni correction was 0.382 for the two-sample t-test. The expression levels of *miR-375* did not return to a level similar to that of the Benign group at day 10 post-operative (p=0.638).

Figure 5.5 *miR-375* expression levels in the benign, pre-op and post-op cancer groups



The boxes show the interquartile range and median and the whiskers indicate the range. The difference in mean expression levels did not reach statistical significance in the post op setting (p=0.638).

5.5 Discussion

Mean expression levels of *miR-141* were significantly reduced post-operatively. Previous studies have also highlighted that the expression levels of oncomirs return to baseline after oncological surgery, this has been identified in patients undergoing radical prostatectomy, mastectomy and colonic resection^{113,120,126}.

Mahn *et al* analysed the expression of 4 miRNAs in patients with PCa and healthy controls. Three of these 4 miRNAs were significantly upregulated in patients with PCa in comparison to patients with BPH, *miR-26a*, *-195* and *let-7i*. *MiR-26a* could differentiate PCa from BPH with an AUC of 0.703 yet PSA could differentiate between the 2 clinical conditions with a superior AUC of 0.834. Of 10 patients who underwent a RRP, the expression levels of *miR-26a* and *miR-195* were significantly reduced at post-operative day 7, returning to normal expression levels. This further highlights the diagnostic potential of the overall expression profile of many oncomirs and tumour suppressor miRNAs¹²⁰.

A study by Le *et al* identified that the expression levels of four miRNAs were decreased in sera of 10 days post-operative lung cancer patients, especially *miR-21* and *miR-24*, compared with levels in paired pre-operative sera, suggesting they may be used as predictors in post-operative therapy of lung cancer patients^{121,172-174}. The levels of *miR-205*, *miR-30d*, and *miR-24* were still overexpressed in 10 days post-operative sera compared with normal volunteers. Also, this is a similar finding to what was shown in this study, in that only a single miRNA (*miR-141*) out of four dysregulated miRNAs returned to a level similar to that of the benign prostate group. This suggests that circulating miRNAs can possibly be used as a biomarker for post-operative monitoring of cancer patients undergoing surgical excision of tumours.

It is not entirely clear why *miR-141* was the only miRNA of the four that were dysregulated pre operatively to return to a level similar to that of the Benign group after a radical prostatectomy. However, given that miRNA research in bodily fluids

is still in its infancy, perhaps there are a variety of other factors that could account for the persistently dysregulated miRNAs in the post-operative setting¹²⁰. There are a variety of non-malignant causes for dysregulated circulating miRNAs that can have an effect on post-operative patients such as tissue injury and sepsis^{175,176}. These patients had their post-operative miRNA profile re-examined at mean day 10 post-operatively. This could be too soon in the post-operative setting to assess the miRNA profile as the patients could still be undergoing an inflammatory and healing response to the surgery. Also, these patients had their urethral catheters removed at mean day 7 post-operative, this could also account for the persistent dysregulated levels of *let7a*, *miR-145* and *miR-155*. It would be interesting to evaluate the miRNA profile across a variety of post-operative time points to assess the miRNA response and to correlate this with inflammatory markers such as ESR and CRP.

Zhang *et al* identified that *miR-21* was upregulated in patients with hormone refractory PCa and *miR-21* levels were reduced in patients who responded to docetaxel chemotherapy as compared to those hormone refractory patients who were resistant to chemotherapy¹²². This highlights the potential use for miRNAs as biomarkers for treatment response and also as prognostic markers.

Conclusion

The detection of circulating miRNAs allows for the discrimination of PCa patients from patients with benign disease. Given that miRNAs can return to a normal level after surgery, this identifies the potential that circulating miRNAs may serve as biomarkers for detection of PCa and monitoring response to treatment. Our results suggest a solid basis for future blood-based miRNA studies. It would be of interest to evaluate the diagnostic potential of miRNA in large prospective studies.

Chapter 6

Discussion

6.1 Introduction

Since the discovery of miRNAs in 1993, they have emerged as a promising new class of disease biomarkers. MiRNAs may have further potential as the next generation of therapeutics. MiRNAs are small, non-coding RNA molecules that were previously considered to be of little clinical significance. MiRNAs have been implicated in the pathogenesis of all human cancers, including PCa. The ability to detect these molecules in the circulation of men with PCa identifies miRNAs as future potential clinical biomarkers for the detection of disease, predicting response to treatment and for the detection of recurrence.

6.2 Summary and implications of results

This work aimed to investigate the expression and dysregulation of circulating miRNAs in PCa and thus their potential as novel disease biomarkers.

6.2.1 Identification of miRNAs as endogenous controls for RQ-PCR

Currently, the gold standard for determining the expression levels of miRNAs is RQ-PCR. For this information to be both accurate and reliable, correct normalisation must be performed. There are few studies in the literature that have investigated and validated suitable endogenous controls, in particular the different circulatory media of whole-blood, serum and plasma. It is imperative that there is a standardised approach for the isolation, detection and the quantification of miRNA in the circulation.

The study concluded in chapter 3 that suitable endogenous controls were identified for use for the entirety of this work. *MiR-16* and *miR-425* in combination were identified as the most stably expressed miRNAs and were the ideal choice as endogenous controls for the normalisation of miRNA RQ-PCR in the circulation of men with PCa.

6.2.2 Evaluation of circulating miRNAs in prostate cancer

PCa is a malignancy that warrants a new, sensitive, specific biomarker given the limitations of PSA as a diagnostic marker. MiRNAs are tissue specific and can be easily detected and quantified in the circulation, making them an ideal candidate biomarker. This work aimed to evaluate the role of individual miRNAs as potential biomarkers and to assess if using these miRNAs in combination, there was a miRNA expression profile that was unique to PCa.

Of 102 men who were referred to the RAPAC by their GP for the investigation of an elevated PSA or an abnormal DRE, following a TRUS biopsy, 75 were subsequently diagnosed with PCa. 12 candidate miRNAs were selected and quantified in the circulation of these men to assess if there were dysregulated miRNAs in the circulation of men with PCa. Of these 12 candidate miRNAs, four were found to be dysregulated (*let7a*, *miR-141*, *-145* and *-155*). *Let7a* had a sensitivity of 64% and a positive predictive value (PPV) of 70%, however it had low specificity. *MiR-141* had a sensitivity of 76% and a positive predictive value (PPV) of 73%, but again also lacked sufficient specificity. Using the 4 miRNAs in combination improved upon the specificity and the sensitivity, with a PPV of 80% and an AUC of 0.789.

6.2.3 The evaluation of miRNAs post radical prostatectomy

The expression profile of the four dysregulated miRNAs were then investigated in the post-operative setting in 20 men who underwent surgical excision of their prostate. Interestingly, only a single miRNA (*miR-141*) returned to a similar level to that of the benign group at day 10 post-operatively. This suggests that circulating miRNAs can possibly be used as a biomarker for post-operative monitoring of cancer patients undergoing surgical excision of tumours.

6.3 Potential clinical applications

Given the stability of miRNAs in the circulation and the ease of their detection, miRNAs are an ideal biomarker for prostate cancer in clinical practice. The use of miRNAs in combination may result in the identification of miRNA expression profiles, or signatures, that are unique to a variety of malignancies. In the future, unique expression profiles of miRNA signatures may help to identify the exact treatment required by patients diagnosed with PCa. For example, identifying whether a man with PCa would have a more favourable outcome if he were to undergo surgery rather than radiotherapy or brachytherapy.

This is an interesting concept as many men diagnosed with prostate cancer are deemed to have all treatment options open to them and a unique miRNA signature may help to tailor patient care on an individual level. Men with metastatic PCa who were undergoing chemotherapy for HRPC, elevated levels of miR-21 were identified in those men who were resistant to chemotherapy¹²².

6.4 Future work

There are many areas in miRNA research that warrant further investigation based on the finding of this work. From a panel of 12 miRNAs, a unique expression profile of four of these miRNAs was identified in men with PCa. Future work would entail a microarray of all miRNAs in the circulation to investigate if there are more stably expressed miRNAs which may be a more suitable endogenous control. Also, there may be more miRNAs that may be more predictive of the presence of PCa. With a larger cohort and long-term follow up of patients who undergo treatment, different miRNA expression profiles may emerge in time with a miRNA signature for prognosis.

This work also concentrated on a high risk group of patients. These were patients referred to the RAPAC with an elevated PSA. There may be a different miRNA expression profile that can better differentiate those with PCa from men with benign prostatic disease or even healthy controls in the community. Further prospective evaluation of circulating miRNAs in PCa is required to validate these findings and to further explore their potential as a novel biomarker for PCa.

It is still not clear how these molecules are released into the circulation. Future work will be required to identify the exact mechanism of miRNA release into the circulation as these pathways may also be potential therapeutic targets. Further work is warranted to investigate if whole-blood, serum or plasma is the optimum medium for quantifying miRNAs.

Another future avenue to explore is urinary miRNAs. The prostate gland secretes seminal fluid into the urinary tract. This makes urine an ideal medium to investigate for a miRNA expression profile unique to PCa. These miRNAs may not be released into the circulation and may lead to an alternative, superior test for the diagnosis of PCa.

The specific functions and target pathways of many miRNAs are still unknown in carcinogenesis. Given that miRNAs play a pivotal role in a variety of biological processes, the therapeutic application of miRNAs face the challenge of ensuring safety and avoiding any unwanted side-effects. Further studies are required to validate all the existing findings in regards to miRNAs prior to any application in clinical practice.

Future directions related to this projected include the surveillance of men who have had a benign TRUS biopsy. These men may subsequently develop PCa in the future, and there may be miRNAs which would identify those men who are at risk of developing PCa in the future and who should subsequently be under close surveillance.

Another avenue to explore is to identify what treatments these men with PCa underwent. There may be a miRNA signature that would help to identify which treatment modality would best serve the individual patient. Also, biochemical recurrence is an issue in PCa. There may be miRNAs that are superior to PSA in detecting patients with disease relapse.

6.5 Conclusion

In summary, these results provide evidence that circulating miRNAs are stable, tissue specific and are easily quantified. This thesis identifies four dysregulated miRNAs (*let7a*, *miR-141*, *-145* and *-155*) in the circulation of men with PCa. Using these four miRNAs in combination, to create a unique expression profile of miRNAs, identified a sensitive, specific biomarker for the diagnosis of PCa. MiRNAs dysregulated in the circulation of men with PCa are a potential biomarker for the diagnosis, prognosis and for monitoring response to therapy of PCa.

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Appendix 1 Department of Surgery BioBank Forms

1.1 Division of Surgery Patient Consent Form



GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

Patient Information

Introduction

We would like to invite you to participate in a clinical research initiative at Galway University Hospitals to establish a BioBank. The purpose of the BioBank is to set up a resource that can support a diverse range of research programmes intended to improve the prevention, diagnosis and treatment of cancer. You are under no obligation to take part and if, having read the information below, you would prefer not to participate, we will accept your decision without question.

Although major advances have been made in the management of cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research. Galway University Hospitals are actively involved in research that aims to identify markers that will predict how a cancer develops, progresses and responds to a variety of treatments. This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the diagnosis, treatment and outcome for those who have cancer. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

Your Involvement

If you volunteer to participate in our BioBank, there will be no additional risks to you outside those of your standard investigation and treatment. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Procedure

We invite all patients who are undergoing treatment and/or investigation to participate. All samples for research will be taken at the time you are attending the hospital for routine diagnostic tests.

(i) Tissue Samples

By participating, you give us consent to retain small pieces of your tissue obtained at the time of surgery. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland. This will not affect your diagnosis in any way.

(ii) Blood Samples

By participating, you give us consent to take an extra blood sample (equivalent of 4 teaspoonfuls) at the same time that your blood is being taken for routine tests. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland.

(iii) Clinical Information

By participating, you give us consent to store information relating to your diagnosis and treatment on a database. This information is only accessed by personnel directly involved in research within the Surgical Research Unit.

Further Information

If you would like further information about our BioBank, your participation and your rights, please contact the Surgical Research Unit (Tel: 091 524390).

If you would like further information about research projects that may be conducted, please contact your Consultant.

Thank you in anticipation of your assistance. Please read and sign the Consent section.

I have read the attached information sheet on the above project, dated _____

Please Initial Box

Appendix 1.1: Continued – Consent form Page 2

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. I understand I may withdraw from the study at any time.

(Name of sponsor :)

PARTICIPANT'S NAME:

CONTACT DETAILS:

PARTICIPANT'S SIGNATURE:

DATE:

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:

NAME OF CONSENTER, PARENT, OR GUARDIAN:

SIGNATURE:

RELATION TO PARTICIPANT:

DECLARATION OF INVESTIGATOR'S RESPONSIBILITY

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME:










SIGNATURE:

DATE:










(Keep the original of this form in the investigator's file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).

1.2 Discipline of Surgery Biobank Specimen Request Form

Affix patient label here ↓ **BioBank Specimen Form** 19/10/2009 EH
 Form must be completed & accompany specimens to Dept of Surgery, Clinical Science Ins. (Ext. 4202)

Surname:	First Name:	Referral Reason (Please circle): Breast - Colorectal - Prostate - Skin – Lung - Other _____																																																					
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Appendix 2 Reagents and Equipment

Company	Reagents & Equipments	Catalogue / Part / Model Number
Agilent Technologies Inc. 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA.	Agilent 2100 Bioanalyzer RNA 6000 Nano Assay Kit Guide RNA 6000 Nano LabChip Series II Assay Small RNA Assay Small RNA Kit Guide	- G2941-90126 5065-4476 5067-1548 G2938-90093
Applied Biosystems Lingley House, 120 Birchwood Boulevard, Warrington, WA3 7QH, UK.	High Capacity cDNA Reverse Transcription Kit Megaplex™ PreAmp Primers Megaplex™ Reverse Transcription Human Pool A MicroAmp Optical Adhesive Film TaqMan® Low Density Array TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG TLDA Universal PCR Master Mix, No AmpErase® UNG	4374967 4399233 4399966 4313663 4398965 4352042 4324018
Eppendorf Ltd. Endurance House, Vision Park, Histon, Cambridge, CB24 9ZR, UK.	Eppendorf 5417C Micro Centrifuge	-
Greiner Bio-One Inc. 4238 Capital Drive, Monroe, NC 28110, USA.	Vacurette EDTA K3E blood bottles	-
Invitrogen Corp. 5791 Van Allen Way, PO Box 6482, Carlsbad, CA 92008, USA.	dNTPs Random Primers RNaseOUT SuperScript™ III	18427-088 48190-011 10777-019 18080-085
Kinematica AG Inc. Luzernerstrasse 147a, CH-6014 Lucerne, Switzerland.	Polytron Bench-top Homogeniser	PT1600E
Milestone Medical Technologies Inc. 6475 Technology Avenue, Suite F, Kalamazoo, MI 49009, USA.	MicroMed T/T Mega Histoprocessing Labstation	-
Molecular Research Center Inc. 5645 Montgomery Road, Cincinnati, OH 45212, USA.	Tri Reagent® BD-RNA Isolation for Blood Derivative Protocol	TB 126
MWG Biotech Anzingerstrasse 7a, 85560 Ebersberg,	MiRNA-specific Primers (<i>let-7a</i> , <i>miR-10b</i> , <i>-16</i> , <i>-21</i> , <i>-34a</i> , <i>-125b</i> , <i>-141</i> , <i>-143</i> , <i>-145</i> , <i>-155</i> , <i>-375</i> and <i>miR-425</i>)	-

Germany.		
Nanodrop Technologies Inc. 3411 Silverside Road, Bancroft Building, Wilmington, DE 19810, USA.	Nanodrop 1000® Spectrophotometer	-
Qiagen Ltd. QIAGEN House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK.	Buffer RDD, DNase I Qiazol RNAlater® RNeasy® MinElute Cleanup Kit RNeasy® Mini Kit	79254 79306 76106 74204 74106
Sarstedt AG & Co. Sarstedtstraße, Postfach 1220, 51582 Nümbrecht, Germany.	Sarstedt tubes	-
Slee Technik Lise-Meitner-Strasse 1, 55129 Mainz, Germany.	Slee Cut 4055 Microtome	-

P27 Circulating microRNAs: Novel biomarkers for prostate cancer in the screening setting

Kelly BD^{1,2}, Miller N¹, Sweeney KJ¹, Durkan GC², Rogers E², Walsh K², Kerin MJ¹
¹Department of Surgery, Clinical Science Institute, National University of Ireland, Ireland
²Department of Urology, Galway University Hospital, Ireland

Introduction

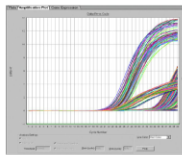
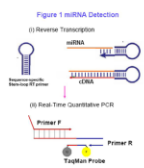
MiRNAs are small non-coding endogenous RNA molecules that vary in length from 18-25 nucleotides. There are numerous dysregulated miRNAs that are implicated in the pathogenesis of cancer. MiRNAs play pivotal roles in the expression of up to 30% of human genes (1) MiRNAs regulate gene expression at the transcriptional and post-transcriptional level. It is hypothesised that a single miRNA can have as many as 200 target sites and that a single mRNA may have the ability for different miRNAs to bind at its sites (2). Dysregulation of miRNA has been associated with the development of cancer and approximately up to 50% of miRNA genes are located in cancer-related genomic locations (3). The discovery of miRNAs in the blood of patients with a variety of malignancies makes them an ideal, novel biomarker for the diagnosis of prostate cancer.

Objective

The aim of this project was to determine if a panel of circulating miRNAs can distinguish patients with prostate cancer in a high risk group.

Methods

RNA was extracted from whole-blood samples of 88 patients, 55 with prostate cancer and 33 benign samples from patients attending a prostate cancer assessment clinic. Reverse-transcription was performed using stem-loop primers and expression levels of 12 candidate miRNAs were determined using real-time quantitative PCR. MiRNA expression levels were then correlated with clinicopathological data.



	Benign	Cancer	Histology	Numbers
Numbers	33	55	Benign	26
Age:			PIN	7
Mean	68	69	3+3	20
Range	(52 – 84)	(49 – 83)	3+4	12
years		years	4+3	11
PSA:			4+4	5
Mean	11.72	12.33	4+5	7
Range	(3.9 – 20.6)	(4.6 – 40.5)		
µg/L		µg/L		

Results

Circulating miRNAs were detected and quantified in all subjects. The analysis of miRNA mean expression levels revealed that circulating levels of the tumour-suppressor *let-7a* ($p=0.02$) along with the oncogenic *miR-141* ($p=0.01$) could differentiate prostate cancer patients from patients with benign disease (Figure 3). The sensitivities of *miR-141* and *let-7a* were 83.3% and 86.5%, the specificities were calculated at 45.5% and 30.3% respectively. Using logistical regression analysis, 4 miRNAs can be used in combination to reveal a unique expression profile for patients with prostate cancer (Figure 4), with an AUC of 0.814.

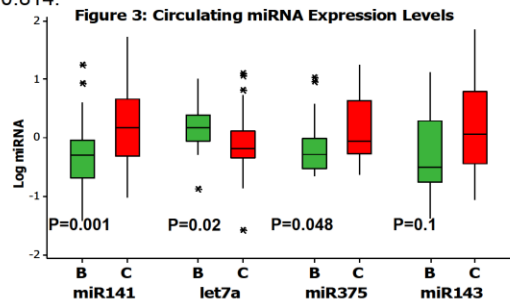
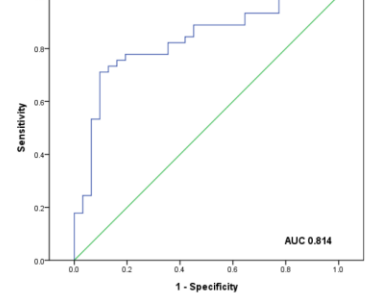


Figure 4: *let7a* & *miR-141* & *miR-143* & *miR-375* in Combination



Conclusion

These findings identify significant differences in expression levels of oncogenic and tumour-suppressor miRNAs in the bloodstream of prostate cancer patients. This highlights their potential use as novel biomarkers of prostate cancer as an adjunct to PSA.

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P32 Circulating miR-141 as a diagnostic biomarker for prostate cancer and for monitoring response to treatment

Kelly BD^{1,2}, Miller N¹, Sweeney KJ¹, Durkan GC², Rogers E², Walsh K², Kerin MJ¹

¹Department of Surgery, Clinical Science Institute, National University of Ireland, Ireland

²Department of Urology, Galway University Hospital, Ireland



Introduction

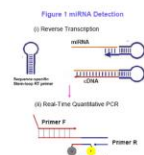
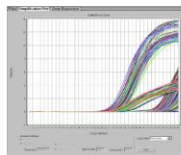
MiRNAs are small non-coding endogenous RNA molecules that vary in length from 18-25 nucleotides. There are numerous dysregulated miRNAs that are implicated in the pathogenesis of cancer. MiRNAs play pivotal roles in the expression of up to 30% of human genes. MiRNAs regulate gene expression at the transcriptional and post-transcriptional level. It is hypothesised that a single miRNA can have as many as 200 target sites and that a single mRNA may have the ability for different miRNAs to bind at its sites. Dysregulation of miRNA has been associated with the development of cancer and approximately up to 50% of miRNA genes are located in cancer-related genomic locations. The discovery of miRNAs in the blood of patients with a variety of malignancies makes them an ideal, novel biomarker for the diagnosis of prostate cancer.

Objective

The aim of this project was to determine if a panel of circulating miRNAs can distinguish patients with prostate cancer in a high risk group, and to ascertain if the dysregulated miRNAs returned to normal expression levels after a radical prostatectomy.

Methods

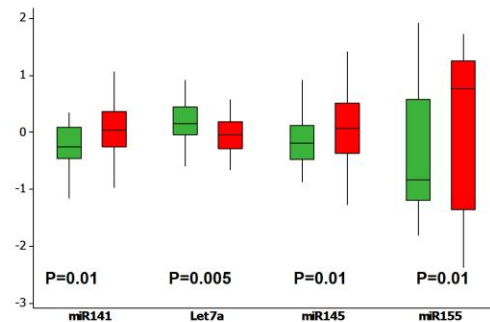
RNA was extracted from whole-blood samples from 102 patients, 75 with prostate cancer and 27 benign samples from patients attending a prostate cancer assessment clinic. Samples were reverse-transcribed using stem-loop primers and expression levels of 12 candidate miRNAs were determined using real-time quantitative PCR. MiRNA expression levels were then correlated with clinicopathological data. Of 20 men that underwent a radical prostatectomy, blood was taken pre operatively and post operatively at mean day 10 (range 5-21).



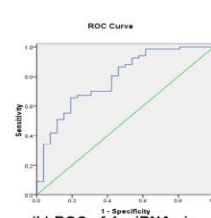
	Benign	Cancer	Histology	Numbers (102)
Numbers	27	75	Benign	27
Age:				
Mean	65 years	64 years	3+3	28
Range	(48 – 80 years)	(48 – 85 years)	3+4	20
PSA:			4+3	14
Mean	11.6 µg/L	11.55 µg/L	4+4	6
Range	(1 – 77)	(1.42 – 52.24)	4+5	7

Results

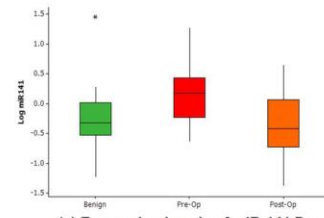
Circulating miRNAs were detected and quantified in all subjects. The analysis of miRNA mean expression levels revealed that circulating levels of the tumour-suppressor let-7a (p=0.005, AUC 0.678) along with the oncogenic miR-141 (p=0.01 AUC 0.655) could differentiate prostate cancer patients from patients with benign disease. Using logistics regression analysis, 4 miRs can be used in combination to reveal a unique expression profile for patients with prostate cancer with an AUC of 0.783. Of 20 patients that underwent a radical prostatectomy, the expression levels of miR-141 returned to normal at day 10 post operatively.



(a) Boxplots of Dysregulated miRNA



(b) ROC of 4 miRNAs in Combination AUC 0.783



(c) Expression Levels of miR-141 Pre and Post operatively

Conclusion

Our findings identify a unique expression profile of miRNAs detectable in the blood of prostate cancer patients. This identifies their use as a diagnostic biomarker for prostate cancer and for monitoring response to treatment.

Appendix 5 A review of expression profiling of circulating microRNAs in men with prostate cancer.

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A review of expression profiling of circulating microRNAs in men with prostate cancer

Brian D. Kelly^{*,†}, Nicola Miller^{*}, Nuala A. Healy^{*}, Killian Walsh[†] and Michael J. Kerin^{*}

**Discipline of Surgery, Clinical Science Institute, National University of Ireland Galway, and †Department of Urology, Galway University Hospital, Galway, Ireland*

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- MicroRNAs (miRNAs) are small non-coding RNAs that are involved in gene regulation and expression. These molecules have been implicated in the pathogenesis of several malignancies.
- MiRNAs have the potential to be both a tissue- and blood-based biomarker for cancer diagnosis, classification, prognosis and response to treatment.
- The levels of dysregulated miRNAs in the circulation of individuals make miRNAs an ideal biomarker for the detection of prostate cancer.
- The present study provides a comprehensive review of the expression

What's known on the subject? and What does the study add?

MicroRNAs are small, single-stranded RNA molecules that are stable in the circulation. These molecules have been identified as potential circulating biomarkers for several malignancies.

In this review we provide a comprehensive overview of the publications that have identified dysregulated microRNAs in the circulation of men with prostate cancer.

profiling of miRNAs in the circulation of men with prostate cancer.

- Further research is necessary to identify if circulating miRNAs have the potential to be a biomarker for prostate cancer in clinical practice.

KEYWORDS

miRNA, circulation, prostate cancer

OVERVIEW OF MICRORNAS (MIRNAS)

MiRNAs are small non-coding endogenous RNA molecules that vary in length from 18 to 25 nucleotides. There are numerous dysregulated miRNAs that are implicated in the pathogenesis of cancer. MiRNAs play a role in the expression of up to 30% of human genes [1]. MiRNAs can be up- or down-regulated. The up-regulation of oncogenic miRNAs (also known as oncomirs) and the down-regulation of tumour suppressor miRNAs are demonstrated in many malignancies [2–5]. MiRNAs are located within the introns and exons of protein-coding genes and they are also located in the intergenic region. Transcription of the intronic and exonic miRNAs appears to occur in parallel with transcription of a host gene. However, transcription of miRNAs located at the intergenic regions appear to be transcribed independently [6].

MiRNAs regulate gene expression at both the transcriptional and post-transcriptional

level. A single miRNA can have several mRNA targets that are involved in the oncogenic process. However, a single mRNA can be regulated by many different miRNAs that bind to its sites [7]. Dysregulation of miRNAs have been associated with the development of cancer and not surprisingly up to 50% of miRNA genes are located in cancer-related genomic locations [8].

The biosynthesis of miRNA is a complicated process. The transcription of a miRNA gene begins with initiation of transcription by RNA Polymerase II. This results in the formation of a large molecule, pri-miRNA [9]. The enzyme Drosha and the nuclear protein DGCR8 then cleaves a miRNA precursor (pre-miRNA), which is exported from the nucleus to the cytoplasm by Exportin5 [10,11]. Drosha and DGCR8 form a large complex, which is known as the microprocessor complex [12]. In the cytoplasm the pre-miRNA is then cleaved by Dicer and an RNA transactivator binding protein into a small dsRNA duplex (miRNA:miRNA*). This duplex molecule is

composed of a mature miRNA and a complementary strand (miRNA*). The mature miRNA is subsequently incorporated in a further complex, named RNA-induced silencing complex (RISC). The miRNA* strand is then usually eliminated by cleavage. The mature miRNA molecule is then free to interact with various mRNA targets, resulting in either cleavage or inhibiting protein synthesis [13].

EXPRESSION PROFILING OF CIRCULATING MIRNA IN PATIENTS WITH PROSTATE CANCER

MiRNAs are an ideal molecule for a blood-based biomarker for the detection of cancer, as they are dysregulated in carcinogenesis and are highly stable in both tissue and in blood samples [5,14–16]. Various studies have documented the differential expression of miRNAs in the circulation of patients with cancer when compared with healthy controls, making miRNA an ideal non-invasive biomarker

[17–19]. Mitchell *et al.* [14] also confirmed that tumour-derived miRNAs are present in the circulation (present in both serum and plasma) at sufficient levels to be used as a suitable biomarker. MiRNAs are resistant to endogenous ribonuclease (RNase) activity as well as variations in temperature and pH [14,20].

The exact mechanisms whereby miRNAs are released into the circulation have been debated. One potential theory is that miRNAs simply leak into the circulation via tissue degradation in a passive, energy-independent process. However, there is increasing evidence that miRNAs are actively secreted into the circulation within exosomes and microvesicles and perhaps may even be selected to be transferred to distant cells [19,21,22]. MiRNAs are also present within Ago complexes in the circulation. MiRNAs in the circulation contained within microvesicles or within Ago complexes may originate from different cell types and may actually reflect a tissue-specific miRNA expression profile [23].

There has been wide variability in results when miRNAs from patients' serum has been used to differentiate between those with or without prostate cancer. Mitchell *et al.* [14] analysed serum samples from a cohort of 50 individuals, 25 patients with metastatic prostate cancer and 25 male, age-matched controls. This revealed increased expression of miR-100, -125b, -141, -143 and -296 in the serum of the metastatic prostate cancer group. MiR-141 had the greatest differential expression in the prostate cancer group in comparison with the control group. In fact, it had a 46-fold overexpression. Serum levels of miR-141 could detect individuals with prostate cancer with a high accuracy, with 100% specificity and 60% sensitivity with an area under the curve (AUC) of 0.907. This identified a blood-based PCR analytical tool for the detection of prostate cancer. Brase *et al.* [24] later identified miR-141 to be upregulated and could be used to differentiate between metastatic and localised prostate cancer. However, Mahn *et al.* [25] encountered difficulties with detecting miR-141 in the circulation, this is surprising given that Mitchell *et al.* [14] and Brase *et al.* [24] found it to be expressed early in prostate cancer sera.

However, Lodes *et al.* [26] identified a unique miRNA signature for prostate cancer based on an extraction technique from serum samples. In an analysis comprising of 13 samples (five from patients with prostate cancer, eight from age-matched controls) 15 upregulated miRNAs were identified. Brase *et al.* [24] investigated the expression profile of miRNAs in the progression of prostate cancer from organ-confined disease to metastatic disease. After an initial screening study of 21 patients (14 with localised prostate cancer, seven with metastatic prostate cancer), subsequent validation studies were performed with serum samples to investigate the expression profile of five dysregulated miRNAs. MiR-375 was useful to identify those patients with metastatic disease but also a positive lymph node status. MiR-141 was also identified as upregulated in the serum of patients with higher grade tumours.

MiR-21 has previously been identified as an oncomir, being upregulated in several cancers. A study by Zhang *et al.* [27] into the expression levels of miR-21 and disease progression reported many interesting results. The serum of 56 patients were included in that study; 20 with localised prostate cancer, 20 with androgen-dependent prostate cancer, 10 with hormone-refractory prostate cancer (HRPC) and six with BPH. Patients with HRPC expressed higher levels of miR-21. Of the patients with HRPC who subsequently received chemotherapy, those who were resistant to chemotherapy had higher levels of miR-21 than those who were sensitive to chemotherapy, but there was no validation of these results in a separate group. Although numbers in the study were small, this identifies the potential of miRNAs as not only a biomarker for diagnosis, but also for disease progression and for response to treatment.

Yaman Agaoglu *et al.* [28] investigated the expression profile of three miRNAs; miR-21, -141 and -221 in plasma. In all, 71 patients were included in the study, 51 with prostate cancer and 20 healthy controls. There was a significant upregulation of miR-21 and miR-221. Levels of all three miRNAs were upregulated in the patients with metastatic disease as compared with those with localised prostate cancer, again confirming that miRNAs have the potential to be used as biomarkers for diagnosis and disease

progression. Zheng *et al.* [29] also identified that miR-221 was upregulated in patients with prostate cancer. In addition, miR-221 was also found to be significantly elevated in patients with androgen-dependent prostate cancer.

Moltzahn *et al.* [30] also identified a miRNA signature that could be used to diagnose prostate cancer and also correlate with disease progression in the serum of 48 patients, 36 with prostate cancer and 12 controls. Five miRNAs were identified to be upregulated and four down-regulated. Three miRNAs, miR-93, -106a and -24 were significantly dysregulated in patients with metastatic disease again identifying that there is a miRNA expression profile unique to both localised prostate cancer and also to metastatic prostate cancer.

Heneghan *et al.* [31] analysed the expression profile of patients with various malignancies including prostate, colon, breast, renal and melanoma. The study showed a significant down-regulation of the tumour suppressors miR-145 and -155 in prostate cancer. Whilst the down-regulation of these two miRNAs were also seen in several of the malignancies included in the study, a significant upregulation of let-7a in prostate and breast cancer, but an upregulation of miR-195 was exclusive to breast cancer. Using three miRNAs in combination; miR-195, -155 and let-7a could be used a biomarker for breast cancer with a sensitivity of 94%. This process of using a combination of miRNAs to identify a miRNA signature unique to a particular cancer has the potential to be used as a blood-based biomarker across other malignancies.

Mahn *et al.* [25] analysed the expression of four miRNAs in 37 patients with localised prostate cancer, eight with metastatic prostate cancer, 18 with BPH and 20 healthy controls. Three of these four miRNAs were significantly upregulated in patients with prostate cancer compared with patients with BPH, miR-26a, -195 and let-7i. MiR-26a could differentiate prostate cancer from BPH with an AUC of 0.703, yet PSA could differentiate between the two clinical conditions with a superior AUC of 0.834. However, in patients with an elevated PSA level, miR-26b could differentiate those with cancer from those with BPH with an AUC of 0.918, this included 37 patients with prostate cancer and seven patients with

TABLE 1 Studies investigating the expression profiles of circulation miRNAs in prostate cancer

Upregulated	Down-regulated	Study no. of patients	Study design	Validation	Blood type	Reference
100, 125b, 141, 143, 296	-	50	25 metastatic PCa vs 25 controls	Yes	Serum	Mitchell et al. [14] 2008
16, 92a, 103, 107, 197, 34b, 328, 485-3p, 486-5p, 92b, 574-3p, 636, 640, 766, 885-5p	-	13	5 PCa vs 8 controls	Yes	Serum	Lodes et al. [26] 2009
9*, 141, 200b, 375, 516a	-	21	7 metastatic PCa vs 14 PCa	Yes	Serum	Brase et al. [24] 2011
Let7a	145, 155	83	20 PCa vs 63 controls	No	Whole blood	Heneghan et al. [31] 2010
21	-	56	50 PCa vs 6 BPH	No	Serum	Zhang et al. [27] 2011
21, 221	-	71	51 PCa vs 20 controls	No	Plasma	Yaman Agaoglu et al. [28] 2011
93, 106a, 874, 1207-5p, 1274a	24, 26b, 30c, 223	48	36 PCa vs 12 controls	No	Serum	Molzahn et al. [30] 2011
221	-	43	28 PCa vs 20 controls	No	Plasma	Zheng et al. [29] 2011
26a, 195, let7f	-	83	45 PCa vs 38 controls	No	Serum	Mahn et al. [25] 2011
141, 298, 346, 375	-	50	25 metastatic PCa vs 25 controls	Yes	Serum	Selth et al. [38] 2011

BPH. These three miRNAs along with the fourth miRNA, miR-32, could be used in combination to further enhance their diagnostic potential with an AUC of 0.758. Of 10 patients who underwent a radical prostatectomy, the expression levels of miR-26a and miR-195 were significantly reduced at postoperative day 7, returning to normal expression levels. This further highlights the diagnostic potential of the overall expression profile of many oncomirs and tumour-suppressor miRNAs. This is not the first time that expression levels of miRNAs have returned to normal for patients undergoing oncological surgery. This has previously been described in breast cancer and patients with colorectal cancer [18,32].

Whilst miRNAs have enormous potential as biomarkers for prostate cancer, work to elucidate their role as systemic markers of this disease remains in its infancy. To date there are a limited number of studies that have investigated circulating levels of miRNAs in men with prostate cancer, with study numbers varying from 13 to 83 patients. Studies range from comparing localised prostate cancer to controls, and some comparing patients with metastatic prostate cancer with controls, with only four studies having a validation cohort.

Currently RNA is being extracted from a various circulating media, including whole blood, serum and plasma using various

different extraction techniques. There is no established consensus on what is the optimum media from which to isolate RNA. Pritchard et al. [33] have recently identified that a variety of circulating miRNAs are highly expressed in one or more blood cell types. They suggested that acceptable ranges for blood cell counts should be established for miRNAs that are vulnerable to blood cell effects. While most articles to date have focused on free or exosomal miRNAs extracted from either serum or plasma, Heneghan et al. [34] have highlighted an RNA extraction technique from whole blood using a trizol-based extraction technique that results in a higher yield of miRNAs than serum or plasma samples. The validity of this process was shown by the fact that there was no significant difference in the white cell count, haemoglobin or haematocrit levels between the cancer and control groups of patients in this article [32,34]. With this lack of consistency about starting material and methodology across results they must be viewed with caution.

Urinary miRNAs are a promising tumour marker in urothelial cancer and miRNAs have also been identified in other bodily fluids, e.g. peritoneal fluid and saliva. This highlights the potential that these molecules have as potential biomarkers across a variety of media [35-37]. However, this is a relatively novel discovery and many further studies are required in this area to elucidate

the true potential of urinary miRNAs to act as biomarkers for prostate cancer. Table 1 summarises studies investigating the expression profiles of circulation miRNAs in prostate cancer [14,24-28,30,31,38].

CONCLUSION

MiRNAs dysregulated in the circulation of men with prostate cancer are a potential biomarker for the diagnosis, prognosis and for monitoring response to therapy of prostate cancer. However, given the few studies, the lack of validation and the disparity of results, much larger scale clinical studies are necessary to determine if circulating miRNAs have the potential to act as biomarkers for prostate cancer in clinical practice.

CONFLICT OF INTEREST

None declared.

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Correspondence: Brian D. Kelly, Department of Surgery, National University of Ireland Galway, Clinical Science Institute, Newcastle Road, Galway, Ireland.
e-mail: drbriankelly@hotmail.com

Abbreviations: miRNA, microRNA; miR, mature miRNA; AUC, area under the curve; HRPC, hormone-refractory prostate cancer.