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Systematic analysis of prognostic miRNAs and isomiRs in prostate cancer

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Declaration

I declare that the work presented in this thesis is my own, unless otherwise stated. All other work is appropriately referenced and acknowledged to my best knowledge.

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Abstract

There are no reliable prognostic indicators to distinguish between indolent and aggressive prostate cancer (PCa). Consequently, 42–66% of patients with indolent PCa are over-treated. Additionally, 15-45% of patients treated with radical prostatectomy (RP) experience biochemical recurrence (BCR) within 5-years, highlighting an urgent need for reliable prognostic biomarkers.

MiRNAs (miRs) and isomiRs (miR isoforms) are non-coding regulatory RNAs that hold ideal biomarker properties such as detection in circulation, tissue and tumour specific expression profiles, and correlation with PCa development and progression. I hypothesised that miR species (canonical miRs and isomiRs) can be utilised as biomarkers for reliable PCa prognostication.

A novel database of prognostic PCa miRs was built by performing a systematic review of relevant publications in the PubMed database. MiRs significantly associated with BCR were also identified following a meta-analysis of six datasets. MiR-148a-3p and miR-582-4p were identified as potential biomarker candidates as they were consistently prognostic in both the review and meta-analysis.

The ability of miR species to predict BCR post-RP was tested with elastic net regularisation models using The Cancer Genome Atlas PCa dataset (recurrent=61, non-recurrent=330). Models based on a combination of isomiRs and clinical markers achieved marginally greater predictive power (AUC=0.795) than the model solely based on clinical markers (AUC=0.748), demonstrating that isomiRs could contribute additional prognostic value to the clinical markers currently used.

The mechanism by which miR-27a-3p, a PCa-specific putative oncomiR, promotes tumour growth was investigated using RNA-seq data from LNCaP tumour xenograft models treated with a miR-27a-3p inhibitor (n=3) and control (n=3). 11 significantly dysregulated genes involved in apoptosis and

oncogenic signalling were identified as likely mir-27a-3p targets.

This study has not only furthered our understanding of the importance of miRs in PCa, but also identified potential prognostic miR biomarkers and showed the inclusion of miR species increases the utility of current markers.

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List of Acronyms

ADAR Adenosine deaminase acting on RNA

ADT Androgen deprivation therapy

AGO2 Argonaute 2

ANOVA Analysis of variance

AR Androgen Receptor

ARV Androgen Receptor splice variant

ASO-NTC Antisense oligonucleotide of non-targeting control

ASO-27a Antisense oligonucleotide or miR-27a-3p

AUC Area under the receiver operating characteristic curve

BCR Biochemical recurrence

BMI Body mass index

BPFS Biochemical progression/ recurrence-free survival

BPH Benign prostatic hyperplasia

CCP Cell cycle progression

CFFS Clinical failure-free survival

cfDNA Cell-free DNA

CI Confidence interval

c-miRs Circulating miRs

CNV Copy number variation

Cox PH Cox proportional hazards

cpm counts per million

CRPC Castration-resistant prostate cancer

CRPC FS Castration resistant prostate cancer-free survival

CSS Cancer-specific survival

CTC Circulating tumour cell

CV Clinical variable

DFS Disease-free survival

DSS Disease-specific survival

DHT 5α-dihydrotestosterone

DRE Digital rectal examination

EAU European Association of Urology

ERSPC European Randomized Study of Screening for Prostate Cancer

EN Elastic Net

FEM Fixed-effects model

GC Genomic classifier

GDC Genomic Data Commons

GEO Gene Expression Omnibus

GPS Genomic prostate score

HIF-1 Hypoxia inducible factor-1

HR Hazard ratio

ISUP International Society of Urological Pathology

KLK Kallikrein

KM Kaplan-Meier

kb kilobase

KW Kruskal-Wallis

LHRH Luteinizing hormone-releasing hormone

MFS Metastasis-free survival

miR microRNA

MP-MRI multi-parametric magnetic resonance imaging

MRE miRNA responding element

NICE National Institute for Healthcare and Excellence

nt nucleotide

ORF Open reading frame

OLS Ordinary least squares

OS Overall survival

PCA Principal component analysis

PCa Prostate Cancer

PCR Polymerase chain reaction

PFS Progression-free survival

PIP2 Phosphatidylinositol-4,5-bisphosphate

PIP3 Phosphatidylinositol (3,4,5)-trisphosphate

PI3K Phosphoinositide 3-kinase

PLCO trial Prostate, Lung, Colorectal and Ovarian trial

pre-miR precursor miRNA

PS Percentage survival

PSA Prostate-specific antigen

RP Radical prostatectomy

REM Random-effects model

REMARK Reporting recommendations for tumour marker prognostic studies

RFS recurrence/ relapse free survival

RISC RNA-induced silencing complex

SNP Single-nucleotide polymorphism

TCGA The Cancer Genome Atlas

TCGA-PRAD The Cancer Genome Atlas - Prostate Adenocarcinoma

TMM Trimmed mean of M-values

TNM Tumour, Node, Metastasis

TRBP Transactivating response RNA-binding protein

TRUS Transrectal ultrasound

UTR Untranslated region

$\mathbf{X^2}$ Chi-squared

Chapter 1

Introduction

1.1 Prostate Cancer

1.1.1 Prostate cancer epidemiology

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second most lethal cancer just after lung cancer in men in the UK [1]. Between 2015 and 2017, there were over 40,000 cases diagnosed every year, which accounted for 26% of all new cancer cases, and 11,700 deaths, which accounted for 14% of all cancer deaths, in men in the UK [1]. Diagnosis rates have increased in the UK over the last decade by 4%, while the mortality rate has decreased substantially by 10% [1].

Worldwide, approximately 1.2 million men were diagnosed with PCa (22.8% of all cancer diagnoses) in 2018, making it the second most common cancer in men worldwide after lung cancer [2]. Similarly the same year, there were an estimated 128,222 deaths (3.3% of total male cancer deaths), making it the eighth most common cause of cancer death in men worldwide [2]. The disease burden is not equally distributed worldwide (Figure 1.1). PCa incidence is higher in the more developed parts of the world such as North America, Northern Europe, Western Europe and Australia/ New Zealand [3]. In contrast, mortality rates are highest in men of African descent with Southern African, Caribbean and Middle African men having the highest mortality rates [3]. East, Southeast and South Central Asian men have the lowest incidence and mortality rates [3].

1.1.2 The prostate gland

The prostate is an exocrine gland of the male reproductive system, located just below the bladder, in front of the rectum and surrounding the urethra (Figure 1.2). It is approximately 20g in weight and secretes thick and alkaline prostatic fluid, which along with sperm from the testicles and seminal vesicle fluid from the seminal vesicles, make up the components of semen [4]. The prostatic fluid makes up to 30% of total fluid ejaculated. It contains Zn^{2+} ions, citric acid and various proteins such as phosphatases, polyamines and Kallikreins (KLKs), which are serine proteases and include prostate-specific antigen (PSA) [5, 6]. These molecules are required for the proper functioning of sperm cells as

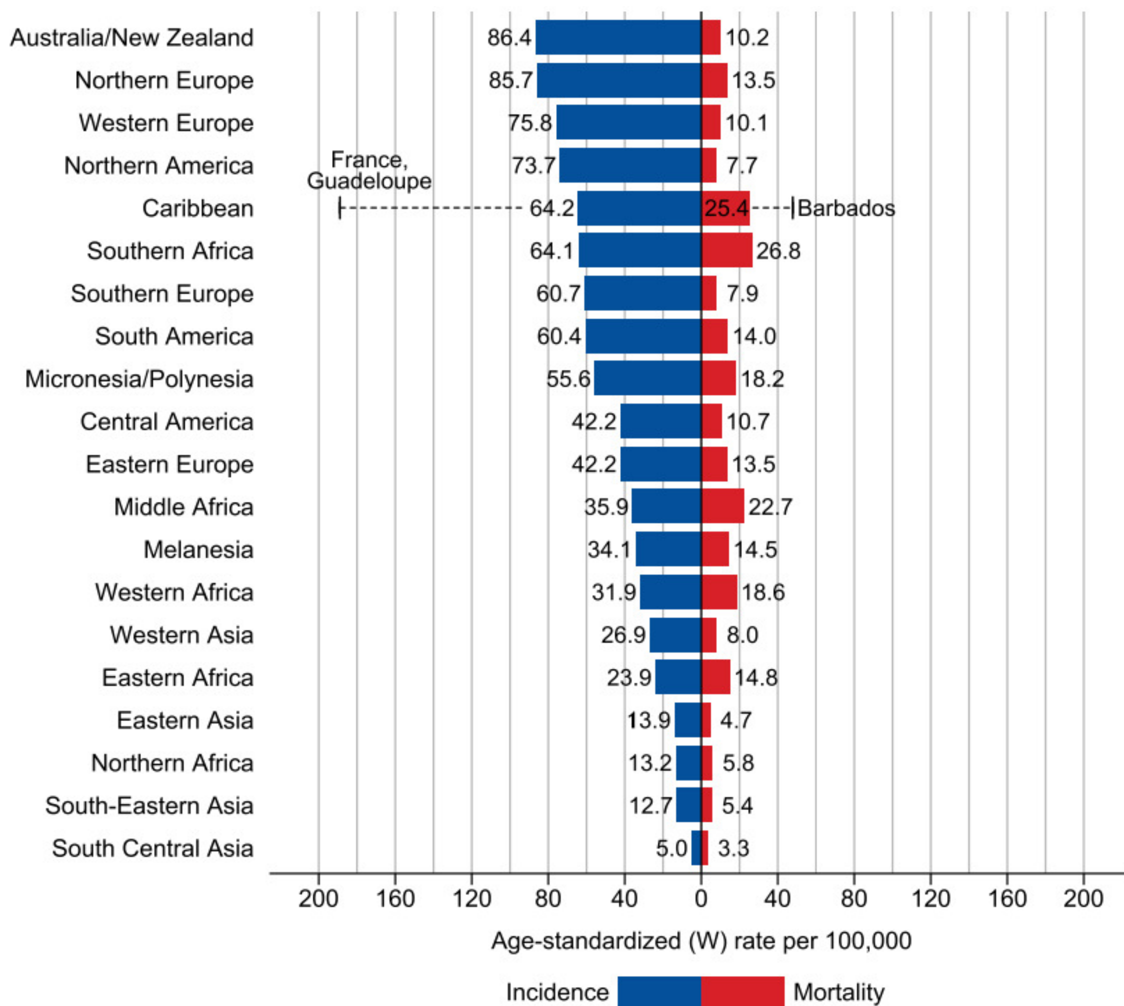

Figure 1.1: Region-specific age standardised incidence and mortality rates for prostate cancer in 2018. Figure extracted from Bray *et al.* (2018) with permission of the rights holder, International Agency for Research on Cancer [3].

they are responsible for regulating semen coagulation, liquefaction, providing nutrition for sperm and aiding sperm motility [4, 5]. The alkalinity of the secretion prolongs the lifespan of sperm as it helps neutralise the acidity of the vaginal tract [6]. Also, the muscles of the prostate contract during ejaculation, closing off the opening between the bladder and urethra, preventing retrograde ejaculation [7].

Anatomically, the prostate can be divided into three zones in humans: transitional, central and peripheral zones (Figure 1.2) [8]. The transition zone is the innermost zone that surrounds the urethra and makes up 5-10% of the gland [6]. Approximately 10-20% of PCa originate in this zone [9]. This region also enlarges with age and due to its immediate proximity to the urethra, this enlargement can cause a non-malignant disease, benign prostatic hyperplasia (BPH) [6, 8]. The central zone surrounds

the ejaculatory ducts, which run from the seminal vesicles to the prostatic urethra and makes up around 20-25% of the gland [6]. Approximately 2.5% of PCa originate in this zone [10]. This zone also begins to enlarge with age. The peripheral zone forms the outer layer of the prostate and makes up around 70% of the gland [6]. It is the most common zone where PCa develops, accounting for 70-80% of PCa cases [6, 8, 9].

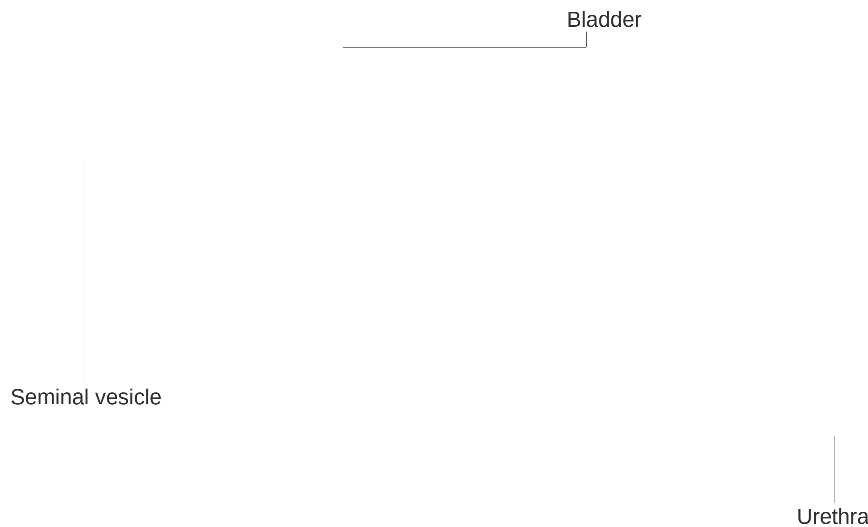

Figure 1.2: Anatomy of the prostate. The prostate is comprised of three main zones: central zone (a), transitional zone (c) and peripheral zone (d) and two additional zones: fibromuscular zone (b) and periurethral gland region (e). The fibromuscular band of tissue separates the transition zone from the remaining glandular compartments. The periurethral gland region is a narrow area with short ducts adjacent to the prostatic urethra. Figure adapted from De Marzo *et al.* (2007) with permission of the rights holder, Springer Nature [11].

Risk factors for prostate cancer

Like most cancers, age, race and family history of the disease are important risk factors in PCa. In 2017, 55% of PCa cases occurred in men aged 70 and over, and more than 80% mortality were in men aged 70 and over [1]. Age as a risk factor relates to the accumulation of genetic and epigenetic alterations and increased exposure to carcinogens, in turn leading to DNA damage and genomic instability over time.

In terms of race as a risk factor, the incidence is highest in men of African origin. In England, black men are at double the risk of being diagnosed and dying from PCa compared to white men [12]. Black men have diagnosis and mortality rates of 29.3% and 8.7% respectively, whilst white men have lower diagnosis and mortality rates of 13.3% and 4.2% respectively [12]. Asian men have the lowest risk of being diagnosed and dying from PCa with diagnosis and mortality rates of 7.9% and 2.3% respectively [12]. Black men also have an earlier onset of disease and higher PSA levels at onset [13]. The reasons for racial disparities are complex. It likely involves genetic factors, which may lead to differences in physiology, tumour biology and treatment response; and environmental factors such as socioeconomic status and lifestyle differences, affecting access to healthcare and contributing to late diagnosis with clinically advanced-stage PCa [14].

Family studies have shown that first-degree relatives of men with PCa have approximately twice the risk of developing PCa compared to the general population; this risk increased three-fold if the men had two affected relatives [15, 16]. Furthermore, the risk for first-degree relatives of men diagnosed with PCa before 60 years of age increases four-fold compared to the general population [16]. These familial studies show strong evidence of genetic predisposition to PCa.

In fact, approximately 5% of cases represent hereditary PCa. Studies conducted to elucidate the genetic components contributing to susceptibility to PCa have identified aberrations in various PCa-specific and DNA repair genes. *HOXB13* is a gene that codes for a transcription factor essential for embryonic and prostate development. A meta-analysis of *HOXB13* mutation in men of European descent showed that men with G84E mutation in *HOXB13* gene had four-fold increased cancer risk in comparison to non-carriers of the mutation [17]. Additionally, men with this mutation and family history had five-fold increased cancer risk compared to men with the mutation but no family history. About 1.4% of the European population carry this mutation, and this frequency increases to 3.1% in carrier men with a family history of PCa with early diagnosis (< 55 years of age) [18]. *BRCA1*, *BRCA2* and *ATM* are tumour suppressor genes that code for proteins involved in the DNA damage

response pathways and are important for the maintenance of genomic stability. The frequencies of germline mutations for these genes ranged between 0.41-0.64% for *BRCA1*, 0.82-5.7% for *BRCA2* and 0.41-1.92% for *ATM* respectively [19–22]. Additionally, men with *BRCA1* and *BRCA2* mutations respectively conferred 3.75 and 5-fold higher relative risks of PCa compared to men without mutations [21, 23]. Carriers of these mutations usually experienced earlier age of onset and a more advanced, aggressive form of cancer with worse prognoses compared to men without the mutations. Like most cancers, a combined influence of hereditary variations in many genes and environmental factors impact the risk of a person developing PCa.

1.1.3 Development of prostate cancer: Androgen signalling

The prostate gland requires androgen hormones for normal development and functioning. As such, the key pathway implicated in the development and progression of PCa is the androgen receptor (AR) signalling pathway (Figure 1.3). In a normal prostate, circulating androgen testosterone produced by the testes is converted to a more potent androgen 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT exerts its biological effects through binding to and activating the AR, a steroid and nuclear receptor located in the cytoplasm. Activated ARs homodimerise and are transported to the nucleus where they bind to androgen response elements in the promoter region of target genes, thus recruiting co-regulatory proteins and facilitating transcription or repression of those genes [24, 25]. Many of these target genes are involved in regulating cell proliferation, differentiation and apoptosis of epithelial cells, playing a pivotal role in tissue maintenance and homeostasis. Dysregulation of this pathway is a key driver of prostate tumorigenesis and progression.

Patients with high-risk/ locally advanced and metastatic tumours (i.e. inoperable) are treated with androgen deprivation therapy (ADT) to deactivate the AR signalling pathway, often in combination with other treatments such as radical radiotherapy and chemotherapies [26]. ADT may involve orchiectomy, administration of luteinizing hormone-releasing hormone (LHRH) agonists and antagonists to suppress the production of androgens, or administration of anti-androgens to competitively bind to and block

ARs in cancer cells [27]. Drug-based ADT usually starts with the LHRH agonist/ antagonist approach and is often combined with anti-androgens to achieve combined androgen blockade. Combined androgen blockade blocks both androgen production and action to attain maximal treatment effectiveness. Although ADT is initially successful in reducing androgen levels and/ or AR activity and prostate tumour growth, patients eventually relapse and develop a much more aggressive ADT resistant form of PCa. This recurrent form have been referred to as androgen-independent PCa or castration-resistant PCa (CRPC). Although these tumours are resistant to ADT, they continue to depend on alternative mechanisms of androgen/ AR action for survival and growth. Thus, these tumours are not completely "androgen-independent"; as such, androgen-independent PCa is a misnomer for the recurrent form of PCa [28, 29]. For clarity, ADT resistant, recurrent cancer will be referred to as CRPC in this thesis.

The progression to CRPC has been attributed to various molecular alterations which abnormally activate AR signalling, such as gain-of-function *AR* mutations, amplifications, *AR* splice-variant expression and aberrant AR co-regulator activities. Indeed, while only 2% of primary tumours carry *AR* mutations and 0-5% carry *AR* amplifications, mutations and amplifications increase remarkably to 18% and 52-63% respectively in metastatic and CRPC cases [30–32]. Alterations to the *AR* gene often result in increased sensitivity to low levels of endogenous androgens and/ or alternative hormones, leading to inappropriate activation and amplification of AR response [25]. Overexpression of *AR* splice variants (ARVs) has also been observed frequently in CRPC. ARVs are abnormally truncated isoforms that lack the ligand-binding domain, thus activating AR reporter genes even in the absence of androgens [33].

1.1.4 Genomic profile of prostate cancer

PCa is a heterogeneous disease at both clinical and molecular level. Several studies have aimed to characterise the underlying genomic heterogeneity in patients with localised and metastatic tumours. They report a low mutational burden in PCa with approximately 1 mutation per megabase in primary tumours and 2-4.4 mutations per megabase in metastatic tumours [34–36]. In contrast, there

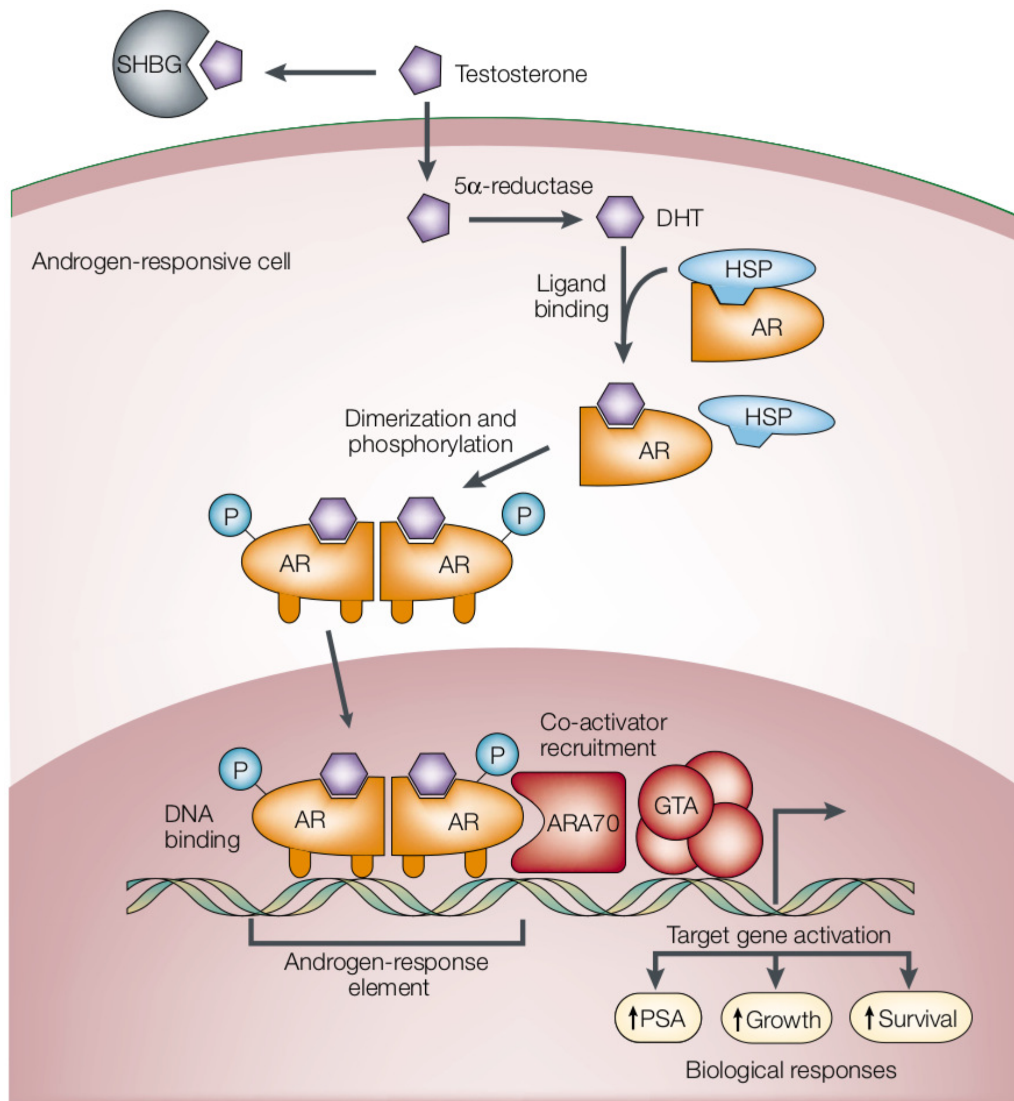

Figure 1.3: The androgen receptor signalling pathway. Testosterone enters prostate cells and is converted to a more potent form: dihydrotestosterone (DHT), by enzyme 5α-reductase. DHT binds to inactive androgen receptor (AR) in the cytoplasm, resulting in dissociation of the AR from chaperone proteins and its subsequent phosphorylation. The activated AR homodimerises and translocates to the nucleus where it binds to androgen response elements in the promoter regions of AR target genes. The AR recruits co-activators and co-repressors and facilitates activation or repression of target genes. Figure extracted from Feldman *et al.* (2001) with permission of the rights holder, Springer nature [25].

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are higher rates of genomic rearrangements and copy number variations (CNVs), which suggests the development and progression of PCa is primarily due to the accumulation of large-scale CNVs and fusion gene formations [31, 32, 34–37].

In primary PCa, the most widely reported genomic alterations are in the ETS gene family fusions; almost 60% of primary tumours exhibiting a fusion involving one of the ETS family genes *ERG*, *ETV1*,

ETV4 and *FLI1* [35]. This gene family is one of the largest families of transcription factors and regulate cell proliferation, differentiation, angiogenesis, inflammation and apoptosis [38]. These gene fusions primarily involve fusion with regulatory regions of prostate-specific and androgen-responsive genes such as *TMPRSS2* and *SLC45A3* [35, 38–40].

Focal deletions and mutations of *PTEN* (17%), *TP53* (3.4-8%), *BRCA2* (3%), *SPOP* (8-11%) and *FOXA1* (2.3-3%) are also observed in primary tumours. These genes are key tumour suppressor genes implicated in various cancers. *PTEN* codes for a lipid and protein phosphatase that is responsible for dephosphorylation of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to Phosphatidylinositol 4,5-bisphosphate (PIP2). This inhibits the downstream oncogenic PI3K/AKT signalling pathway, which leads to inhibition of several cellular processes required for tumour development such as cell proliferation, migration and survival [41]. *TP53* codes for a transcription factor which plays a key role in the regulation of various genes involved in DNA repair, cell cycle progression, apoptosis, or senescence in response to cellular stress leading to DNA damage [42]. As previously stated, *BRCA2* codes for a protein involved in double-strand DNA damage repair and is responsible for the maintenance of genomic stability [43]. *SPOP* codes for a substrate adaptor for a ubiquitin ligase CRL3 and recruits substrates to CRL3 for ubiquitination and subsequent proteasomal degradation [44]. Its substrate includes AR [44]. *FOXA1* codes for a transcription factor involved in endodermal organogenesis, metabolism and homeostasis and are pioneer factors (transcription factors that can condense chromatin and negatively or positively regulate transcription) for AR [45, 46]. Besides deletions of tumour suppressor genes, focal amplifications of 8q24.21 locus spanning *MYC* (8%) are also observed in primary tumours [35, 47]. *MYC* is a proto-oncogene that codes for a transcription factor which is activated upon various mitogenic signals and regulates various processes such as cell cycle progression, cellular transformation and apoptosis.

Metastatic tumours have a similar molecular landscape to primary tumours, albeit at different magnitudes. ETS gene family fusions collectively occur at 56% frequency, while alterations in tumour sup-

pressors genes *TP53*, *PTEN*, *BRCA2*, *SPOP* and *FOXA1* occur at higher frequencies (53.3%, 40.7%, 13.3%, 8% and 12% respectively) in comparison to primary tumours [32]. Focal amplification of the oncogene *MYC* is also high in metastases (13%) [32]. Besides, there are genomic modifications almost exclusive to metastases such as mutation and focal amplification of *AR* (58-63%); alteration in *RB1* (9%), a tumour suppressor gene which codes for a protein that regulates cell growth by suppressing cell cycle progression; and focal deletion in 11q23 spanning *ZBTB16* (10%), a transcriptional repressor that induces epigenetic changes, including histone modifications and DNA methylation [31, 32, 35, 48]. Several studies have characterised the genomic landscape of primary and metastatic prostate, however, the functional relevance of many of these genomic events are still not well understood.

1.1.5 Prostate cancer diagnosis

The PSA test is one of the diagnostics tests currently used to diagnose PCa. It detects the level of PSA protein in the blood. A serum PSA level above the 'normal' threshold of 4.0 ng/ml is considered suggestive of potential prostate malignancy [49]. An elevated PSA level can be indicative of PCa but is nevertheless, not an exclusive symptom of PCa. It can be observed in patients with benign prostate conditions such as prostatitis, BPH, urinary tract infection or even in healthy males [50, 51]. Thus, the test does not necessarily indicate PCa development and results in a high proportion of false-positives [52, 53].

Besides diagnosis, PSA test is also used for PCa screening in US. However, PSA screening test is not currently offered in the UK and its use for screening is highly debated. Several trials have tested the efficacy of the test in PCa screening. The European Randomized Study of Screening for Prostate Cancer (ERSPC) was a randomised trial conducted in eight European countries where more than 162,000 men recruited were randomly assigned to PSA test screening group or control group without any screening [54]. This trial showed that the PSA screened group had a 21% reduction in PCa-specific mortality during 11-years of follow-up [55]. However, a study conducted by Gosselaar *et al.* on the Rotterdam cohort of the ERSPC trial showed that 50% of PCa diagnosed through PSA screening

showed clinically and pathologically low-risk features, similar to the pathology of incidental cancers found at autopsy [56]. The overall survival for these patients was 70%, while none died of PCa. Similarly, another PSA screening trial, the Prostate, Lung, Colorectal and Ovarian (PLCO) trial, which screened more than 76,000 men over a period of ten years, showed no difference in PCa-specific mortality with PSA testing [57]. These trials demonstrate that although the PSA test is useful in PCa screening, most of the cancers detected are indolent tumours, which are clinically asymptomatic and therefore lead to over-diagnosis. Additionally, the increased incidence of PCa seen worldwide (Section 1.1.1) is attributed partly to the widespread application of the PSA test.

In addition to the PSA test, a digital rectal examination (DRE), where the prostate is examined for any abnormalities such as lumps by inserting a finger in the rectum, is another diagnostic procedure. Patients with positive diagnostic test results will be referred for a more invasive prostate tissue biopsy which is the only method to give a definitive diagnosis. This can be performed either using a transrectal ultrasound (TRUS), where a needle is inserted into the prostate through the rectum or a transperineal ultrasound, where a needle is inserted into the prostate through the skin behind the scrotum. Traditionally, 10-12 tissue samples are obtained for TRUS and 18-28 samples are obtained for transperineal biopsies. However, the optimal number of samples that should be taken during prostate biopsies is still debated; increasing the number of samples taken could improve the detection rate of PCa at the risk of increasing the side-effects.

Beside biopsies, imaging tests such as MRI and CT scans are also for PCa diagnosis and have proven to be beneficial for early detection. In 2017 a clinical trial, PROMIS, was performed in 740 men with elevated PSA where multi-parametric MRI (MP-MRI) was performed prior to biopsy [58]. This clinical trial reported MP-MRIs diagnosed 5% fewer indolent cancers (5% reduction in over-diagnosis of clinically insignificant cancers) than the biopsies. Additionally, the MP-MRI diagnosis could reduce unnecessary biopsies of 27% of patients.

1.1.6 Prostate cancer prognosis: one of the main clinical challenges

The natural course of PCa is variable. It manifests as either a low-risk, indolent tumour that is asymptomatic and localised to the prostate, or a high-risk, aggressive tumour that eventually metastasises and proves lethal if untreated. Approximately 42-66% of patients present the indolent form of PCa [52, 59]. Currently, there are no reliable methods to distinguish between indolent and aggressive disease. The National Institute for Health and Care Excellence (NICE) and the European Association of Urology (EAU) guidelines recommend using risk stratification systems that incorporate clinicopathological variables serum PSA at diagnosis, Gleason score and clinical tumour stage to predict disease severity/ prognosis and inform disease management decisions [26, 60].

Risk stratification based on clinicopathological variables

As stated above, risk stratification of PCa patients is based on three clinical factors: PSA level at diagnosis, Gleason score and clinical tumour stage [26, 60]. All of these are determined at diagnosis. To determine the Gleason score, the biopsies taken for definitive diagnosis are graded according to the Gleason grading system by a pathologist. This system categorises patients according to their histological features. A Gleason score can be between 1 (most differentiated, essentially normal) and 5 (least differentiated). In this system, two grades are assigned; the primary grade is the dominant pattern of the tumour and the secondary grade is the next most common pattern. These two grades are subsequently combined to give an overall Gleason score which ranges between 2 (1+1) and 10 (5+5). The score reflects aggressiveness and extent of de-differentiation of the cancer cells, with higher numbers indicating greater aggressiveness and poor differentiation. Scores ≤ 5 are insignificant and are not reported. A Gleason score of 6, 7 and 8-10 signify that cells are well-differentiated, moderately differentiated and poorly differentiated and thus have low, intermediate and high risks.

In addition to the overall Gleason score, a new grading system defined by the International Society of Urological Pathology (ISUP), where overall Gleason scores are further categorised into groups ranging from 1 to 5, has been used in newer studies for prognostication. In the classic Gleason score 7, consist-

ing of 3+4=7 and 4+3=7, are categorised into the same prognostic risk group. However, research has shown that these two groups are prognostically different; group 3+4=7 are mostly well-differentiated cancer and have a more intermediate favourable prognosis, while group 4+3=7 are mostly poorly differentiated cancer and have an intermediate unfavourable prognosis [61, 62]. Accordingly, Gleason score 7 is divided into two groups: ISUP Grade 2 (3+4=7) and ISUP Grade 3 (4+3=7), in the ISUP grading system to account for the different prognoses (Table 1.1).

ISUP Grade	**Gleason score**	**Definition**	**Risk group/ prognosis**
1	≤ 6	individual discrete well-formed glands	low
2	3+4=7	predominantly well-formed glands with a lesser component of poorly-formed/fused/cribriform glands	intermediate favourable
3	4+3=7	predominantly poorly-formed/fused/cribriform glands with a lesser (>5%) component of well-formed glands	intermediate unfavourable
4	4+4=8, 3+5=8, 5+3=8	only poorly-formed/fused/cribriform glands OR predominantly well-formed glands with a lesser component lacking glands OR predominantly lacking glands with a lesser component of well-formed glands	high
5	4+5=9, 5+4=9, 5+5=10	lacks gland formation (or with necrosis) with or without poorly-formed/fused/cribriform glands	high

Table 1.1: Histological definitions of the ISUP Gleason Grade group categories. Table adapted from Epstein *et al.* (2016) with permission of the rights holder, Elsevier [61].

Besides histopathology of the biopsy samples to determine the Gleason scores, results from the imaging tests are used to determine the clinical tumour stage according to the Tumour, Node, Metastasis (TNM) system, which is used to describe the location and spread of cancer. In TNM staging, the T stage is a measure of the size and extent of the primary tumour inside and in the periphery of the prostate, N stage is a measure of the spread of cancer to nearby lymph nodes and M stage is a measure of metastasis of cancer to other parts of the body. The first site of metastases is lymph nodes adjacent to the primary tumours. This is followed by metastases to the bone (84.40%), distant lymph nodes (10.6%), liver (10.60%) and thorax (9.10%) [63]. Rarely, prostate tumours may spread to the brain (3.10%), adrenal glands and kidneys (1%), digestive system (1.6%), and retroperitoneum (0.9%) [63]. The TNM scoring system is further detailed in Table 1.2.

Tumour stage		Definition
stage	score	
T	TX	primary tumour cannot be assessed
	T0	no evidence of primary tumour
	T1	clinically inapparent tumor neither palpable nor visible by imaging
	T1a	tumour is found in less than 5% of the removed tissue
	T1b	tumour is found in more than 5% of the removed tissue
	T1c	tumours are found by biospy performed after a raised PSA level
	T2	tumour is detectable with a DRE or imaging but is confined to the prostate
	T2a	tumour is found in only half of one side of the prostate
	T2b	tumour is found in more than half of one side of the prostate
	T2c	tumour is found in both sides of the prostate
	T3	Tumor extends through the prostate capsule
	T3a	extraprostatic extension (unilateral or bilateral)
	T3b	tumour has invaded seminal vesicle(s)
	T4	tumour has invaded adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder or pelvic wall
N	NX	regional lymph nodes were not measured
	N0	no regional lymph node metastasis
	N1	metastasis in regional lymph nodes(s)
M	M0	no distant metastasis
	M1	distant metastasis
	M1a	metastasis in non-regional lymph nodes
	M1b	metastasis in the bones
	M1c	metastasised in other sites such as lungs, liver or brain with or without bone disease

Table 1.2: Definitions of the Tumour, Node, Metastasis staging system for prostate cancer. Information extracted from Edge *et al.* (2010) with permission of the rights holder, Elsevier [64].

Once Gleason score and tumour stage are determined, these variables and PSA at diagnosis are used to categorise patients into risk groups. The criteria for determining risks of patients according to NICE and EAU guidelines are summarised in Table 1.3. Patients then follow personalised treatment/ disease management regimes depending on their risk.

Level of risk	**PSA**		**Gleason score**		**Clinical tumour stage**
low risk	$<$10 ng/ml	AND	≤ 6	AND	T1-T2
intermediate rsk	10-20 ng/ml	OR	7	OR	T2b
high risk	$>$20 ng/ml	OR	8-10	OR	$\geq$ T2c
	any PSA	AND	any GS	AND	T3-T4

Table 1.3: Prostate cancer risk stratification based on Gleason score, clinical tumour stage and serum PSA at diagnosis. Information adapted from the National Institute for Health and Care Excellence (NICE) and European Association of Urology guidelines with permission of the rights holders, NICE and Elsevier [26, 60]. Abbreviations: GS=Gleason score; PSA=Prostate Specific Antigen.

As previously mentioned, raised PSA level is not PCa disease-specific and results in a high proportion of false positives. In addition, the PSA test detects asymptomatic indolent disease, leading to over-diagnosis. Another problem is that Gleason score and tumour stage measurements are subject to sampling and random errors as the biopsies may miss tumours, resulting in a high proportion of misdiagnoses. More than 30% TRUS biopsies are false negatives and higher than 45% of cancers patients have their Gleason scores underestimated [58, 65]. MRI-guided diagnoses are superior to ultrasound-guided biopsies; however, they also suffer from more than 50% false positives [58]. Although the clinical variables are good indicators of disease severity and correlate with patient survival, they are unreliable prognostic markers and may not represent true disease state.

Due to the unreliable diagnostic tests and risk stratification system, there are high rates of over-diagnosis and over-treatment of patients with indolent tumours. Patients with indolent tumours should be offered less invasive treatments such as active surveillance (monitoring of patients closely where diagnostic tests such as the PSA test, prostate biopsies and imaging tests are performed routinely to track tumour growth/ aggressiveness) and watchful waiting (less intensive monitoring of PCa with fewer diagnostic tests, thus avoiding surveillance-related risks and side effects). However, these patients

with indolent tumours may follow the same highly invasive treatment procedures as patients with aggressive disease with no significant survival benefit. These treatments include radical prostatectomy (RP), radiotherapy and ADT. Watchful waiting and active surveillance, although less invasive, can give rise to complications such as infections, sepsis, rectal bleeding and acute urinary retention due to the invasive biopsy procedures [66]. On the other hand, RP, radiotherapy and ADT treatments for treating aggressive disease are associated with extreme side effects such as sexual dysfunction, urinary incontinence, impaired rectal/ bowel function, hernia, scarring of the urethra, and thromboembolic or cardiovascular events leading to poorer quality of life and increasing disease burden [67–69].

1.1.7 Prostate cancer relapse: another clinical challenge

In addition to the problem of over-treatment of indolent cases, a substantial proportion of patients with aggressive disease experience disease relapse. This is because the PCa risk stratification system (along with other clinical variables such as family history, age at diagnosis and co-morbidity) is also used to devise treatment strategy for the patients according to their risk [26]. Although these clinicopathological variables are used to devise treatment strategy, they are not indicative of treatment response; currently, there are no prognostic markers that are reliable predictors of treatment response. Consequently, approximately 15-45% of patients treated with RP, one of the first lines of curative treatments for localised PCa, experience biochemical recurrence (BCR) within five years [70–74]. Similarly, 30-60% of patients treated with radiotherapy experience BCR between three and ten years [74, 75]. Although it does not always equate to clinical recurrence, BCR is considered an initial event signifying disease progression and has been shown to be associated with increased risk of PCa metastasis and cancer-specific mortality [72, 73, 76–78].

Further, men treated with ADT for advanced disease relapse within one to three years to an incurable disease state, CRPC [50, 79, 80]. The outcome of ADT can be improved if it is combined with other treatment strategies. Results from the STAMPEDE trial, a randomised controlled trial in the UK and Switzerland that is evaluating different combinations of novel treatment strategies with ADT, show

that advanced disease treated with a combination of ADT and the chemotherapy drug docetaxel had a longer time to CRPC (3.03 years) in comparison to just ADT treatment (2.04 years) [81]. Similarly, a meta-analysis of five randomised controlled trials (CHAARTED, GETUG-15, GETUG-12, RTOG 0521 and STAMPEDE) conducted by Vale *et al.* reported that the combination of ADT and docetaxel led to a lower 4-years CRPC free survival (64%) compared to just ADT treatment (80%) [82].

Although results from these trials show improvement in disease outcome for advanced disease with adjuvant treatment strategies, it remains a challenge that there are no biomarkers or methods that can be used to identify (and separately treat) patient subgroups that will successfully respond to specific treatments or are more likely to experience relapse after treatment. The problem of unreliable prognosis and subsequent relapse after treatment are two of the main issues in PCa patient care. They highlight an urgent need for novel biomarkers that can accurately and reliably identify aggressive disease from indolent disease in order to limit over-treatment and facilitate appropriate personalised treatment strategies for PCa disease management.

1.1.8 Prognostic biomarkers currently being evaluated

In the last decade, hundreds of studies have been published addressing the lack of reliable biomarkers in PCa. These studies have introduced promising novel prognostic markers and tests; however, none have yet successfully replaced the established clinicopathological variables used for predicting disease risk. Some of the RNA-based gene biomarker panels that are commercially available for use and potential biomarkers currently being explored for prognostication are briefly discussed in this section before the introduction of miRNAs, a novel class of small non-coding RNAs with promising biomarker potential and the focus of my project.

OncotypeDX Genomic Prostate Score

OncotypeDX Genomic Prostate Score (GPS) is a gene panel for predicting disease aggressiveness (growth and spread) at diagnosis in men with clinically low-/ favourable intermediate-risk PCa (Glea-

son scores 3+3=6, 3+4=7) [83]. It is based on a multi-gene assay consisting of 17 genes (12 genes related to androgen metabolism, cellular organization, proliferation and stromal response, and 5 reference genes) and outputs a GPS between 0 to 100; higher scores indicate a more aggressive disease. The test may be useful in detecting aggressiveness in men with low-/ intermediate-risk PCa and assisting clinicians in deciding between active surveillance and immediate treatment of patients at the time of diagnosis. Currently, OncotypeDX GPS is commercially available only in the United States. The predictive performance of OncotypeDX in independent datasets are reported in Table 1.4.

Prolaris

Prolaris is a gene panel test for predicting disease aggressiveness in men with Gleason scores ≥ 7 [84]. The test calculates a cell cycle progression (CCP) score, which is based on the expression of 31 cell cycle progression genes. The CCP score can be positive or negative values and a score of ≥ 2 indicates an aggressive tumour. This test combines clinicopathological information with the CCP scores to generate either the ten-year risk of BCR (from RP specimens) or the ten-year PCa-specific mortality risk (from biopsy samples). Similar to the OncotypeDX GPS test, the Prolaris test can be used to decide between active surveillance and active treatment options, but in men with intermediate-/ high-risk PCa. In the UK, this test is commercially available in private clinics only. Its predictive performance in external validation datasets are reported in Table 1.4.

Decipher

The Decipher test is a gene panel test for predicting clinical metastasis within five years of RP in men with high-risk pathology after RP, i.e. PSA > 20, Gleason score ≥ 8, pathological tumour stage T3b [85]. The Decipher test is a 'genomic classifier' (GC), a machine learning model built on a random forest algorithm, and is based on the expression profile of 22 different genes [85]. It outputs a GC score that ranges from 0 to 1; cases with scores > 0.6 are considered at high risk of progression. The Decipher test may be useful in predicting progression post-RP and improve the treatment decision-

making process for high-risk patients. The Decipher test is currently only commercially available in the United States. The predictive performance of Decipher in external validation datasets are reported in Table 1.4.

Biomarker panels	Endpoint	Sample size	AUC/ C-index (% CI)	Ref
Oncotype DX	adverse pathology	402	0.720 (n/s)	[86]
		732	0.730 (n/s)	[87]
Prolaris	biochemical recurrence	366	0.842 (n/s)	[84]
	PCa specific mortality	337 (cases: 68)	0.878 (n/s)	[84]
Decipher	metastasis	186 (cases: 63)	0.75 (0.67-0.83)	[85]
		235	0.84 (0.61-0.93)	[88]

Table 1.4: Predictive performance of the three commonly genomic biomarker panels in validation/ external datasets. n/s represents not specified.

1.2 A new class of biomarkers: MicroRNAs

MicroRNAs (miRs) are small, non-coding regulatory RNA molecules of approximately 22 nucleotides (nt) in length. They negatively regulate gene expression primarily at the post-transcriptional level. They do so by binding to complementary sequences in the 3' untranslated region (UTR) of target mRNAs via their preserved 'seed sequence' region, which in turn represses translation of the target mRNAs [89]. The first miR was discovered in 1993 in the nematode *Caenorhabditis elegans* and was implicated in post-embryonic development [90]. Since then, the discovery of miRs in other species, including humans, have increased exponentially. The most recent version of miRBase database (version 22), an archive of miR annotations and sequences for all species, reported 4,800 mature miRs in humans [91]. Due to their regulatory role, these molecules have been implicated in various developmental, cellular and physiological processes and their dysregulation has been associated with various diseases including PCa [89, 92]. Consequently, miRs have been investigated for their potential as diagnostic, prognostic and treatment predictive biomarkers in PCa in the last two decades. These regulatory molecules present promising biomarker alternatives to the unreliable clinical markers for PCa prognosis.

1.2.1 MicroRNAs: biogenesis

In the canonical biogenesis pathway, miR coding genes are transcribed by RNA polymerase II to produce a primary miRNA transcript (group 1 in Figure 1.4) [89, 93]. Primary miRNAs are several kilobases (kb) in length and have at least one region that folds into a hairpin structure. They are cleaved by the Microprocessor, a complex of RNAse III enzyme Drosha and RNA binding protein DGCR8 to produce a ∼70 nt long stem-loop precursor miRNA (pre-miR) transcripts with a 2 nt overhang at its 3' end [89, 93]. Pre-miRs are exported to the cytoplasm by Exportin-5 and further cleaved by another RNAse III enzyme, Dicer, near the loop producing a miRNA duplex of ∼22 nts in length. This duplex has a 2 nt overhang at the newly generated 3' end as a result of offset cuts made by Dicer [89, 93]. Both strands of the duplex can act as a functional miR. However, only one strand, the guide strand, is incorporated to the RNA-induced silencing complex (RISC) while the remaining strand is often degraded. Strand selection usually depends on the thermodynamic stability of the 5' ends of the duplex; the strand that has relatively unstable base pairing at the 5' end becomes the guide strand [93, 94]. As mature miRs can be generated from both the 5' and 3' arm of the pre-miR, miRs have -5p and -3p suffixes at the end of their names to denote their arm of origin.

Besides the canonical pathway, miRs can also be produced via various alternative biogenesis pathways where the action of Drosha, Exportin-5 or Dicer are not required. Young-Kook *et al.* proposed six biogenesis pathways including the canonical pathway (referred to as Group 1) in humans (Figure 1.4) [95]. Group 2 biogenesis pathway requires mono-uridylation (non-templated addition of a single uridine at the 3' end) of pre-miRs by enzymes TUT7 and/ or TUT4 because the pre-miRs have a 1 nt overhang at their 3' end instead of typical 2 nt overhang [95–97]. Following mono-uridylation, they are processed by Dicer in a typical manner to generate the mature miR. The pre-miRs of miRs produced via the group 3 biogenesis pathway are directly generated through transcription by RNA polymerase II and are 7-methylguanosine capped at the 5' end [93, 98, 99]. The pre-miR bypasses Drosha cleavage and is directly exported into the cytoplasm by Exportin-1 where it is processed by Dicer to generate the mature form. Alternatively, miRs produced via the group 4 biogenesis pathway depend on Drosha

cleavage but are independent of Dicer cleavage. This is because, in this pathway, Drosha cleavage produces a pre-miR with high stem complementarity and a short stem length which is less than 21 bp, too small for Dicer recognition [100, 101]. Instead, the pre-miR is directly cleaved by Argonaute 2 (AGO2), an endonuclease which is a component of the RISC [100, 101]. The cleaved product, ac-pre-miR, is further cleaved by 3'- 5' exoribonuclease PARN before being loaded into the RISC [89, 100]. MiRs produced via group 5 and group 6 biogenesis pathways are also Drosha independent and originate from spliced-out introns (group 5) or other small non-coding RNAs such as small nucleolar RNAs and tRNAs (group 6) [93, 102].

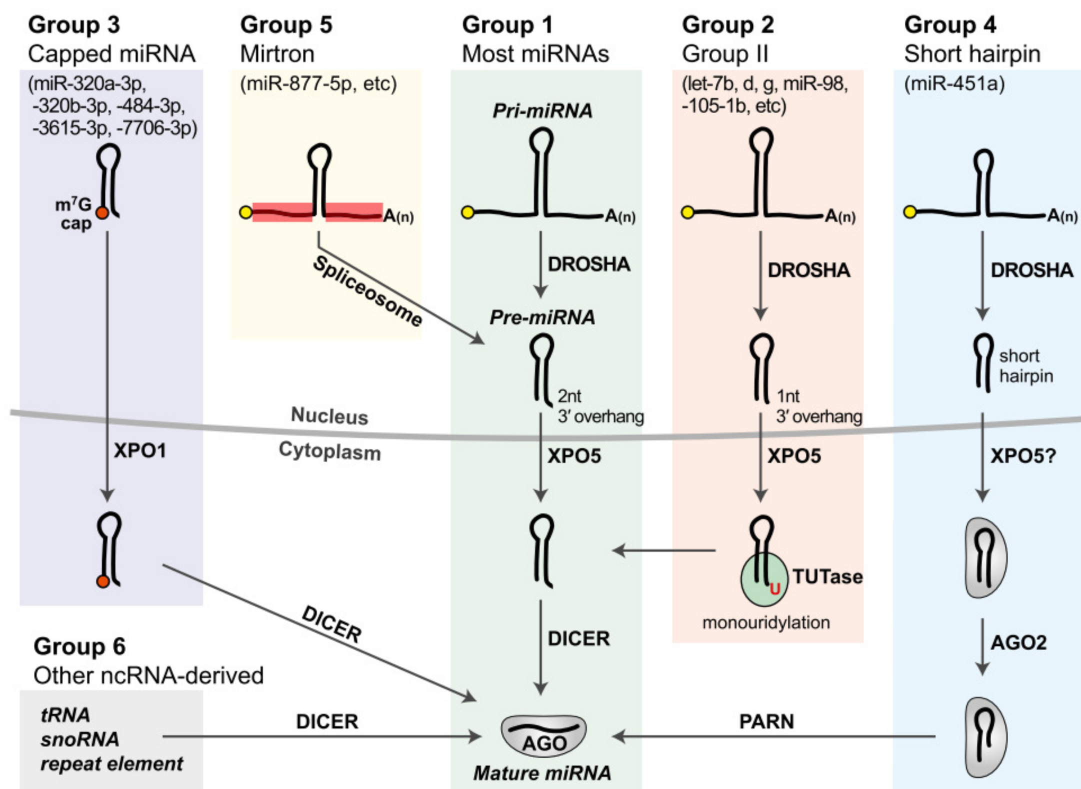


Figure 1.4: Canonical and alternative miRNA biogenesis pathways. The canonical miRNA biogenesis pathway (Group 1) requires the action of Drosha, Exportin-5 and Dicer, while alternative biogenesis pathways (Group 2-6) bypass action of these key proteins or require additional processing by other proteins such as TUTases, PARN and AGO2. Figure extracted with permission from Young-Kook *et al.*(2016) [95].

1.2.2 microRNA mechanism of action

Mature miRs mediate their functionality by associating with the RISC (Figure 1.5). The RISC is a multi-protein complex made up of Dicer, transactivating response RNA-binding protein (TRBP) and AGO2. As previously mentioned, Dicer is responsible for cleavage of pre-miR to produce a miR duplex of ~22 nt long, TRBP associates with Dicer and stabilises it, and AGO2 is an endonuclease and as such is the catalytic centre of the RISC [93, 103, 104]. The RISC incorporates single-stranded RNA fragments, such as miRs, as a template for binding to complementary mRNA and preventing their translation.

MiR target recognition requires Watson-Crick base pairing of the miR seed sequence to complementary sequence/ miRNA responding elements (MREs) in target mRNAs. In canonical binding, the seed region of miR, nts 2-7 from 5' end, base-pairs with complementary MRE sites of target mRNAs (Figure 1.6). This canonical seed region is termed a 6mer site and it confers marginal repression [105, 106]. The seed region is not just limited to 6mer sites. MiR-mRNA binding and repression can be further enhanced by the presence of an adenosine in the mRNA opposite miRNA nt 1 (7mer-A1 site) or base-pairing of mRNA to miR nts 2-8 (7mer-m8 site) [106]. The presence of both 7mer-A1 and 7mer-m8 sites, termed an 8mer site, confers the most efficacious binding; however, most miR targets harbour 7mer sites [106]. Complete or partial complementary paring between the miR and target mRNA results in translational repression of the mRNA either by preventing translation initiation or by promoting mRNA de-adenylation and degradation [107]. Target mRNA can also be directly cleaved by AGO2 in the RISC when there is a near-perfect complementary match between the miR and mRNA [106, 108, 109].

MREs are usually located within the 3' UTRs of target mRNAs. The 3'UTR is the section of an mRNA directly after the translation termination codon and contains various regulatory regions such as MREs, AU-rich elements, and polyadenylation signals, that post-transcriptionally influence gene expression. As the 3'UTR of genes can be very long often reaching lengths over 1 kb, it can contain

MREs for multiple miRs. Targeting has also been shown to occur in the 5'UTR (the section of an mRNA directly upstream from the initiation codon) and open reading frames (ORFs) of mRNAs. Although these are less frequent and less effective than 3'UTR targeting due to the translational machinery displacing the RISC complex as it moves from the 5'UTR of the transcript along the ORF [106].

As the seed sequences are very short, many miRs share identical seed sequences even if they originate from different genomic loci. This property leads to the miRs being functionally redundant or pleiotropic. As such, miR-mRNA targeting has a many-to-many relationship, where a specific miR can target multiple mRNAs and a specific mRNA is regulated by multiple miRs [110].

Target repression

Target degradation

Figure 1.5: Mechanism of miRNA action. The RISC is a multi-protein complex consisting of Dicer, TRBP and AGO2. The Dicer cleaves the pre-miR to generate a miR duplex of $\sim$ 22 nt long. The Dicer then associates with AGO2 and TRBP to transfer the miR duplex. The strand with a less thermodynamically stable 5' end is loaded into the RISC (guide strand; red in the figure) the AGO2 protein degrades the remaining strand (passenger strand). The RISC will recognise and pair with mRNAs that have complementary sequence to the seed sequence of the guide strand. Depending on the degree of complementarity, the mRNAs are either translationally repressed or cleaved by AGO2. Figure adapted from Bartel (2018) with permission of the rights holder, Elsevier [89].

Figure 1.6: The different types of seed sites in a miRNA. Seed sites of miRNAs are comprised of nucleotides between positions 2-8 from the 5'end. The most effective seed sites are 8mer sites and the least effective seed sites are 6mer sites. 7mer-m8 and 7mer-A1 sites are more effective than 6mer but less effective than 8mer, and are the most common types of seed sites. Figure extracted from Bartel (2018) with permission of the rights holder, Elsevier [89].

1.2.3 Isoforms of microRNAs

Each strand of pre-miRs was initially believed to produce only one mature miR, however, deep-sequencing studies have shown that pre-miRs can give rise to more than just the 3' and 5' canonical miRs. These variants of canonical miRs are termed isomiRs and differ in length and/ or sequence compared to their canonical form. IsomiRs can be categorised into four main isotypes: 3' end templated isomiRs, 5' end templated isomiRs, within-sequence non-templated isomiRs and 3' end non-templated isomiRs (Figure 1.7) [89, 111, 112]. The templated isomiRs differ only in length and can have base additions or deletions at their respective 5' or 3' ends. Any base additions in the templated isomiRs match the bases in the pre-miR at the corresponding position. Conversely, non-templated isomiRs can differ in length and/ or sequences and base changes can occur at the 3' end or anywhere within the sequence. Templated isomiRs are usually generated by imprecise cleavage by Drosha or Dicer [89, 111]. Non-templated isomiRs are generated by RNA-editing enzymes such as ADARs, which convert base adenine to inosine (read as guanine by the ribosome), or cytidine deaminases, which convert cytosine to uracil [89, 111, 113, 114]. Formation of isomiRs has also been attributed to nucleotidyl transferases and exonucleases which extend (adenylate and uridylate) or trim the 3' end [93, 96, 111, 115].

The biological significance of isomiRs has not yet been exhaustively characterized. Nevertheless, many studies have shown that these molecules are loaded into the RISC and thus maintain functionality [113, 116–118]. Changes in miR sequences leading to changes at the 3' end are associated with impacting miR processing (strand selection), stability and efficiency of target repression [94, 111, 115]. Changes at the 5' end alter the seed sequence (seed-shifting), which can lead to drastic changes in target repertoire and potentially distinct functional consequences in comparison to the canonical form [111, 119]. Accordingly, most frequently observed isomiRs have variation at the 3'end, while 5' ends are more conserved [89].

IsomiR nomenclature

IsomiR nomenclature follows the rules defined in *miraligner*, a command-line tool for isomiR mapping and annotation [120, 121]. Here, miR name is followed by the changes the miR contains at the four isotype positions i.e. miR name: within seq non-templated change : 3' end non-templated change : 5' end templated change : 3' end templated change. IsomiR nomenclature is better illustrated in Figure 1.7 with miR-21-5p as an example.

*
*
*

Figure 1.7: Isotypes and isomiR nomenclatures. IsomiRs can be categorised into 4 isotypes: 5' end templated (row 3, 4 in the figure), 3' end templated (row 5, 6), 3' end non-templated (row 7) and within sequence non-templated (row 8). IsomiR nomenclature is demonstrated in the above figure using miR-21-5p as an example. **IsomiR annotation** follows-miR name: within sequence non-templated change : 3' end non-templated change : 5' end templated change : 3' end templated change. Uppercase represents base additions, lowercase represents base deletions. For within sequence non-templated changes, the number is the position where substitution occurs, followed by the new base and the original base. Figure adapted with permission from Ms M Drozdz (2019) [122].

1.2.4 microRNA species and cancers

MiRs are estimated to regulate more than 60% of all protein-coding genes in mammals [109]. Due to their important regulatory function, several studies have already demonstrated an association of miR dysregulation with various diseases, including different types of cancers. Focusing on cancers, over 50% of miR-encoding loci are found in cancer-associated genomic regions or fragile sites [123]. As such, there is a wide dysregulation of miR expression in tumour growth and progression. One of the first studies to demonstrate the association between miRs and cancer was conducted by Calin *et al.* in 2002, where they showed deletion or down-regulation of miR-15 and miR-16 in B-cell chronic lymphocytic leukaemia [124]. Subsequently, the group confirmed that miR-15 and miR-16 induced

apoptosis by targeting anti-apoptotic factor *BCL2* in leukaemia [125]. Since then, a large number of studies have examined and reported dysregulation of miRs, including isomiRs in various cancers [126–134]. The role of miRs in cancers is tissue-specific and context-dependent so they can function as both oncomiRs or tumour suppressors. For example, miR-125b is upregulated in leukaemias, pancreatic and prostate cancers and considered to have oncogenic activity; while it is downregulated in many solid tumours such as bladder, ovarian and breast cancers, and considered to have a tumour suppressor role [135, 136]. The association of dysregulated miR species (miRs and isomiRs) with tumour growth, progression and treatment response have also been reported in various cancers [132, 133, 137–145]. Their tissue-specific properties, as well as an association with tumour growth, disease outcome and treatment response, have led to the hypothesis that miR species have great potential as disease biomarkers and therapeutic targets.

1.2.5 Properties of microRNA species

MiR species have properties that make them ideal biomarker candidates for investigation. Firstly, as mentioned above, the expression profile of these molecules are tissue-specific, allowing for disease specificity. They also show different expression profile between tumour and normal tissues at different stages of tumour progression and in response to treatment; this can be utilised for an indication of disease state or monitoring/ predicting disease and treatment outcomes.

Secondly, miR species are much more stable than mRNAs. They have been estimated to be highly stable with an average half-life from several hours to as long as 5 days in-vivo, higher than mRNAs [109]. They are also stable in storage in fresh-frozen paraffin-embedded samples compared to mRNAs [146, 147].

Thirdly, miRs exist stably in cell-free form in circulation (serum and plasma), potentially by incorporating themselves into microvesicles (exosomes and apoptotic bodies) or associating with other molecules to form RNA-protein complexes and thereby protecting themselves against RNAse activity

[130, 148, 149]. As circulating miRs (c-miRs) can originate from several sources, including normal tissues, blood cells and tumour tissues, it is still unclear what proportion of c-miRs originate from tumour cells. There have been studies that have shown evidence for tumour-derived c-miRs. For example, Brase *et al.* showed high expression of miR-375 and miR-141 in both serum and tumour tissue samples of PCa patients [150]; Mitchell *et al.* demonstrated that tumour-derived miRs can be detected in circulation by developing tumour xenograft models injected with 22Rv1, an androgen-sensitive PCa cell line. They showed that miR-629-3p and miR-660-5p, expressed in 22Rv1 cell line but without known mouse homologs were readily detected in the plasma of xenografts but not in control mice [149]. These studies demonstrate that c-miRs can originate from tumours. C-miRs may be more useful biomarkers than tumour tissue-derived miRs. This is because they can provide more accurate information about the disease state as c-miRs avoid the heterogeneity one might encounter when sampling tumours directly. MiRs have also been detected in biofluids including urine and saliva [142, 149, 150]. Thus, stable detection of miRs in circulation and biofluids allow for non-invasive sample collection in the clinics.

Finally, compared to protein-based biomarkers, miRs have a specific advantage in that low abundance miRs in samples can be readily amplified and profiled using technologies such as quantitative real-time PCR, small RNA sequencing and miR microarrays and do not require the development of detection agents or antibodies (as needed for protein biomarkers) [151]. All these properties of miR species make them excellent biomarker candidates for investigation.

1.2.6 microRNA species in prostate cancer

One of the first studies to profile the expression of miRs in PCa patients was done by Porkka and colleagues in 2007 [152]. They identified 51 miRs with differential expression between BPH (n=4) and PCa tissue samples (n=9). Since then, various studies have profiled miR expression in PCa tissues and identified miRs with diagnostic and prognostic potential. Szczyrba and colleagues used deep sequencing to compare miR profiles of primary tumour (n=10) and normal prostate tissues (n=10)

[153]. They reported 33 differentially expressed miRs; this included significant upregulation of miR-375, miR-148a and miR-200c, and significant downregulation of miR-145 and miR-143 in prostate tumours. Schaefer *et al.* compared normal and matched tumour tissue samples (n=76) and reported 15 differentially expressed miRs. Of these, upregulated miR-183 and downregulated miR-205 classified 84% of tumour samples correctly, showing diagnostic potential, whilst upregulated miR-96 correlated with cancer recurrence, showing prognostic potential [154]. Besides tissues, researchers have also profiled and compared miRs extracted from serum and plasma. In 2008, Mitchell *et al.* profiled miRs in the serum of healthy (n=25) and metastatic PCa (n=25) patients and identified miR-141, which is significantly overexpressed in tumour samples, to have the greatest potential as a diagnostic biomarker [149]. Brase *et al.* compared serum-derived miRs between localised PCa (n=14) and metastatic samples (n=7) and identified overexpressed miR-141 and miR-375 as markers for high-risk tumours [150].

Similarly, isomiR expression profiles have also been studied in PCa. IsomiRs were first reported to be detected in PCa in a study by Watahiki *et al.* in 2011 [155]. However, these molecules were not further explored until recently. In 2016, Koppers-Lalic and colleagues examined miR species extracted from urine and showed that isomiRs of miR-21, miR-204 and miR-375 were significantly dysregulated in PCa patients (n=9) compared to healthy controls (n=4) [142]. The group also showed that isomiRs of these three miRs had improved diagnostic performance compared to their canonical counterparts. Magee *et al.* also characterised and demonstrated significant dysregulation of isomiR profiles in PCa [156]. In addition, they reported isomiRs that were significantly dysregulated between tumour and normal samples exclusively in black and white cohorts, demonstrating race-specific property of isomiRs.

Besides dysregulation of miR species at various stages of PCa, there is also growing evidence for dysregulation of miRs in response to treatments, which thus, could be novel biomarkers for therapy response in PCa [130, 148]. The PhD work by Dr Akifumi Shibakawa from the Bevan lab also identified eight circulating miRs (miR-132-3p, miR-141-3p, miR-200a-3p, miR-200c-3p, miR-210-3p, miR-30a-5p, miR-34a-5p and miR-375) which were significantly upregulated in responders to cabazitaxel

chemotherapy (n=21) in comparison to non-responders (n=21) [157]. Zhang and colleagues showed that oncomiR miR-21 expression levels were significantly higher in CRPC patients than androgen-dependent patients [158]. They additionally showed, with a small sample size of 10 patients, CRPC patients (n=4) resistant to docetaxel-based chemotherapy had higher levels of miR-21 in comparison to drug-responsive patients (n=6) [158]. Similarly, in a study by Lin *et al.* higher levels of miR-200c and miR-200b, and lower levels of miR-146a were observed in non-responders to docetaxel treatment (n=36) in comparison to responders (n=61) [159]. Although validation of these miRs in a separate cohort did not yield a significant association to docetaxel response, these studies highlight validation of findings in external datasets, along with the biomarker performance, as important factors to consider in biomarker development [160].

The literature is filled with studies that have successfully characterised the expression of miR species and identified potential miR based biomarkers in tissues and biofluids at different stages of prostate disease progression and in response to treatment. However, a huge caveat to these studies and a major problem in miR biomarker discovery is that these studies often report conflicting and/ or inconsistent findings. This could be due to various limitations such as different study design, protocols, low sample numbers and clinical heterogeneity in patient samples. As such, there is no general consensus of robust miR candidates for further investigation as biomarkers in PCa. Thus, although these discovery research are promising, larger prospective studies and validation of findings in independent datasets are required before their approval for investigation as biomarkers in clinical trials.

1.2.7 miRNA-27a-3p and prostate cancer

MiR-27a-3p is a part of a cluster of three miRs: miR-23a, miR-27a and miR-24-2, located on chromosome 19p13.1. It is a well-studied miR that has been termed an "oncomiR" due to its upregulation and pro-survival role in various cancers. In ER-negative breast cancer MDA-MB-231, miR-271-3p over-expression has shown to increase cell proliferation by suppressing *ZBTB10* and *Myt-1*, which are important for cell cycle progression at G2/M stage [161]. In gastric cancer and osteosarcoma

cell line models, elevated expression of the miR promoted proliferation, migration and invasion; these studies showed that miR-27a-3p conferred its pro-survival activity by targetting genes *SFRP1* via the wnt/B-catenin signalling pathway in gastric cancer and *MAP2K4* in the JNK signalling pathway in osteosarcoma [162, 163]. In a lung cancer cell model, overexpression of miR-27a-3p promoted proliferation by downregulating transcription factor *FOXO1*, and promoted the G1/S cell cycle transition by decreasing the cell cycle inhibitors p21 and p27 and increasing the cell cycle regulator cyclin D1 [164]. Similarly, the miR-27a-3p expression was elevated in ovarian cancer and overexpression of the miR in HO8910 and OV90 cell models promoted migration and invasion by targeting the transcription factor *FOXO1* via the Wnt/ b-catenin signalling pathway [165]. Additionally in ovarian cancer, miR-27a-3p expression may lead to the development of chemotherapy resistance partly by targeting *HIPK2*, a tumour suppressor involved in suppressing *VEGF* activation and inducing apoptosis [166]. These studies demonstrate the role of miR-27a-3p in promoting EMT/ disease progression and confer an oncomiRic role in solid cancers.

MiR-27a-3p has also been reported to be frequently dysregulated in PCa, however, its direction of dysregulation is inconsistent between studies. A study by Porkka *et al.* showed that miR-27a-3p levels were significantly downregulated in PCa tissue samples (n=9) in comparison to BPH samples (n=4) [152]. Similarly, Wan *et al.* demonstrated significant downregulation of miR-27a-3p in prostate tumours compared to normal samples in three publicly available datasets [167]. In contrast, Volinia *et al.* reported miR-27a-3p levels to be significantly upregulated in prostate tumours (n=56) compared to normal tissues (n=7) [126]. Nam *et al.* also found miR-27a-3p upregulation in patients who developed metastasis after RP (n=19) in comparison to non-recurrent cases (n=19) [168]. Upregulation of miR-27a-3p has also been reported in sera of PCa patients [169, 170]. Additionally, miR-27a-3p has also been identified as a biomarker and included in miR panels for PCa diagnostics and prognostication [168, 170].

Due to its frequent dysregulation at various stages of PCa, miR-27a-3p is of particular interest to

the Bevan lab; it has been investigated for its potential as a biomarker and a therapeutic target by members of the lab. Work done by Fletcher *et al.*, from the Bevan group, into the mechanism of miR-27a-3p in PCa, showed miR-27a-3p and AR (a steroid hormone receptor which is a key driver of growth in PCa carcinogenesis and progression, (Section 1.1.3)) regulate each other in a positive feedback loop mechanism [171]. Specifically, they demonstrated that AR induced transcription of the miR-23a/27a/24-2 cluster, encoding miR-27a-3p, as well as increased processing of the primary-mir-23a/27a/24-2 cluster by Drosha, resulting in an increase in mature miR-27a levels. In turn, miR-27a-3p suppressed the AR co-repressor Prohibitin, leading to an increase in AR activity [171]. Chromatin immunoprecipitation assays demonstrated enriched AR binding to the regulatory regions of miR-27a-3p encoding DNA in the presence of androgens, culminating in an upregulation of miR-27a-3p expression in androgen-dependent LNCaP cell lines [172]. The inconsistent reporting of the diagnostic/prognostic capabilities of miR-27a-3p expression between studies may be reflective of miR-27a-3p regulation by AR. As such, miR levels may change with respect to AR status and activity as prostate tumours progress and transition from hormone-dependent to castration-resistant phenotype.

Even though miR-27a-3p has been reported to be dysregulated in PCa by various publications, its precise role in PCa biology and progression remains yet to be fully elucidated. Its frequent dysregulation in clinical samples, implication with a key driver of PCa (AR), and potential as a therapeutic target highlight its importance in PCa and prompt the need for a thorough investigation of its role in PCa.

1.3 Hypothesis and Aims

The shortcomings of current clinical prognostic markers have lead to an over-treatment of a high proportion of patients with indolent tumours and the absence of reliable prognostic biomarkers for predicting treatment response have led to inappropriate treatment strategies and a high rate of disease relapse in patients with aggressive disease. These problems highlight the need to develop more effective prognostic biomarkers in PCa that can predict disease progression more reliably and aid clinicians in

devising patient-specific personalised treatments and therapies. MiR species present ideal candidates for investigation as prognostic biomarkers for PCa. In this study, I hypothesised that:

i) dysregulated miR and isomiR profiles in the prostate are associated with prostate malignancy

ii) dysregulated miRs and isomiRs in PCa provide additional value as prognostic biomarkers in comparison/ addition to the standard clinical markers.

With these hypotheses, I aimed to:

i) elucidate the role of putative oncomiR miR-27a-3p and its mechanism of action in PCa

ii) identify consistently reported prognostic miRs in PCa by systematically reviewing all relevant publications in the scientific literature to date

iii) assess and compare the prognostic performance of miR species in predicting disease progression with the performance of clinical prognostic markers.

Chapter 2

Identification of prognostic miRNA biomarkers in prostate cancer

2.1 Background

The first extensive miR expression profiling in prostate cancer (PCa) cell lines, xenograft samples and clinical tumour samples was published by Porkka *et al.* in 2007 [152]. Since then, numerous studies have characterised miR expression profile in PCa tissues and biofluids at various stages of the disease and examined their prognostic potential [142, 150, 151, 153, 154, 173–178]. A major caveat to these studies is that they often report inconsistent results, possibly due to differences in study designs, methodologies, tumour content and clinical diversity. Thus, there is no consensus to date on the miRs that truly associate with disease progression and have the potential to be utilised as a prognostic biomarker for PCa. Attempts at meta-analyses to combine results from multiple studies and appraise the current miR biomarker landscape are limited to only a handful of publicly available datasets [179]. A systematic review, which does not require the disclosure of sensitive clinical data, may be more useful in examining prognostic miR biomarker landscape in PCa and subsequently identifying consistent patterns across the studies. There has been no systematic review covering the topic of prognostic miR biomarkers in PCa as yet.

In this chapter, I aimed to review the relevant publications in the scientific literature to date and identify consistently reported miRs with potential as prognostic biomarkers in PCa. Firstly, a systematic review was performed on studies that investigated the prognostic potential of individual miRs or miR panels in PCa. Here, a broad, comprehensive approach was taken in which any publications evaluating prognostic miRs were included, irrespective of methodological or clinical diversity such as differences in study design, profiling technologies, sample source or clinical trial endpoints. The findings of the review revealed a considerable number of publications that examined the association of tumour tissue-derived miRs with biochemical recurrence (BCR) in patients who have undergone radical prostatectomy (RP). The only meta-analysis addressing prognostic miRs in PCa was performed in 2017 [179]. Thus, secondly, to account for any new public datasets after the first and only meta-analysis, an updated meta-analysis was performed on studies with publicly accessible global miR expression datasets. Based on the results of the systematic review, the aim was redefined to

focus on identifying miRs that are prognostic of BCR in patients that have undergone RP. Here, only tissue-specific miRs were considered as the majority of publications in the systematic review ($\sim$ 88%) addressed tissue-derived miRs. This approach minimised possible heterogeneity introduced from considering different sample types.

2.2 Methods

2.2.1 Methodology for systematic review

Search strategy

A methodological search of electronic database PubMed was performed in order to identify relevant studies published between January 2007 and December 2019. The search was conducted on 24th of January 2020. The keywords searched were "**prostate cancer microRNAs prognosis relapse outcome**". This search included both free words and MeSH terms, which ensured all publications with the keywords and related terms in their title or body were included in the search result. The MeSH term associated with the keywords were:
("micrornas"[MeSH Terms] OR "micrornas"[All Fields] OR "mirnas"[All Fields] OR "miRs"[All fields] OR "microrna"[All Fields] OR "mirna"[All Fields] OR "miR"[All fields]) AND ("prostatic neoplasms"[MeSH Terms] OR ("prostatic"[All Fields] AND "neoplasms"[All Fields]) OR "prostatic neoplasms"[All Fields] OR ("prostate"[All Fields] AND "cancer"[All Fields]) OR "prostate cancer"[All Fields]) AND ("prognosis"[MeSH Terms] OR "prognosis"[All Fields] OR "recurrence"[MeSH Terms] OR "recurrence"[All Fields] OR "relapse"[All Fields] OR "mortality"[Subheading] OR "mortality"[All Fields] OR "survival"[All Fields] OR "survival"[MeSH Terms] OR "outcome"[All Fields]).

Study eligibility

Studies were selected according to the following criteria:

i) the study measured the expression of miRs in tissues or biofluids (circulation, urine, saliva) of PCa patients (not xenograft or other animal models)

ii) the study investigated the association of miRs with outcome with a survival analysis: Cox proportional hazards (Cox PH) regression model or Kaplan-Meier (KM) analysis, and appropriate test statistics such as hazard ratio (HR), associated 95% confident intervals (CI) and log-rank p-values were reported in the main text or supplementary section.

Studies were excluded if:

i) the study tested the prognostic role of miR host genes or target genes instead of the miR itself

ii) the study tested the prognostic role of miR in combination with non-miR markers such as clinical factors, genes or proteins

iii) the study was in a different language with no English translation available

iv) the study was a meta-analysis, review, comment, letter or duplicate publication.

Using the criteria described above, the title and abstract of the studies obtained from the keyword search were screened. Studies that were clearly not relevant to the review were removed. This was followed by full-text screening to identify studies with relevant prognostic information. This systematic review was conducted in accordance with the (PRISMA) guidelines [180].

2.2.2 Data extraction

The following data were extracted from each eligible study: EntrezUID, surname of first author, year published, title, miR/s investigated, sample size, sample type, detection method, outcome endpoint, endpoint definition, test type (Cox PH/ KM), effect estimates: HR, 95% CI or log-rank p-value, Cox PH test type (univariate/ multivariate), adjusted variables (if multivariate Cox PH). If the study performed both Cox PH model and KM analysis, only the results for Cox PH model was extracted.

Statistical analysis

For the miRs that had multiple entries and the same endpoint, and had their Cox PH test statistics reported, a meta-analysis was performed to calculate the summary effect size (pooled HR). For the miR entries originating from the same study, a fixed-effects model (FEM) approach was employed

for the meta-analysis. For the miR entries from different studies, we expected the true effect size to vary across studies due to biological and technological diversity; therefore, a random-effects model (REM) approach was employed. Low miR expression was set as the reference group, so for entries with high miR expression as the reference group, reciprocal of HR and 95% CI were calculated. Reference group standardisation allowed consistent interpretation of HR between all studies that have performed Cox PH: miRs with HR > 1 had a negative association, miRs with HR $= 0$ had no association, and miRs with HR < 1 had a positive association with disease outcome. Between study heterogeneity was assessed using Cochran's Q-test and Higgins I^2 statistic. Significance for the Q-test was set to p-value < 0.05. As the number of studies considered in the meta-analysis for each miR was very low publication bias was not assessed. The meta-analysis and heterogeneity tests were performed in statistical software *R* using package *metafor* (version 2.4.0) [181].

MiRNA annotation

As the search spanned across more than a decade, the miR annotation was outdated in many of the studies. This is due to growing research in the last decade or so providing a better understanding of miR biogenesis, evolution and functionality, and discoveries of novel miRs, requiring revision and update of miR annotations [182]. For such cases, the article was screened in order to obtain strand information for the miR of interest. If strand information was not stated in the article, the miR was assumed to be the dominant strand. The miR name was then cross-referenced with its entry in the miRBase database and updated to the most recent version (version 22) [91]. MiR names were left unchanged if the dominant/ passenger strand in miRBase was not specified.

2.2.3 Methodology for meta-analysis

Search strategy

The meta-analysis employed a search strategy similar to the systematic review. A methodological search of electronic database PubMed was performed in order to identify relevant studies published between January 2007 and December 2019. The search was conducted on 23rd of April 2020. The

keywords searched were "**prostate cancer relapse microRNA expression**". The MeSH term associated with the keywords were:

("prostatic neoplasms"[MeSH Terms] OR ("prostatic"[All Fields] AND "neoplasms"[All Fields]) OR "prostatic neoplasms"[All Fields] OR ("prostate"[All Fields] AND "cancer"[All Fields]) OR "prostate cancer"[All Fields]) AND ("recurrence"[MeSH Terms] OR "recurrence"[All Fields] OR "relapse"[All Fields]) AND ("micrornas"[MeSH Terms] OR "micrornas"[All Fields] OR "mirna"[All Fields]) AND ("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields] OR "expression"[All Fields])

Study eligibility

Studies were selected according to the following criteria:

i) the study measured miR expression in tissues of PCa patients who underwent RP and no other curative therapy (no studies with miR profiled in circulation)

ii) the study generated global miR expression profile dataset which was available in public data repositories

iii) the study contained follow-up data, i.e. BCR status of patients and time to BCR.

Studies were excluded if:

i) the study was in a different language with no English translation available

ii) the study was a meta-analysis, review, comment, letter or duplicate publication.

Using the criteria described above, title and abstract screening were performed on the studies obtained from the keyword search in order to remove any irrelevant articles. This was followed by full-text screening to select studies with public PCa miR expression data and clinical information. For studies with public expression datasets and insufficient follow-up information, corresponding authors were directly contacted for additional clinical information. Studies that investigated global miR expression profile without generating novel data were also included to examine if the datasets they used were

eligible for the meta-analysis. This meta-analysis was conducted in accordance with the (PRISMA) guidelines [180].

Data extraction and normalisation

Five studies, which included six datasets, were eligible for the meta-analysis (Table 2.5). The workflow for study selection and study characteristics of eligible datasets are described in results section 2.3.2. For The Cancer Genome Atlas - Prostate Adenocarcinoma (TCGA-PRAD) dataset, access for raw miR-sequencing (level 1) data was applied through the National Institute of Health database of Genotypes and Phenotypes (https://www.ncbi.nlm.nih.gov/gap/). Once access was granted, the raw miR-sequencing data and associated clinical data were downloaded from the Genomic Data Commons (GDC) data portal via the GDC data transfer tool (version 1.6.0) and Bioconductor package *TCGAbiolinks* (version 2.12.6) [183, 184]. Raw miR expression data was normalised using the trimmed mean of M-values (TMM) method using the *edgeR* package (version 3.26.8) [185]. MiRs were then filtered to include only those with normalised read counts ≥ 1 counts per million (cpm) in at least 80% of samples.

For rest of the datasets, normalised miR expression data and associated clinical data were obtained from the National Centers for Biotechnology Information Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). For GSE21036, clinical information was supplemented with data obtained from the data repository in the Memorial Sloan-Kettering Cancer Centre - computational biology centre website (https://cbio.mskcc.org/cancergenomics/prostate/data/). For GSE26245 and GSE26247, clinical information was supplemented with clinical data provided in the supplementary section of their corresponding paper [186]. For GSE46738 and GSE88958, the corresponding authors directly provided follow-up data (Leite K., written communication, 27 June 2018; Ozen M., written communication, 18 January 2019). The normalised datasets were then standardised according to z-score transformation. MiR annotation in each dataset was also updated to miRBase version 22 using package *miRBaseConverter* (version 1.8.0) [187].

Statistical analyses

In addition to follow-up information, clinical variables used for PCa risk stratification: PSA at diagnosis, Gleason score and clinical tumour stage, were available in five of the six datasets. Only, GSE88958 did not contain tumour stage information. Additionally, GSE21036 reported clinical tumour stage information, while the rest of the datasets reported pathological tumour stage information. To examine the prognostic potential of the miRNAs, firstly, a univariate Cox PH analysis was performed in each of the six datasets, where the only predictor being tested for association with disease relapse was miR expression. Secondly, a multivariate Cox PH analysis was performed in each of the five datasets with all three clinical variables available. Here, the Cox PH model included miR expression as the main predictor with PSA at diagnosis, Gleason score and tumour stage as confounders. Cox PH regressions were performed using R package *survival* (version 3.1.12) [188]. An analysis of variance (ANOVA), Kruskal-Wallis (KW) and Chi-squared (X^2) tests were also performed to test whether the distribution of the clinical variables differed between the datasets.

Following univariate/ multivariate Cox PH regression, a REM meta-analysis was performed to estimate the overall effect size (pooled HR) of the miRs across the studies. The meta-analysis was performed only for miRs that were present in all the datasets. Subsequently, a total of 162 and 164 miRs were evaluated for the univariate and multivariate analyses, respectively. The significance threshold was set at p-value < 0.05. Considering the low number of studies included in the meta-analysis, publication bias was not assessed.

2.3 Results

2.3.1 Prognostic miRNAs in prostate cancer: A systematic review

Study selection

A total of 992 studies were retrieved from the initial literature search. The title and abstract screening removed 800 non-relevant studies such as meta-analyses, book chapters, reviews and other irrelevant

publications. Full-text screening removed 64 studies for various reasons such as inaccessibility of full text, insufficient reporting of results, no prognostic test performed and containing mistakes such as incorrect CIs or female PCa sample population. Ultimately, 128 studies were eligible for the review. These studies included 215 entries for individually prognostic miRs (containing 120 unique miRs) and 18 entries for miR signatures panels (containing 8 unique miR signatures). The workflow for the study selection is detailed in Figure 2.1.

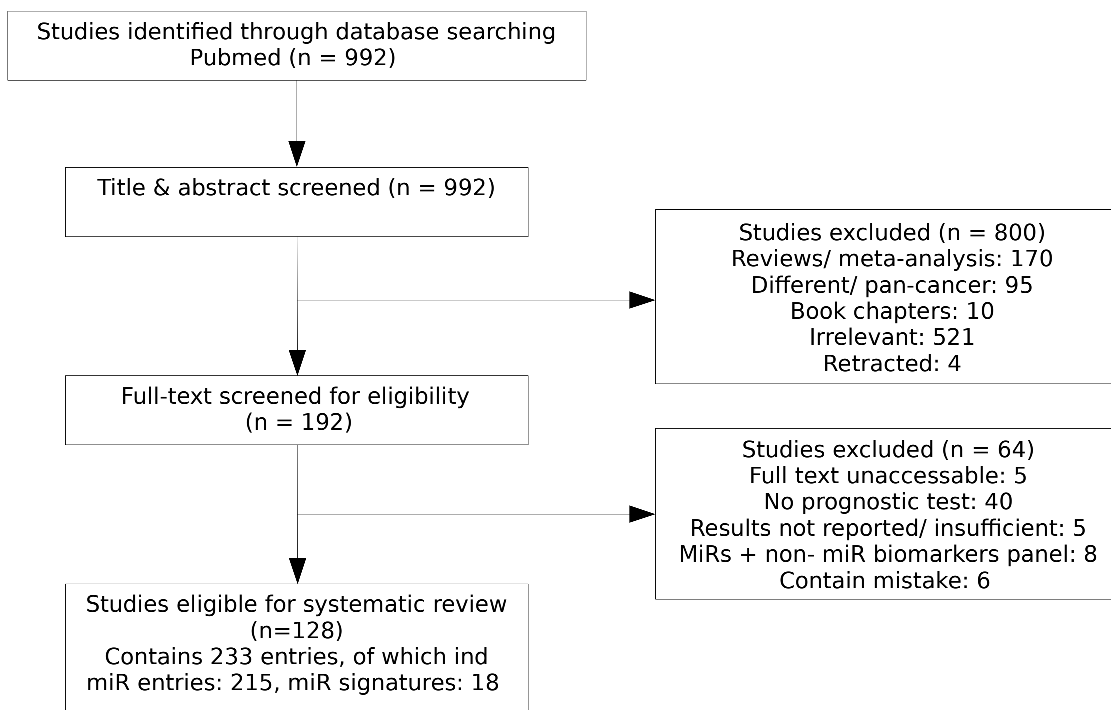


Figure 2.1: Workflow for selecting eligible studies for the systematic review.

Study characteristics

The majority of the miR biomarkers were detected using variations of the polymerase chain reaction (PCR) technique: quantitative PCR, reverse transcription-PCR and quantitative reverse transcription-PCR. Less frequent detection methods were microarrays, (small-)RNAseq, NanoString, *in situ* hybridization, mass spectrometry and BeadChip based technologies. This review included biomarkers extracted from a variety of sources such as tissues (n=204), blood (whole blood, peripheral blood, serum, plasma) (n=23), exosomes (n=2), urine (n=2) and cells (epithelial and stromal, fibroblast) (n=2). The prognostic ability of 178 entries was tested using Cox PH regression and 55 entries tested

using KM analysis and log-rank test. The different endpoints used by the studies are listed in Table 2.1. The most common endpoint used as a surrogate for progression was biochemical recurrence-free survival (BPFS) (44.64%), followed by overall survival (OS) (20.17%). The remaining endpoints each accounted for less than 10% of the studies in the review. A total of 177 entries (75.97%) had sample size ≥ 50. The median sample size was 93, and the range was 16 to 846. Only 14 entries did not report their sample sizes. The study characteristics, statistical results, endpoint definitions and additional variables included in the survival analysis (if a multivariate Cox PH was performed) are summarised in Appendix Tables B.1 and B.2 for individually prognostic miRs and in Appendix Tables B.3 and B.4 for prognostic miR signatures.

Endpoint	Abbreviation	No. of entries (%)
bone metastasis-free survival	bone MFS	8 (3.43)
biochemical progression/ recurrence-free survival	BPFS	104 (44.64)
clinical failure-free survival	CFFS	7 (3.00)
castration resistant prostate cancer-free survival	CRPC FS	6 (2.58)
cancer-specific survival	CSS	6 (2.58)
disease-free survival	DFS	11 (4.72)
disease-specific survival	DSS	2 (0.86)
metastasis-free survival	MFS	6 (2.58)
overall survival	OS	47 (20.17)
progression-free survival	PFS	5 (2.17)
percentage survival (survival rate)	PS	13 (5.58)
recurrence/ relapse-free survival	RFS	18 (7.73)

Table 2.1: Progression endpoints considered in the systematic review. There were 12 different endpoints considered. After verifying endpoint definitions in respective studies, endpoints with redundant meanings were categorised into the same group. If the studies did not provide definitions or the definitions were different between studies, endpoint with redundant meanings were not categorised together.

Individually prognostic miR biomarkers in PCa

There was a total of 215 entries in the systematic review that report 120 unique individual prognostic miR markers (Appendix Table B.1). Majority of the studies reported the association of miR expression with disease progression. Six studies reported the association of miR single-nucleotide polymorphisms (SNPs) or miR methylation with disease progression [189–194]. These were miR-146a-5p (rs2910164), miR-423-3p (rs6505162), miR-23a (rs3745453) and miR-605 (rs2043556) SNPs and miR-129-5p, miR-

205 and miR-34b/c methylation. Within the 120 unique miRs, 15 miR families and 12 miR clusters were represented. These miR families and clusters are reported in Tables 2.2 and 2.3. MiR-17 was the biggest miR family represented in the review with five miR members. Similarly, the miR-17/92 cluster was also one of the biggest clusters represented along with miR-183/96/182; both clusters had more than three members in the review.

mir family	miR members
let-7	let-7b-5p, let-7c
mir-10	miR-100-5p, miR-10b-5p
mir-130	miR-130b-3p, miR-301a-3p
mir-148	miR-148a-3p, miR-152-3p
mir-15	miR-15b-5p, miR-195-5p
mir-154	miR-409-3p, miR-410-3p
mir-17	miR-106b-5p, miR-17-5p, miR-20a-5p, mir-20b-5p, miR-93-5p
mir-182	miR-182-5p, miR-182-3p
mir-221	miR-221-3p, miR-222-3p
mir-23	miR-23a-3p, miR-23b-3p
mir-27	miR-27a-3p, miR-27b-3p
mir-34	miR-34b-3p, miR-34c-5p
mir-3622	miR-3622a-5p, miR-3622b-5p
mir-582	miR-582-3p, miR-582-5p
mir-8	miR-141-3p, miR-200b-3p

Table 2.2: The mir families represented within the individually prognostic miRs in the systematic review. In total, 15 mir families were represented in the review, of which mir-17 was the most represented miR family with five miR members.

miR cluster	miR members
miR-143/145	miR-143-5p, miR-145-5p
miR-17/92	miR-17-5p, miR-19a-3p, miR-20a-5p
miR-183/96/182	miR-182-5p, miR-182-3p, miR-183-3p, miR-96-5p
miR-221/222	miR-221-3p, miR-222-3p
miR-224/452	miR-224-5p, miR-452-5p
miR-23b/27b/24-1	miR-23b-3p, miR-27b-3p
miR-23a/27a/24-2	miR-27a-3p, miR-23a-3p
miR-34b/c	miR-34b-3p,miR-34c-5p
miR-3622a/b	miR-3622b-5p,miR-3622a-5p
miR-370/410	miR-409-3p, miR-410-3p
miR-424(322)/503	miR-503-5p, miR-424-3p
miR-106/25	miR-93-5p, miR-106b-5p

Table 2.3: The mir clusters represented within the individually prognostic miRs in the systematic review. In total, 12 miR clusters were represented in the review, of which miR-17 /92 and miR-183/96/182 were the most represented miR clusters with three and four miR members respectively.

There were a total of 44 unique miRs with multiple entries in the review. These miRs were either evaluated against different endpoints/ cohorts in the same study or were evaluated more than once in separate studies. Of these, 36 miRs had Cox PH output available. A REM meta-analysis was performed for these miRs against the same endpoint and their overall association determined (Figure 2.2). Seven miRs, let-7b-5p, miR-128a-3p, miR-188-5p, miR-224-5p, miR-23a-3p, miR-23b-3p and miR-34b/c, consistently and significantly associated with progression, irrespective of different end-

points. High expression of miR-34b/c and miR-23a-3p associated with disease progression, while high expression of rest of the miRs associated with favourable disease outcome. The Q-test for heterogeneity was not significant for these miRs (where meta-analysis was performed), suggesting an absence of heterogeneity between the datasets. However, Q-test has low power to detect heterogeneity when the number of datasets in the meta-analysis is low. The I^2 statistic, which also tests for heterogeneity, ranged between 0.00 - 4.30%, suggesting absence of statistical heterogeneity. Interestingly, for three miRs, miR-21-5p, miR-222-3p and miR-30c-5p, association with progression differed depending on the endpoints:

i) high miR-21-5p expression significantly associated with shorter time to BPFS but longer time to RFS

ii) high miR-222-3p expression significantly associated with shorter time to BPFS and RFS but longer time to CRPC FS

iii) high miR-30c-5p expression significantly associated with longer time to BPFS but shorter PS.

These inconsistent results may suggest these miRs have dual roles at different stages of disease progression. However, it is more likely these results are due to clinical and methodological heterogeneity between the studies such as different comorbidities, sample sizes, and endpoint definitions. A forest plot was also generated for the remaining miRs with single entries (Appendix Figure A1).

In summary, considering the effect sizes of both Cox PH and KM outputs for the 120 unique miRs, 57 miRs negatively associated with progression, 43 miRs positively associated with progression and 20 miRs had an inconsistent direction of association. Only four miRs, let-7b-5p, miR-152-3p, miR-195-5p and miR-224-5p, significantly and consistently associated with progression, irrespective of different endpoints, in multiple patient cohorts in the same study or at least two independent studies. Additionally, although insignificant, miRs-145-5p had a consistent trend in association with progression in five independent studies. Low expression of these five miRs associated with shorter time to disease progression. These miRs are reported in Table 2.4 and are the strongest prognostic biomarker

candidates for PCa based on current literature.

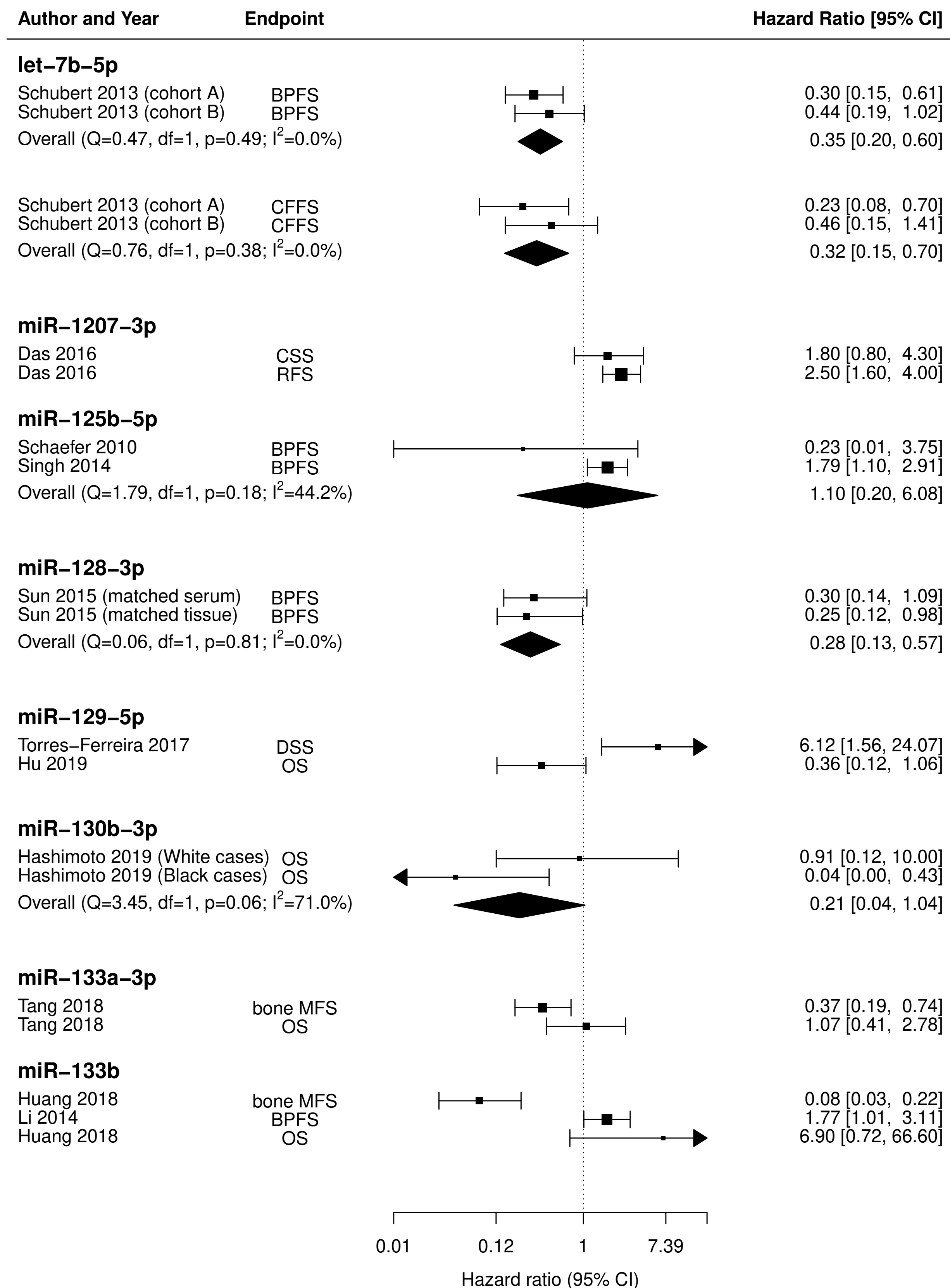
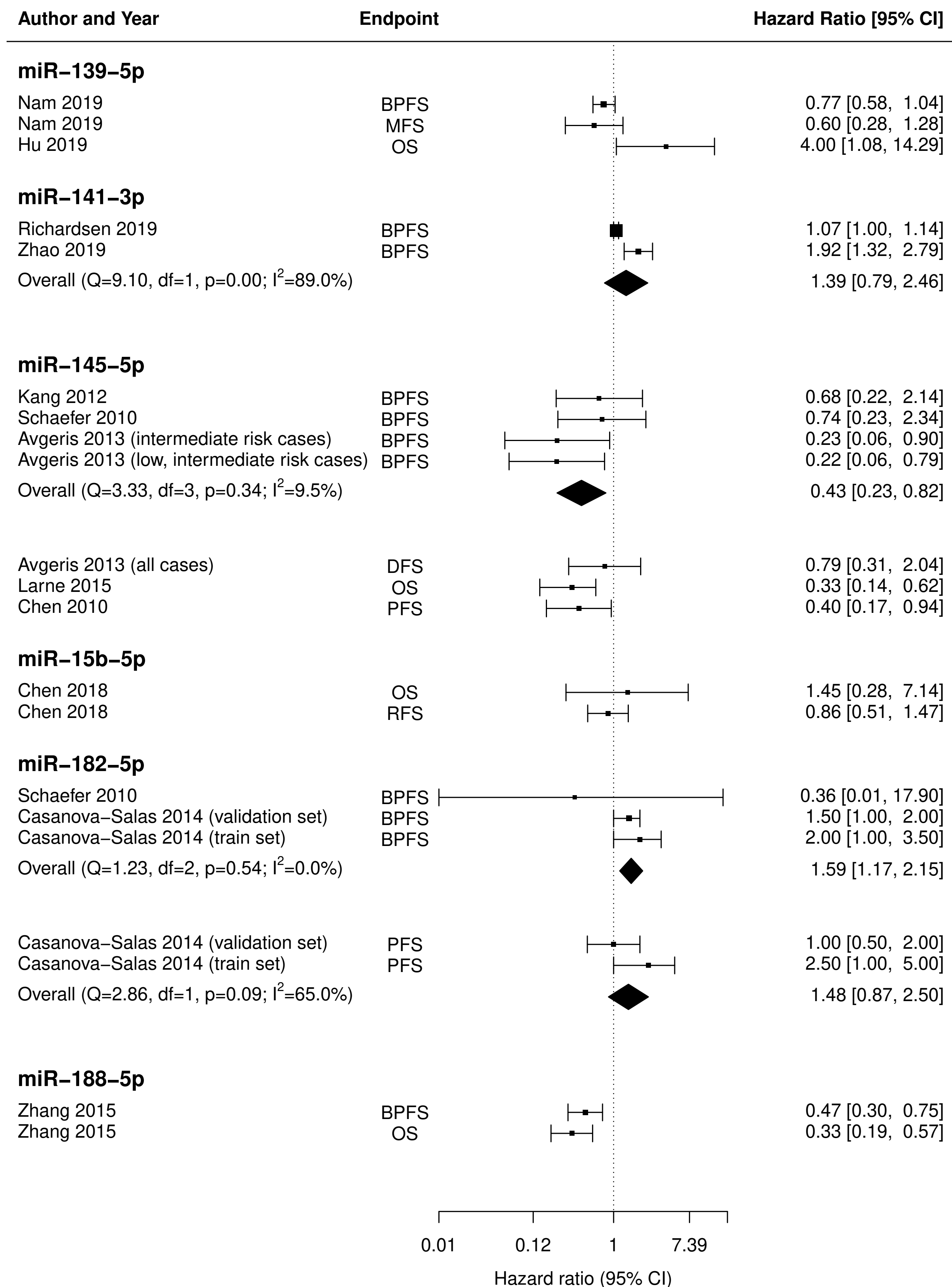


Figure 2.2: Forest plot for all miRs with multiple entries in the systematic review. For those miRs with multiple entries and the same progression endpoints, a random-effects model was performed to get an overall effect estimate (pooled hazard ratio). For the full form of the abbreviated endpoints, refer to Table 2.1. **Figure continued.**



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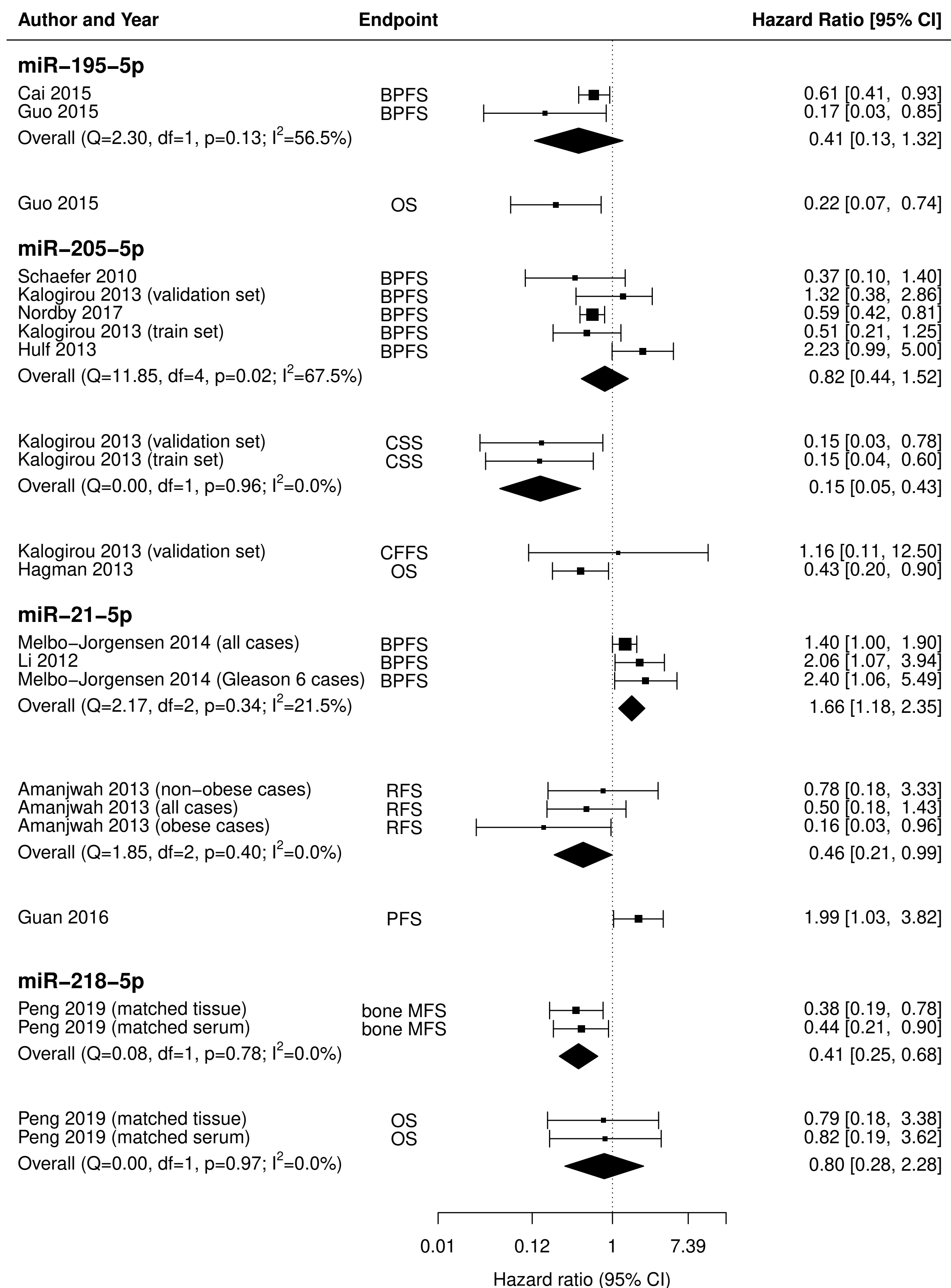

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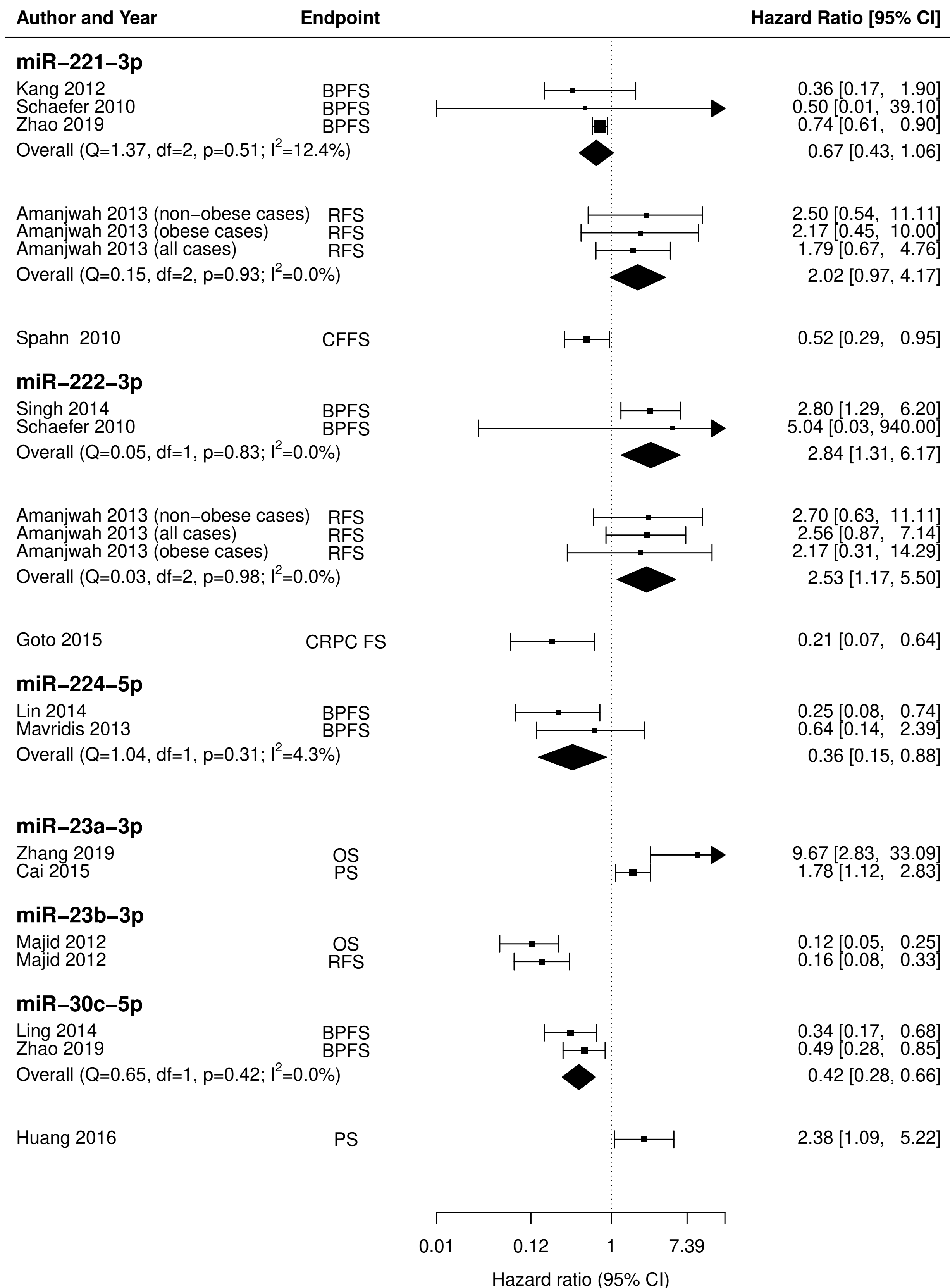

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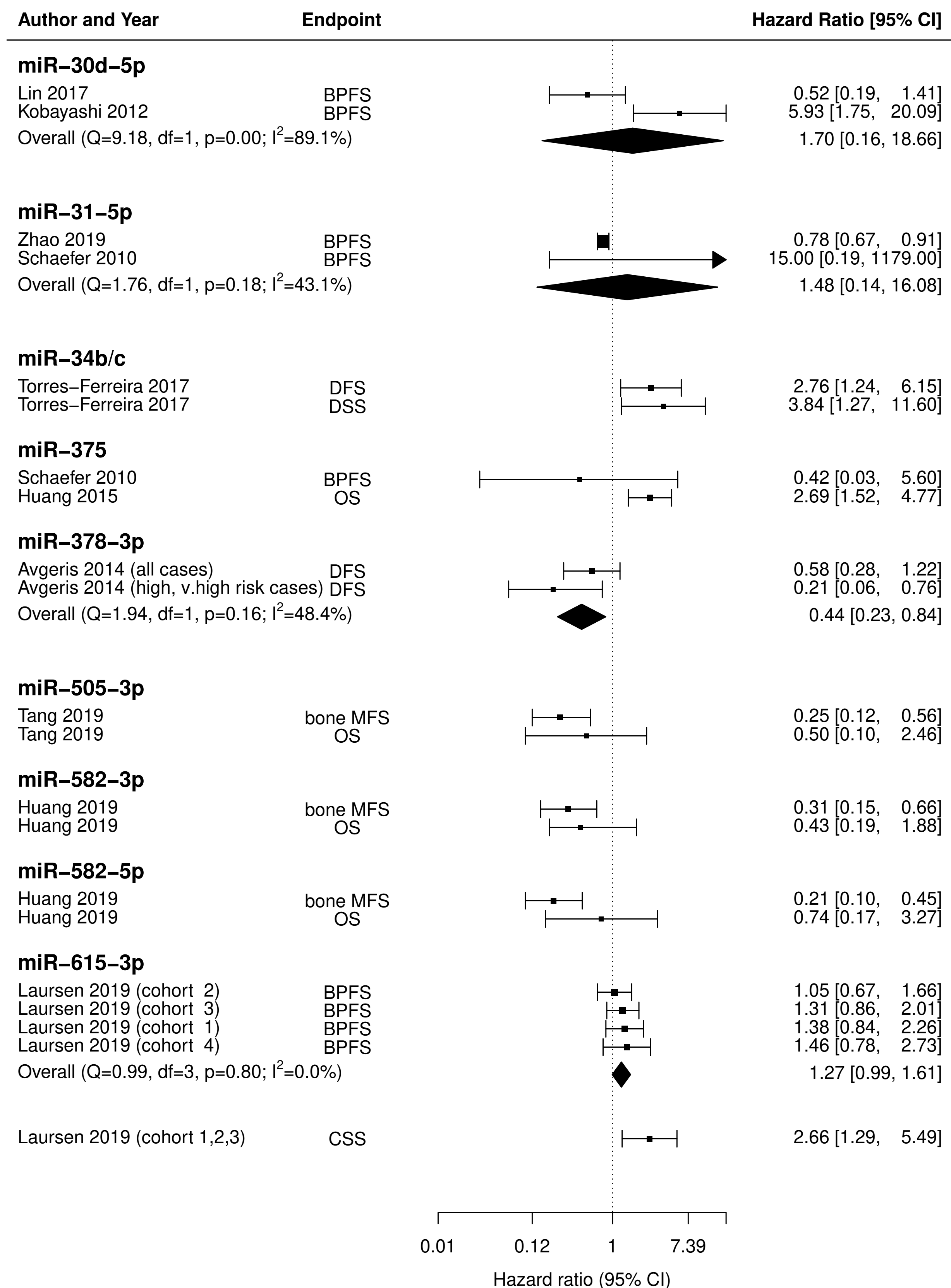

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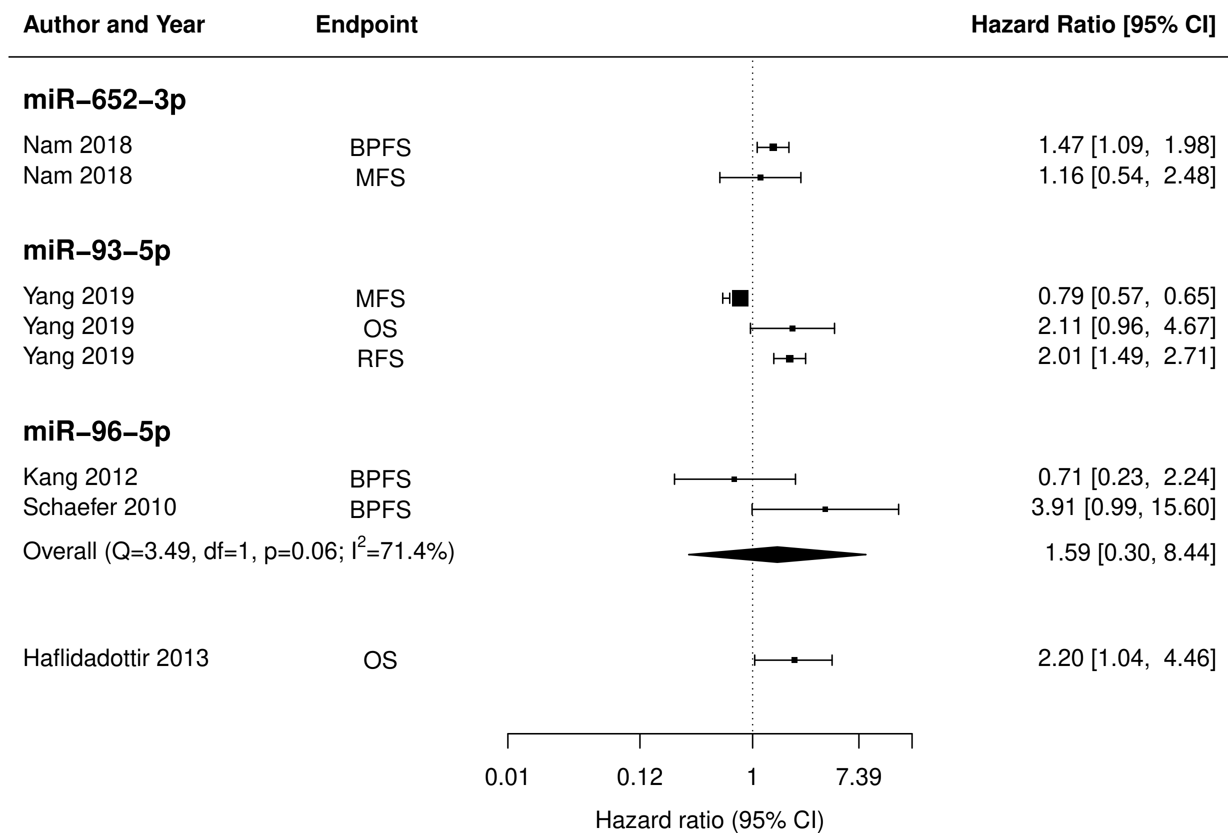


Figure 2.2: Forest plot for all miRs with multiple entries in the systematic review. For those miRs with multiple entries and the same progression endpoints, a random-effects model was performed to get an overall effect estimate (pooled hazard ratio). For the full form of the abbreviated endpoints, refer to Table 2.1.

Prognostic miR	Prognostic test: test: endpoint	HR (95% CI)	p	Reference group	Association after reference standardisation	Sample size	Sample type	PMID	Ref
let-7b-5p	multivariate: BPFS	0.44 (0.193-1.022)	0.05	low	negative	98 (cohort A)	tissue	23798998	[195]
	multivariate: BPFS	0.30 (0.15-0.61)	<0.010	low	negative	92 (cohort B)	tissue		
	multivariate: CFFS	0.23 (0.08-0.70)	<0.010	low	negative	92 (cohort B)	tissue		
	multivariate: CFFS	0.46 (0.15-1.41)	0.17	low	negative	98 (cohort A)	tissue		
miR-145-5p	multivariate: BPFS	4.47 (1.27-15.74)	0.020	high	negative	36 (low + intermediate risk)	tissue	23703249	[196]
	multivariate: BPFS	4.43 (1.11-17.61)	0.035	high	negative	29 (intermediate risk)	tissue		
	multivariate: PFS	0.40 (0.17-0.94)	0.036	low	negative	106	tissue	20332243	[197]
	univariate/ KM: OS	3.00 (1.60-7.00)	<0.010	high	negative	49	tissue	25969144	[198]
	univariate: BPS	0.74 (0.23-2.34)	0.609	low	negative	76	tissue	19676045	[154]
	univariate: BPFS	0.68 (0.22-2.14)	0.510	low	negative	73	tissue	22864280	[199]
	multivariate: DFS	1.26 (0.49-3.27)	0.629	high	negative	73	tissue	23703249	[196]
miR-152-3p	KM: BPFS	-	<0.001	low	negative	n/s (MSKCC)	tissue	25004396	[200]
	multivariate: DFS	0.23 (0.07-0.72)	0.012	low	negative	494 (TCGA)	tissue	29599847	[201]
miR-195-5p	multivariate: BPFS	5.96 (1.18-30.02)	0.031	high	negative	140	tissue	26338045	[202]
	multivariate: OS	4.46 (1.35-14.72)	0.014	high	negative	140	tissue		
	multivariate: BPFS	0.61 (0.41-0.93)	0.022	low	negative	107 (MSKCC)	tissue	26080838	[203]
	KM: BPFS	-	0.009	low	negative	131 (MSKCC)	tissue	30032144	[204]
	KM: RFS	-	0.049	low	negative	98 (MSKCC)	tissue	26650737	[205]
	KM: DFS	-	<0.010	low	negative	n/s (MSKCC)	tissue	27175617	[206]
miR-224-5p	multivariate: BPFS	0.25 (0.08-0.74)	0.010	low	negative	114	tissue	24382668	[207]
	multivariate: BPFS	0.64 (0.14-2.39)	0.525	low	negative	58	tissue	23136246	[208]

Table 2.4: The miRs with consistent direction of association to disease progression, irrespective of different endpoints, that have been validated in multiple cohorts or independent studies. The KM, univariate and multivariate tests stand for Kaplan-Meier analysis, and univariate and multivariate Cox PH regressions respectively. For the test entry"univariate/ KM", both univariate Cox PH and KM analysis were performed, but the p-value for the univariate Cox PH regression was not reported. Thus, the HR and 95% CI corresponds to outputs of the univariate Cox PH regression and the p-value corresponds to the KM log-rank test. For studies that performed multivariate analysis, the different variables adjusted for are reported in Table B.2. The values in the "Prognostic test" and "Reference group" columns refer to the statistics and the reference group used for comparison as reported in respective papers. In contrast, the "Association after reference standardisation" column refers to the association of the miRs to progression after standardising the comparisons to "low" miR expression as the reference group. n/s represents not-specified.

Prognostic miR signatures as biomarker panels in PCa

Eight prognostic miR signatures, comprising of 36 unique miRs, were reported as prognostic in eight independent studies (Appendix Table B.3). The majority of these studies performed independent clinical validations and/ or have large sample sizes ($\gtrsim 100$), making their findings robust. Interestingly, only Feng *et al.* (2017) investigated a panel of miRs that were biologically related, i.e. the miRs in the signature panel were part of miR-17/92 cluster [209]. The remaining studies grouped miRs into signature panels if they were significantly differentially expressed between recurrent and non-recurrent cases or had significant predictive power to distinguish between recurrent and non-recurrent cases.

Within the eight signatures, only miRs let-7a-5p and miR-223 were present in multiple miR signatures. In Mihelich *et al.* [210], both miR-223 and let-7a-5p were grouped into a panel with five other miRs as their expression levels were significantly down-regulated in recurrent patients compared to non-recurrent patients. In Nam *et al.* [211] miR-223, and in Fredsoe *et al.* [212] let-7a-5p, were grouped into signature panels for their predictive power to significantly distinguish between recurrent and non-recurrent PCa cases. Interestingly, although prognostic as part of miR signatures, neither let-7a-5p nor miR-223 has been reported as individually prognostic predictors. However, 16 out of the 36 unique miRs in the signature panels (miR-10b-5p, miR-130b-3p, miR-139-5p, miR-145-5p, miR-17-5p, miR-19a-3p, miR-200b-3p, miR-20a-5p, miR-221-3p, miR-23a-3p, miR-301a-3p, miR-326, miR-374b-5p, miR-375, miR-652-3p and miR-96-5p) were reported as individually prognostic in multiple studies (Appendix Table B.1). For 11 out of the 16 miRs (miR-10b-5p, miR-130b-3p, miR-145-5p, miR-17-5p, miR-19a-3p, miR-23a-3p, miR-301a-3p, miR-326, miR-374b-5p, miR-652-3p and miR-96-5p), the individual association with progression in corresponding studies were consistent with the direction of expression in signature panel studies. These 16 miRs also include three members of the miR-17/92 cluster panel (miR-17-5p, miR-19a-3p and miR-20a-5p) evaluated in the study by Feng *et al.* [209]. The consistent association of the miR-17/92 cluster and its members in independent studies suggest they have a biological role in PCa progression and support them as potential biomarker panel candidates for prognostication in PCa.

2.3.2 Identification of miRNA biomarkers for prostate cancer recurrence following radical prostatectomy: A meta-analysis of six public datasets

Study selection

A total of 185 studies were retrieved from the initial literature search. After title and abstract screening 164 ineligible articles such as meta-analyses, reviews and studies based on non-tissue datasets or non-PCa studies were removed. Full-text of the remaining articles were screened; 16 studies were removed as their datasets were not publicly available (n=7), did not have follow-up information (n=5), could not share clinical information due to patient confidentiality (n=2), contained inconsistent clinical information (n=1) or categorised as duplicate as it utilised public dataset already included in this meta-analysis (n=1). Ultimately, five studies, containing six datasets, were eligible for the meta-analysis. The workflow for the selection of studies is detailed in Figure 2.3 and the study characteristics are reported in Table 2.5. Two of the datasets, GSE21036 and TCGA-PRAD were publicly available datasets cited in Schaefer *et al.* (2010), while the rest of the datasets were novel data generated by the authors of the paper [154].

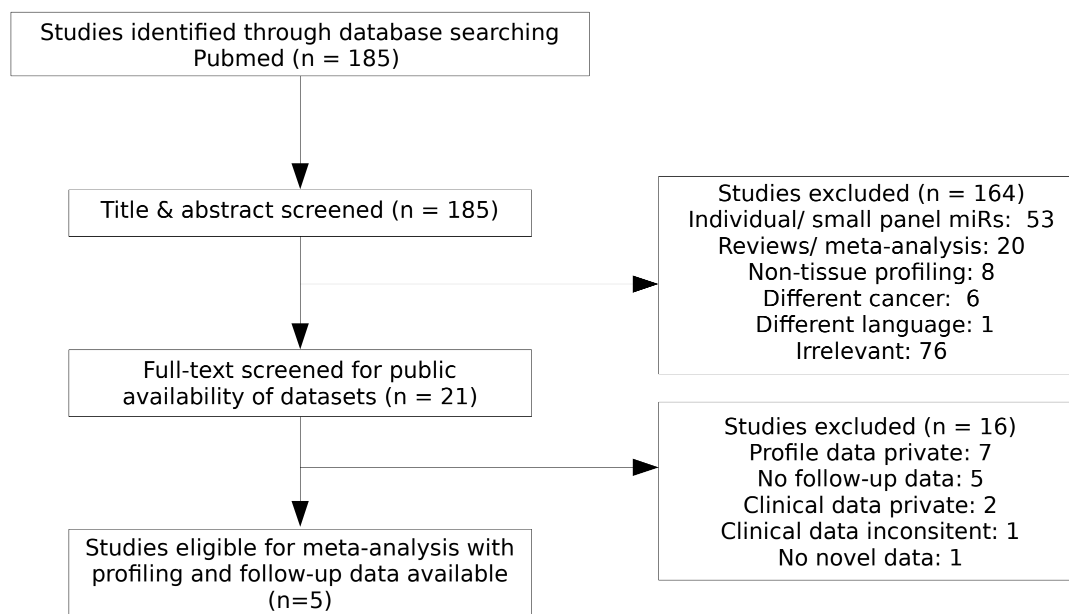


Figure 2.3: Workflow for selecting eligible datasets for the meta-analysis.

Author, Year	study ID	profiling technology	endpoint definition	sample collection	sample type	sample size with follow-up	No. of miRs	ref
Taylor, 2010	GSE21036	Agilent-019118 Human miRNA Microarray 2.0 G4470B	PSA $\geq$ 0.2 ng/ml on two occasions	RP	tissue	99	373	[31]
Long, 2011	GSE26245	Illumina Custom Prostate Cancer DASL Panel miRNA	two detectable PSA readings (> 0.2 ng/mL)	RP	tissue FFPE	63	733	[186]
Long, 2011	GSE26247	Illumina Custom Prostate Cancer DASL Panel miRNA	two detectable PSA readings (>0.2 ng/mL)	RP	tissue FFPE	40	1145	[186]
Leite, 2015	GSE46738	Affymetrix Multispecies miRNA-1 Array	PSA >0.2 ng/ml	RP	tissue frozen	51	847	[213]
Suer, 2018	GSE88958	Agilent 8x15 K Human V3 microRNA Microarray V3	PSA $\geq$ 0.2 ng/ml on two occasions	RP	tissue	30	851	[178]
TCGA	TCGA-PRAD	Illumina GAIIx or HiSeq 2000 miRNA Sequencing	PSA > 0.2 ng/ml at two or more occasions	RP	tissue frozen	349	328	[35]

Table 2.5: Characteristics of the studies included in the meta-analysis. These five studies contained six prostate cancer datasets eligible for the meta-analysis. All of which had global miR expression profiled from tumour tissue samples collected during radical prostatectomy. Abbreviations: FFPE=Fresh-frozen paraffin-embedded; PSA=Prostate specific antigen; RP=radical prostatectomy.

Sample characteristics of eligible datasets

MiRs were profiled from tissue samples collected from men who underwent RP in all datasets. The endpoint for the datasets was BCR, which was defined by majority of the datasets as a rise in serum PSA levels $\geq$ 0.2ng/ml on two or more occasions, as per the European Association of Urology (EAU) guidelines [214]. Only GSE36738 did not specify the number of rising PSA measurements required to classify a BCR event. As mentioned in Section 2.2.3, the majority of the datasets contained accompanying clinical variables: age at diagnosis, PSA at diagnosis, Gleason score and tumour stage. Only GSE88958 did not contain tumour stage information. The sample characteristics for these studies are provided in Table 2.6.

There were significant differences in age and PSA at diagnosis of patients between the six datasets (ANOVA $p < 0.05$ and KW test $p < 0.05$ respectively) (Figure 2.4a, 2.4b). There were also significant differences in the proportion of samples with different Gleason scores, tumour stages and BCR events between the datasets (X^2 test $p < 0.05$) (Figure 2.4c, 2.4d, 2.4e). TCGA-PRAD contained one of the highest proportions of aggressive tumour samples with Gleason scores $\geq$ 8 and T3+T4 stages at 40.88% and 60.47% frequencies, respectively. However, it contained the lowest median PSA at diagnosis (0.12 ng/mL) and the lowest proportion of samples experiencing BCR (14.55%). The majority of the samples in the TCGA-PRAD cohort originate from data centres in the US, a country that offers PSA screening to men between 55 and 69 years of age. This routine screening often leads to early diagnosis, especially of indolent tumours, and could potentially explain the younger age and low PSA levels at diagnosis seen in this cohort [56, 57]. Early detection could also lead to early intervention, thus explaining the low proportion of samples experiencing BCR in this cohort.

Similarly, GSE21036 also originated from the US. This dataset contained the youngest cohort with a median age of 57 years at diagnosis, the second-lowest median for PSA at diagnosis (5.6 ng/mL) and the second-lowest proportion of samples with BCR events (19.19%). It also had less aggressive cases compared to other datasets with Gleason scores $\geq$ 8 and T3+T4 stages at 12.24% and 30.30%,

respectively. The lower age and PSA at diagnosis, and lower proportion of BCR samples could be, again, due to the patients taking the PSA screening and early intervention.

GSE46738 contained the oldest cohort with a median age of 66 years at diagnosis. Accordingly, this dataset had aggressive disease with the highest proportion of Gleason scores ≥ 8 (44%) and second-highest T3+T4 proportion (50%), suggesting that patients in this cohort diagnosis had aggressive PCa due to diagnosis at a later age. The proportion of BCR samples in this cohort was not high (24%) compared to the rest of the datasets. This is probably because patients diagnosed at an older age with highly aggressive disease are usually offered passive treatments instead of curative treatments. The remaining three datasets: GSE88958, GSE26247 and GSE26245, generally had an older age at diagnosis (≥ 63 yrs) and low to medium levels of proportion of samples with aggressive histopathology compared to the rest of the datasets. These three datasets also had the top three highest median PSA level at diagnosis (≥ 8) and proportion of BCR samples ($> 30\%$) among the six datasets.

Characteristics		TCGA-PRAD	GSE88958	GSE46738	GSE26247	GSE26245	GSE21036
number of samples	**total**	433	30	50	40	63	99
	BCR	63	19	12	13	25	19
	non-BCR	370	11	38	27	38	80
follow-up time (months)	**median**	28.73	69.83	25.50	49.50	49.97	46.39
	range	0.00-165.17	1.38-118.16	1.35-120.20	1.00-164.00	0.99-163.99	1.35-128.42
PSA at diagnosis (ng/mL)	**$<$10**	364	12	36	14	41	80
	10-20	3	4	13	17	18	12
	$>$ 20	26	3	0	4	2	6
	NA	40	11	1	5	2	1
age at diagnosis	**median**	61	63	66	64	63	57
	range	41-78	40-75	49-77	49-82	45-79	37-83
tumour stage	**version**	pathological	NA	pathological	pathological	pathological	clinical
	T1	2	NA	0	0	12	0
	T2	168	NA	22	27	42	69
	T3	251	NA	28	10	6	25
	T4	9	NA	0	3	3	5
	$\geq$ 6	44	8	15	12	18	32
	7	212	17	13	18	39	54
	$\leq$ 8	177	5	22	10	6	12

Table 2.6: Sample characteristics of the datasets included in the meta-analysis. Abbreviations: BCR=Biochemical recurrence; NA=Not available;PSA=Prostate specific antigen.

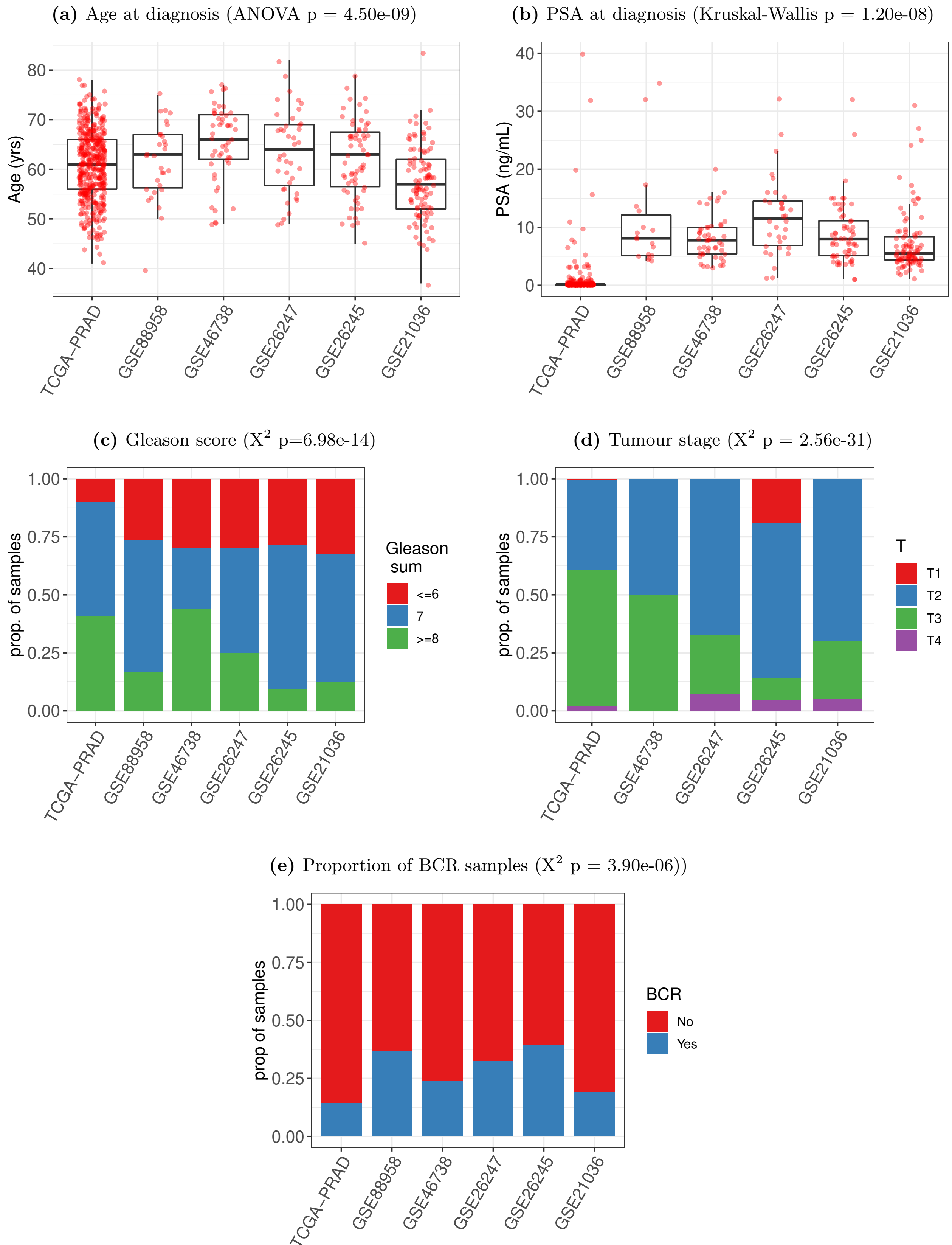


Figure 2.4: Clinical characteristics of the datasets included in meta-analysis. There were significantly different distributions between the five datasets for all five clinical variables examined. Abbreviations: ANOVA=Analysis of variance; BCR=Biochemical recurrence; PSA=Prostate specific antigen; X^2=Chi-squared test.

Association of clinical features with disease relapse

Whilst clinical features PSA level at diagnosis, tumour stage and Gleason score are established prognostic factors in PCa; age is one of the key risk factors strongly associated with PCa incidence and mortality. In the UK, $\sim$ 55% of PCa cases and $\sim$ 86% PCa specific mortality occurred in men aged 70 and over [1]. The association of these features with disease relapse, defined as BCR, were tested in each dataset and a REM meta-analysis model was employed to summarise the overall effect across the datasets. The results are reported as forest plots in Figure 2.5. Although non-significant, higher age at diagnosis associated with higher risk of BCR (Figure 2.5a). Similarly, higher PSA levels at diagnosis also showed a non-significant association with BCR (Figure 2.5b). Higher Gleason score ($\geq$8) and higher tumour stages (T3+T4) had significant and stronger association with poor disease outcome (Figure 2.5c, 2.5d). As, Gleason score, tumour stage and PSA at diagnosis are the standard prognostic features as per the National Institute for Healthcare and Excellence (NICE) and EAU guidelines, the multivariate Cox PH models testing the association of miR expression with BCR were adjusted for these three confounding variables [26, 60].

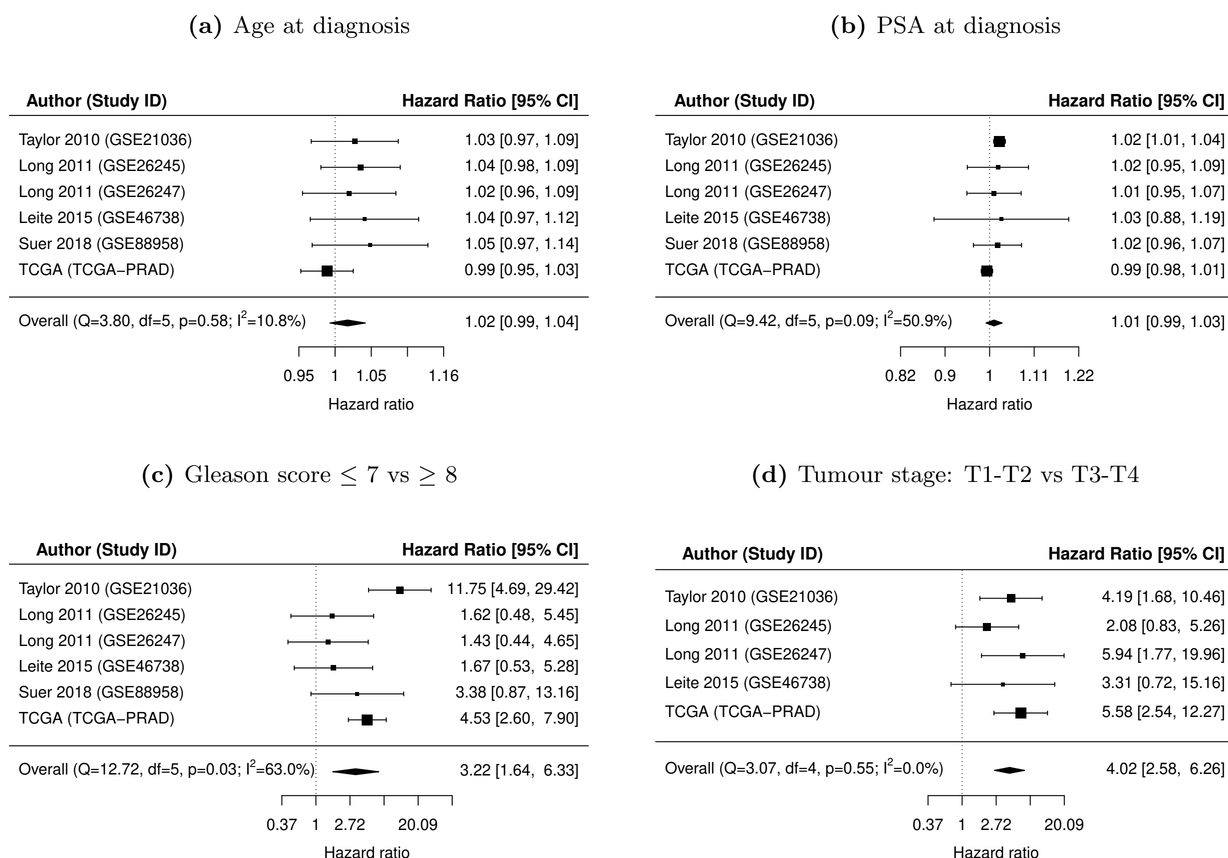


Figure 2.5: Association of clinical characteristics with biochemical recurrence. Whilst age and PSA show a non-significant association with biochemical recurrence, there were significant overall associations of higher Gleason score and tumour stage with biochemical recurrence. "Overall" refers to the overall effect estimate, i.e. pooled hazard ratio. The "Q", "df" and "p" values refer to the statistics for Q-test for heterogeneity and the "I^2" value refers to the outcome of I^2 test for heterogeneity. Abbreviations: PSA=Prostate specific antigen.

MiRNAs that consistently associate with disease recurrence: a univariate analysis

Univariate Cox PH regression followed by a REM meta-analysis was performed for 162 miRs that were common in all six datasets. Pooled HR estimates for 18 miRs were significantly associated with BCR (Figure 2.6). Out of these, 17 miRs (let-7a-5p, miR-125b-5p, miR-133a-3p, miR-135a-5p, miR-148a-3p, miR-155-5p, miR-203a-3p, miR-204-5p, miR-218-5p, miR-222-3p, miR-26b-5p, miR-30a-3p, miR-30c-5p, miR-30e-3p, miR-374a-5p, miR-455-5p and miR-582-5p) had negative association, while only miR-425-3p had positive association with BCR. The Q-test for heterogeneity was not significant for any of the miRs and I^2 statistic ranged from 0-40%, suggesting moderate levels of heterogeneity between the datasets.

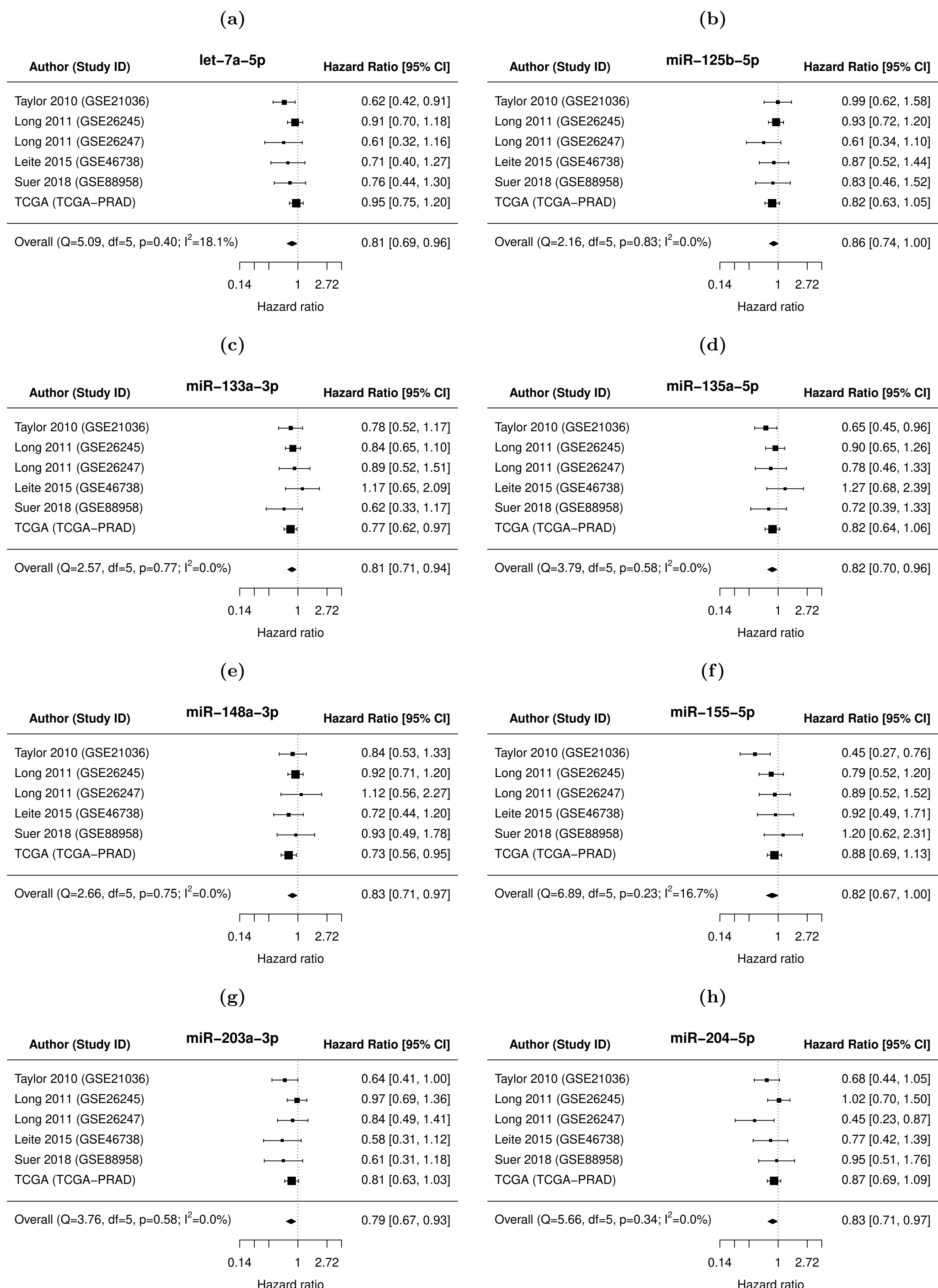
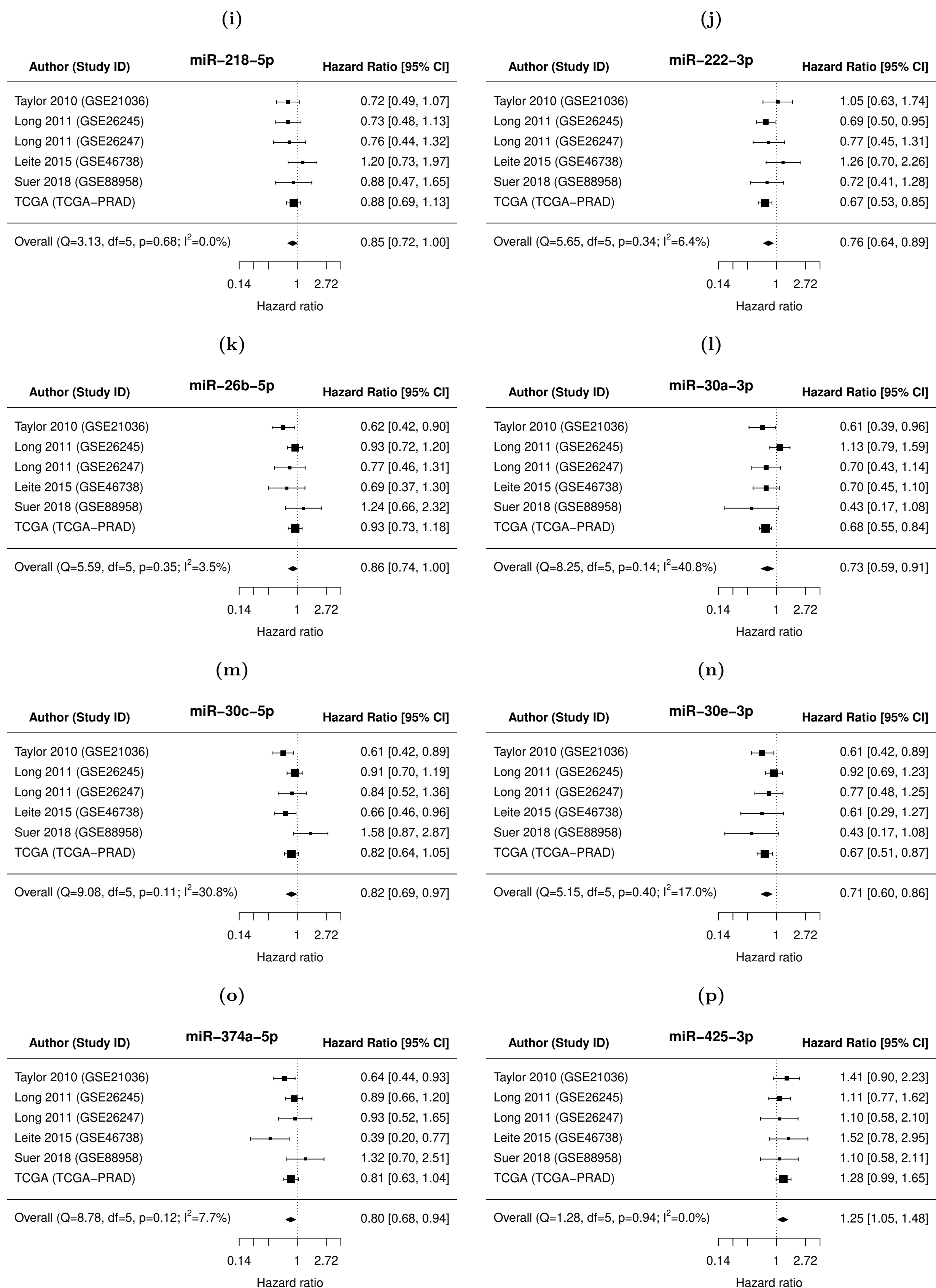


Figure 2.6: Association of miR expression with biochemical recurrence: an univariate analysis. The overall associations of these 18 miRs were significantly associated with biochemical recurrence. The expression of all but miR-425-3p associated negatively with recurrence. "Overall" refers to the overall effect estimate, i.e. pooled hazard ratio. The "Q", "df" and "p" values refer to the statistics for Q-test for heterogeneity and the "I^2" value refers to the outcome of I^2 test for heterogeneity. **Figure continued.**



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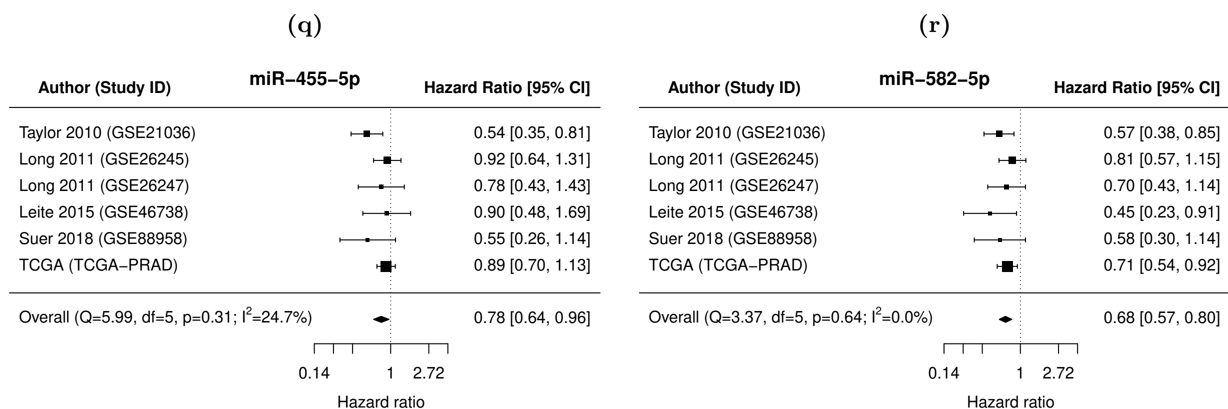

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MiRNAs that consistently associate with disease recurrence: a multivariate analysis with adjustment for prognostic clinical markers

Multivariate Cox PH regression followed by a REM meta-analysis was performed for 164 miRs that were common in the five datasets considered for the multivariate analysis. The variables adjusted in this multivariate analysis were clinical markers Gleason score, tumour stage and serum PSA at diagnosis. The meta-analysis revealed only 16 miRs were significantly associated with BCR (Figure 2.7). 13 miRs (let-7a-5p, miR-1-3p, miR-148a-3p, miR-203a-3p, miR-20a-5p, miR-221-3p, miR-26b-5p, miR-30a-3p, miR-30c-5p, miR-30e-3p, miR-30e-5p, miR-374a-5p and miR-582-5p) had negative association and three miRs (miR-130b-3p, miR-181b-5p and miR-425-3p) had positive association with BCR. The Q test for heterogeneity between samples for these miRs were non-significant and the I^2 value ranged from 0-30%. These values represent absence to moderate heterogeneity between the datasets. The slight reduction in heterogeneity in the multivariate analysis compared to the univariate analysis suggests that heterogeneity between the datasets was partly explained by clinical diversity, i.e. tumour stage, Gleason score and serum PSA. Overall, ten miRs (let-7a-5p, miR-148a-3p, miR-203a-3p, miR-26b-5p, miR-30a-3p, miR-30c-5p, miR-30e-3p, miR-374a-5p, miR-425-3p and miR-582-5p) were significantly prognostic in both univariate and multivariate meta-analyses (Table 2.7).

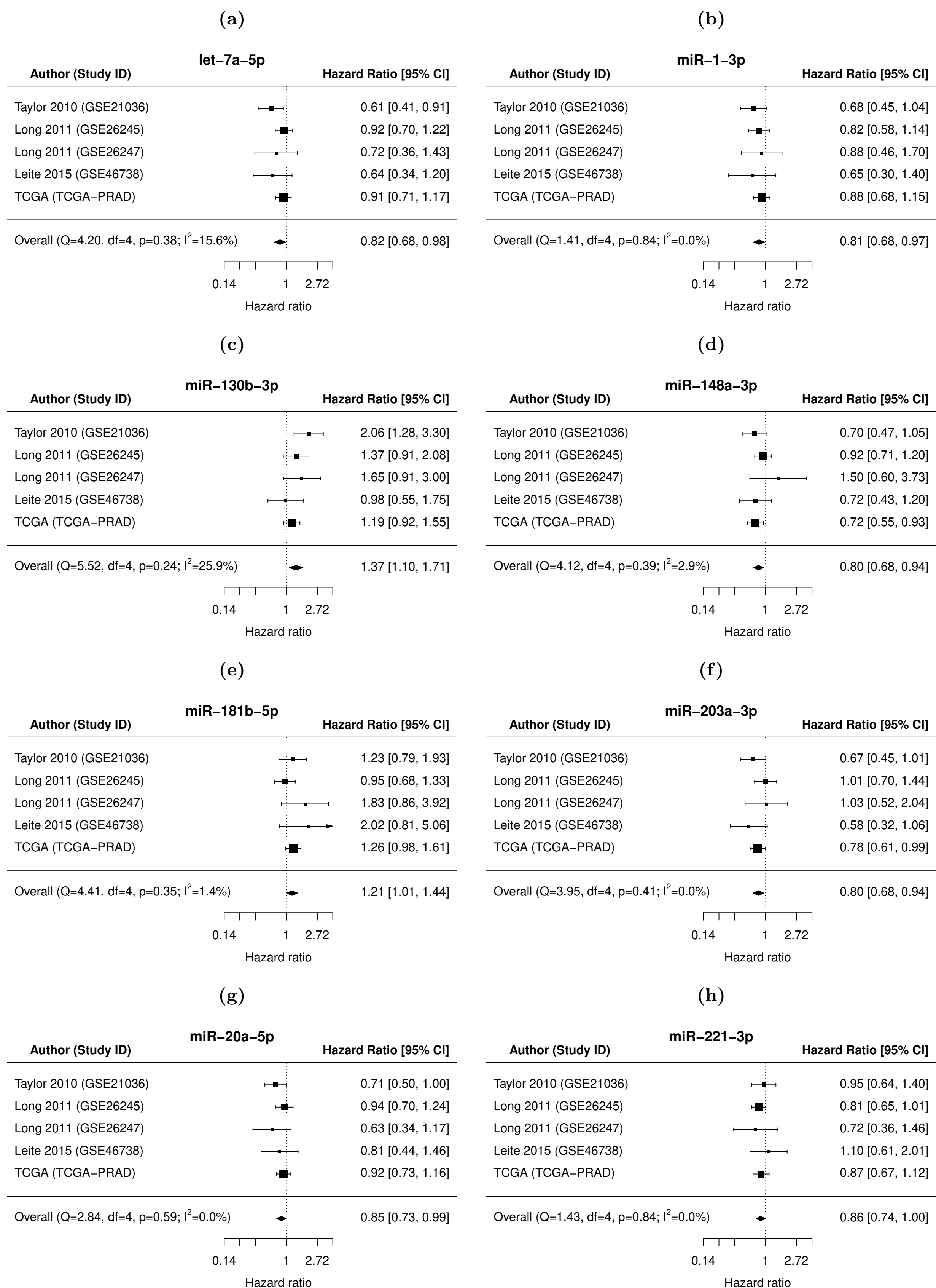
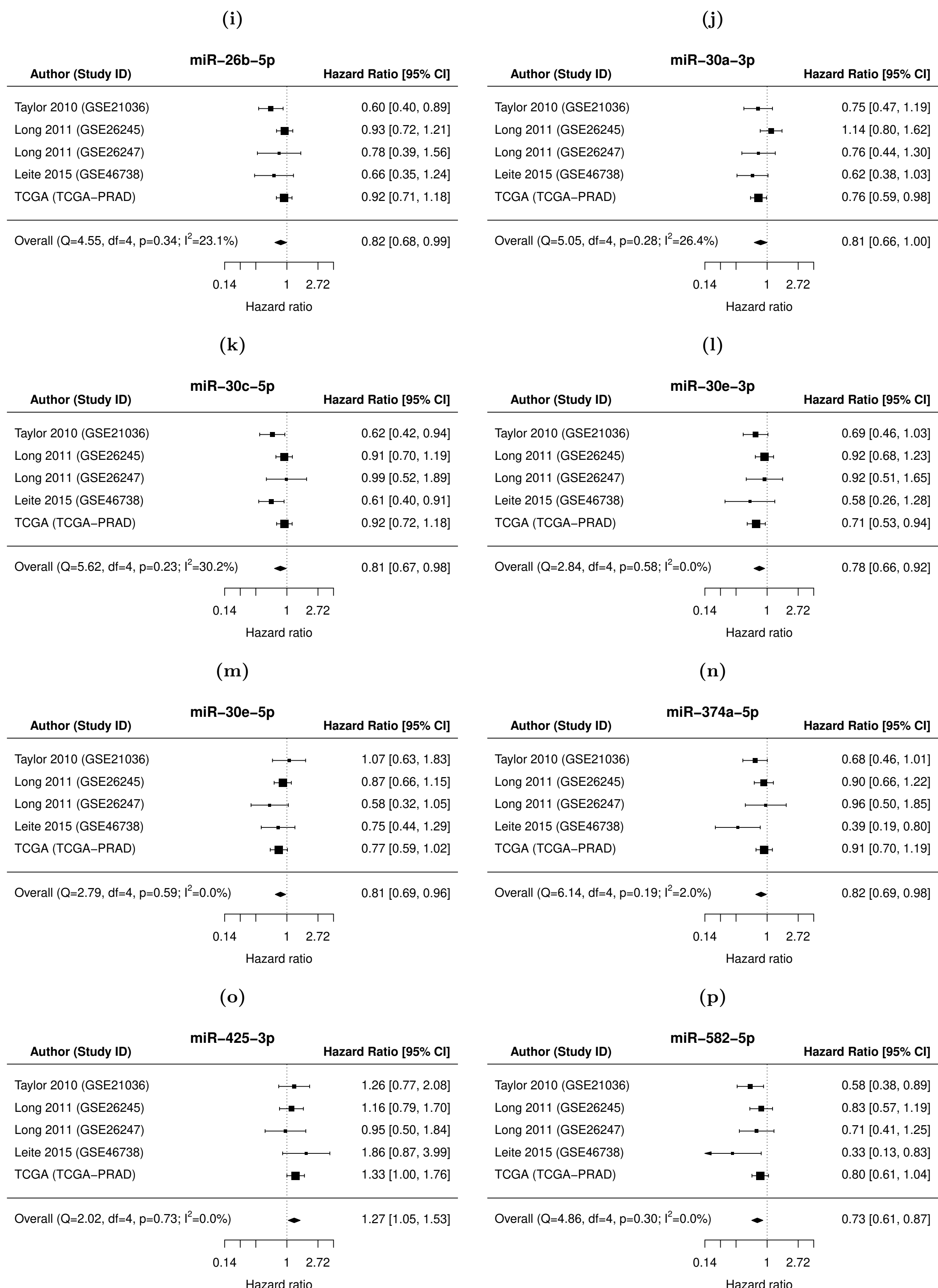


Figure 2.7: Association of miR expression with biochemical recurrence: a multivariate analysis. In total 16 miRs were significantly associated with biochemical recurrence even after adjustment for prognostic clinical markers Gleason score, tumour stage and serum PSA at diagnosis. The expression of all but three miRs (miR-130b-3p, miR-181b-5p, miR-425-3p) associated negatively with recurrence. "Overall" refers to the overall effect estimate, i.e. pooled hazard ratio. The "Q", "df" and "p" values refer to the statistics for Q-test for heterogeneity and the "I^2" value refers to the outcome of I^2 test for heterogeneity. **Figure continued.**



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miRs	univariate Cox PH		multivariate Cox PH		systematic review				
	pooled HR	**CI (95%)**	**pooled HR**	**CI (95%)**	**test: endpoint**	**HR**	**CI (95%)**	**sample size**	**ref**
let-7a-5p	0.81	0.69-0.86	0.82	0.68-0.98					
miR-148a-3p	0.83	0.71-0.97	0.80	0.68-0.94	multivariate: BPFS	0.60	0.44-0.81	207	[215]
miR-203a-3p	0.79	0.67-0.93	0.80	0.68-0.94	KM: PS	2.52	1.11-4.88	44	[216]
miR-26b-5p	0.86	0.74-1.00	0.82	0.68-0.99					
miR-30a-3p	0.73	0.59-0.91	0.81	0.66-1.00					
miR-30c-5p	0.82	0.69-0.97	0.81	0.67-0.98	multivariate: BPFS	0.34	0.17-0.68	103	[217]
					multivariate: BPFS	0.49	0.28-0.85	207	[215]
					univariate: PS	2.38	1.09-5.22	44	[216]
miR-30e-3p	0.71	0.60-0.86	0.78	0.66-0.92					
miR-374a-5p	0.80	0.68-0.94	0.82	0.69-0.98					
miR-425-3p	1.25	1.05-1.48	1.27	1.05-1.53					
miR-582-5p	0.68	0.57-0.80	0.73	0.61-0.87	KM: bone MFS	0.21	0.10-0.45	94	[218]

Table 2.7: Ten miRs significantly associated with biochemical recurrence in both univariate and multivariate meta-analyses. Four miRs: miR-148a-3p, miR-203a-3p, miR-30c-5p and miR-582-5p, have been identified as prognostic in independent publications, although the direction of association with progression is not consistent for miR-203a-3p and miR-30c-5p between my findings and the independent publications. KM, univariate and multivariate tests refer to Kaplan-Meier analysis, univariate Cox PH regression and multivariate Cox PH regression respectively. In the multivariate Cox PH, the adjusted variables were Gleason score, tumour stage, and PSA. A total of 5 and 6 datasets were included in the univariate and multivariate meta-analyses, respectively. Abbreviations: KM=Kaplan-Meier. For the full form of the abbreviated endpoints, refer to Table 2.1.

2.3.3 MiRNAs with consistent association with prostate cancer recurrence: validation between systematic review and meta-analysis

In the systematic review, five miRs, let-7b-5p, miR-145-5p, miR-152-3p, miR-195-5p and miR-224-5p, were identified as consistently individually prognostic, of which the latter four miRs were evaluated in the multivariate meta-analysis. However, the association of these four miRs with BCR were insignificant and inconsistent in the meta-analysis (Figure 2.8).

In the meta-analysis, ten miRs, let-7a-5p, miR-148a-3p, miR-203a-3p, miR-26b-5p, miR-30a-3p, miR-30c-5p, miR-30e-3p, miR-374a-5p, miR-425-3p and miR-582-5p, were validated as significantly prognostic of BCR post-RP. Among these, only four miRs (miR-148a-3p, miR-582-5p, miR-30c-5p and miR-203a-3p) were identified as individually prognostic in the systematic review (Table 2.7). The direction of association of miR-148a-3p and miR-582-5p with progression endpoints BPFS and bone MFS respectively in the review were consistent with the direction of association of the miRs with BCR in the meta-analysis [215, 218]. MiR-30c-5p was reported as prognostic in three independent studies; Ling *et al.* and Zhao *et al.* reported negative association of miR-30c-5p expression with BPFS, which were consistent with the results from the meta-analysis [215, 217]. However, the findings of Huang *et al.* was inconsistent as they reported a positive association of miR-30c expression with PCa patient survival [216]. For miR-203a-3p, its association with PCa patient survival was also conflicting with the findings of the meta-analysis [216]. The inconsistencies for miR-30c-5p and miR-203a-3p could potentially be due to differences in endpoints or statistical approaches, such as inclusion of different confounder variables in the multivariate models. Potential sources of heterogeneity are discussed in Section 2.4.

Although there were no overlaps between miRs identified as of interest in the systematic review and meta-analysis, two miRs: miR-148a-3p and miR-582-5p, were identified as consistently predictive of BCR in the meta-analysis and had at least one publication in the systematic review verifying their association [215, 218]. Therefore, these two miRs are ideal candidates to follow-up as individual

prognostic markers for PCa.

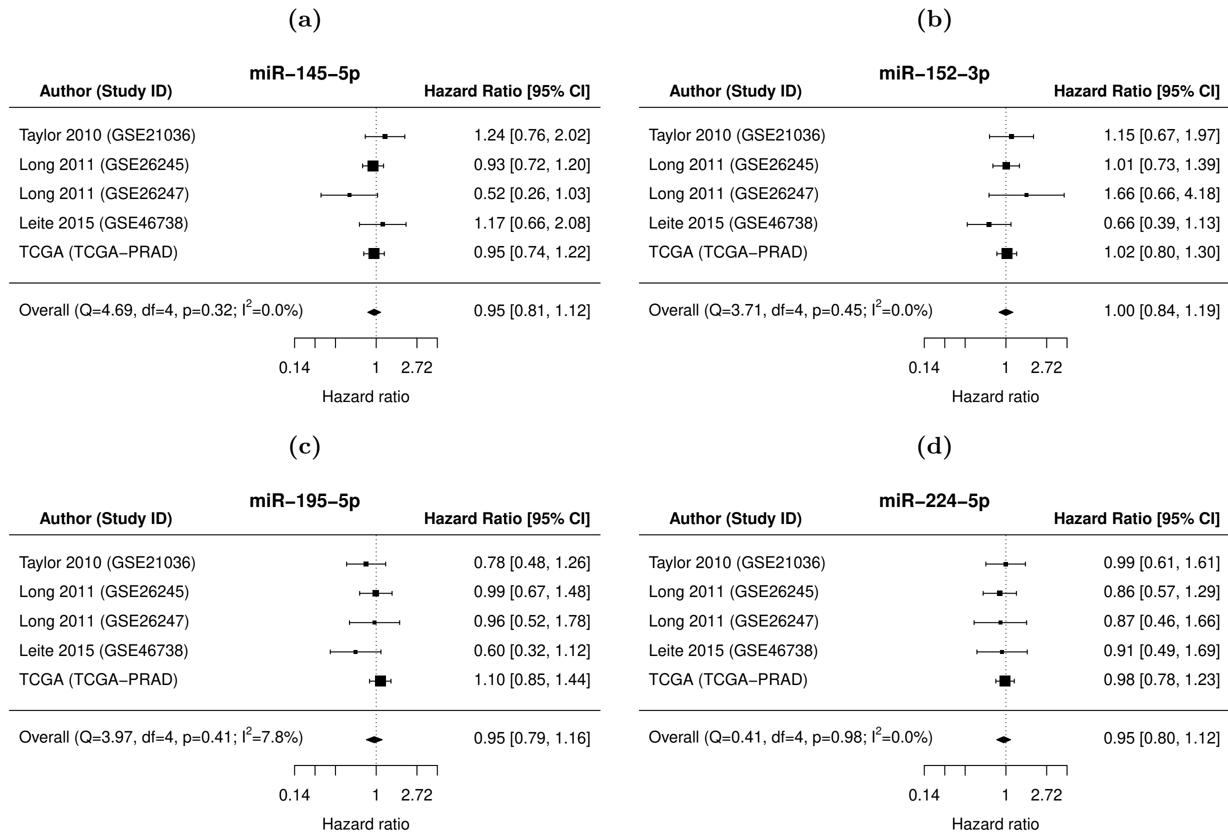

Figure 2.8: The association of four miRs identified as prognostic in the systematic review with biochemical recurrence: a multivariate Cox PH analysis. This analysis was adjusted for prognostic clinical markers Gleason score, tumour stage and serum PSA at diagnosis. None of these four miRs were significantly associated with biochemical recurrence in the meta-analysis. Let-7b-5p could not be evaluated in the meta-analysis as it was not profiled in all five datasets. "Overall" refers to the overall effect estimate, i.e. pooled hazard ratio. The "Q", "df" and "p" values refer to the statistics for Q-test for heterogeneity and the "I^2" value refers to the outcome of I^2 test for heterogeneity.

2.4 Discussion

In this work, I addressed the problem of inconsistent and conflicting reports of prognostic miRs in PCa in the literature with (i) a systematic review which summarised and identified consistently reported prognostic miR markers in PCa. I next performed (ii) a meta-analysis of six publicly available datasets which identified tumour tissue-derived miRs consistently associated with BCR in post-RP samples. Two miRs: miR-148a-3p and miR-582-5p, were identified as independently prognostic of PCa progression in the review and meta-analysis despite significant heterogeneity between studies and thus, presented as novel promising biomarkers for PCa progression.

2.4.1 miR-148a-3p

MiR-148a-3p is one of the commonly dysregulated miRs in human cancers. Its downregulation has been observed in various cancers such as bladder, oesophageal, gastric, breast, colorectal and ovarian cancers [219–225]. Upregulation of miR-148a has also been detected in osteocarcinoma and glioblastoma [226, 227]. In PCa, miR-148a-3p has been reported to be both up- and down-regulated. Upregulation of miR-148a-3p levels has been shown in prostate tumours tissues in comparison to adjacent normal tissues [153]. Upregulation was also observed in serum and urine of PCa patients in comparison to healthy controls [177, 228]. In contrast, downregulation of miR-148a-3p has been reported in CRPC cell lines PC3 and DU145 [229, 230]. Similarly, in PCa patients, downregulation of the miR has been reported in CRPC cases compared to BPH cases and in high-grade tumours compared to low-grade tumours [152, 231].

Although expression of miR-148a-3p is variably reported in the literature, studies investigating its biological role in PCa generally suggest a tumour suppressive role. Sengupta *et al.* showed downregulation of miR-148a-3p in CRPC and identified DNA methylatransferase *DNMT1*, a gene upregulated in several cancers, as a target of the miR [230]. They reported that the two molecules exhibit a negative loop in PCa: while *DNMT1* enzyme methylates the miR promoter and silences miR expression, miR-148a directly targets *DNMT1*, whose repression leads to induction of apoptosis and repression of cell proliferation and migration. They also demonstrated that ectopic expression of miR-148a-3p repressed anti-apoptotic *BCL2* in PC3 cells promoting apoptosis. Suppression of *DNMT1* by miR-148-3p has been reported in pancreatic, liver, bladder, oesophageal and gastric cancers [219–222, 232, 233]. Targeting of *BCL2* by miR-148a-3p has also been reported in colorectal and pancreatic cancers [224, 234]. Additionally, a study by Fujita *et al.* showed miR-148a-3p expression increased chemosensitivity in PC3 cells by directly targeting mitogen-and stress-activated protein kinase, *MSK1* [229]. These studies demonstrate that miR-148a-3p plays a role in promoting anti-survival, tumour suppressive phenotype via similar mechanisms in various cancers including PCa and its loss is not only a good indicator of tumour progression but also shows potential to serve as a biomarker for therapeutic

response in PCa.

MiR-148a-3p is a highly abundant miR which has been detected and successfully profiled in PCa patients from various sources including tumour tissues, urine and circulation [155, 177, 228, 235]. Its high abundance and detection in circulation allows for non-invasive sample collection and monitoring in the clinics, adding to its value as a biomarker. Its consistent association with progression and ideal biomarker properties make it a potential candidate for further investigation as a clinical prognostic biomarker. However, its role in PCa at various stages of the disease still remains uncertain and needs to be elucidated; understanding the role of miR-148a-3p at various stages of PCa will allow for its utilisation as a biomarker at disease stages where it is the most effective.

2.4.2 miR-582-5p

miR-582-5p is a poorly investigated miR in oncology. Similar to miR-148a-3p, it is reported to act as both an oncogene and a tumour suppressor in various cancers. In gastric, bladder, non-small cell lung cancers and endometrial carcinoma, miR-582-5p levels are downregulated and shown to suppress proliferation, migration, invasion and promote apoptosis [236–239]. Conversely, in colorectal cancer and pituitary adenomas, it is over-expressed and promotes proliferation [240, 241].

The clinical significance of miR-582-5p in PCa is not yet elucidated and the literature presents conflicting evidence. Most recent research on miR-582-5p in PCa investigated the role of the miR in promoting bone metastasis [218]. In this study, lower miR-582-5p expression was reported in PCa tissues with bone metastasis compared to PCa tissues without bone metastasis. The study reported that lower miR-582-5p expression was significantly associated with shorter bone MFS. They also demonstrated that over-expression of the miR in mice model bearing PC3 tumour xenografts repressed bone metastasis and over-expression in PCa cell lines PC3, VCaP and C42B repressed tumour invasiveness and migration. Mechanistically, the study proposed that miR-582-3p exerted its anti-invasion and migration properties by directly inhibiting components of the TGFβ signalling pathway: *SMAD2*,

TGFBRI and *TGFBRII*, and subsequently the pathway itself. Maeno *et al.* developed AR-positive, androgen-independent xenograft model KUCaP2 and cell line AILNCaP#1 and observed upregulation of miR-582-5p in these models in comparison to their androgen dependent counterparts [242]. Their study also demonstrated suppression of the miR decreased cell proliferation in AILNCaP#1, suggesting an oncomiRic role of miR-582-5p in the transition of PCa from hormone- sensitive to more aggressive castration-resistant phenotypes. These limited studies on miR-582-3p report conflicting roles of the miR in tumour progression. Their findings may indicate a dual role of the miR at different stages of progression from invasion and metastasis to the bones, to transition from androgen-dependent to aggressive CRPC. MiR-582-5p is a novel miR that has been identified as a potential prognostic candidate for PCa. However, its exact role in PCa tumour progression is poorly understood and prompts further research along with its investigation as a biomarker.

2.4.3 Limitations

One of the major issues highlighted by this project is the inconsistent findings between studies and datasets despite their common aim to identify prognostic miR biomarkers in PCa. Inconsistent summary effects between studies, which can potentially lead to inaccurate conclusions, is termed statistical heterogeneity or heterogeneity, and arises from clinical and methodological heterogeneity at any point during the study. Due to the nature of retrospective cohort studies, clinical heterogeneity, which encompasses factors such as race, family history, co-morbidity, treatment history, time to outcome and differential loss of follow up between studies, was unavoidable. In the systematic review, a potential contributor of clinical heterogeneity was outcome endpoints. There were 12 endpoints (Table 2.1) in the systematic review that were considered surrogates of disease progression. Although there may be a correlation between different endpoints and irrefutable clinical progression, the occurrence of these endpoints does not warrant clinical progression, thus introducing the potential for inaccurate conclusions. Different endpoints were combined if they had redundant meaning and unambiguous, matching definitions. However, a large proportion of studies did not provide definitions for their chosen endpoints. For some studies that did specify endpoint definitions, there was still definition

heterogeneity between studies; this is evident in studies by Hulf *et al.* and Nordby *et al.* where both studies examined the association of miR-205 with BPFS but used different criteria to define BPFS [138, 193]. As such, even if some studies had similar/ redundant endpoints, they were not combined.

In the meta-analysis, only studies examining association with BPFS as their surrogate for disease progression was considered to minimise endpoint heterogeneity. However, BPFS may not have been the most suitable surrogate endpoint for disease progression. The ICECaP study, a large scale meta-analysis that aimed to determine clinically relevant endpoints for localised PCa, determined MFS, and not PSA-based endpoints, as the most appropriate surrogate for PCa specific survival [243, 244]. Ideally, MFS would be used as the endpoint of interest for the meta-analysis. However, studies in the literature frequently use BPFS instead of MFS. This is evident in the systematic review, where almost half the studies (44%) considered BPFS, while only 6% of studies considered bone-/metastasis FS as endpoints. As BPFS was used as the surrogate endpoint, the prognostic miRs identified in the meta-analysis may not be the most reliable predictors of disease progression. Moving forward, studies should consider evidence-based clinically relevant endpoints.

To minimise heterogeneity from different sample sources, only samples originating from tumour tissues of patients who underwent RP were considered. Nonetheless, this approach could not ensure a comparable level of tumour content in the samples. Datasets TCGA-PRAD, GSE88958 and GSE21036 included samples with at least 60-70% tumour content in the tissues, while the rest of the datasets did not specify their percentage tumour content. Differences in baseline severity also existed in samples between the datasets (Figures 2.4). To reduce their impact on heterogeneity, clinical confounders were included as predictors in multivariate Cox PH analyses.

Methodological heterogeneity, attributing to differences in study design, sample preparation methods, sample types, profiling technologies and threshold values for a positive result, were also present in the analyses. Besides these factors, one of the sources of methodological heterogeneity that may have

influenced my results were the different statistical tests, either Cox PH regression or KM analysis, employed by different studies. The KM analysis only allows categorical variables as predictors (e.g. miR expression needs to be categorised into high vs low expression), which can lead to weakening or loss of potential signal. It also cannot adjust for multiple predictors. Whereas, Cox PH regression is more flexible, allowing for both categorical and continuous variables as predictors. Besides, multiple predictors can be added into a Cox PH model, thus allowing for adjustment of confounding variables. For this reason, when a study in the systematic review reported outcome of both Cox PH and KM analyses, the Cox PH results were prioritised and reported. However, even with adjustment for confounders in the Cox PH regression, there was potential for further heterogeneity to be introduced as different studies adjusted for different confounders. For example, Amankwah *et al.*, Melbo-Jorgensen *et al.* and Guan *et al.* examined association of miR-21-5p with progression using a multivariate Cox PH model but each study considered different confounders in their models B.2 [245–247].

In the meta-analysis, heterogeneity was controlled for as much as possible. This was done by firstly, standardising the expression dataset with z-scores and secondly, by only including datasets in the multivariate analysis if they had all three standard clinical variables (PSA at diagnosis, Gleason score and tumour stage) present. The multivariate Cox PH analysis in each of the five datasets were adjusted for those confounding clinical variables, ensuring that the association of miRs with BCR could be interpreted independently of them. Although appropriate measures were taken to reduce heterogeneity, it cannot be completely eliminated. This calls for the need for standardisation of methodology and protocols in the field of biomarker discovery in order to derive more accurate conclusions from future investigations.

Besides heterogeneity, another major limitation in the meta-analysis was the limited number of publicly available datasets. Numerous studies generate novel miR expression data, but most do not make their data publicly available. This led to the inclusion of only six datasets for the meta-analysis. Additionally, a caveat to the studies included in the meta-analyses was that the proportion of samples

that experienced BCR were disproportionately lower than the samples that did not (Figure 2.4e). Insufficient and unbalanced datasets are a major problem of working with biomedical data, reducing the power of the study and potentially leading to biased, inaccurate conclusions specific to the cohorts being studied rather than the general population.

2.4.4 Conclusion

This is the first systematic review and only the second meta-analysis, updated with newer datasets and larger sample sizes compared to the first meta-analysis performed in 2017, to focus on prognostic miR markers in PCa [179]. It revealed considerable research undertaken in the field of biomarkers discovery in PCa and catalogued a novel database of all PCa prognostic miRs reported so far. These findings present a valuable reference point for future studies. This investigation also highlighted a lack of validation or inconsistent evidence for miRs frequently suggested to have prognostic biomarker potential. Only miR-148a-3p and miR-582-5p were consistently associated with disease progression in multiple publications and datasets, indicating reliability in predicting prognosis. Nevertheless, their biological significance in PCa progression is still uncertain. Further research to verify their biological roles is warranted to support investigations into their performance as prognostic PCa biomarkers.

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Appendix A

Appendix figures

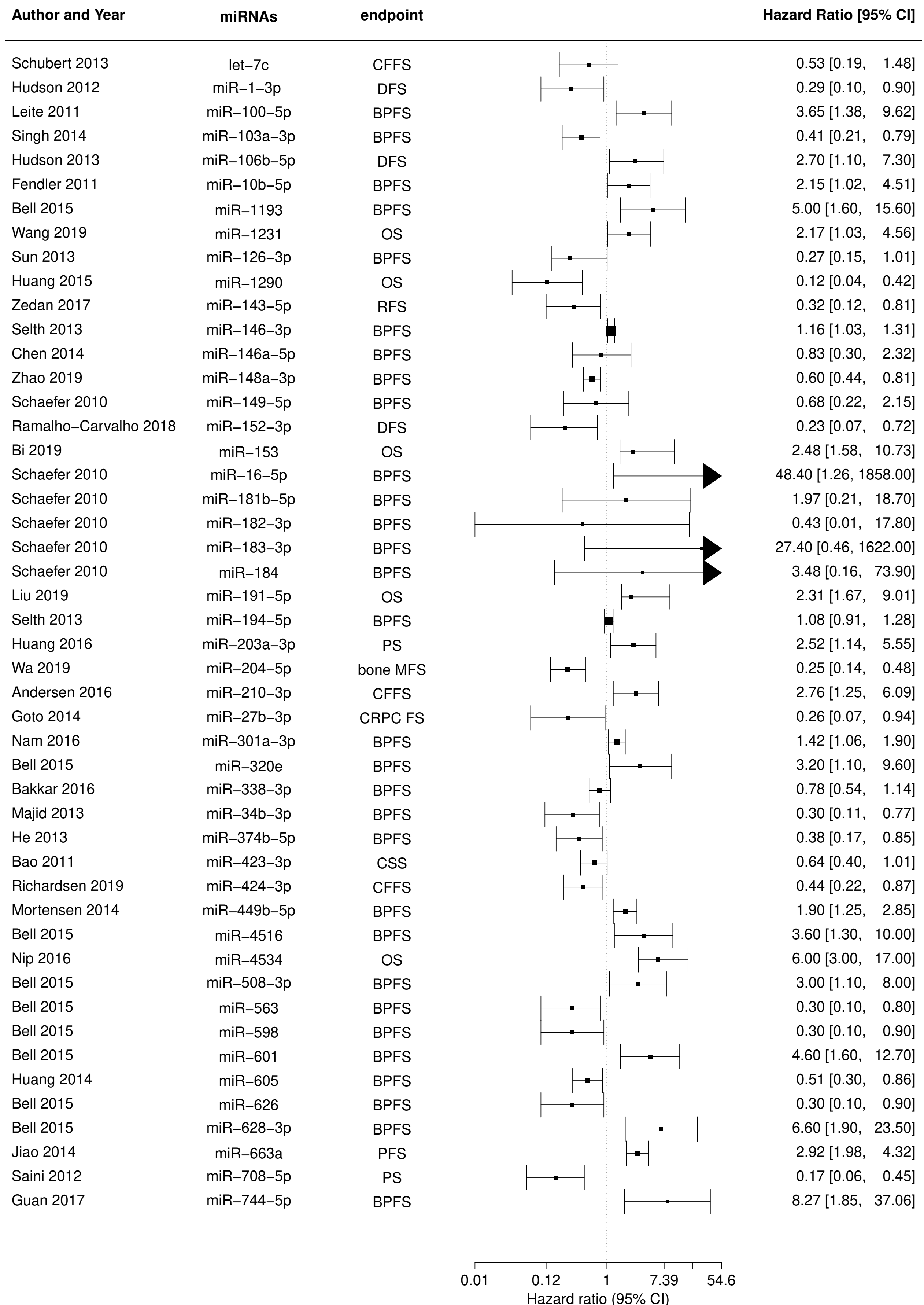

Figure A1: Forest plot for miRs with single entries in the systematic review. For the full form of the abbreviated endpoints, refer to Table 2.1.

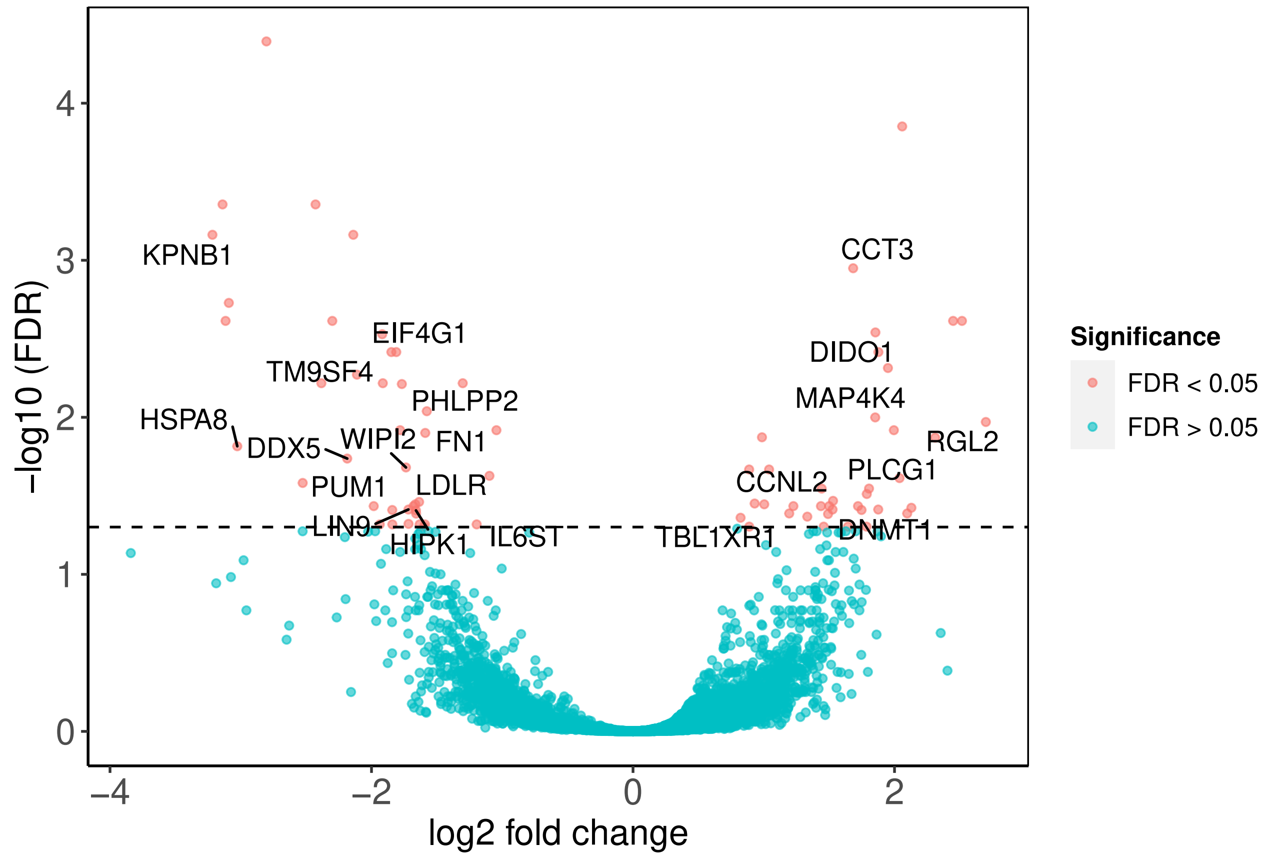

Figure A2: Volcano plot of the differentially expressed genes in ASO-27a the RNAseq dataset. Generalised linear model and FDR methods were used to perform the differential expression analysis and obtain the multiple test corrected p-values. Genes with positive and negative log2 fold changes are upregulated and downregulated respectively in ASO-27a treated group compared to ASO-NTC treated group. Genes that are predicted miR-27a-3p targets are labelled. Gene ITSN2, a significantly dysregulated and putative miR-27a-3p target gene, has been omitted from this plot due to its very low FDR p-value which skewed the plot. For an unedited volcano plot refer to Figure **??**.

Appendix B

Appendix tables

Prognostic miR	Prognostic test: test: endpoint	HR (95% CI)	p	Reference group	Association after reference standardisation	Sample size	Sample type	PMID	Ref
let-7b-5p	multivariate: BPFS	0.44 (0.193-1.022)	0.05	low	negative	98 (cohort A)	tissue	23798998	[195]
	multivariate: BPFS	0.30 (0.15-0.61)	<0.010	low	negative	92 (cohort B)	tissue		
	multivariate: CFFS	0.23 (0.08-0.70)	<0.010	low	negative	92 (cohort B)	tissue		
	multivariate: CFFS	0.46 (0.15-1.41)	0.17	low	negative	98 (cohort A)	tissue		
let-7c	multivariate: CFFS	0.53 (0.19-1.48)	0.22	low	negative	98 (cohort A)	tissue	23798998	[195]
miR-1-3p	univariate/ KM: DFS	0.29 (0.10-0.90)	0.008	low	negative	99 (MSKCC)	tissue	22210864	[248]
miR-100-5p	multivariate: BPFS	3.65 (1.38-9.62)	0.009	low	positive	49	tissue	21255804	[249]
miR-103a-3p	multivariate: BPFS	0.41 (0.21-0.79)	0.008	low	negative	93	serum	24583788	[250]
miR-106b-5p	univariate/ KM: DFS	2.70 (1.10-7.30)	0.014	low	positive	113 (MSKCC)	tissue	22986525	[251]
miR-10b-5p	multivariate: BPFS	2.15 (1.02-4.51)	0.044	low	positive	52	tissue	21769427	[252]
miR-1193	multivariate: BPFS	5.00 (1.60-15.60)	0.006	low	positive	43	tissue	25760964	[253]
miR-1207-3p	multivariate: RFS	2.50 (1.60-4.00)	<0.010	low	positive	368	tissue	27267842	[254]
	multivariate: CSS	1.80 (0.80-4.30)	0.060	low	positive	368	tissue		
miR-1231	multivariate: OS	2.17 (1.03-4.56)	0.041	low	positive	118	tissue	31822000	[255]
miR-125b-5p	multivariate: BPFS	1.79 (1.10-2.91)	0.018	low	positive	93	serum	24583788	[250]
	multivariate: BPFS	0.23 (0.01-3.75)	0.304	low	negative	76	tissue	19676045	[154]
miR-126-3p	multivariate: BPFS	3.68 (0.99-6.83)	0.010	high	negative	128	tissue	24350576	[256]
miR-128-3p	multivariate: BPFS	3.96 (1.02-8.12)	0.010	high	negative	128	tissue	26339409	[257]
	multivariate: BPFS	3.32 (0.92-6.91)	0.010	high	negative	128	serum		
miR-129-5p	multivariate: OS	2.77 (0.93-8.17)	0.048	high	negative	84	peripheral blood	31788094	[258]
	multivariate: DSS	6.12 (1.56-24.07)	0.009	low methylation	positive	180 (cohort 1)	tissue	28143614	[194]
miR-1290	univariate: OS	8.04 (2.36-27.33)	<0.001	high	negative	100	exosome (blood)	25129854	[259]
miR-1303	KM: OS	-	0.031	high	positive	30	tissue	31772644	[260]
miR-130b-3p	multivariate: OS	22.4 (2.30-222.40)	0.008	high	negative	36 (African Americans)	tissue	31266828	[261]

	multivariate: OS	1.1 (0.10-8.30)	0.910	high	negative	57 (European American)	tissue		
miR-133a-3p	multivariate: bone MFS	0.37 (0.19-0.74)	0.005	low	negative	223 (TCGA)	tissue	30021600	[262]
	multivariate: OS	1.07 (0.41-2.78)	0.886	low	positive	245 (TCGA)	tissue		
miR-133b	multivariate: BPFS	1.78 (1.01-3.11)	0.040	low	positive	135	tissue	24610824	[263]
	univariate: bone MFS	0.08 (0.03-0.22)	0.001	low	negative	176	tissue	30006541	[264]
	multivariate: OS	6.90 (0.72-66.60)	0.095	low	positive	202	tissue		
miR-139-5p	multivariate: OS	0.25 (0.07-0.93)	0.038	high	positive	84	peripheral blood	31788094	[258]
	multivariate: BPFS	0.77 (0.58-1.04)	0.091	low	negative	540	tissue	31269290	[265]
	multivariate: MFS	0.60 (0.28-1.28)	0.188	low	negative	540	tissue		
miR-141-3p	multivariate: BPFS	1.07 (1.00-1.14)	0.05	low	positive	463	epithelial and stromal	30674952	[266]
	multivariate: BPFS	1.92 (1.32-2.79)	0.001	low	positive	207	tissue	31640261	[215]
miR-143-5p	univariate: RFS	0.32 (0.12-0.81)	0.016	low	negative	49	tissue	28628624	[267]
	KM: OS	-	0.047	high	positive	n/s (TCGA)	tissue	30027097	[268]
miR-145-5p	multivariate: BPFS	4.47 (1.27-15.74)	0.020	high	negative	36 (low + intermediate risk)	tissue	23703249	[196]
	multivariate: BPFS	4.43 (1.11-17.61)	0.035	high	negative	29 (intermediate risk)	tissue		
	multivariate: PFS	0.40 (0.17-0.94)	0.036	low	negative	106	tissue	20332243	[197]
	univariate/ KM: OS	3.00 (1.60-7.00)	<0.010	high	negative	49	tissue	25969144	[198]
	univariate: BPS	0.74 (0.23-2.34)	0.609	low	negative	76	tissue	19676045	[154]
	univariate: BPFS	0.68 (0.22-2.14)	0.510	low	negative	73	tissue	22864280	[199]
	multivariate: DFS	1.26 (0.49-3.27)	0.629	high	negative	73	tissue	23703249	[196]
miR-146-3p	multivariate: BPFS	1.16 (1.03-1.31)	0.017	low	positive	16	serum	23846169	[176]
miR-146a-5p	KM: BPFS	-	0.048	low	negative	98 (MSKCC)	tissue	26306811	[269]
miR-146a-5p rs2910164	multivariate: BPFS	0.83 (0.30-2.32)	0.722	CC vs GG/GC	negative	72	peripheral blood	25526182	[189]
miR-148a-3p	multivariate: BPFS	0.60 (0.44-0.81)	0.001	low	negative	207	tissue	31640261	[215]
miR-149-5p	multivariate: BPFS	0.68 (0.22-2.15)	0.510	low	negative	76	tissue	19676045	[154]
miR-150-5p	KM: OS	-	0.035	low	negative	86	tissue	30009782	[270]

miR-152-3p	KM: BPFS	-	<0.001	low	negative	n/s (MSKCC)	tissue	25004396	[200]
	multivariate: DFS	0.23 (0.07-0.72)	0.012	low	negative	494 (TCGA)	tissue	29599847	[201]
miR-153	multivariate: OS	2.48 (1.58-10.73)	0.019	low	positive	143	tissue	31490362	[271]
miR-15b-5p	univariate: OS	0.69 (0.14-3.51)	0.658	high	positive	387 (TCGA)	tissue	29363862	[272]
	multivariate: RFS	1.16 (0.68-1.98)	0.583	high	negative	387 (TCGA)	tissue		
miR-16-5p	KM: BPFS	-	0.003	low	negative	n/s (MSKCC)	tissue	30032144	[204]
	multivariate: BPFS	48.4 (1.26-1858)	0.037	low	positive	76	tissue	19676045	[154]
miR-17-5p	KM: BPFS	-	0.013	high	positive	268 (TCGA)	tissue	31122242	[273]
miR-181b-5p	multivariate: BPFS	1.97 (0.21-18.70)	0.553	low	positive	76	tissue	19676045	[154]
miR-182-3p	multivariate: BPFS	0.43 (0.01-17.80)	0.658	low	negative	76	tissue	19676045	[154]
miR-182-5p	KM: OS	-	0.002	high	positive	63	tissue	27179774	[274]
	KM: OS	-	0.012	high	positive	52	tissue	23383207	[275]
	multivariate: BPFS	2.00 (1.00-3.50)	0.009	low	positive	204	tissue	24518785	[276]
	multivariate: PFS	2.50 (1.00-5.00)	0.013	low	positive	204	tissue		
	univariate: BPFS	1.50 (1.00-2.00)	0.147	low	positive	137	tissue		
	multivariate: PFS	1 (0.50-2.00)	0.387	low	positive	137	tissue		
	multivariate: BPFS	0.36 (0.01-17.90)	0.608	low	negative	76	tissue	19676045	[154]
miR-183-3p	KM: OS	-	0.001	high	positive	n/s (TCGA)	tissue	30027097	[268]
	multivariate: BPFS	27.40 (0.46-1622)	0.112	low	positive	76	tissue	19676045	[154]
miR-184	multivariate: BPFS	3.48 (0.16-73.90)	0.423	low	positive	76	tissue	19676045	[154]
miR-186-5p	KM: PS	-	0.028	low	negative	38	tissue	27121312	[277]
miR-188-5p	multivariate: BPFS	2.11 (1.34-3.33)	0.001	high	negative	180	tissue	25714029	[278]
	multivariate: OS	3.01 (1.74-5.21)	<0.001	high	negative	180	tissue		
miR-190a	KM: DFS	-	0.035	low	negative	35	tissue	26314494	[279]
miR-191-5p	multivariate: OS	2.31 (1.67-9.01)	0.027	low	positive	146	tissue	31335671	[280]
miR-192-5p	KM: BPFS	-	0.007	high	positive	n/s (TCGA)	tissue	30544100	[281]
miR-194-5p	multivariate: BPFS	1.08 (0.91-1.28)	0.399	low	positive	16	serum	23846169	[176]
miR-195-5p	multivariate: BPFS	5.96 (1.18-30.02)	0.031	high	negative	140	tissue	26338045	[202]
	multivariate: OS	4.46 (1.35-14.72)	0.014	high	negative	140	tissue		
	multivariate: BPFS	0.61 (0.41-0.93)	0.022	low	negative	107 (MSKCC)	tissue	26080838	[203]
	KM: BPFS	-	0.009	low	negative	131 (MSKCC)	tissue	30032144	[204]
	KM: RFS	-	0.049	low	negative	98 (MSKCC)	tissue	26650737	[205]
	KM: DFS	-	<0.010	low	negative	n/s (MSKCC)	tissue	27175617	[206]
miR-19a-3p	KM: BPFS	-	0.034	high	positive	328 (TCGA)	tissue	29416742	[282]

miR-200b-3p	KM: BPFS	-	0.049	low	negative	51	tissue	25409297	[283]
miR-203a-3p	univariate/ KM: PS	2.52 (1.14-5.55)	0.023	low	positive	44	tissue	26499781	[216]
miR-204-5p	KM: bone MFS	0.25 (0.14-0.48)	<0.001	low	negative	136	serum	31678733	[284]
miR-205-5p	multivariate: BPFS	1.70 (1.23-2.36)	0.001	high	negative	535	tissue	29176717	[138]
	univariate/ KM: OS	2.33 (1.11-4.88)	0.030	high	negative	49	tissue	23571738	[285]
	multivariate: BPFS	2.23 (0.99-5.00)	0.05	low methylation	negative	149	tissue	22869146	[193]
	multivariate: CSS	6.88 (1.66-28.53)	0.001	high	negative	105 (cohort train)	tissue	24173237	[286]
	multivariate: CSS	6.55 (1.29-33.10)	0.023	high	negative	78 (cohort validation)	tissue		
	multivariate: BPFS	1.96 (0.80-4.80)	0.141	high	negative	105 (cohort train)	tissue		
	multivariate: CFFS	0.86 (0.08-9.16)	0.900	high	positive	78 (cohort validation)	tissue		
	multivariate: BPFS	0.76 (0.35-2.62)	0.472	high	positive	78 (cohort validation)	tissue		
	univariate: BPFS	0.37 (0.10-1.40)	0.128	low	negative	76	tissue	19676045	[154]
miR-20a-5p	KM: BPFS	-	<0.001	high	positive	268 (TCGA)	tissue	31122242	[273]
miR-20b-5p	KM: BPFS	-	0.180	high	positive	268 (TCGA)	tissue	31122242	[273]
miR-21-5p	multivariate: BPFS	2.40 (1.06-5.49)	0.037	low	positive	167 (Gleason == 6)	stromal	25401698	[246]
	multivariate: BPFS	1.40 (1.0-1.90)	0.089	low	positive	170 (all cohort)	stromal		
	multivariate: BPFS	2.06 (1.08-3.94)	0.029	low	positive	168	tissue	22341810	[287]
	multivariate: PFS	1.99 (1.03-3.82)	0.040	low	positive	85	tissue	27040772	[247]
	multivariate: RFS	6.15 (1.04-36.48)	0.045	high	negative	65 (obese)	tissue	23353719	[245]
	multivariate: RFS	1.99 (0.70-5.64)	0.200	high	negative	65 (obese + non-obese)	tissue		
	multivariate: RFS	1.28 (0.3-5.49)	0.740	high	negative	45 (non-obese)	tissue		
miR-210-3p	multivariate: CFFS	2.76 (1.25-6.09)	0.012	low	positive	535	fibroblast	27824162	[288]
miR-212-3p	KM: PS	-	<0.050	low	negative	72	tissue	29917185	[289]
miR-218-5p	univariate/ KM: bone MFS	0.44 (0.21-0.90)	0.015	low	negative	107	serum	30870834	[290]
	univariate/ KM: bone MFS	0.38 (0.19-0.78)	0.009	low	negative	107	tissue		
	univariate/ KM: OS	0.82 (0.19-3.62)	0.875	low	negative	109	serum		
	univariate/ KM: OS	0.79 (0.18-3.38)	0.757	low	negative	109	serum		

miR-221-3p	multivariate: CRFS	0.53 (0.29-0.95)	0.032	low	negative	92	tissue	19585579	[291]
	KM: CRPC FS	-	0.012	high	positive	45 (Gleason >= 8)	whole blood	24760272	[292]
	multivariate: BPFS	0.74 (0.61-0.90)	0.002	low	negative	207	tissue	31640261	[215]
	univariate: BPFS	0.36 (0.17-1.90)	0.570	low	negative	73	tissue	22864280	[199]
	KM: CRPC FS	-	0.147	low	negative	52	tissue	26325107	[293]
	multivariate: BPFS	0.5 (0.01-39.10)	0.757	low	negative	76	tissue	19676045	[154]
	multivariate: RFS	0.56 (0.21-1.50)	0.250	high	positive	63 (all cases)	tissue	23353719	[245]
	multivariate: RFS	0.40 (0.09-1.84)	0.240	high	positive	44 (non-obese)	tissue		
	multivariate: RFS	0.46 (0.10-2.22)	0.330	high	positive	19 (obese)	tissue		
miR-222-3p	multivariate: BPFS	2.80 (1.29-6.20)	0.009	low	positive	93	serum	24583788	[250]
	multivariate: CRPC FS	0.21 (0.07-0.64)	0.006	low	negative	52	tissue	26325107	[293]
	multivariate: BPFS	5.04 (0.03-940)	0.544	low	positive	76	tissue	19676045	[154]
	multivariate: RFS	0.39 (0.14-1.15)	0.090	high	positive	60 (all cases)	tissue	23353719	[245]
	multivariate: RFS	0.37 (0.09-1.59)	0.180	high	positive	42 (non-obese)	tissue		
	multivariate: RFS	0.46 (0.07-3.19)	0.440	high	positive	18 (obese)	tissue		
miR-224-5p	multivariate: BPFS	0.25 (0.08-0.74)	0.010	low	negative	114	tissue	24382668	[207]
	multivariate: BPFS	0.64 (0.14-2.39)	0.525	low	negative	58	tissue	23136246	[208]
miR-23a-3p	multivariate: PS	1.78 (1.12-2.83)	0.015	low	positive	123	tissue	25714010	[294]
miR-23a-3p rs3745453	multivariate: OS	9.67 (2.83-33.09)	0.001	CT/TT vs CC	positive	156	peripheral blood	31876746	[190]
miR-23b-3p	univariate/ KM: OS	8.10 (4.00-19.00)	<0.001	high	negative	151	tissue	23074286	[295]
	univariate/ KM: RFS	6.20 (3.00-13.00)	<0.001	high	negative	151	tissue		
	KM: OS	-	0.042	high	positive	n/s (TCGA)	tissue	30027097	[268]
miR-26a-3p	KM: OS	-	0.038	low	negative	140	tissue	27449037	[296]
miR-27a-3p	KM: PS	-	<0.050	high	positive	60	serum	30250598	[169]
miR-27b-3p	multivariate: CRPC FS	0.26 (0.07-0.94)	0.041	low	negative	49	tissue	25115396	[297]
miR-301a-3p	multivariate: BPFS	1.42 (1.06-1.90)	0.019	low	positive	609	tissue	26990571	[298]
miR-30c-5p	multivariate: BPFS	0.34 (0.17-0.68)	0.002	low	negative	103	tissue	24452717	[217]
	univariate: PS	2.38 (1.09-5.22)	0.015	low	positive	44	tissue	26499781	[216]
	multivariate: BPFS	0.49 (0.28-0.85)	0.011	low	negative	207	tissue	31640261	[215]
miR-30d-5p	multivariate: BPFS	5.93 (1.75-20.09)	0.003	low	positive	56	tissue	23231923	[299]

	multivariate: BPFS	1.94 (0.71-5.29)	0.198	high	negative	113 (MSKCC)	tissue	28241827	[300]
miR-31-5p	multivariate: BPFS	0.78 (0.67-0.91)	0.001	low	negative	207	tissue	31640261	[215]
	multivariate: BPFS	15 (0.19-1179)	0.224	low	positive	76	tissue	19676045	[154]
miR-320e	multivariate: BPFS	3.20 (1.10-9.60)	0.034	low	positive	43	tissue	25760964	[253]
miR-326	KM: OS	-	0.027	low	negative	58	tissue	30243091	[301]
	KM: BPFS	-	0.020	low	negative	58	tissue		
miR-335-5p	KM: OS	-	0.339	low	negative	20	tissue	23456549	[302]
	KM: MFS	-	0.185	low	negative	20	tissue		
	KM: BPFS	-	0.713	low	negative	20	tissue		
miR-338-3p	univariate/ KM: BPFS	0.78 (0.54-1.14)	0.020	low	negative	25 (MSKCC)	tissue	26907180	[303]
miR-34b-3p	univariate/ KM: BPFS	3.30 (1.30-8.70)	0.020	high	negative	74	tissue	23147995	[304]
miR-34b/c	multivariate: DFS	2.76 (1.24-6.15)	0.013	low methylation	negative	74	tissue	28143614	[194]
	multivariate: DSS	3.84 (1.27-11.60)	0.017						
miR-34c-5p	KM: PS	-	<0.001	low	negative	49	tissue	21351256	[305]
miR-3607-5p	KM: PS	-	0.046	low	negative	100	tissue	24817628	[306]
miR-3622a-5p	KM: OS	-	0.049	low	negative	124 (TCGA)	tissue	28498363	[307]
miR-3622b-5p	KM: BPFS	-	0.032	low	negative	124	tissue	27611943	[308]
	KM: OS	-	0.262	low	negative	94	tissue		
miR-373-3p	KM: OS	-	0.038	low	negative	56	tissue	30338790	[309]
miR-374b-5p	multivariate: BPFS	0.38 (0.17-0.85)	0.018	low	negative	99	tissue	24191917	[310]
miR-375	univariate: OS	2.69 (1.52-4.77)	<0.001	low	positive	100	exosomes (blood)	25129854	[259]
	multivariate: BPFS	0.42 (0.03-5.60)	0.544	low	negative	76	tissue	19676045	[154]
miR-378-3p	multivariate: DFS	4.79 (1.31-15.52)	0.018	gain vs loss	negative	27 (high + v. high risk)	tissue	25153390	[311]
	multivariate: DFS	1.72 (0.82-3.63)	0.152	gain vs loss	negative	26	tissue		
miR-379-5p	KM: DFS	-	0.012	high	positive	107 (MSKCC)	tissue	25324143	[312]
miR-409-3p	KM: DFS	-	<0.001	high	positive	107 (MSKCC)	tissue	24963047	[313]
miR-410-3p	KM: OS	-	0.011	high	positive	82	tissue	29969630	[314]
miR-423-3p rs6505162	multivariate: CSS	0.64 (0.40-1.01)	0.054	CC vs CA/AA	negative	601	peripheral blood	21149617	[191]
miR-424-3p	multivariate: CFFS	0.44 (0.22-0.87)	0.018	low	negative	404	tissue	31337863	[315]

miR-4288	KM: OS	-	0.070	low	negative	74	tissue	30874288	[316]
miR-4319	KM: OS	-	<0.050	low	negative	40	tissue	29633185	[317]
miR-449b-5p	multivariate: BPFS	1.90 (1.25-2.85)	0.003	low	positive	163	tissue	25416653	[318]
miR-4516	multivariate: BPFS	3.60 (1.30-10.00)	0.013	low	positive	43	tissue	25760964	[253]
miR-452-5p	KM: CRPC FS	-	0.041	low	negative	52	tissue	27070713	[319]
miR-4534	univariate/ KM: OS	6.00 (3.00-17.00)	0.040	low	positive	84	tissue	27634912	[320]
miR-455-5p	KM: RFS	-	0.006	low	negative	107 (MSKCC)	tissue	31111062	[321]
miR-466	KM: RFS	-	0.010	low	negative	75	tissue	28125091	[322]
miR-4723-5p	KM: PS	-	0.043	low	negative	57	tissue	24223753	[323]
miR-500a-5p	KM: OS	-	<0.050	high	positive	148	tissue	28631332	[324]
miR-503-5p	KM: PS	-	<0.010	low	negative	82	tissue	27267060	[325]
miR-505-3p	univariate/ KM: bone MFS	0.25 (0.12-0.56)	0.002	low	negative	81	tissue	30365141	[326]
	univariate/ KM: OS	0.50 (0.10-2.46)	0.002	low	negative	127	tissue		
miR-508-3p	multivariate: BPFS	3.00 (1.10-8.00)	0.030	low	positive	43	tissue	25760964	[253]
miR-515-5p	KM: OS	-	0.018	low	negative	96	tissue	30685303	[327]
miR-548c-3p	KM: RFS	-	0.039	low	negative	n/s (MSKCC)	tissue	25234358	[328]
miR-563	multivariate: BPFS	0.30 (0.10-0.80)	0.023	low	negative	43	tissue	25760964	[253]
miR-573	KM: MFS	-	0.041	low	negative	55	tissue	26451614	[329]
miR-582-3p	univariate/ KM: bone MFS	0.31 (0.15-0.66)	0.002	low	negative	94 (TCGA)	tissue	30852380	[218]
	univariate/ KM: OS	0.43 (0.19-1.88)	0.26	low	negative	157 (TCGA)	tissue		
miR-582-5p	univariate/ KM: bone MFS	0.21 (0.10-0.45)	<0.001	low	negative	94 (TCGA)	tissue	30852380	[218]
	univariate/ KM: OS	0.74 (0.17-3.27)	0.696	low	negative	157 (TCGA)	tissue		
miR-598	multivariate: BPFS	0.30 (0.10-0.90)	0.030	low	negative	43	tissue	25760964	[253]
miR-601	multivariate: BPFS	4.60 (1.60-12.70)	0.004	low	positive	43	tissue	25760964	[253]
miR-605 rs2043556	multivariate: BPFS	1.96 (1.16-3.30)	0.010	GG vs AA/AG	positive	846	peripheral blood	24740842	[192]
miR-615-3p	multivariate: CSS	2.66 (1.29-5.49)	0.008	low	positive	734 (cohort 1+2+3)	tissue	31539518	[330]
	multivariate: BPS	1.38 (0.84-2.26)	0.210	low	positive	239 (cohort 1)	tissue		
	multivariate: BPS	1.05 (0.67-1.66)	0.820	low	positive	222 (cohort 2)	tissue		
	multivariate: BPS	1.31 (0.86-2.01)	0.210	low	positive	273 (cohort 3)	tissue		

	multivariate: BPS	1.46 (0.78-2.73)	0.240	low	positive	387 (cohort 4)	tissue		
miR-626	multivariate: BPFS	0.30 (0.10-0.90)	0.039	low	negative	43	tissue	25760964	[253]
miR-628-3p	multivariate: BPFS	6.60 (1.90-23.50)	0.004	low	positive	43	tissue	25760964	[253]
miR-652-3p	multivariate: BPFS	1.47 (1.09-1.98)	0.013	low	positive	585	tissue	29721191	[331]
	multivariate: MFS	1.16 (0.54-2.48)	0.710	low	positive	585	tissue		
miR-663	multivariate: PFS	2.92 (1.98-4.32)	<0.001	low	positive	127	tissue	24243035	[332]
miR-7-5p	KM: CRPC FS	-	0.004	high	positive	45 (Gleason >= 8)	whole blood	24760272	[292]
miR-708-5p	KM: PS	6.00 (2.20-16.40)	0.006	high	negative	134	tissue	22552290	[333]
miR-744-5p	multivariate: BPFS	8.27 (1.85-37.06)	0.006	low	positive	98 (MSKCC)	tissue	28107193	[334]
miR-93-5p	KM: RFS	2.01 (1.49-2.71)	<0.001	low	positive	n/s (TCGA)	tissue	30582208	[335]
	KM: MFS	0.79 (0.65-0.57)	0.701	low	negative	n/s (TCGA)	tissue		
	KM: OS	2.11 (0.96-4.67)	0.064	low	positive	n/s (TCGA)	tissue		
miR-95-3p	KM: OS	-	0.012	high	positive	n/s (TCGA)	tissue	30027097	[268]
miR-96	KM: OS	2.20 (1.04-4.46)	0.039	low	positive	50	tissue	23951320	[336]
	multivariate: BPFS	3.91 (0.99-15.60)	0.053	low	positive	76	tissue	19676045	[154]
	uni: BPFS	0.71 (0.23-2.24)	0.560	low	negative	73	tissue	22864280	[199]

Table B.1: A table of all individual miRNAs that have been investigated for their prognostic potential in PCa so far, built by performing a systematic review of relevant publications in the Pubmed database. KM, univariate and multivariate tests stand for Kaplan-Meier test, and univariate and multivariate Cox PH regressions respectively. For test entries"univariate/ KM", both univariate Cox PH and KM analysis were performed but there was no associated p-value for the Cox analysis. Thus the HR and 95% CI corresponds to outputs of the univariate Cox PH and the p-value corresponds to KM log-rank test. The values in the "Prognostic test" and "Reference group" columns refer to the statistics and the reference group used for comparison as reported in respective papers. In contrast, the "Association after reference standardisation" column refers to the association of the miRs to progression after standardising the comparisons to "low" miR expression as the reference group. Refer to B.2 for endpoint definitions and adjusted variables included in the multivariate analyses. n/s represents not-specified. Refer to Table 2.1 for the full form of the abbreviated endpoints.

PMID	miR	Endpoint	Endpoint definition	variables in multivariate Cox PH analysis					Ref
				Gleason	T stage	PSA	age	others	
23798998	let-7b-5p	BPFS	PSA $\geq$ 0.2 ng/ml on 2 consecutive follow-up visits	x	x	x			[195]
23798998	let-7b-5p	CFFS	clinical failure declared when either local or distant metastases histologically proven or confirmed by CT or bone scan	x	x	x			[195]
23798998	let-7c	CFFS	clinical failure declared when either local or distant metastases histologically proven or confirmed by CT or bone scan	x	x	x			[195]
22210864	miR-1-3p	DFS	no definition	x			x		[248]
21255804	miR-100-5p	BPFS	PSA $\geq$ 0.2 ng/ml			x		% tumour volume	[249]
24583788	miR-103a-3p	BPFS	serum PSA of 0.2 ng/mL or greater (obtained 6 weeks – 3 months post-operatively), with a second confirmatory level of PSA greater than 0.2 ng/mL	x		x	x	body-mass index	[250]
22986525	miR-106b-5p	DFS	no definition						[251]
21769427	miR-10b-5p	BPFS	the first post-operative PSA of >0.1 ng/ml, as confirmed by at least 1 subsequent increasing value (persistent PSA increase) after achieving undetectable PSA post-operatively, defined as a detection limit of <0.04 ng/ml	x	x	x		surgical margin status	[252]
25760964	miR-1193	BPFS	recurrence after salvage radiation at least twice consecutively following the nadir	x				lymph node status	[253]
27267842	miR-1207-3p	RFS	time from the date of PCa diagnosis to PCa recurrence or non-recurrence death, whichever comes first		x		x		[254]
27267842	miR-1207-3p	CSS	PCa death		x		x		[254]
31822000	miR-1231	OS	no definition	x	x	x	x	differentiation, lymph node met	[255]
24583788	miR-125b-5p	BPFS	serum PSA of 0.2 ng/mL or greater (obtained 6 weeks – 3 months postoperatively), with a second confirmatory level of PSA greater than 0.2 ng/mL (N=31, classified as progressors)	x		x	x	body-mass index	[250]

19676045	miR-125b-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
24350576	miR-126-3p	BPFS	the period between surgical treatment and the measurement of two successive values of serum PSA level $\geq$ 0.2 ng/ml		x			lymph node met, angiolymphatic invasion	[256]
26339409	miR-128-3p	BPFS	the period between surgical treatment and the measurement of two successive values of serum PSA level $\geq$ 0.2 ng/ml		x			lymph node met, angiolymphatic invasion	[257]
31788094	miR-129-5p	OS	no definition	x	x	x		met,TNM,miR139	[258]
28143614	miR-129-5p	DSS	the time elapsed since diagnosis until death or the last follow-up	x					[194]
25129854	miR-1290	OS	no definition						[259]
31772644	miR-1303	OS	no definition						[260]
31266828	miR-130b-3p	OS	the time of surgery until time of the death or last follow-up	x	x	x	x	PSA failure	[261]
31266828	miR-130b-3p	OS	the time of surgery until time of the death or last follow-up	x	x	x	x		[261]
30021600	miR-133a-3p	bone MFS	no definition	x	x	x	x	lymph node status	[262]
30021600	miR-133a-3p	OS	no definition	x	x	x	x	lymph node status	[262]
24610824	miR-133b	BPFS	the time from the date of surgery to that of BCR (postoperative serum PSA concentration $\geq$ 0. 2 ng/mL)	x		x		RB1CC1 gene, surgical tumour margins	[263]
30006541	miR-133b	bone MFS	no definition						[264]
30006541	miR-133b	OS	no definition	x	x	x	x	lymph node status	[264]
31788094	miR-139-5p	OS	overall survival rate	x	x	x		lymph node met, distant met, miR-129	[258]
31269290	miR-139-5p	BPFS	PSA increase of at least 0.2 ng/mL on at least two separate consecutive measurements that are at least 3 months apart	x	x	x	x	surgical margin status, lymph node status	[265]

31269290	miR-139-5p	MFS	lesions within the bone identified on radionuclide bone scan and lymphadenopathy or visceral lesions identified by computed tomography imaging of the abdomen, pelvis and chest	x	x	x			[265]
30674952	miR-141-3p	BPFS	the time from surgery to PSA threshold (no definition of PSA threshold given)	x	x		x	positive surgical margins, apical positive surgical margins, perineural infiltration	[266]
31640261	miR-141-3p	BPFS	no definition					miR-30c-5p, miR-30d-5p, miR-31-5p, miR-148a-3p, miR-221-3p	[215]
28628624	miR-143-5p	RFS	the time from surgery to BCR or death of any cause						[267]
30027097	miR-143-5p	OS	no definition						[268]
20332243	miR-145-5p	PFS	the time from definitive diagnosis to any of the following events after initial treatment: prostate-specific antigen elevation, local progression, metastasis, or disease-specific death as failure of treatment	x	x	x			[197]
23703249	miR-145-5p	BPFS	two consecutive measurements of serum PSA $\geq$ 0.2 ng/ml	x	x	x	x	digital rectal examination	[196]
23703249	miR-145-5p	BPFS	two consecutive measurements of serum PSA $\geq$ 0.2 ng/ml	x	x	x	x	digital rectal examination	[196]
25969144	miR-145-5p	OS	no definition						[198]
22864280	miR-145-5p	BPFS	PSA $\geq$ 0.2 ng ml at two consecutive follow-up visits						[199]
23703249	miR-145-5p	DFS	interval between the radical prostatectomy and the time of biochemical relapse, or the time period between the surgery and the most recent measurement of serum PSA for the patients who did not present biochemical recurrence	x	x	x	x	digital rectal examination	[196]

19676045	miR-145-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery						[154]
23846169	miR-146-3p	BPFS	biochemical disease progression with a serum PSA concentration of 0.2 ng/ml increasing over a 3-month period	x	x	x		surgical margin status, seminal vesicle invasion	[176]
26306811	miR-146a-5p	BPFS	no definition						[269]
25526182	miR-146a-5p	BPFS	post-operative PSA level $\geq$ 0.2 ng/mL	x	x	x	x	positive surgical margins, perineural infiltration	[189]
31640261	miR-148a-3p	BPFS	no definition					miR-30c-5p, miR-30d-5p, miR-31-5p, miR-141-3p, miR-221-3p	[215]
19676045	miR-149-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery						[154]
30009782	miR-150-5p	OS	no definition						[270]
25004396	miR-152-3p	BPFS	no definition						[200]
29599847	miR-152-3p	DFS	the date of the radical prostatectomy to the date of relapse, or date of last follow-up or death if relapse-free	x	x	x	x	surgical margin status, lymph node status	[201]
31490362	miR-153	OS	no definition	x	x	x	x	TNM staging, family history, lymph node met, bone met, type of surgery	[271]
29363862	miR-15b-5p	OS	no definition						[272]
29363862	miR-15b-5p	RFS	no definition	x	x	x			[272]
30032144	miR-16-5p	BPFS	no definition						[204]

19676045	miR-16-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
31122242	miR-17-5p	BPFS	no definition						[273]
19676045	miR-181b-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
19676045	miR-182-3p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
27179774	miR-182-5p	OS	no definition						[274]
24518785	miR-182-5p	BPFS	PSA 0.4 ng/ml or greater during followup	x	x	x		lymph node status, surgical margin status	[276]
24518785	miR-182-5p	PFS	local (prostatic fossa), regional (lymph nodes) or distant (metastasis) progression	x	x	x		lymph node status, surgical margin status	[276]
23383207	miR-182-5p	OS	no definition						[275]
24518785	miR-182-5p	BPFS	PSA 0.4 ng/ml or greater during followup						[276]
24518785	miR-182-5p	PFS	local (prostatic fossa), regional (lymph nodes) or distant (metastasis) progression	x				surgical margin status	[276]
19676045	miR-182-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
30027097	miR-183-3p	OS	no definition						[268]
19676045	miR-183-3p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]

19676045	MiR-184	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
27121312	miR-186-5p	PS	patient survival						[277]
25714029	miR-188-5p	BPFS	the period between surgical treatment and the measurement of two successive values of serum PSA level $\geq$ 0.2 ng/ml	x		x		seminal vesicle invasion	[278]
25714029	miR-188-5p	OS	no definition	x	x	x			[278]
26314494	miR-190a	DFS	no definition						[279]
31335671	miR-191-5p	OS	no definition	x	x	x	x	pelvic lymph node met, bone met, surgical margin status	[280]
30544100	miR-192-5p	BPFS	no definition						[281]
23846169	miR-194-5p	BPFS	biochemical disease progression with a serum PSA concentration of 0.2 ng/ml increasing over a 3-month period	x	x	x		surgical margin status, seminal vesicle invasion	[176]
26080838	miR-195-5p	BPFS	no definition	x	x	x	x		[203]
26650737	miR-195-5p	RFS	no definition						[205]
27175617	miR-195-5p	DFS	no definition						[206]
30032144	miR-195-5p	BPFS	no definition						[204]
26338045	miR-195-5p	BPFS	no definition	x				lymph node met	[202]
26338045	miR-195-5p	OS	no definition	x	x				[202]
29416742	miR-19a-3p	BPFS	no definition						[282]
25409297	miR-200b-3p	BPFS	PSA >0.02 ng/mL						[283]
26499781	miR-203a-3p	PS	no definition						[216]
31678733	miR-204-5p	bone MFS	no definition						[284]
22869146	miR-205-5p	BPFS	biochemical disease progression with a serum PSA concentration $\geq$ 0.2ng/ml increasing over a 3-month period or local recurrence on digital rectal examination confirmed by biopsy or by a subsequent rise in PSA	x	x				[193]

29176717	miR-205-5p	BPFS	Post-operative PSA $\geq$ 0.4 or intervention with salvage therapy					CAPRA-S score, tumour size, perineural infiltration, lymphovascular infiltration	[138]
23571738	miR-205-5p	OS	no definition						[285]
24173237	miR-205-5p	CSS	PCa specific death	x	x				[286]
24173237	miR-205-5p	BPFS	PSA $\geq$ 0.2 ng/mL on two consecutive follow-up visits	x	x				[286]
24173237	miR-205-5p	CSS	PCa specific death	x	x				[286]
24173237	miR-205-5p	CFFS	histologically proven local recurrence or distant metastasis confirmed by CT or bone-scan	x	x				[286]
24173237	miR-205-5p	BPFS	PSA $\geq$ 0.2 ng/mL on two consecutive follow-up visits	x	x				[286]
19676045	miR-205-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery						[154]
31122242	miR-20a-5p	BPFS	no definition						[273]
31122242	miR-20b-5p	BPFS	no definition						[273]
22341810	miR-21-5p	BPFS	Post-operative serum PSA 0.2 ng/ml or greater	x	x	x	x	surgical margin status, lymph node metastasis, capsular invasion	[287]
23353719	miR-21-5p	RFS	either an elevated PSA level ($\geq$ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]
25401698	miR-21-5p	BPFS	PSA>0.4 ng/ mL and rising in a minimum of two different blood samples postoperatively	x	x			non-apical positive surgical margin, apical positive surgical margin	[246]
27040772	miR-21-5p	PFS	no definition		x				[247]
23353719	miR-21-5p	RFS	either an elevated prostate-specific antigen level ($\geq$ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]

23353719	miR-21-5p	RFS	either an elevated prostate-specific antigen level (≥ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]
25401698	miR-21-5p	BPFS	PSA ≥ 0.4 ng/mL and rising in a minimum of two different blood samples post-operatively	x	x			non-apical positive surgical margin, apical positive surgical margin	[246]
27824162	miR-210-3p	CFFS	symptomatic, locally advanced progression or metastasis to bone, visceral organs or lymph nodes verified by radiology	x	x	x	x	tumour size, perineural infiltration, lymphovascular infiltration, non-apical positive surgical margin	[288]
29917185	miR-212-3p	PS	no definition						[289]
30870834	miR-218-5p	bone MFS	no definition						[290]
30870834	miR-218-5p	OS	no definition						[290]
30870834	miR-218-5p	bone MFS	no definition						[290]
30870834	miR-218-5p	OS	no definition						[290]
19585579	miR-221-3p	CFFS	histologically proven local recurrence or distant metastasis confirmed by CT or bone scan	x	x	x	x		[291]
24760272	miR-221-3p	CRPC FS	castration resistance was evaluated through PSA recurrence, which was defined as two consecutive increasing PSA values of more than 1.0 ng/mL and differing by more than 0.2 ng/mL						[292]
31640261	miR-221-3p	BPFS	no definition					miR-30c-5p, miR-30d-5p, miR-31-5p, miR-141-3p, miR-148a-3p	[215]
22864280	miR-221-3p	BPFS	PSA ≥ 0.2 ng/ml at two consecutive follow-up visits						[199]
23353719	miR-221-3p	RFS	either an elevated prostate-specific antigen level (≥ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]

23353719	miR-221-3p	RFS	either an elevated prostate-specific antigen level ($\geq$ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]
23353719	miR-221-3p	RFS	either an elevated prostate-specific antigen level ($\geq$ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]
26325107	miR-221-3p	CRPC FS	CRPC is defined as castrate serum testosterone <50 ng/dl or 1.7 nmol/l plus one of the following types of progression: biochemical progression, radiologic progression						[293]
19676045	miR-221-3p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
24583788	miR-222-3p	BPFS	serum PSA of 0.2 ng/mL or greater (obtained 6 weeks – 3 months postoperatively), with a second confirmatory level of PSA greater than 0.2 ng/mL	x		x	x	body-mass index	[250]
26325107	miR-222-3p	CRPC FS	CRPC is defined as castrate serum testosterone <50 ng/dl or 1.7 nmol/l plus one of the following types of progression: biochemical progression, radiologic progression	x	x	x	x	lymph node met, distant met	[293]
23353719	miR-222-3p	RFS	either an elevated prostate-specific antigen level ($\geq$ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]
23353719	miR-222-3p	RFS	either an elevated prostate-specific antigen level ($\geq$ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]
23353719	miR-222-3p	RFS	either an elevated prostate-specific antigen level ($\geq$ 0.2 ng/ml) after surgical treatment, clinical metastasis or disease specific death	x	x				[245]

19676045	miR-222-3p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
24382668	miR-224-5p	BPFS	PSA $\geq$ 0.2 ng/mL on two occasions.	x	x	x	x		[207]
23136246	miR-224-5p	BPFS	the period between surgery and the persistent increase of serum PSA concentrations, evidenced by 2 consecutive PSA results $\geq$ 0.2 ng/mL	x	x	x			[208]
31876746	miR-23a-3p	OS	no definition		x		x	CRPC occurance time, survival time, outcome, body-mass index, tobacco smoking, family history of cancer, alcohol consumption	[190]
25714010	miR-23a-3p	PS	no definition	x	x			distant met	[294]
23074286	miR-23b-3p	OS	no definition						[295]
23074286	miR-23b-3p	RFS	no definition						[295]
30027097	miR-23b-3p	OS	no definition						[268]
27449037	miR-26a-3p	OS	no definition						[296]
30250598	miR-27a-3p	PS	no definition						[169]
25115396	miR-27b-3p	CRPC FS	CRPC is defined as castrate serum testosterone <50 ng/dl or 1.7 nmol/l plus one of the following types of progression: biochemical progression, radiologic progression	x	x	x	x	lymph node met, distant met	[297]
26990571	miR-301a-3p	BPFS	PSA increase $\geq$ 0.2 ng/ml on at least two occasions, at least 3 months apart	x	x	x	x	surgical margin status, lymph node status	[298]
24452717	miR-30c-5p	BPFS	the time interval between the initial surgery and the day of postoperative PSA 0.2 ng/ml or greater	x	x	x		surgical margin status	[217]
26499781	miR-30c-5p	PS	no definition						[216]

31640261	miR-30c-5p	BPFS	no definition					miR-30d-5p, miR-31-5p, miR-141-3p, miR-148a-3p, miR-221-3p	[215]
23231923	miR-30d-5p	BPFS	continuous elevation with a PSA level >0.2 ng/mL	x	x	x	x	SOCS1	[299]
28241827	miR-30d-5p	BPFS	no definition		x	x	x		[300]
31640261	miR-31-5p	BPFS	no definition					miR-30c-5p, miR-30d-5p, miR-141-3p, miR-148a-3p, miR-221-3p	[215]
19676045	miR-31-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
25760964	miR-320e	BPFS	recurrence after salvage radiation at least twice consecutively following the nadir	x				lymph node status	[253]
30243091	miR-326	OS	the period from radical prostatectomy to death or at the end of the last follow-up						[301]
30243091	miR-326	BPFS	two continuous values of serum PSA level $\geq$ 0.2 ng/ml after radical prostatectomy						[301]
23456549	miR-335-5p	OS	no definition						[302]
23456549	miR-335-5p	MFS	no definition						[302]
23456549	miR-335-5p	BPFS	no definition						[302]
26907180	miR-338-3p	BPFS	time from radical prostatectomy to PSA recurrence						[303]
23147995	miR-34b-3p	BPFS	the first postoperative PSA value greater than 0.1 ng/mL, confirmed by at least 1 undetectable PSA level (detection limit <0.04 ng/mL) after surgery						[304]
28143614	miR-34b/c	DFS	the date of the radical prostatectomy or other curative treatment to the date of biochemical relapse, date of last follow-up, or death if relapse-free		x				[194]

28143614	miR-34b/c	DSS	the time elapsed since diagnosis until death or the last follow-up		x				[194]
21351256	miR-34c-5p	PS	survival time was measured from the time of TURP						[305]
24817628	miR-3607-5p	PS	no definition						[306]
28498363	miR-3622a-5p	OS	no definition						[307]
27611943	miR-3622b-5p	BPFS	no definition						[308]
27611943	miR-3622b-5p	OS	no definition						[308]
30338790	miR-373-3p	OS	no definition						[289]
24191917	miR-374b-5p	BPFS	no definition	x	x	x			[310]
25129854	miR-375	OS	no definition						[259]
19676045	MiR-375	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
25153390	miR-378-3p	DFS	no definition	x	x	x	x	digital rectal examination	[311]
25153390	miR-378-3p	DFS	no definition	x	x	x	x	digital rectal examination	[311]
25324143	miR-379-5p	DFS	no definition						[312]
24963047	miR-409-3p	DFS	no definition						[313]
29969630	miR-410-3p	OS	no definition						[314]
21149617	miR-423-3p	CSS	no definition					KIF3C SNP, PALLD SNP,GABRA1 SNP, SYT6 SNP, ZDHHC7 SNP	[191]
31337863	miR-424-3p	CFFS	clinically palpable tumor recurrence or metastasis verified by radiology	x				vascular infiltration	[315]
30874288	MiR-4288	OS	no definition						[316]
29633185	miR-4319	OS	no definition						[317]
25416653	miR-449b-5p	BPFS	two consecutive measurements of PSA >0.2 ng/mL	x	x	x	x	surgical margin status	[318]
25760964	miR-4516	BPFS	The recurrence after salvage radiation at least twice consecutively following the nadir	x				lymph node status	[253]

27070713	miR-452-5p	CRPC FS	CRPC described as castrate serum levels of testosterone (testosterone <50 ng/dl); Three consecutive rises of prostate-specific antigen (PSA), 1 wk apart, resulting in two 50% increases over the nadir with PSA >2.0 ng/ml; Antiandrogen withdrawal for at least 4 wk for flutamide and for at least 6 wk for bicalutamide; PSA progression, despite consecutive hormonal manipulations; Progression of osseous lesions			[319]
27634912	miR-4534	OS	no definition			[320]
31111062	miR-455-5p	RFS	no definition			[321]
28125091	miR-466	RFS	no definition			[322]
24223753	miR-4723-5p	PS	no definition			[323]
28631332	miR-500a-5p	OS	no definition			[324]
27267060	miR-503-5p	PS	no definition			[325]
30365141	miR-505-3p	bone MFS	no definition			[326]
30365141	miR-505-3p	OS	no definition			[326]
25760964	miR-508-3p	BPFS	The recurrence after salvage radiation at least twice consecutively following the nadir	x	lymph node status	[253]
30685303	miR-515-5p	OS	no definition			[327]
25234358	miR-548c-3p	RFS	no definition			[328]
25760964	miR-563	BPFS	The recurrence after salvage radiation at least twice consecutively following the nadir	x	lymph node status	[253]
26451614	miR-573	MFS	no definition			[329]
30852380	miR-582-3p	bone MFS	no definition			[218]
30852380	miR-582-3p	OS	no definition			[218]
30852380	miR-582-5p	bone MFS	no definition			[218]
30852380	miR-582-5p	OS	no definition			[218]
25760964	miR-598	BPFS	The recurrence after salvage radiation at least twice consecutively following the nadir	x	lymph node status	[253]
25760964	miR-601	BPFS	The recurrence after salvage radiation at least twice consecutively following the nadir			[253]

24740842	miR-605	BPFS	the period of time elapsed between the date of RP and two consecutive PSA values of at least 0.3 ng/ml, one PSA value of at least 0.3 ng/ml followed by androgen-deprivation therapy or radiation therapy, and a single last-recorded PSA value of at least 0.3 ng/ml after RP	x	x	x	x	surgical margin status	[192]
31539518	miR-615-3p	CSS	PCa specific death					Capra-S score	[330]
31539518	miR-615-3p	BPFS	PSA $\geq$ 0.2 ng/mL					Capra-S score	[330]
31539518	miR-615-3p	BPFS	PSA $\geq$ 0.2 ng/mL					Capra-S score	[330]
31539518	miR-615-3p	BPFS	PSA $\geq$ 0.2 ng/mL					Capra-S score	[330]
31539518	miR-615-3p	BPFS	PSA $\geq$ 0.2 ng/mL					Capra-S score	[330]
25760964	miR-626	BPFS	The recurrence after salvage radiation at least twice consecutively following the nadir	x				lymph node status	[253]
25760964	miR-628-3p	BPFS	The recurrence after salvage radiation at least twice consecutively following the nadir	x				lymph node status	[253]
29721191	miR-652-3p	BPFS	PSA increase of at least 0.2 ng/mL on at least two separate consecutive measurements that are at least 3 months apart	x	x	x	x	margin status	[331]
29721191	miR-652-3p	MFS	lesions within the bone identified on radionuclide bone scan and lymphadenopathy or visceral lesions identified by computed tomography imaging of the abdomen, pelvis and chest.	x	x	x			[331]
24243035	miR-663a	PFS	histologically proven local recurrence or distant metastasis confirmed by CT or bone scan	x	x	x	x		[332]
24760272	miR-7-5p	CRPC FS	castration resistance was evaluated through prostate-specific antigen (PSA) recurrence, which was defined as two consecutive increasing PSA values of more than 1.0 ng/mL and differing by more than 0.2 ng/mL						[292]
22552290	miR-708-5p	PS	no definition						[333]

28107193	miR-744-5p	BPFS	no definition	x	x	x	x	lymph node invasion, surgical margin status, extracapsular extension, seminal vesicle invasion	[334]
30582208	miR-93-5p	RFS	no definition						[335]
30582208	miR-93-5p	MFS	no definition						[335]
30582208	miR-93-5p	OS	no definition						[335]
30027097	miR-95-3p	OS	no definition						[268]
23951320	miR-96-5p	OS	no definition						[336]
19676045	miR-96-5p	BPFS	post-operative PSA value >0.1 lg/l confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit <0.04 lg/l) after surgery	x	x	x	x	surgical margin status	[154]
22864280	miR-96-5p	BPFS	PSA $\geq$ 0.2 ng ml at two consecutive follow-up visits						[199]

Table B.2: A table of endpoint definitions and adjusted variables included in multivariate Cox PH analyses for the studies in the systematic review (accompanying table for B.1). For the full form of the abbreviated endpoints, refer to Table 2.1. “x” represents the variable was included in the multi-variate analysis. Abbreviations: BMI=Body mass index; CRPC=Castration Resistant Prostate Cancer; DRE=Digital Rectal Examination; PSA=Prostate specific antigen; SNP=Single-nucleotide polymorphism, TNM=Tumour, node, metastasis

Prognostic miR	Prognostic test			Sample size	Sample type	PMID	Ref
	test: endpoint	**HR (95% CI)**	**p**				
miR-185-5p, miR-221-3p, miR-326	multivariate: BPFS	1.36 (1.03-1.79)	0.031	126 (cohort 1)	tissue	27120795	[337]
	multivariate: BPFS	1.28 (1.00-1.64)	0.048	110 (cohort 2)	tissue		
	multivariate: BPFS	1.91 (1.26-2.91)	0.012	99 (cohort 3)	tissue		
let-7a-5p, miR-125-5p,miR-151a-5p	multivariate: BPFS	0.61 (0.41-0.90)	0.013	122 (cohort 1)	urine	28753866	[212]
	multivariate: BPFS	0.47 (0.28-0.77)	0.003	133 (cohort 2)	urine		
miR-10b-5p, miR-133a, miR-23a-3p, miR-374b-5p	multivariate: BPFS	2.43 (1.45-4.07)	0.008	123 (cohort PCA123)	tissue	30010760	[338]
	multivariate: BPFS	1.44 (1.04-2.00)	0.029	352 (cohort PCA352)	tissue		
	multivariate: BPFS	1.89 (1.08-3.32)	0.027	476 (cohort PCA476)	tissue		
	multivariate: CSS	2.43 (1.45-4.07)	0.021	352 (cohort PCA476)	tissue		
miR-145-5p, miR-183-5p, miR-96-5p, miR-221-5p	univariate: PS	6.50 (n/s)	0.001	49 (cohort 1)	tissue	23184647	[339]
	univariate: PS	6.20 (n/s)	0.001	71 (cohort 2)	tissue		
	univariate: BPFS	2.70 (n/s)	0.007	71 (cohort 2)	tissue		
miR-139-5p, miR-223, miR-301a-3p, miR-454-3p, miR-652-3p	multivariate: BPFS	2.60 (1.80-3.60)	<0.001	491	tissue	26516365	[211]
	multivariate: MFS	4.30 (1.60-11.10)	0.002	491	tissue		
miR-132-3p, miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-375, miR-429	multivariate: OS	3.20 (1.81-5.91)	<0.001	97 (cohort 1)	plasma	28278515	[160]
	multivariate: OS	3.30 (1.64-6.63)	0.001	85 (cohort 2)	plasma		
miR-17-5p, miR-18a-5p, miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-92a-3p	KM: BPFS	-	<0.050	29	tissue	29163712	[209]
let-7a-5p, miR-106a-5p, miR-107, miR-130b-3p, miR-26b-5p, miR-223, miR-451a	KM: BPFS	-	0.031	100	serum	25874774	[210]

Table B.3: A table of signature miRNAs of prognostic importance in PCa identified in the systematic review. KM, univariate and multivariate tests stand for Kaplan-Meier test, and univariate and multivariate Cox PH regressions respectively. The “Association” column was obtained after standardising the comparisons to low as the reference. TCGA and MSKCC represent cohorts provided by the respective data repositories. Refer to Table B.4 for endpoint definitions and adjusted variables included in the multivariate analyses. n/s represents not-specified. Refer to Table 2.1 for the full form of the abbreviated endpoints.

PMID	Endpoint	Endpoint definition	Variables in multivariate Cox PH analysis					Ref
			Gleason	T stage	PSA	age	others	
27120795	BPFS	PSA cut-off $\geq$ 0.2 ng/ml	x	x	x	x	SMS	[337]
28753866	BPFS	PSA $\geq$ 0.2 ng/ml	x		x			[212]
30010760	BPFS	a postoperative PSA test $\geq$ 0.2 ng/ml					Capra-s	[338]
23184647	PS	patient/ percent survival						[339]
26516365	BPFS	a PSA increase of at least 0.2 ng/mL on at least two separate consecutive measurements that are at least 3 months apart	x	x	x			[211]
28278515	OS	the time from initiation of the first cycle of docetaxel to the time of death or last follow-up			x		haemoglobin, alkaline phosphatase	[160]
29163712	BPFS	no def						[209]
25874774	BPFS	no def						[210]

Table B.4: A table of endpoint definitions and adjusted variables for the studies in the systematic review (accompanying table for B.3). "x" represents the variable was included in the multi-variate analysis. For the full form of the abbreviated endpoints, refer to Table 2.1. Abbreviations: BMI=Body mass index; CRPC=Castration Resistant Prostate Cancer; DRE=Digital Rectal Examination; PSA=Prostate specific antigen; SNP=Single-nucleotide polymorphism, TNM=Tumour, node, metastasis

Coefficients of features selected for the final models

Features	Coefficients
(Intercept)	0.507
Gleason group 2 VS Gleason group 1	0.0703
Gleason group 5 VS Gleason group 1	-0.72
T2 stage VS T1 stage	0.59
T3 stage vs T1 stage	-0.515

Table B.5: The coefficients of features for clinical variables (CVs) model (model i). Only variables Gleason group and tumour stage were predictive of recurrence.

Features	Coefficients
(Intercept)	-1.11
hsa-miR-200a-5p:14CA:0:0:0	-0.00434
hsa-miR-214-5p:0:0:0:0	-1.01
hsa-let-7a-3p:0:0:0:0	0.254
hsa-miR-132-3p:0:A:0:0	0.117
hsa-miR-320a:0:0:a:0	0.826
hsa-miR-143-3p:3GA:AA:0:tc	0.0554
hsa-let-7f-1-3p:0:T:0:c	-0.46
hsa-miR-629-5p:0:0:0:T	0.106
hsa-let-7f-5p:0:AG:0:t	-0.514
hsa-miR-151a-3p:0:TA:0:gg	-0.452
hsa-miR-126-3p:0:A:0:g	-0.786
hsa-miR-15b-5p:0:0:0:0	-0.404
hsa-miR-222-3p:0:0:0:0	0.352
hsa-miR-106b-3p:0:0:A:c	0.455
hsa-miR-29a-3p:8TA:0:0:a	-0.0173
hsa-miR-664a-3p:0:0:0:a	0.564
hsa-miR-143-3p:0:TA:0:A	0.516
hsa-miR-151a-3p:3GA:A:0:A	0.771
hsa-miR-379-5p:4CT:0:0:0	-0.307
hsa-miR-148a-3p:16GA:0:0:t	0.000229
hsa-miR-99b-5p:13TC:0:0:0	0.00244
hsa-miR-99b-5p:15CA:0:0:0	-0.0358
hsa-miR-223-3p:0:0:0:a	-0.383
hsa-miR-30e-3p:8AT:T:0:c	-0.0885
hsa-miR-25-3p:19TC:0:0:0	-0.0564
hsa-miR-152-3p:0:A:0:0	0.0743
hsa-miR-30a-3p:9TG:0:c:0	-0.0923
hsa-let-7f-5p:2TG:0:0:0	0.0396
hsa-miR-30e-3p:0:CC:0:gc	0.919
hsa-miR-28-3p:0:C:0:ga	-0.0207
hsa-miR-340-5p:0:0:0:0	-0.654
hsa-miR-21-5p:4GC:A:0:0	-0.131
hsa-miR-19a-3p:0:0:0:0	0.161
hsa-miR-30e-5p:11TC:0:0:0	0.677
hsa-miR-197-3p:0:A:0:c	-0.482
hsa-miR-181a-3p:0:0:0:0	-0.0993

hsa-miR-22-3p:0:T:0:T	0.0721
hsa-miR-182-5p:14CG:0:0:ct	-0.0082
hsa-miR-30a-5p:0:A:t:0	-0.0691
hsa-miR-30a-3p:1TC:0:0:gc	-0.128
hsa-miR-30e-3p:0:A:c:c	0.0272
hsa-miR-182-5p:14AG:0:0:ct	-0.17
hsa-miR-151a-3p:3GA:A:0:g	0.00717
hsa-miR-10b-5p:0:0:0:T	0.158
hsa-miR-3065-3p:0:0:0:0	0.0839
hsa-miR-361-5p:0:GA:0:c	0.048
hsa-miR-29a-3p:6GC:0:0:a	-0.402
hsa-let-7a-5p:6GT:GT:0:tt	-0.227
hsa-miR-143-3p:7TG:A:0:tc	0.155
hsa-miR-17-5p:4CA:0:0:0	0.26
hsa-miR-223-3p:0:G:0:0	-0.219
hsa-let-7f-5p:6CT:0:0:t	0.653
hsa-miR-625-3p:0:0:0:ca	0.24
hsa-miR-148a-3p:7TC:0:0:t	0.102
hsa-miR-10b-5p:16GA:0:0:g	0.0687
hsa-miR-101-3p:9GT:0:0:a	-0.125
hsa-miR-143-3p:7CG:GT:0:c	-0.0387
hsa-miR-199b-5p:0:0:0:ttc	0.167
hsa-miR-29a-3p:0:A:C:tta	0.139
hsa-miR-25-3p:0:AT:0:0	-0.223
hsa-miR-2355-5p:0:0:0:T	0.147
hsa-miR-22-3p:0:AA:0:t	-0.377
hsa-miR-148a-3p:0:0:G:0	0.127
hsa-miR-1307-3p:0:0:0:GT	0.0484
hsa-miR-99b-5p:16TC:0:0:0	0.00807
hsa-miR-532-5p:0:0:0:t	0.0597
hsa-miR-145-5p:16TG:0:0:0	0.0305
hsa-miR-361-5p:0:0:0:0	0.376
hsa-miR-99b-5p:8TA:0:0:g	-0.47
hsa-miR-128-3p:0:AG:0:0	-0.125
hsa-miR-590-5p:0:0:0:0	0.00877
hsa-miR-92a-3p:14TC:0:0:0	0.231
hsa-miR-10b-5p:0:A:t:g	-0.727
hsa-let-7c-5p:0:GT:0:tt	-0.045
hsa-miR-148a-3p:7GC:0:0:0	0.219
hsa-miR-101-3p:4TA:T:0:0	-0.169
hsa-miR-21-5p:12GA:0:0:0	-0.0966
hsa-miR-378a-3p:0:A:0:gc	0.286
hsa-miR-10a-5p:0:0:0:T	0.385
hsa-let-7b-5p:2CG:0:0:tt	-0.2
hsa-miR-182-5p:12GT:0:0:act	-0.0272
hsa-miR-148a-3p:16TA:0:0:0	0.012
hsa-miR-182-5p:16CA:0:0:t	-0.0217
hsa-let-7b-5p:0:AT:0:tt	-0.306
hsa-miR-10b-5p:8AT:CT:0:gtg	0.241
hsa-miR-30e-3p:0:AA:0:gc	-0.000759
hsa-miR-582-3p:0:0:t:0	0.412
hsa-miR-23b-3p:0:AGA:0:cc	-0.21
hsa-miR-182-5p:0:0:t:0	-0.981

hsa-miR-1296-5p:0:0:0:0	0.328
hsa-miR-99b-5p:0:A:c:0	0.498
hsa-let-7a-5p:0:0:G:0	-0.157
hsa-miR-143-3p:14AT:TT:0:0	-0.0644
hsa-miR-7-1-3p:0:0:0:0	0.399
hsa-let-7d-3p:0:A:0:t	0.0856
hsa-miR-375:9TG:0:T:0	-0.0186
hsa-miR-143-3p:10AG:AT:0:c	-0.636
hsa-miR-1287-5p:0:0:0:0	0.261
hsa-miR-29c-3p:0:GT:0:tta	0.477
hsa-miR-140-3p:0:0:0:AC	-0.0886
hsa-miR-744-5p:0:A:0:ca	-0.178
hsa-miR-151a-3p:0:TT:0:g	-0.201
hsa-miR-151a-3p:0:TT:0:A	0.268
hsa-miR-30b-5p:0:A:0:0	-0.0503
hsa-miR-101-3p:0:C:G:0	0.306
hsa-miR-23a-3p:9TG:0:0:0	-0.0836
hsa-miR-29b-3p:0:A:0:tt	0.0114
hsa-miR-22-3p:17CA:A:0:t	0.0313
hsa-miR-363-3p:0:0:a:0	-0.213
hsa-let-7c-5p:1AT:0:0:0	-0.343
hsa-let-7c-3p:0:0:0:c	0.0712
hsa-miR-200b-3p:0:T:0:a	-0.233

Table B.6: The coefficients of features for all miR species model (model ii). All together 112 variables miR species were predictive of recurrence.

Features	**Coefficients**
(Intercept)	0.692
hsa-miR-30e-3p:0:CC:0:gc	0.107
hsa-miR-30e-5p:11TC:0:0:0	0.000179
hsa-miR-99b-5p:8TA:0:0:g	-0.0367
hsa-miR-21-5p:12GA:0:0:0	-0.0968
hsa-miR-21-5p:6GT:0:0:C	-0.0274
hsa-miR-143-3p:7TG:0:0:A	0.0667
T2 stage VS T1 stage	0.55
T3 stage VS T1 stage	-0.267
Gleason group 5 VS Gleason group 1	-0.519

Table B.7: The coefficients of features for all miR species and clinical variables (model iii). Altogether 8 variables: 6 isomiRs and clinical variables Gleason group and tumour stage, were predictive of recurrence.

Features	Coefficients
intercept	0.22
hsa-miR-148a-3p:0:AC:0:t	-0.0109
hsa-miR-148a-3p:11TA:0:0:0	-0.0498
hsa-miR-148a-3p:8TA:0:0:0	-0.02
hsa-miR-148a-3p:11TA:A:0:t	-0.0125
hsa-miR-148a-3p:6TG:T:0:0	-0.0295
hsa-miR-148a-3p:14TG:A:0:t	0.0681
hsa-miR-148a-3p:6TG:0:0:CT	-0.00236
hsa-miR-148a-3p:5AT:CT:0:gt	0.04
hsa-miR-148a-3p:0:0:G:0	0.0554
hsa-miR-148a-3p:16CA:0:0:t	-0.0177
hsa-miR-148a-3p:7GC:0:0:0	0.0777
hsa-miR-148a-3p:16CA:0:0:0	-0.0267
hsa-miR-148a-3p:0:C:0:gt	0.0272
hsa-miR-148a-3p:17TC:0:0:0	0.104
hsa-miR-148a-3p:8TA:0:0:t	-0.0779
T2 stage VS T1 stage	0.583
T3 stage VS T1 stage	-0.611
Gleason group 2 VS Gleason group 1	0.195
Gleason group 5 VS Gleason group 1	-0.729
PSA at diagnosis	0.000431

Table B.8: The coefficients of features for model based on miR-148a-3p isomiRs and clinical variables (model iv). Altogether 18 variables: 15 isomiRs of miR-148a-3p, and clinical variables Gleason group, tumour stage and serum PSA, predictive of recurrence.

Features	Coefficients
(Intercept)	0.422
hsa-miR-582-5p:0:0:0:t	0.0779
T2 stage VS T1 stage	0.658
T3 stage VS T1 stage	-0.635
Gleason group 2 VS Gleason group 1	0.314
Gleason group 4 VS Gleason group 1	-0.0516
Gleason group 5 VS Gleason group 1	-0.764
PSA at diagnosis	0.00105

Table B.9: The coefficients of features for model based on miR-582-5p isomiRs and clinical variables (model v). Altogether 4 variables: hsa-miR-582-5p:0:0:0:t isomiR and clinical variables Gleason group, tumour stage and serum PSA, predictive of recurrence.

Features	Coefficients
(Intercept)	0.329
T2 stage VS T1 stage	0.54
T3 stage VS T1 stage	-0.312
Gleason group 5 VS Gleason group 1	-0.59

Table B.10: The coefficients of features for model based on isomiRs of miR-148a-3p and miR-582-5p and clinical variables (model vi). Only clinical variables Gleason group and tumour stage were predictive of recurrence.

Features	Coefficients
(Intercept)	0.443
miR-222-3p	0.134
miR-664a-3p	0.0725
miR-27a-3p	-0.0571
miR-181a-3p	-0.0703
T2 stage VS T1 stage	0.507
T3 stage VS T1 stage	-0.413
Gleason group 5 VS Gleason group 1	-0.597

Table B.11: The coefficients of features for model based on parent miRs signature and clinical variables (model vii) Altogether 6 variables: 4 parent miRs, and clinical variables Gleason group and tumour stage, were predictive of recurrence.

Features	Coefficient
(Intercept)	0.199
T2 stage VS T1 stage	0.605
T3 stage VS T1 stage	-0.182
Gleason group 5 VS Gleason group 1	-0.546

Table B.12: The coefficients of features for model based on clusters signature and clinical variables (model viii). Only Gleason group and tumour stage, and no clusters signature were predictive of recurrence.

Features	Coefficients
intercept	0.641
3' end templated AND within seq non-templated	-0.927
5' end templated AND 3' end non-templated AND within seq non-templated	0.174
T2 stage VS T1 stage	0.685
T3 stage VS T1 stage	-0.595
Gleason group 2 VS Gleason group 1	0.0859
Gleason group 5 VS Gleason group 1	-0.899
PSA at diagnosis	0.00019

Table B.13: The coefficients of features for model based on isotypes signature and clinical variables (model ix). Altogether 5 variables: 2 isotype signatures, and clinical variables Gleason group, tumour stage and serum PSA, were predictive of recurrence.

Features	Coefficients
(Intercept)	0.659
-1	-1.07
T2 stage VS T1 stage	0.679
T3 stage VS T1 stage	-0.611
Gleason group 2 VS Gleason group 1	0.0627
Gleason group 5 VS Gleason group 1	-0.946

Table B.14: The coefficients of features for model based on 3' end size variations signature and clinical variables (model x). Only 3 features: signature group of isomiRs with 1 base deletion at their 3' end, and clinical variables Gleason group and tumour stage, were predictive of recurrence.

Features	Coefficients
(Intercept)	6.13
0	-5.48
-1	0.555
1	4.12
2	-0.305
-3	0.399
-2	-1.83
pT.grpT2	-0.701
pT.grpT3	-2.56
pT.grpT4	-2.32
Gleason group 2 VS Gleason group 1	-3.14
Gleason group 3 VS Gleason group 1	-3.69
Gleason group 4 VS Gleason group 1	-3.95
Gleason group 5 VS Gleason group 1	-5
PSA at diagnosis	0.0108

Table B.15: The coefficients of features for model based on 5' end size variations signature and clinical variables (model xi). Altogether 9 variables: signature groups of isomiRs with 0 base changes, 1 base and 2 base additions, -2 and -3 base deletions at their 5' end; and clinical variables Gleason group, tumour stage and PSA at diagnosis, were predictive of recurrence.

Features	Coefficients
(Intercept)	0.344
AGCTGAT	-0.0534
GAGATTA	0.0363
CTTTCAG	0.0347
TTGGCAG	-0.00444
ACCCGTT	-0.059
T2 stage VS T1 stage	0.421
T3 stage VS T1 stage	-0.354
Gleason group 5 VS Gleason group 1	-0.46

Table B.16: The coefficients of features for model based on 7mer-m8 seeds signature and clinical variables (model xii). Altogether 7 variables: 5 7mer-m8 seed signature groups, and clinical variables Gleason group and tumour stage,were predictive of recurrence.

Features	Coefficients
(Intercept)	0.343
AGCTGA	-0.058
GAGATT	0.0455
CTTTCA	0.0489
T2 stage VS T1 stage	0.426
T3 stage VS T1 stage	-0.354
Gleason group 5 VS Gleason group 1	-0.47

Table B.17: The coefficients of features for model based on 6mer seeds signature and clinical variables (model xiii). Altogether 5 variables: 3 6mer seed signature groups, and clinical variables Gleason group and tumour stage, were predictive of recurrence.

Features	Coefficients
(Intercept)	0.53
iso.5p	0.0362
iso.5p+mism	0.0815
T2 stage VS T1 stage	0.615
T3 stage VS T1 stage	-0.589
Gleason group 2 VS Gleason group 1	0.225
Gleason group 5 VS Gleason group 1	-0.758
PSA at diagnosis	0.000361

Table B.18: The coefficients of features for model based on miR-148a-3p isotypes signature and clinical variables (model xiv). Altogether 5 variables: 2 miR-148a-3p isotypes signatures, and clinical variables Gleason group, tumour stage and PSA at diagnosis, were predictive of recurrence.

gene symbol	Ensembl transcript ID	log2FC	p-value	FDR p-value	miR-27a-3p target
ITSN2	ENST00000361999	-3.35	5e-18	1.86e-13	Y
DDX3X	ENST00000646319	-2.8	2.17e-09	4.04e-05	N
SRP14	ENST00000558720	2.06	1.13e-08	0.000141	N
SMARCA4	ENST00000643296	-3.14	5.27e-08	0.000441	N
PLLP	ENST00000613167	-2.43	5.92e-08	0.000441	N
KPNB1	ENST00000540627	-3.22	1.21e-07	0.000688	Y
UNC13B	ENST00000619578	-2.14	1.29e-07	0.000688	N
CCT3	ENST00000368259	1.68	2.41e-07	0.00112	Y
NKRF	ENST00000649446	-3.09	4.51e-07	0.00187	N
DSN1	ENST00000373740	2.45	7.13e-07	0.00243	N
ERO1A	ENST00000554019	-2.3	7.18e-07	0.00243	N
ATP6V0E2	ENST00000615196	-3.12	8.37e-07	0.00243	N
CLPTM1	ENST00000546079	2.52	8.49e-07	0.00243	N
DIDO1	ENST00000354665	1.85	1.08e-06	0.00288	Y
IDE	ENST00000650060	-1.92	1.19e-06	0.00296	N
SMIM7	ENST00000593409	-1.85	1.65e-06	0.00384	N
ARF1	ENST00000540651	1.88	1.82e-06	0.00384	N
EIF4G1	ENST00000427845	-1.81	1.86e-06	0.00384	Y
LSS	ENST00000630761	1.95	2.48e-06	0.00486	N
SGSM1	ENST00000400358	-2.11	2.87e-06	0.00535	N
RECQL	ENST00000421138	-2.38	3.52e-06	0.00607	N
HNRNPU	ENST00000640056	-1.3	3.73e-06	0.00607	N
TM9SF4	ENST00000217315	-1.91	3.74e-06	0.00607	Y
PHLPP2	ENST00000393524	-1.77	3.95e-06	0.00614	Y
HBB	ENST00000335295	-1.58	6.38e-06	0.00915	N
MAP4K4	ENST00000456652	1.85	7.26e-06	0.01	Y
RGL2	ENST00000487403	2.7	8.05e-06	0.0107	Y
GOLGA2	ENST00000421699	-1.04	9.72e-06	0.0121	N
HPS4	ENST00000429411	2	9.86e-06	0.0121	N
SPIN3	ENST00000638289	-1.78	1e-05	0.0121	N
FN1	ENST00000446046	-1.59	1.08e-05	0.0126	Y
RPL8	ENST00000529163	0.986	1.19e-05	0.0134	N
COL3A1	ENST00000487010	2.31	1.23e-05	0.0135	N
HSPA8	ENST00000533238	-3.03	1.44e-05	0.0153	Y
DDX5	ENST00000578804	-2.19	1.77e-05	0.0183	Y
WIPI2	ENST00000404704	-1.74	2.07e-05	0.0209	Y
RACK1	ENST00000512805	0.888	2.2e-05	0.0215	N
KCTD3	ENST00000495537	1.04	2.25e-05	0.0215	N
FOLH1	ENST00000533034	-1.1	2.53e-05	0.0236	N
MAPKBP1	ENST00000456763	2.04	2.68e-05	0.0244	N
TMPO	ENST00000393053	-2.53	2.95e-05	0.0262	N
SCFD1	ENST00000463622	1.44	3.32e-05	0.0284	N
PLCG1	ENST00000609257	1.81	3.36e-05	0.0284	Y
RNF208	ENST00000392827	1.79	3.72e-05	0.0308	N
CCNL2	ENST00000482621	1.53	4.2e-05	0.034	Y
SPATS2	ENST00000549412	-1.64	4.36e-05	0.0346	N
TBC1D14	ENST00000446947	0.93	4.56e-05	0.0354	N
HNRNPK	ENST00000351839	1	4.79e-05	0.0358	N
LDLR	ENST00000558518	-1.66	4.8e-05	0.0358	Y
AC073111.5	ENST00000641234	1.72	5.14e-05	0.0368	N
PLA2G4B	ENST00000458483	1.5	5.18e-05	0.0368	N
CCDC106	ENST00000588740	1.44	5.23e-05	0.0368	N

PUM1	ENST00000257075	-1.98	5.37e-05	0.0368	Y
ALG11	ENST00000649651	-1.68	5.46e-05	0.0368	N
LSR	ENST00000427250	1.23	5.53e-05	0.0368	N
DHX38	ENST00000567142	2.13	5.76e-05	0.0377	N
M6PR	ENST00000539143	1.88	6.11e-05	0.0387	N
LIN9	ENST00000481685	-1.72	6.2e-05	0.0387	Y
N4BP2L2	ENST00000446957	1.53	6.22e-05	0.0387	N
CMC2	ENST00000565108	-1.84	6.39e-05	0.0389	N
DNMT1	ENST00000586588	1.75	6.48e-05	0.0389	Y
HIPK1	ENST00000340480	-1.66	6.63e-05	0.0392	Y
GCH1	ENST00000536224	2.1	7.18e-05	0.041	N
RNF40	ENST00000324685	1.19	7.21e-05	0.041	N
TTC14	ENST00000465065	-1.66	7.38e-05	0.041	N
SMN2	ENST00000628642	1.49	7.53e-05	0.0413	N
MAN1B1	ENST00000474902	1.33	7.95e-05	0.0429	N
TBL1XR1	ENST00000636864	0.823	8.19e-05	0.0436	Y
WDR4	ENST00000398208	-1.72	9.09e-05	0.0477	N
MPP7	ENST00000496637	-1.59	9.55e-05	0.0481	N
NCAPH	ENST00000435975	-1.63	9.6e-05	0.0481	N
IL6ST	ENST00000336909	-1.2	9.71e-05	0.0481	Y
ABCA2	ENST00000371605	-1.84	9.75e-05	0.0481	N
TPM3	ENST00000473036	-1.94	9.8e-05	0.0481	N
PHTF2	ENST00000275575	1.64	0.000107	0.0497	N
EWSR1	ENST00000360091	0.889	0.000107	0.0497	N
MPV17	ENST00000486898	-1.6	0.000107	0.0497	N
QARS	ENST00000494984	1.46	0.000108	0.0497	N
AADAT	ENST00000515480	1.79	0.000109	0.0497	N

Table B.19: Differentially expressed genes (n=79) between the ASO-27a and ASO-TNC treated groups. Putative miR-27a-3p targets (n=22), identified after consulting six miR targets databases, are labelled with a Y (yes) or N (no) in the column titled “miR-27a-3p target”.

Appendix C

Appendix methods

Signature sets for isomiR signature-based models

IsomiRs grouped into isotype signatures

For the isotypes biological criteria (criteria iii; model ix), there were a total of 16 modules the isomiRs could be grouped into, according to the different combinations of the four main isotypes. These were:

i) no changes (canonical)

ii) 5' end templated isotype

iii) 3' end templated isotype

iv) 3' end non-templated isotype

v) within seq non-templated isotype

vi) 5' end templated AND 3' end templated isotypes

vii) 5' end templated AND 3' end non-templated isotypes

viii) 5' end templated AND within seq non-templated isotypes

ix) 3' end templated AND 3' end non-templated isotypes

x) 3' end templated AND within seq non-templated isotypes

xi) 3' end non-templated AND within seq non-templated isotypes

xii) 5' end templated, 3' end templated AND 3' end non-templated isotypes

xiii) 5' end templated, 3' end non-templated AND within seq non-templated isotypes

xiv) 5' end templated, 3' end non-templated AND within seq non-templated isotypes

xv) 3' end templated, 3' end non-templated AND within seq non-templated isotypes

xvi) 5' end templated, 3' end templated, 3' end non-templated AND within seq non-templated isotypes

IsomiRs grouped into signatures based on identical size variations at their 3' end

For the identical size variations at the 3' end biological criteria (criteria iv; model x), there were seven signatures the isomiRs could be grouped into. These were:

i) no changes at the 3' end compared to their canonical form

ii) any 1 base added at the 3' end compared to their canonical form

iii) any 2 bases added at the 3' end compared to their canonical form

iv) any 3 bases added at the 3' end compared to their canonical form

v) any 1 base deleted at the 3' end compared to their canonical form

vi) any 2 bases deleted at the 3' end compared to their canonical form

vii) any 3 bases deleted at the 3' end compared to their canonical form

IsomiRs grouped into signatures based on identical size variations at their 5' end

For the identical size variations at the 5' end biological criteria (criteria v; model xi), there were six signatures the ismiRs could be grouped into. These were:

i) no changes at the 5' end compared to their canonical form

ii) any 1 base added at the 5' end compared to their canonical form

iii) any 2 bases added at the 5' end compared to their canonical form

iv) any 1 base deleted at the 5' end compared to their canonical form

v) any 2 bases deleted at the 5' end compared to their canonical form

vi) any 3 bases deleted at the 5' end compared to their canonical form

IsomiRs of miR-148a-3p grouped into signatures according to their isotypes

For miR-148a-3p isomiRs grouped according to isotypes (criteria viii; model xiv), miRs were grouped according to the different combinations of the four main isotypes of miR-148a-3p. These were:

i) all miR species that are not miR-148a-3p isomiRs

ii) canonical miR-148a-3p

iii) miR-148a isomiRs with 5' end templated isotype

iv) miR-148a isomiRs with 3' end templated isotype

v) miR-148a isomiRs with 3' end non-templated isotype

vi) miR-148a isomiRs with within seq non-templated isotype

vii) miR-148a isomiRs with 5' end templated AND 3' end templated isotypes

viii) miR-148a isomiRs with 5' end templated AND within seq non-templated isotypes

ix) miR-148a isomiRs with 3' end templated AND 3' end non-templated isotypes

x) miR-148a isomiRs with 3' end templated AND within seq non-templated isotypes

xi) miR-148a isomiRs with 3' end non-templated AND within seq non-templated isotypes

xii) miR-148a isomiRs with 3' end templated, 3' end non-templated AND within seq non-templated isotypes

Appendix D

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