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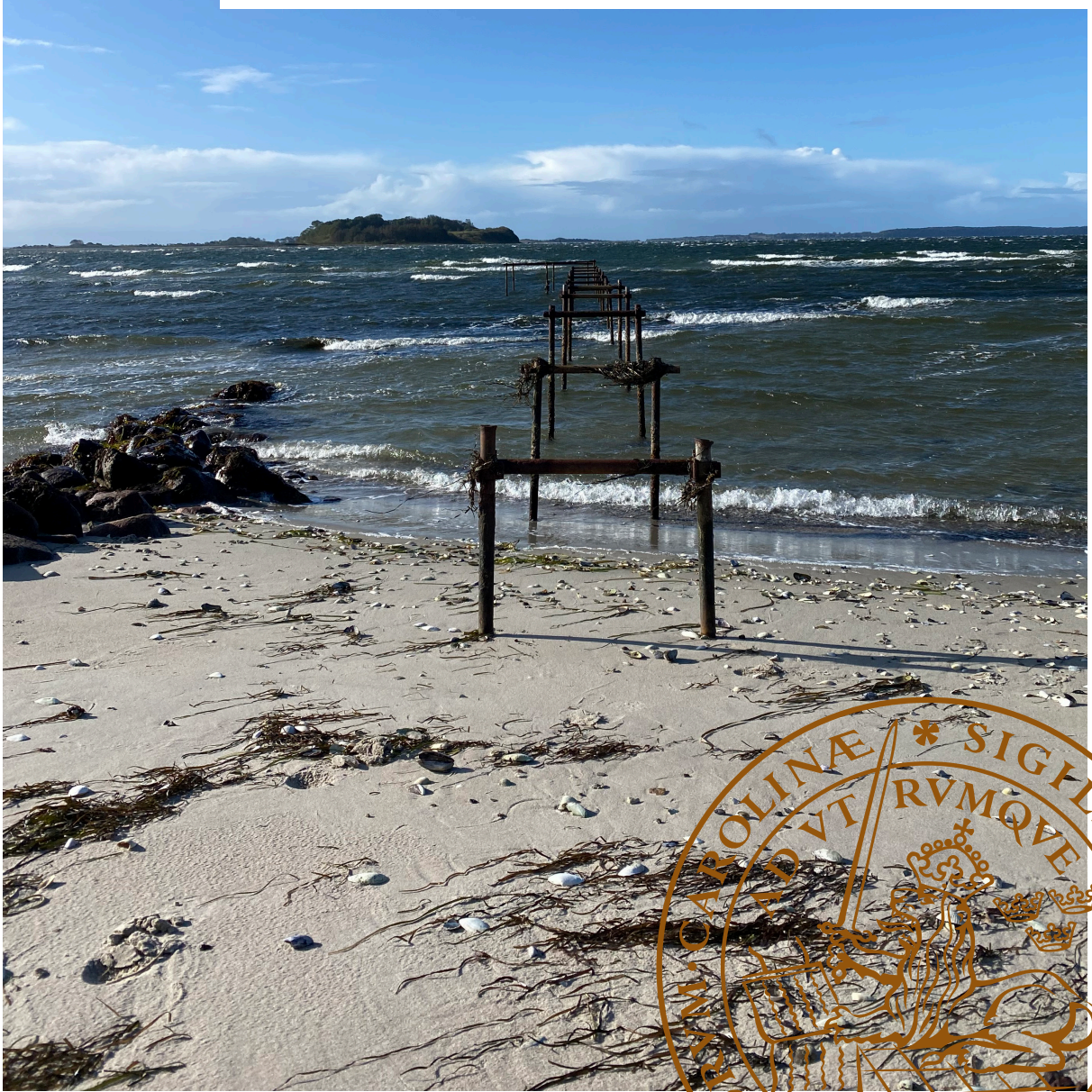

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James Cassidy

DOCTORAL DISSERTATION

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Abstract:

Prostate cancer (PCa) is a leading cause of death in men worldwide. If PCa remains localised, it is potentially curable; however, if it has metastasised, which typically results in spread to the bone, then there are currently no curative treatments. One potentially new avenue for PCa treatment is using microRNA (miRNA) molecules. In this thesis, we look at the therapeutic potential of miRNA-379 (miR-379), which we have previously shown could drive metastatic spread to the bone.

In Paper I, we found using a series of in vitro functional experiments, that miR-379 overexpression inhibited cell growth, migration, colony formation, and adhesion to bone cells. This was done in both a normal media setting and an osteoblast-conditioned media (OBCM) setting to mimic the bone environment in vitro. We also performed intracardiac injections in vivo and found that miR-379 upregulation inhibits metastatic spread to the bone. As a result, miR-379 may be a suitable candidate for blocking metastatic spread to the bone.

In Paper II, we investigated the biological role of miR-379 to better understand the pathways and their relevance in PCa homing to the bone. We performed cytokine arrays to and found that prostate-specific antigen (PSA) was consistently enriched in an anti-miR-379 setting. We performed an immunoprecipitation and found that miR-379 does not directly bind to PSA, but the effect is indirect through the androgen receptor (AR), a key regulator of PSA. We have validated that miR-379 overexpression reduces protein expression of PSA and AR and that in patients, there is a negative correlation between miR-379 and both serum PSA and AR.

In Paper III, we investigated the biological impact of adenosine-to-inosine (A-to-I) miR-379 editing on PCa development. We compared the unedited and edited isoforms on functional assays and found that there was a difference that is dependent on the AR status of cell lines. We also investigated the effect of editing on the expression of epithelial and mesenchymal markers and found that both isoforms increased cell-cell adhesion. Differences in unedited and edited targets were also explored using in silico tools, and a difference was seen in both targets and their respective pathways.

In conclusion, this thesis suggests that miR-379 can inhibit metastatic spread to the bone. We also explore some of the pathways involved in miR-379 dysregulation and the impact of A-to-I editing on the miRNA.

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Table of Contents

Abstract

Prostate cancer (PCa) is a leading cause of death in men worldwide. If PCa remains localised, it is potentially curable; however, if it has metastasised, which typically results in spread to the bone, then there are currently no curative treatments. One potentially new avenue for PCa treatment is using microRNA (miRNA) molecules. In this thesis, we look at the therapeutic potential of miRNA-379 (miR-379), which we have previously shown could drive metastatic spread to the bone.

In Paper I, we found using a series of in vitro functional experiments, that miR-379 overexpression inhibited cell growth, migration, colony formation, and adhesion to bone cells. This was done in both a normal media setting and an osteoblast-conditioned media (OBCM) setting to mimic the bone environment in vitro. We also performed intracardiac injections in vivo and found that miR-379 upregulation inhibits metastatic spread to the bone. As a result, miR-379 may be a suitable candidate for blocking metastatic spread to the bone.

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In conclusion, this thesis suggests that miR-379 can inhibit metastatic spread to the bone. We also explore some of the pathways involved in miR-379 dysregulation and the impact of A-to-I editing on the miRNA.

Popular science summary

Prostate cancer (PCa) is responsible for a huge number of deaths among men worldwide. Since it primarily affects more elderly men, it is a disease whose incidence is only rising, with the trend seen particularly in Western countries with an ageing population. Intriguingly, most men with PCa will not die of the disease since it is typically slow-growing, and men will usually succumb to other age-related causes of death. There are several treatments available to men, and these can be curative or halt the disease progression; however, a fraction of men will develop a more aggressive form of the cancer. This aggressive PCa will often metastasise to distant sites, most typically the bone, and unfortunately for these patients, the prognosis is very bleak. There are currently no curative treatments available to these patients, so the need for newer therapeutic options is vital. We focus on a set of molecules called miRNAs, which have been shown to be implicated in many human diseases, including PCa. We have also previously shown that one miRNA, miR-379, can drive the spread of cancer to the bone when it is downregulated. With this logic, we sought to investigate if, by increasing the levels of miR-379, we could stop aggressive PCa from spreading to the bone. To investigate this, we started by testing if miR-379 could stop the spread of PCa to the bone and test its functional effects on cell growth, invasion, colony formation, and adhesion, which, are commonly associated as key cancer processes.

In Paper I, we found that miR-379 could indeed stop metastatic spread to the bone in mouse models. Furthermore, many characteristic markers of cancer are decreased when we performed experiments on cells with miR-379, namely, decreasing cell growth, invasion, colony formation, and adhesion to bone cells. We are still not sure of the pathways impacted by miR-379, however, so we sought to explore this and some of the targets. We found that when we took bone cells and grew them in a condition with reduced levels of this miR-379, a particularly interesting protein was found called GDF-15. Since this has been shown to also be involved in driving cancer in a bone setting, we speculated that one of the ways that reduced levels of miR-379 can lead to bone metastasis is through the increased levels of GDF-15.

In Paper II, we describe our investigations into other potential targets and pathways of our miRNA of interest, miR-379. We have also looked at which proteins are expressed more in an artificial bone environment and found that

PSA was consistently increased in this setting when miR-379 was reduced. PSA is the most common marker used for screening men, but it has also been reported in other papers to be involved in PCa disease progression. We tested to see if miR-379 could directly impact PSA expression; however, this was not the case. Instead, we believe that miR-379 acts directly on AR expression, which in turn is involved in the regulation of PSA. When we look at patient material, we see the same relationship: when we have reduced levels of miR-379, we have an increase in both PSA and AR, supporting this idea. We are currently trying to further understand the role that miR-379 plays with these proteins so that we can understand the mechanisms of its action and hopefully exploit this knowledge to aid in the treatment of PCa.

Our work on miR-379 is also not as straightforward as perhaps the first two papers would suggest. This is because the miRNA can exist in two different forms: the 'normal' unedited form, which we have been focusing on, but also an edited form. This edited form is almost identical, apart from one small change. In Paper III, we wanted to see if this change has an impact on how the miRNA works and if it has different targets or pathways, it might be involved in. We used four different types of cells: two were reliant on hormone signalling, and two could survive without the need for hormones. We did notice that depending on the cells we used, we found different results and that their dependence on hormones seemed to be determining this. The cell lines that did not need hormones showed increased cell growth with the addition of the unedited miR-379 but not the edited form. We also found that both the unedited and edited forms of miR-379 increased the 'stickiness' of themselves to their own cells. We used computer prediction tools to also try and guess the targets of both unedited and edited miR-379 and saw that, for the most part, they had distinct targets, with 260 unedited specific targets, 296 edited specific targets, and four shared targets. The pathways that these targets are involved in are also different, suggesting that they could play different biological roles.

In conclusion, this thesis shows that miR-379 could potentially be used as a future therapy as it can stop the spread of PCa to the bone. There is, however, a lot more to learn about the mechanisms by which it works and the impact of miR-379 editing. Overall, the use of miRNAs as tools to help treat cancer has an exciting future, and we hope that this research can in some way contribute to this.

Populärvetenskaplig sammanfattning

PCa är ansvarig för ett stort antal dödsfall bland män över hela världen. Eftersom det främst påverkar äldre män är det en sjukdom vars förekomst bara ökar, med en trend speciellt synlig i västerländska länder med en åldrande befolkning. Förvånansvärt kommer de flesta män med PCa inte att dö av sjukdomen eftersom den vanligtvis är långsamväxande, och män kommer vanligtvis att falla offer för andra åldersrelaterade dödsorsaker först. Det finns flera behandlingar tillgängliga för män, och dessa kan vara botande eller stoppa sjukdomens progression. Dock kommer en del män att utveckla en mer aggressiv form av cancer. Denna aggressiva PCa sprider sig ofta till avlägsna platser, oftast i skelettet, och tyvärr är prognosen mycket dyster för dessa patienter. Det finns för närvarande inga botande behandlingar tillgängliga för dessa patienter, så behovet av nyare terapeutiska alternativ är avgörande. Vi fokuserar på en uppsättning molekyler som kallas miRNAs, vilka har visat sig vara inblandade i många mänskliga sjukdomar, inklusive PCa. Vi har även tidigare visat att en miRNA, miR-379, kan driva spridningen av cancer till skelettet när den är nedreglerad. Med denna logik försökte vi undersöka om vi genom att öka nivåerna av miR-379 kunde förhindra att aggressiv PCa sprider sig till skelettet. För att undersöka detta började vi med att testa om miR-379 kunde stoppa spridningen av PCa till skelettet och testa dess funktionella effekter på celltillväxt, invasion, kolonibildning och adhesion, vilka vanligtvis är förknippade med grundläggande cancerprocesser.

I artikel 1 fann vi att miR-379 faktiskt kunde stoppa metastatisk spridning till skelettet i mössmodeller. Dessutom minskade många karakteristiska markörer för cancer när vi utförde experiment på celler med miR-379, nämligen minskad celltillväxt, invasion, kolonibildning och adhesion till benceller. Vi är fortfarande osäkra på vilka vägar som påverkas av miR-379, så vi försökte utforska detta och några av målen. Vi fann att när vi tog benceller och odlade dem i en miljö med reducerade nivåer av miR-379, hittades en särskilt intressant protein som kallas GDF-15. Eftersom det har visats att detta även är involverade i att driva cancer i en ben miljö, spekulerade vi om ett av de sätt som minskade nivåer av miR-379 kan leda till benmetastas är genom ökade nivåer av GDF-15.

I artikel II undersöker vi för närvarande fler potentiella mål och vägar för vår intressanta miRNA, miR-379. Vi har också undersökt vilka proteiner som är mer uttryckta i en konstgjord ben miljö och funnit att PSA konsekvent ökade i denna miljö när miR-379 var reducerad. PSA är den vanligaste markören som används för att undersöka män, men det har också rapporterats i andra artiklar

att det är involverat i PCa-sjukdomens progression. Vi testade för att se om miR-379 direkt kunde påverka PSA-uttrycket; det var dock inte fallet. Istället tror vi att miR-379 verkar direkt på AR-uttrycket, vilket i sin tur är involverat i regleringen av PSA. När vi tittar på patientmaterial ser vi samma förhållande: när vi har minskade nivåer av miR-379 har vi en ökning av både PSA och AR, vilket stöder denna idé. Vi försöker för närvarande ytterligare förstå den roll som miR-379 spelar med dessa proteiner så att vi kan förstå mekanismerna för dess handling och förhoppningsvis utnyttja denna kunskap för att hjälpa till vid behandlingen av PCa.

Vårt arbete med miR-379 är inte heller så enkelt som kanske de första två papperna skulle föreslå. Detta beror på att miRNA kan existera i två olika former: den 'normala' oredigerade formen, som vi har fokuserat på, men också en redigerad form. Denna redigerade form är nästan identisk, förutom en liten förändring. I artikel III ville vi se om denna förändring påverkar hur miRNA fungerar och om det har olika mål eller vägar det kan vara involverat i. Vi använde fyra olika typer av celler: två var beroende av hormon signalering, och två kunde överleva utan behov av hormoner. Vi märkte att beroende på de celler vi använde, fann vi olika resultat och att deras beroende av hormoner verkade bestämma detta. Cellinjerna som inte behövde hormoner visade ökad celltillväxt med tillsatsen av den oredigerade miR-379 men inte den redigerade formen. Vi fann också att både den oredigerade och redigerade formen av miR-379 ökade deras "klibbighet" till sina egna celler. Vi använde dator förutsägelse verktyg för att också försöka gissa målen för både oredigerade och redigerade miR-379 och såg att de för det mesta hade distinkta mål, med 260 oredigerade specifika mål, 296 redigerade specifika mål och fyra delade mål. De vägar som dessa mål är involverade i är också olika, vilket antyder att de kan spela olika biologiska roller.

Sammanfattningsvis visar denna avhandling att miR-379 potentiellt kan användas som en framtida terapi eftersom den kan stoppa spridningen av PCa till benet. Det finns dock mycket mer att lära om de mekanismer genom vilka den fungerar, och om miR-379-redigeringens påverkan. Övergripande har användningen av miRNA som verktyg för att hjälpa till att behandla cancer en spännande framtid, och vi hoppas att denna forskning på något sätt kan bidra till detta.

Populærvidenskabelig oversigt

Verden over er PCa ansvarlig for et stort antal dødsfald blandt mænd. PCa rammer primært ældre mænd, hvorfor stigningen af tilfælde især ses i de vestlige lande med en aldrende befolkning. Bemærkelsesværdigt vil de fleste mænd med PCa ikke dø af sygdommen, da den typisk vokser langsomt og mænd vil normalt dø af andre aldersrelaterede dødsårsager. Der er flere behandlinger til rådighed for mænd, og disse kan være helbredende eller standse sygdomsprogressionen; dog vil en brøkdel af mænd udvikle en mere aggressiv form for kræft. Denne aggressive PCa vil ofte lave metastaser til andre dele af kroppen, typisk knoglerne. For disse patienter er prognosen meget dyster. Der er i øjeblikket ingen helbredende behandlinger til rådighed for disse patienter, så behovet for nyere terapeutiske muligheder er afgørende. Vi fokuserer på et sæt molekyler kaldet miRNA'er, som har vist sig at være impliceret i mange menneskelige sygdomme, herunder PCa. Vi har også tidligere vist, at ét miRNA, miR-379, kan drive spredningen af kræft ind til knoglerne, når det nedreguleres. Med denne logik forsøgte vi at undersøge, om vi ved at øge niveauerne af miR-379, kunne forhindre aggressiv PCa i at sprede sig til knoglerne. For at undersøge dette startede vi med at teste, om miR-379 kunne stoppe spredningen af PCa til knoglerne og teste dens funktionelle virkninger på cellevækst, invasion, kolonidannelse og vedhæftning, som ofte er forbundet som vigtige kræftprocesser.

I artikel I fandt vi, at miR-379 kunne stoppe metastatisk spredning til knoglerne i musemodeller. Desuden blev mange karakteristiske markører for kræft reduceret, når vi udførte eksperimenter på celler med miR-379; især reduceret cellevækst, invasion, kolonidannelse og vedhæftning til knogleceller. Vi er dog stadig ikke sikre på de veje, der påvirkes af miR-379, så vi forsøgte at udforske dette og nogle af målene. Vi fandt, at når vi tog knogleceller og dyrkede dem i en tilstand med reducerede niveauer af denne miR-379, blev der fundet et særligt interessant protein kaldet GDF-15. Da dette har vist sig også at være involveret i at drive kræft ind i knoglerne, spekulerede vi på, om en af måderne, hvorpå reducerede niveauer af miR-379 kan føre til knoglemetastase, er gennem de øgede niveauer af GDF-15.

I artikel II undersøger vi i øjeblikket flere potentielle mål og veje for vores aktuelle miRNA, miR-379. Vi har også set på, hvilke proteiner der syntetiseret mest i et kunstigt knoglemiljø og fundet ud af, at PSA konsekvent blev øget, når miR-379 blev reduceret. PSA er den mest almindelige markør, der anvendes til screening af mænd, men det er også blevet rapporteret i andre

papirer at være involveret i PCa sygdomsprogression. Vi testede for at se, om miR-379 direkte kunne påvirke PSA-ekspression; Dette var imidlertid ikke tilfældet. I stedet mener vi, at miR-379 virker direkte på AR-udtryk, som igen er involveret i reguleringen af PSA. Når vi ser på patientmateriale, ser vi den samme sammenhæng: Når vi har et reduceret niveau af miR-379, har vi en stigning i både PSA og AR, hvilket understøtter denne idé. Vi forsøger i øjeblikket yderligere at forstå den rolle, som miR-379 spiller med hensyn til disse proteiner, så vi kan forstå mekanismerne for dets virkning og forhåbentlig udnytte denne viden til at hjælpe med behandlingen af PCa.

Vores arbejde med miR-379 er ikke så ligetil, som de to første papirer maaske antyder. Dette skyldes, at miRNA'et kan eksistere i to forskellige former: den 'normale' uredigerede form, som vi har fokuseret på, men også en redigeret form. Denne redigerede form er næsten identisk, bortset fra en lille ændring. I papir III ønskede vi at se, om denne ændring har indflydelse på, hvordan miRNA'et fungerer, og om det har forskellige mål eller veje, det kan være involveret i. Vi brugte fire forskellige typer celler: to var afhængige af hormonsignalering, og to kunne overleve uden behov for hormoner. Vi bemærkede, at afhængigt af hvilke celler, vi brugte, fik vi forskellige resultater, og at cellernes afhængighed af hormoner syntes at bestemme dette. De cellelinjer, der ikke havde brug for hormoner, viste øget cellevækst med tilføjelsen af den uredigerede miR-379, men ikke den redigerede form. Vi fandt også, at både de uredigerede og redigerede former af miR-379 øgede 'klæbrigheden' af sig selv til deres egne celler. Vi brugte computerforudsigelsesværktøjer til at gætte målene for både uredigeret og redigeret miR-379 og så, at de for det meste havde forskellige mål med 260 uredigerede specifikke mål, 296 redigerede specifikke mål og fire delte mål. De veje, som disse mål er involveret i, er også forskellige, hvilket tyder på, at de kan spille forskellige biologiske roller.

Afslutningsvis viser denne afhandling, at miR-379 potentielt kan bruges som en fremtidig terapi, da det kan stoppe spredningen af PCa til knoglerne. Der er dog meget mere at lære om de mekanismer, hvormed det fungerer, og virkningen af miR-379-redigering. Samlet set har brugen af miRNA'er som værktøjer til behandling af kræft en spændende fremtid, og vi håber, at denne forskning på en eller anden måde kan bidrage til dette.

List of Papers

Paper I

Expression of microRNA-379 reduces metastatic spread of prostate cancer (2023) James R. Cassidy, Gjendine Voss, Kira Rosenkilde Underbjerg, Margareta Persson, and Yvonne Ceder

Paper II

MicroRNA-379 influences PSA expression in metastatic prostate cancer (manuscript) James R. Cassidy, Margareta Persson, Gjendine Voss, Kira Rosenkilde Underbjerg, Tina Catela Ivkovic, Anders Bjartell, Anders Edsjö, Hans Lilja, and Yvonne Ceder

Paper III

Functional consequences of A-to-I editing of miR-379 in prostate cancer cells (2023) Gjendine Voss, James R. Cassidy, and Yvonne Ceder

Author's contribution to the papers

Paper I

Experimental design, running of experiments, data analysis, figure design, writing of manuscript

Paper II

Running of experiments, data analysis, figure design, writing of manuscript

Paper III

Running of experiments, data analysis, figure design, writing of manuscript

Abbreviations

ADAR	adenosine deaminase acting on RNA
ADT	androgen deprivation therapy
AGO	argonaute
AR	androgen receptor
A-to-I	adenosine-to-inosine
BPH	benign prostatic hyperplasia
CRPC	castration resistant PCa
DHT	dihydrotestosterone
DGCR8	DiGeorge syndrome critical region 8
DRE	digital rectal examination
EMT	epithelial-to-mesenchymal transition
fPSA	free PSA
HoLEP	holmium laser enucleation of the prostate
HSP	heat shock proteins
isomiR	miRNA isoform
KLK2	Human kallikrein-related peptidase 2
MET	mesenchymal-to-epithelial transition (MET)
miRNA	microRNA
NSCLC	non-small cell lung cancer
OBCM	osteoblast-conditioned medium
PARP	poly ADP-Ribose polymerase
PCa	prostate cancer
PIN	prostatic intraepithelial neoplasia
Pre-miRNA	precursor-miRNA
pri-miRNA	primary-miRNA
PSA	prostate-specific antigen

PTHrP	parathyroid hormone-related
RISC	RNA-induced silencing complex
RNA pol	RNA polymerase
Runx2	Runt-related transcription factor 2
TGF-β	Transforming growth factor beta
TMPRSS2	Transmembrane protease, serine 2
TNM	tumour, lymph Node, metastasis
tPSA	total PSA
TURP	transurethral resection of the prostate

Introduction

Prostate Cancer

Prostate anatomy and function

The prostate is an accessory gland in the male reproductive system located below the bladder and in front of the rectum, with the proximal section of the urethra passing through the centre of it. The prostate is comparable to a walnut in terms of shape and size, and although the weight varies between young men at approximately 20 grams [1], it typically gets larger after the age of 40. In pathology, the prostate is often divided into three functionally distinct zones: the central zone, peripheral zone, and transitional zone [2] (Figure 1). The peripheral zone is the largest of the three, accounting for around 70% of the prostate's volume. The second largest is the central zone, which accounts for 25% of the gland's volume with the final 5% being accounted for by the transitional zone. Despite the transitional zone's small volume, it is the site from which benign prostatic hyperplasia (BPH) exclusively originates [3]. There is also a disproportionally higher incidence of PCa development in the transitional zone, with approximately 20-25% of PCa originating from this zone [4]. Only around 3% of carcinomas arise from the central zone, but these are typically aggressive in nature and have a poor prognosis for patients [5]. The peripheral zone is both the largest in volume and also the most common site for PCa to develop (70-75%), and has a characteristically worse prognosis than transitional zone cancers, which often do not result in any clinical symptoms [6].

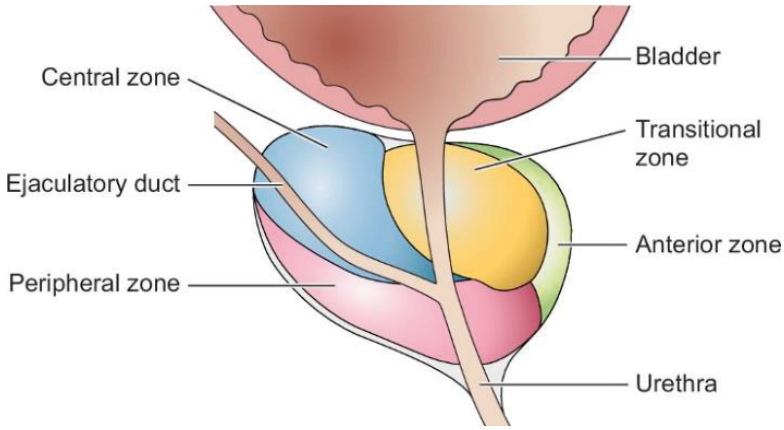

Figure 1.
Prostate anatomy and zones. The anatomical location of the prostate relative to the bladder and the three zones of the prostate are shown. The image was taken from a paper by Giacomini *et al*. [7]

The function of the prostate is primarily to produce the prostatic fluid, which is composed of several components, including spermine, citric acid, zinc, and PSA. Prostatic fluid accounts for only around 10% of the seminal fluid, with the majority being produced in the seminal vesicles [8]. The prostatic fluid is essential in semen as it promotes sperm motility. During ejaculation, smooth muscles inside the prostate contract and drive the seminal fluid through the urethra. These muscles are important for the prostate to control the flow of urine down the urethra as well as ensuring that there is no mixing of urine and seminal fluid. The prostate is important in the production of PSA [9] and hormones such as dihydrotestosterone (DHT) [10], which play an essential role in the sexual development of men. There are several other lesser-known roles that the healthy prostate serves in men. It is thought that prostatic fluid is useful in protecting against urinary tract infections due to its antiseptic properties [11]. The prostate is important for a normal sex life as the cavernous nerve, the main autonomic nerve for regulating erections, runs to the penis from the tip of the prostate [12]. When treating for PCa or BPH, this can sometimes lead to the cavernous nerve being damaged, and erectile dysfunction can occur as a result.

Prostatitis, BPH, and PIN

The three most common causes of prostatic disease are PCa, BPH, and prostatitis. PCa and BPH are urological conditions that typically arise later in life for men; however, prostatitis affects men of all ages. Prostatitis, in a

nutshell, is the inflammation of the prostate and is typically characterised by difficulties and pain when urinating, as well as pain in the groin region. The National Institutes of Health have classified prostatitis into four distinct categories (I-IV) [13]. Type I refers to prostatitis caused by acute bacterial infection, and Type II is caused by chronic bacterial infection. Type III and IV are non-bacterial and simply refer to the symptomatic (III) and asymptomatic (IV) conditions of the patient. It is estimated that more than 90% of men with prostatitis do not have any bacterial infection [14], with type III being by far the most prevalent. For these patients, there are several treatment options, such as anti-inflammatory drugs, which significantly reduce symptoms [15]. Prostatitis has also been linked to PCa, as there is a positive correlation between the two diseases [16]. It is thought that this association is through inflammatory cytokines, reactive oxygen and nitrogen species, and other inflammatory mediators that can promote PCa development [17, 18].

BPH is very common in elderly men, and the prevalence of this disease is rising in concordance with the ageing population [19]. Autopsy studies have shown that the prevalence of BPH in men rises from 8% in men in their 40's up to 80% for men in their 90's [20] and it is the most common benign tumour in men. There have been suggestions that ethnicity plays a contributing factor in BPH, but the results are variable [21]. It is established that genetics plays a large role in susceptibility to BPH, and relatives of men with BPH have a much higher risk and are more likely to develop symptoms earlier in life [22]. Other lifestyle factors, such as diet [23], and in particular alcohol intake [24], are also thought to increase the risk of BPH, with an emphasis on reduced intake of red meat and alcohol. BPH develops from the non-cancerous proliferation of epithelial and stromal cells in the transitional zone of the prostate. This growth leads to an enlarged prostate, and since the transitional zone surrounds the urethra, this leads to compression, which can have clinical repercussions. Most often, patients develop bladder outflow obstruction, which can cause urinary retention and infection [3]. This can drastically reduce quality of life for patients who may suffer from the frequent need for urination and nocturia, as well as difficulty producing a solid stream when urinating. BPH can also affect ejaculation, with a report that around 20% of men with BPH can experience pain during orgasm [25]. When men present with symptoms of BPH, they should seek attention from a urologist. Although exact protocols vary between countries for how BPH is diagnosed, typically a self-report such as the International Prostate Symptom Score questionnaire [26] and an assessment of urine flow are common, followed by a digital rectal examination (DRE). After

this, blood may also be taken to measure levels of PSA; however, on its own, it cannot distinguish between potential BPH or PCa.

The treatment options for patients with BPH depend primarily on the severity of the symptoms. If the patient only has slight discomfort, then the urologist may suggest watchful waiting with an emphasis on self-management through changes in diet and lifestyle [27]. However, if the patient is dealing with more severe discomfort, then intervention is required. Certain drugs can be administered, such as alpha-blockers, which can be effective in helping better control the flow of urine [28]. Should this not provide satisfactory relief for the patient, surgical intervention may be required. There is usually more consideration required by the urologist when discussing this with a patient since complications can arise, and given that typically elderly men suffer from BPH, an invasive treatment may not always be suitable. The gold standard for surgical intervention has been transurethral resection of the prostate (TURP) [29] for a long period of time. In short, TURP is a process whereby excess prostatic tissue is cut away using tools inserted into the urethra. However, with the advent of newer technologies such as holmium laser enucleation of the prostate (HoLEP), many urologists are hailing this as the new gold standard. HoLEP, which uses light beams to remove excess prostatic tissue, has reportedly a lower risk of complications such as erectile dysfunction and may also be better suited for patients who have particularly enlarged prostates [30].

Prostatic intraepithelial neoplasia (PIN) is a neoplastic growth that is seen as the precursor to PCa [31]. Low-grade PINs are seen as clinically insignificant, and many healthy men have them even at a young age. Since PINs do not significantly elevate PSA, they are only detected during biopsies [32]. If high-grade PINs are detected, this is highly predictive for the future progression of PCa.

Prostate cancer

PCa is the main disease associated with the prostate and mortality. In men, PCa has the highest estimated number of new cancer cases for 2023 and the second highest number of cases for cancer mortality, accounting for 29% and 11%, respectively, in the U.S. [33]. As with BPH, PCa typically affects older men, and thus the incidence of the disease is also rising since life expectancy has increased in the past decades. The number of cases in the United Kingdom

between 2016 and 2018 is shown in Figure 2 and is a good representation of the PCa distribution across different age groups. The use of PSA testing has also resulted in an overdiagnosis of men with PCa [34]. This can lead to complications from treatment that may not have been necessary. Recent approaches to better stratify patients and avoid this threat of overdiagnosis include the use of MRI-directed targeted biopsies [35]. This approach, as opposed to systematic biopsies, can also avoid unnecessary biopsies. This more measured approach will better tip the ratio of benefits to harm in PSA testing. Risk factors for men to develop PCa are like those of BPH, with age, genetics, lifestyle, and diet playing a role in the relative risk of succumbing to the disease. Unlike BPH, race is thought to play a significant role in the mortality of the disease. British men from an Afro-Caribbean background had, for example, a 3-fold higher risk of being diagnosed with PCa than British men with a European background [36].

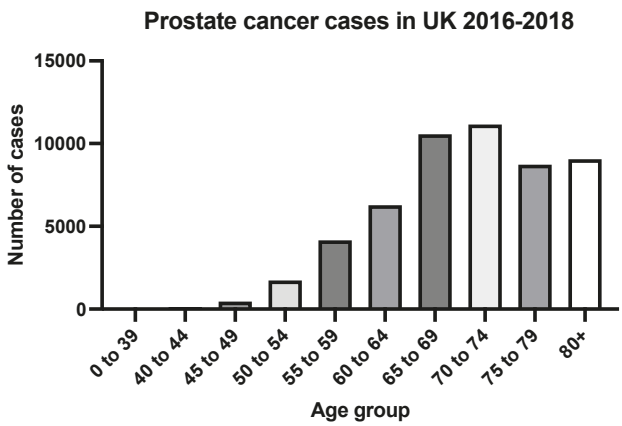

Figure 2.
PCa incidence in the United Kingdom by age group. Number of cases between 2016-2018 is shown with the graph created from data of cancer registration statistics from the office for national statistics [37].

There are different subtypes of PCa, although almost all men with the disease have prostate adenocarcinoma. Prostate adenocarcinoma can arise from either the gland cells that line the prostate, referred to as acinar adenocarcinoma, or from cells that line the ducts of the prostate, and this is called ductal adenocarcinoma [38]. Acinar adenocarcinoma is the most prevalent of the two and thus represents the largest proportion of patients with PCa. There are rare subtypes such as prostate squamous cell carcinoma, and although there is less research on this, it is known to be extremely aggressive and typically leads to

metastatic spread [39]. The cell of origin for PCa has been a topic of conversation for many years for researchers studying the disease. Historically, the leading candidate has been the luminal cells, since during PCa development there is an expansion in luminal cells and basal cells are usually absent [40]. There has been a specific set of rare luminal cells that express Nkx3.1 and are able to perform self-renewal [41], and these have also been proposed to represent the cell of origin.

Androgen receptor signalling

The androgen receptor (AR) signalling pathway is critical for sexual development. It is a receptor that, when bound to by androgens, will dissociate from heat shock proteins (HSP), dimerise, and translocate into the nucleus, whereby it will bind to androgen response elements in the DNA and drive transcription of AR response genes such as PSA. In healthy men, it is critical for the body to adapt to changes in hormones, especially during periods such as puberty [42]. The basic pathway is shown in Figure 3.

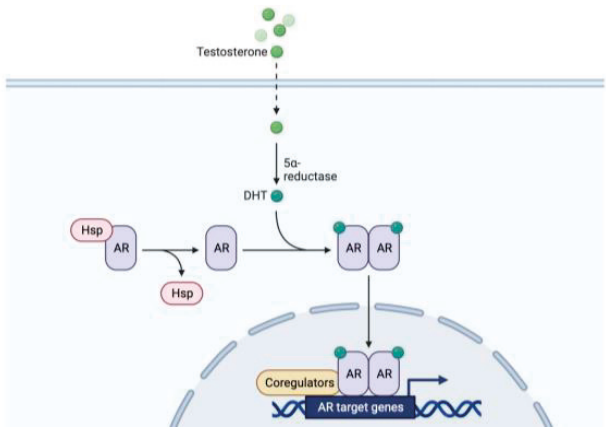

Figure 3.
AR signalling pathway. Testosterone is converted to DHT by 5α-reductase. This subsequently causes AR to dissocatiate from HSP and dimerise. The AR then translocates to the nucleus where it will bind to AR reponse elements on target genes. Figure was generated using BioRender [43].

In the context of PCa development, the AR signalling pathway is also critical. AR is known to drive cancer growth and is essential for PCa initiation [44]. Consequently, androgen deprivation therapy (ADT) has been used as a method for treating patients since the 1940s [45]. However, in many patients, the

cancer will progress after this in what is commonly referred to as castration-resistant PCa (CRPC). The mechanisms of this castration resistance rely on alternative methods for the activation of AR. AR splice variants, for example, lack the ligand binding domain that is targeted by traditional ADTs, thus allowing the AR to be expressed regardless [46]. Other changes, such as AR amplification and alternative activation through signal transduction pathways like MAPK and STAT3 [47], are mechanisms by which patients develop CRPC, highlighting the importance of AR signalling in the progression of the disease.

PCa metastasis

Of the original hallmarks of cancer: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [48], it is only the activation of invasion and metastasis that is specific to malignant tumours. The metastatic process requires several steps and is a very inefficient process, with experimental studies showing that only around 0.01% of cancer cells in circulation can form metastases [49]. Metastases are typically monoclonal [50], so understanding how this single cell can detach from the primary tumour, migrate, and establish itself at a distant site is of great interest. The metastatic cascade is composed of the following steps: local invasion into surrounding tissue, intravasation, survival through circulation, extravasation into target tissue, and finally survival and proliferation [51]. Within each step, there is a complex network of signalling between the cancer cells and their environment for the cells to proceed through this metastatic cascade. This plasticity of cells is essential for cancer development. One key mechanism of plasticity is the cells ability to undergo both epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET). EMT leads to reduced cell-cell adhesion, and as these cells gain a more mesenchymal phenotype, they gain migratory and invasive properties [52]. However, the transition from EMT to MET is not binary, and cells exhibit a phenotype somewhere within the spectrum [53]. When researching cancer, by using markers to try and identify the phenotypic shifts in cells, we can better understand the metastatic process. One epithelial marker often used is E-cadherin, which is a protein that aids adhesion between epithelial cells [54].

When PCa has metastasised there are several sites that are preferentially selected, with the most common being bones, lymph nodes, liver, and lungs.

The site of spread has clinical consequences in terms of the prognosis and treatment options afforded to patients. Metastatic spread to the lymph nodes is not considered to cause a high level of mortality, although the presence of these is a poor prognostic sign [55]. Distant metastases are associated with poor survival, and the most common distant site is the bone. One way to better understand why PCa favourably metastasises to the bone is through the study of its microenvironment. The cells that make up the bone are osteoblasts, osteoclasts, and osteocytes, as shown in Figure 5. In brief, osteoblasts cause ossification, osteoclasts break down bone for remodelling, and osteocytes regulate biomineralization [56]. PCa bone metastases are typically osteoblastic, with unstable ossification leading to a fragile structure that is susceptible to fracturing. PCa is distinct from most bone metastases derived from other solid tumours, which are typically osteolytic in nature [57]. There are likely several mechanisms responsible for this, including the action of PSA since parathyroid hormone-related protein (PTHrP) is known to be a substrate of PSA [58]. Given that the function of PTHrP is to stimulate bone resorption through increased activity of osteoclasts [59], it is unsurprising that when PSA cleaves this protein, it can lead to a more osteoblastic phenotype in PCa patients.

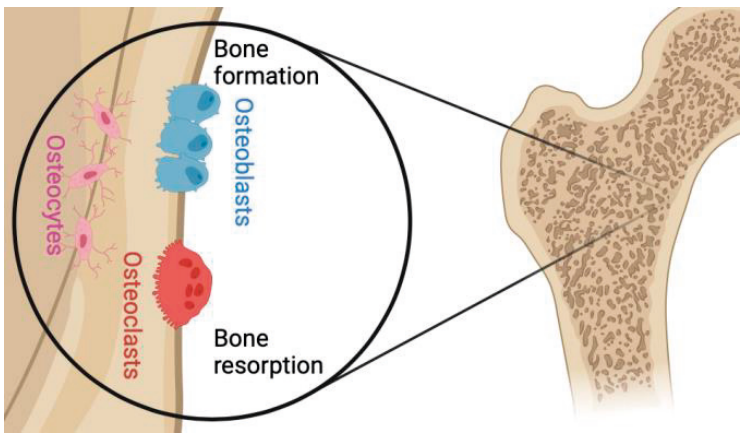

Figure 5.
Major cells that make up bone. The three main bone cells that constiture the bone microenvironment are shown. Figure was generated using BioRender [43].

Since men with metastatic spread to the bone represent the highest fraction of mortality, many treatments are specific to this malignancy. There are currently

no curative treatments; however, therapies are still useful in relieving tumour burden as well as limiting the pain and discomfort experienced by patients, thus improving their quality of life. The most common sites for bone metastases are the spine, hips, and ribs, and this is also an important factor when considering suitable treatment options. Metastatic spread to the spine can cause spinal cord compression, which, if left untreated, can lead to nerve damage and even paralysis. For patients with spinal cord metastasis, surgery and/or radiotherapy can be used. Radium-223 can be used to treat bone metastasis and is considered an internal radiotherapy since cancerous cells in the bone preferentially take up more of the radioactive element than healthy cells due to acting as a calcium mimetic [60]. This treatment is used to impede cancer progression and alleviate pain in men. Another common treatment is the use of bisphosphonates, which reduce the risk of fractures in patients by strengthening bones. The most commonly used bisphosphonate in the clinic is zoledronic acid [61]. Receptor activator of nuclear factor kappa beta ligand (RANKL) is expressed on osteoblasts, and regulates osteoclast formation and bone remodelling [62], and is a target for the treatment of PCa bone metastases. Denosumab, which is also called Xgeva and Prolia, is an antibody against RANKL and has shown positive signs for reducing bone pain and bone metastasis-free survival [63]. There are still no curative treatments for metastatic PCa; however, the development of PCa therapeutics is a constantly evolving field, and novel drugs as well as altering treatment regimens and combinations of therapies will allow PCa patients in the future to live longer and with a better quality of life.

Diagnosis of PCa

If the symptoms of PCa arise, they are like those of BPH, such as difficulty initiating urination and painful ejaculation. However, many men with PCa are asymptomatic as opposed to BPH, and this can be attributed to their development in different zones within the prostate. Screening can therefore be extremely useful in helping identify these patients, and the most common biomarker for this is PSA. There has been some controversy about routine screening of men since PSA tests lead to many false-positive results; however, it is still considered to have reduced the number of PCa mortalities [64]. It is difficult to designate what constitutes a ‘normal’ PSA value, but historically this was anything below 4ng/ml. But there are individuals below this threshold that do have PCa and there many men with twice as much serum PSA that do not have PCa [65]. PSA testing guidelines for urologists vary between countries and even within regions of different countries. In Skåne, Sweden, the

following screening criteria are followed: men between ages 50 and 74 are offered the option of testing if they are not in a hereditary risk group. If men are in a hereditary risk group, and then they are offered testing after the age of 40 [66]. The table below (Table 1) shows the total PSA (tPSA) concentration required for referral by Region Skåne, the healthcare provider in southern Sweden.

Table 1.
tPSA values required for referral for men living in Skåne, Sweden. The cutoff values are taken directly from the 1177/Region Skåne website [67] and refer to values obtained from blood samples.

tPSA limit	Age
3 ng/ml	< 70
5 ng/ml	70-80
7 ng/ml	> 80

The biggest therapeutic challenge for PCa is to be able to successfully distinguish between aggressive cancers that will lead to death and indolent cancers. The sensitivity of PSA is such that only around a third of men with elevated PSA have cancerous growth [68]. There has been suggestion for clinicians to switch from traditional PSA testing to look at the relative ratios of free PSA (fPSA) and tPSA since this has been shown to be a useful indicator of PCa [69], whereby a low ratio of fPSA to tPSA indicates a worse prognosis. The 4K score takes into account fPSA, tPSA, intact PSA, and kallikrein related peptidase 2 [70], and this test has since been FDA-approved in 2021 [71]. To better determine the likelihood of cancer, physicians will typically perform DRE to look for signs of induration and nodularity, with patients who have an unusual DRE and elevated PSA proceeding to have a biopsy. This process too is under scrutiny, with recent research suggesting it is useless in men with only moderately high PSA expression (3-3.9 ng/ml) [72].

Simply put, the process of a prostate biopsy involves needles that are inserted through the rectum, accompanied by an ultrasound probe to extract cells from the prostate. The urologist will usually take a dozen biopsies from different areas of the prostate, and the process takes around 15 minutes. It is not uncommon for patients to have discomfort and even blood when urinating on the days that follow this, but the procedure itself should be painless since a

local anaesthetic is used. Unfortunately, more severe side effects can also occur, such as prostatitis, but this is less common [73]. Since prostate biopsies have a high false negative rate [74], it requires many men to undergo the procedure again. Several techniques have been adapted to try and reduce the rate of false negatives, such as the addition of lateral peripheral biopsies, which improves PCa detection [75]. Biopsy cores are graded by pathologists, and if PCa is detected, then it can be graded using the Gleason score. The Gleason grading system uses two scores from distinct biopsy cores, which can range from a value of one to five, and is named after the physician Donald Gleason, who developed the system in the 1960's [76]. This score is based on the most common malignant cells and the second-most common malignant cells. A score of one indicates small, uniform glands that are well differentiated, with five being very poorly differentiated and barely any gland formation. The TNM (Tumour, Lymph Node, Metastasis) system is also used to assess the stage of PCa development [77]. Stages TI and TII are referred to as early-stage cancer as the tumour is still confined to the prostate. Stage TIII is referred to as locally advanced since the tumour has now spread beyond the capsule surrounding the prostate but only into adjacent tissues, such as the seminal vesicles. Stage TIV has the worst prognosis since the cancer has now spread to other tissues and organs in the body, typically the bone, liver, lungs, and lymph nodes. At this point, the cancer is referred to as advanced PCa. The four tumour stages are shown in Figure 4.

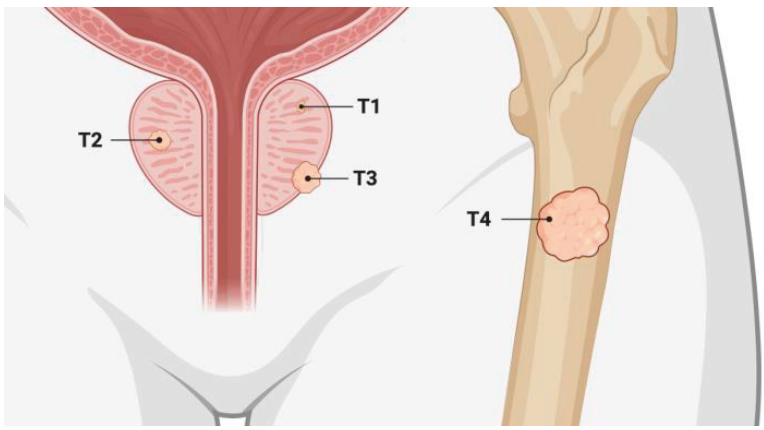

Figure 4.
Tumour grading system for PCa. The four stages of tumour in the TNM system are shown. T1 is too small to be felt during DRE, T2 is when the tumour is larger but still confined to the tumour, T3 the tumour has expanded beyond the prostate gland and T4 it has metastasised to more distant tissues. Figure was generated using BioRender [43].

Treatment of PCa

The course of treatment for suspected PCa is based on many factors. Given that PCa is typically slow-growing and often indolent, then treatment is not necessary for patients with no symptoms and deemed to have indolent PCa. Watchful waiting is a term often used by urologists whereby no treatment is given, but the patient will have active surveillance and check-ups to monitor tumour progression. TURP can alleviate symptoms for patients with issues urinating who have an enlarged prostate, either due to cancerous growth or BPH. Prostatectomy is one of the first treatment lines for localised PCa. The surgical procedure involves the removal of the prostate, and this is most often done with robotic assistance since it has fewer side effects than traditional prostatectomy [78]. Radiotherapy is also a common first-line treatment, and although variants exist, such as brachytherapy, which uses radioactive seeds implanted into the prostate, external beam radiotherapy is typically used. Intensity-modulated radiation therapy uses photons, and due to technical advancements in the field and the instruments used, patients are now able to receive more precise doses of radiation. This means that the radiation can be increased in the tumorous regions and decreased in surrounding tissues, which not only reduces the rate of side effects and the need for many repeated sessions but also increases the effectiveness of the treatment for curing the patient. Both prostatectomy and radiotherapy can be curative, but patients will be monitored after treatment with PSA testing to assess any recurrence of disease. In the case of biochemical recurrence, salvage radiotherapy is the only curative treatment for patients who have already undergone prostatectomy [79], although hormone therapies can often halt further progression.

If the recurrence is persistent and/or the PCa spreads, then hormone therapy and chemotherapy can be applied to try and treat patients. As discussed earlier, it has been well established that PCa, in its early manifestation, is dependent on AR signalling. Therefore, ADT is often seen as the standard first-line treatment for men with metastatic PCa, with abiraterone acetate and enzalutamide being the most frequently used [80]. As mentioned earlier, men with this treatment will over time develop resistance, and patients with CRPC have a very poor prognosis, with a median survival of only 1.86 years in Sweden [81]. There are additional therapeutic options available for these castration-resistant patients, such as docetaxel [82], but these treatments are not curative and aim to reduce the disease burden. Newer therapies have been introduced, such as Poly ADP-Ribose Polymerase (PARP) inhibitors, to try and reduce CRPC in patients with aberrations in DNA repair genes. The most

recently FDA-approved PARP inhibitor treatment is an enzalutamide-talazoparib combination, which has been shown to be significantly more effective than enzalutamide, an ADT drug, alone [83]. There are many treatments available for PCa; however, these can have a range of adverse effects, so the balance between the treatment of the disease and ensuring as good a quality of life as possible needs to be considered. Given that this cancer predominantly affects elderly men, it is often very difficult for them to cope with these side effects, and thus the need for novel therapies is paramount.

MicroRNAs

Discovery and nomenclature

Non-coding RNAs are functional RNA molecules that, despite not being translated into proteins, are vital for the development of all animals, plants, and even some viruses. One subset of these essential non-coding RNAs is miRNA. These miRNAs are single stranded RNA molecules between 19 and 25 nucleotides in length, play an essential role in cellular processes, usually through gene silencing [84]. The first miRNA to be discovered was lin-4 in 1993, and this miRNA was found in *C.elegans*, where it plays a role in larval development [85]. Originally, the term used for lin-4 was simply small RNA or small temporal RNAs; however, in 2001, when lin-4 was found to be evolutionarily conserved in invertebrates and vertebrates, the terminology was switched to miRNA [86]. It has been 30 years since the discovery of the first miRNA, and we now have 1917 miRNAs annotated by miRbase in the human genome [87].

The miRNA nomenclature system follows an intuitive system that is shown in Figure 6. In sinistrodextral order, the first three letters refer to the organism for which the miRNA is present (eg. mmu refers to mice). Then it is further distinguished whether the miRNA is mature or a precursor through the capitalisation of the letter r, with R indicating the mature miRNA and r the precursor. The number simply refers to the order in which the miRNA was discovered. Several miRNAs will have a letter after the number, and this is used in cases where there are multiple miRNAs that vary by only one or two nucleotides. There can also sometimes be an additional number after this, and this refers to identical miRNA sequences that are derived from distinct locations in the genome. Since the pre-miRNA is processed into passenger or guide strands, this can also be distinguished with either a -3p or -5p to show which arm of miRNA precursor they are derived from. The guide strand is the strand that assembles with the argonaute (AGO) protein.

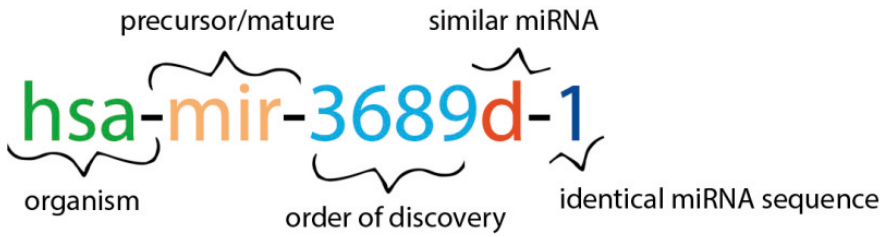

Figure 6.
Naming convention for miRNAs. The nomenclature of miRNAs. The system is based on the species (green), whether or not the miRNA is in a precursor state or is in its single stranded mature form (yellow), the number is based simply on its discovery with more recently discovered miRNAs being larger (light blue), similar miRNAs that differ by one or two nucleotides (red) and if there are identical miRNA sequences transcribed from different genomic regions this is shown as a number at the end (dark blue).

Biogenesis and mechanisms of miRNAs

The biogenesis of miRNAs can occur through several distinct pathways that incorporate different enzymes and cleavage events; however, these will not be discussed, and I will focus purely on the canonical pathway. The canonical pathway involves the transcription of the miRNA gene from primarily RNA polymerase (RNA pol) II into miRNA precursors called primary-miRNAs (pri-miRNAs). These pri-miRNA transcripts can be several kilobases [88] long, if there are several in tandem, and contain 5'cap structures and a poly(A) tail [89]. The pri-miRNAs often contain several hairpin structures, which are approximately 70 nucleotides in length, from which the mature miRNA will eventually derive. This pri-miRNA will then be processed in the nucleus by the microprocessor complex, which is made up of the RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8) and the ribonuclease enzyme Drosha. DGCR8 initially binds the pri-miRNA and recruits Drosha to cleave the pri-miRNA within the nucleus into precursor-miRNA (pre-miRNA) which has a characteristic two nucleotide overhang at the 3' end [90]. This overhang is recognised by Exportin-5, which functions to translocate the pre-miRNA out of the nucleus into the cytoplasm for further processing. Once in the cytoplasm, the second cleavage event can occur through the action of Dicer to produce an RNA duplex with overhangs on both 3' ends. The two strands are the guide strand and passenger strand, in which the guide strand is anchored into the AGO complex, and typically the passenger is degraded after they are unwound through helicase activity. However, the passenger strand is not always degraded, and this can become an active miRNA [91]. The mature miRNA

bound to AGO protein form part of the RNA-induced silencing complex (RISC). The canonical miRNA biogenesis pathway is shown in Figure 7.

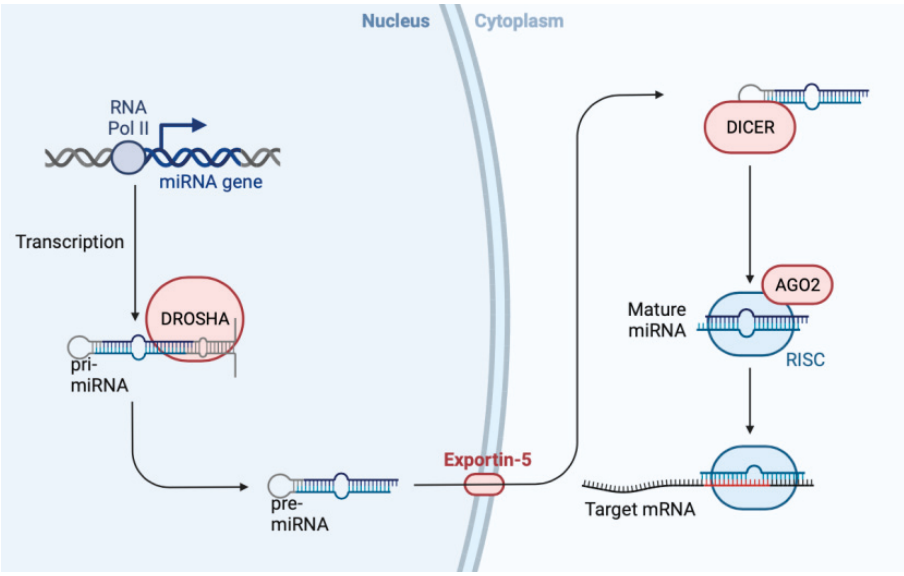

Figure 7.
Canonical miRNA biogenesis pathway. The main pathway for miRNA biogenesis with the key proteins and complexes involved included. Figure was generated using BioRender [43].

The miRNA-RISC complex will typically bind to target mRNA sequences whereby, it usually inhibits mRNA translation, and it is thought that these miRNAs regulate around 60% of all protein coding genes in humans [92]. miRNAs have hundreds of potential mRNA targets, and one mRNA can be inhibited by many different miRNAs. These characteristics are responsible for miRNAs putative role in 'fine-tuning' cellular processes. The mechanism of miRNA binding to target mRNA is through the seed region, a sequence typically located at nucleotides 2–8 from the 5' end of the miRNA [93]. The seed sequence typically has complete complementarity to the converse mRNA strand; however, the remaining bases do not need full complementarity for successful binding, hence the ability of a single miRNA to have multiple targets. There are several different mechanisms by which miRNAs can affect post-transcriptional gene expression, and although this usually results in repression of the target, there are cases where the actions of miRNA can upregulate the gene expression of its target. Repression of target mRNAs is

dependent on the level of complementarity. Limited miRNA-mRNA base pairing leads to translational repression, and if there is complete complementarity between the miRNA and the target mRNA, then cleavage occurs through the action of AGO2 endonuclease activity [94]. The binding of miRNA to the 5'UTR region, however, can lead to increased translation of the target mRNA, as exemplified in a study by Örom *et al*. looking at the effect of miRNA-10a on ribosomal protein mRNAs [95]. If there is complete complementarity between the miRNA and the target mRNA, then cleavage occurs through the action of AGO2 endonuclease activity [94]. Target sites can be predicted i*n silico* using databases such as miRDB, which uses bioinformatic tools and algorithms to predict affinity [96].

miRNA editing

The number of actual mRNAs targeted by a miRNA can be further complicated by the addition of post-transcriptional modification of the miRNA itself. Since the actions of Dicer and Drosha are not always perfectly precise, they can be a source of variation due to aberrant cleavage, leading to isoforms of miRNAs (isomiRs). RNA editing is also a common form of miRNA diversification, and the most studied example of this is A-to-I editing, with around 16% of human pri-miRNAs predicted to be subject to this process [97]. A-to-I editing is whereby ADAR (Adenosine deaminase acting on RNA) enzymes convert the nucleoside adenosine to inosine. Due to the structure of inosine, it can base pair with cytidine, and thus A-to-I editing is comparable to the substitution of guanosine for adenosine. It is also important to bear in mind that the affinity between inosine-cytidine and guanosine-cytidine is not identical, with only two hydrogen bonds between inosine-cytidine as opposed to three, meaning a reduction in the stability of the bonds holding them together [98]. Nonetheless, the impact of A-to-I editing can have a huge biological impact on cellular processes, as isomiRs that have undergone A-to-I editing in the seed region will have a different set of target mRNAs compared to their unedited forms, as shown in Figure 8. A-to-I editing outside the seed region can lead to a change in loading efficiency into the RISC complex, and this can also have a biological impact [99].

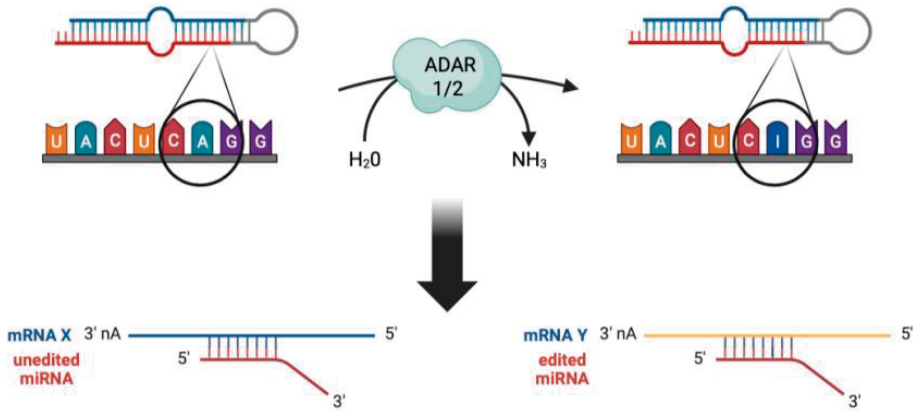

Figure 8.
A-to-I editing of miRNAs. Mechanism by which A-to-I editing of miRNAs is mediated through ADAR and its effect on target mRNAs. Figure was generated using BioRender [43].

In humans, RNA A-to-I editing is mediated through ADAR1 and ADAR2, and although humans also possess the ADAR3 protein, this is only expressed in the brain and does not contain the catalytic deaminase domain present in ADAR1 and ADAR2 [100]. Knockout of ADAR1 and ADAR2 leads to embryonic lethality [101, 102], and aberrant ADAR expression has also been linked with cancer [103]. Overediting through ADAR1 overexpression is well documented in many different cancer types, including liver and lung, as generally having a tumour-promoting phenotype [104]. However, there have also been studies showing that ADAR1 overexpression can have tumour suppressive properties, such as in melanoma, whereby it inhibits growth and metastasis *in vivo* through its action on miRNA-455 [105]. Aberrant A-to-I editing is also referenced in PCa development and is a source of transcriptomic diversity in tumours, and editing of the AR may even be implicated in the progression of CRPC [106]. The impact of editing on miR-379 will be discussed later in the thesis.

miRNAs in PCa

The link between aberrant miRNA expression and cancer was first established in 2002, when a study by Calin *et al*. found that the genomic region containing miR-15 and miRNA-16 was deleted in more than half of the patients with B-cell chronic lymphocytic leukaemia [107]. Since then, there have been numerous reports of different miRNAs and their involvement in cancer

development, with a general trend for miRNAs to play a tumour suppressor role. One example of this in PCa is miR-145, which suppresses the AR as well as PSA, with low miRNA-145 expression being linked to reduced survival in men after TURP [108]. However, there are still many miRNAs that play an oncogenic role if their target mRNA is a tumour suppressor. The tumour suppressor gene PTEN is very often deleted in PCa progression [109]. It is unsurprising then that miRNA-221/-222, who are known to target PTEN, are overexpressed metastatic CRPC in addition to this [110]. Figure 9 summarises some of the miRNAs that have been referenced in papers to play tumour suppressive and oncogenic roles in PCa [111-116].

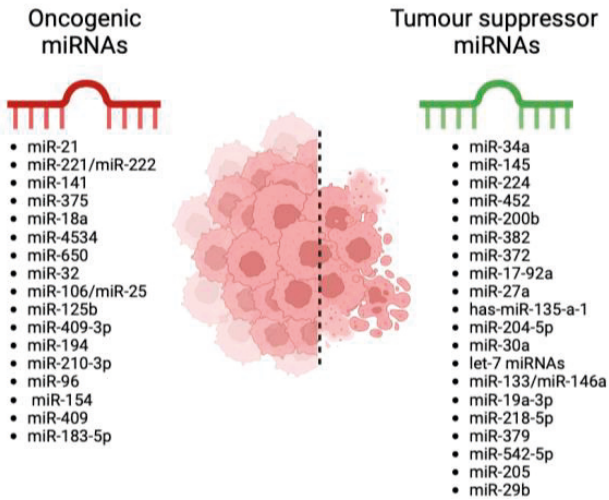

Figure 9.
Oncogenic and tumour suppressive miRNAs in PCa. The miRNAs listed are from several review papers and our own papers [111-116]. Figure was generated using BioRender [43].

One of the key pathways involved in PCa progression is the dysregulation of AR signalling, as mentioned previously in this thesis. There is a bidirectional relationship between miRNAs and the AR since miRNAs can target the AR, but there are also many miRNAs that are regulated by AR signalling [117]. Many miRNAs have been implicated in AR signalling, including miRNA-21, an oncogenic miRNA implicated in several cancer types. It has been shown that inhibiting miRNA-21 could reduce cell growth in AR-dependent PCa cell lines, and when miRNA-21 was overexpressed, tumour growth increased in mice. Furthermore, the study performed bilateral orchiectomy, and despite this, AR-dependent cell lines transduced with miRNA-21 continued to increase

in volume, suggesting that miRNA-21 can drive castration resistance [118]. AR dysregulation through miRNAs can also occur through targeting regulators of AR, such as c-Myc [119]. It has been shown that miRNA-let-7c targets c-Myc for degradation, and in turn, this suppresses AR activity [120]. It is therefore unsurprising to find that miRNA-let-7c is downregulated in CRPC, and by upregulating it *in vivo*, it can reduce tumour burden [121].

miRNAs in the clinic

There has been a lot of excitement for the clinical application of miRNAs, and broadly, the translational aspect of this research focuses on miRNAs as therapeutics and as biomarkers. Many start-up companies have seen this potential, such as the Danish company Santaris Pharma, which has focused on miRNA therapeutics for diseases such as hepatitis C [122]. Many of the bigger companies are also taking note, such as Roche which has since acquired Santaris Pharma, under the name Roche Innovation Center Copenhagen [123]. The potential for miRNA-based therapies does inherently have some limitations, such as their delivery, specificity, and potential side effects, due to their ability to bind multiple mRNA targets. However, this has not stopped several clinical trials taking place for miRNA therapies. One example of this is Remlarsen, a miRNA-29 mimic that prevents the formation of a fibrotic scar and scleroderma [124], and this therapy has passed phase II clinical trials [125]. One of the main challenges for the development of miRNA therapies is the delivery system used. The latest developments can be loosely grouped as either viral-based delivery methods or non-viral-based delivery methods. Different viral vectors have different advantages and disadvantages that need to be considered for the desired miRNA payload to reach the intended tissues. Retroviral vectors, for example, can only transduce dividing cells, whereas lentiviral vectors can transduce both dividing and non-dividing cells [126]. In developing a non-viral vector for delivering a specific miRNA, there are also different considerations that need to take place. Lipid-based nanocarriers are the most commonly used method for delivering miRNAs and are very simple to manufacture at low cost; however, their tissue specificity is not as good as the more expensive extracellular vesicles, which have recently been found to be able to have surface modifications, making them far more precise at delivering the miRNA payload [126].

While progress for miRNAs as therapeutics may be a little slower to reach their clinical potential, their use as biomarkers has been far more promising. In the clinic, miRNAs have the potential to be useful in the diagnosis, prognosis, and

response to therapies for many cancers, including PCa. One key characteristic that make miRNAs good candidates as biomarkers is that, due to their small size and incorporation in proteins, they are often shielded from RNAse enzymes. Most miRNAs in circulation are bound to protein complexes such as AGO, with the remaining fraction enveloped in exosomes [127]. miRNAs have been established as cancer biomarkers since 2007, when researchers found miRNA-155, miRNA-21, and miRNA-221 were able to distinguish different subtypes of diffuse large B-cell lymphoma [128]. I will focus on their clinical applications in PCa since this is the cancer we studied during this PhD. As discussed previously, there are typically several considerations when assessing the course of treatment, such as the PSA score and Gleason grade. Given the stability of miRNAs, they have great potential as additional prognostic tools since they can be detected in many fluids such as blood, saliva, and urine and they can also be stored for extended periods of time with little degradation. Through meta-analysis of miRNA datasets, there are some miRNAs that seem to be commonly upregulated, such as miRNA-484 and miRNA-498, with some also commonly downregulated, such as miRNA-137 and miRNA-221 [129]. Further validation should be carried out to both verify the biological role of the miRNAs as well as their ability to robustly perform as useful prognostic biomarkers. For miRNAs to become more standardised in a clinical setting, there are a few limitations that need addressing. First, the methods used for miRNA detection should be standardised to increase reproducibility between different laboratories and hospitals. This includes the methods by which miRNAs are isolated, whether that be from blood samples, saliva, or urine, for example. Secondly, the same miRNA can have different implications for different cancer types and tissues, and even when we focus specifically on PCa, there can be conflicting reports on specific miRNAs, primarily due to clinical and methodological heterogeneities. As mentioned previously, miRNA editing can also add another layer of complexity, and the predictive ability of a miRNA may be isoform-specific, so this is also an area that needs to be addressed further.

miR-379

General information

The miR-379 gene is located on chromosome 14 on the q arm. The genomic location of miR-379 is chromosome 14:101,022,066-101,022,132. The sequence of the miRNA sequence and its corresponding pre-miRNA sequence are shown in Table 2.

Table 2.
miR-379 information. Sequences of the mature miRNA sequence and the pri-miRNA stem loop are shown, as well as the genomic location of the gene.

Mature miRNA Sequence:	**UGGUAGACUAUGGAACGUAGG**
Stem-loop Sequence	**AGAGAUGGUAGACUAUGGAACGUAGGCGUUAUG AUUUCUGACCUAUGUAACAUGGUCCACUAACUCU**
Chromosome Location	**Chr. 14 - 101022066 - 101022132 [+] on Build GRCh38**

The genomic location of the miR-379 gene is within an evolutionary conserved miRNA cluster, known as the miR-379/miRNA-656 cluster which is located on the chromosome 14q32.31 region [130]. This cluster is imprinted from the maternal allele and is only present in placental mammals [131] and no other vertebrate genomes [132]. In this cluster, miR-379 is located furthest on the 5' side and miRNA-656 on the 3' [132], and it contains 54 miRNAs, 53 of which are in the 5' to 3' direction (including miR-379) and only miRNA-1247 in the 3' to 5' orientation [133]. Transcription of the miR-379/miRNA-656 cluster is under positive regulation of the MEF2 transcription factor [130, 134] and has also been shown to be regulated by glucocorticoids [135] and histone methylation [130]. Within the cluster, there are also the imprinted genes DLK1, RTL1, and DIO3, which are paternally expressed, and MEG3, MEG8, and antisense RTL1, which are maternally expressed [136].

Role of miR-379 in disease

Due to the characteristic nature of miRNAs being able to bind to multiple mRNA targets and pathways, a singular miRNA can often be implicated in multiple disorders. There have been several diseases linked to aberrant miR-379 expression reported in the literature. The knockout of miR-379 using CRISPR-Cas9 in mice does not present any obvious phenotypic deficiencies, and this is likely due to the compensation of other miRNAs and pathways. However, this knockout can reduce the impact of diabetic kidney disease [137], increase the sensitivity of mice to insulin [138], and is implicated in other metabolism-related afflictions such as non-alcoholic fatty liver disease [139]. Higher serum levels of miR-379 have been seen in patients with Duchenne muscular dystrophy compared to age-matched controls [135]. A study found that miR-379 could induce cell cycle arrest in vascular smooth muscle cells and that its downregulation is seen in patients with acute myocardial infarction [140]. Patients with polycystic ovarian syndrome were reported to have lower levels of miR-379 in granulosa cells [141]. This study also found that overexpression of miR-379 inhibited granulosa cell proliferation *in vivo*.

The role of miR-379 is generally seen as tumour suppressive, with many different cancer types reporting that reduced expression can be a driver of disease. The miR-379/miRNA-656 cluster was also identified as playing a tumour suppressor role in several cancer subtypes [142]. I will summarise all the cancers, in alphabetical order, for which a tumour suppressor has been described in Table 3.

Table 3.
Cancers where miR-379 plays a tumour suppresor role.

Cancer type	Information in literature
Bone cancers	In multiple myeloma cells, there is a reduction in miR-379 compared to normal bone marrow-derived plasma cells, and its overexpression could inhibit proliferation and induce apoptosis *in vitro* [143]. There is downregulation of miR-379 in osteosarcoma tissues and cell lines, and furthermore, upon miR-379 overexpression, proliferation and invasion were reduced, and in mouse xenografts, tumour size was reduced [144]. There are significantly reduced levels of

	miR-379 in chronic myeloid leukaemia cells co-cultured with human bone marrow stromal cells, and upon upregulation of miR-379, these cells showed sensitivity to imatinib [145].
Brain cancers	Previous assessments of the miR-379/miRNA-656 found that glioblastoma has the highest proportion of miRNA members in its cluster to be downregulated, at 68%, according to Laddha *et al*. [142]. The miR-379/miRNA-656 cluster was also downregulated between 78% and 88% according to Nayak *et al*., who looked at three different sub-types of gliomas [146], and in oligodendrogliomas, the cluster was also significantly downregulated [130]. Independently, miR-379 has also been shown to be consistently downregulated in glioblastoma [147-150], with Kaplan-Meier plots also showing that its downregulation significantly reduced patient survival [150]. Medulloblastoma. There was only one study by Kaur *et al*. that investigated the miR-379/miRNA-656 cluster and found it to be significantly downregulated in all medulloblastoma subgroups [151].
Breast cancer	There have been several studies implicating both miR-379 and the miR-379/miRNA-656 cluster in breast cancer development. Reduced levels of miR-379 and its cluster have been associated with disease progression [142, 152-155] suggesting a tumour suppressive role. Furthermore, overexpression of miR-379 leads to a reduction in key cancer phenotypes like proliferation, migration, and invasion [156]. There has also been a paper by O'Brien *et al*. whereby they engineered mesenchymal stem cells to secrete extracellular vesicles containing miR-379, which were able to home to the tumour and have a therapeutic effect [155].
Bladder cancer	There has only been one paper by Wu *et al*. investigating the role of miR-379 in bladder cancer and this, like other cancer types, found the miRNA to play a tumour suppressive role [157]. Overexpression of miR-

	379, also lead to a reduction in proliferation, migration, and invasion *in vitro* [157].
Cervical cancer	miR-379 plays a tumour suppressive role in cervical cancer, and its upregulation inhibits proliferation and invasion *in vitro* [158]. Furthermore, HPV 16 is also linked to downregulated miR-379 [159]. HPV 16 is known to significantly increase the risk of cervical as well as vaginal, oropharyngeal, and penile cancer [160].
Endometrial cancer	Expression of miR-379 was found to be significantly reduced in patients endometrial cancer tissue and could potentially be used as a diagnostic marker as there was a significant association between miR-379 expression and tumour stage, grade, and lymph node metastasis [161]. Furthermore, overexpression of miR-379 inhibits the proliferation, migration, and invasion of endometrial cancer cells *in vitro* [162].
Kidney cancers	The miR-379/miRNA-656 cluster was shown to be 61% downregulated in renal clear cell carcinoma [142]. Expression levels of miR-379 were also found to be associated with patient survival in papillary renal cell carcinoma [163]. In hepatocellular carcinoma, miR-379 plays a tumour suppressive role [164, 165]. *In vitro*, miR-379 can inhibit epithelial-to-mesenchymal transition as well as invasion and migration, and *in vivo*, it has been shown to reduce metastatic spread with a reduction in intrahepatic metastatic nodules [164]. Furthermore, miR-379 could increase the sensitivity of hepatocellular carcinoma cell lines to doxorubicin [166].
Laryngeal carcinoma	There has been one paper by Wei *et al*. investigating miR-379 and its impact on laryngeal carcinoma. This paper found that miR-379 plays a tumour suppressor role, with the expression of the miRNA being lower in laryngeal carcinoma cancer tissue and cell lines [167]. *In vitro*, miR-379 can inhibit proliferation, migration, and invasion [167].

Lung cancers	In lung cancer, miR-379 was shown to be reduced in cisplatin-resistant lung cancer cell lines, and when miR-379 mimics were used in cell lines, this increased the sensitivity to cisplatin [168]. Non-Small Cell Lung Cancer (NSCLC) has also been repeatedly linked with aberrant miR-379 expression. These studies found reduced miR-379 expression in NSCLC tissues and cells [169-173]. In vitro, miR-379 was also shown to inhibit cell proliferation but increase cellular apoptosis [170], as well as inhibit cell migration and invasion [173]. By increasing miR-379, it was also possible to reduce NSCLC resistance to chemotherapy [169, 172]. Research looking into mutational hotspots for lung adenocarcinoma also found the miR-379 gene to be over-mutated [174]. A Japanese study found that miR-379 was downregulated in three malignant pleural mesothelioma cell lines. Furthermore, by upregulating its expression, invasion was inhibited and sensitivity to vorinostat treatment was increased [175]. Overall, there are many reports in the literature of miR-379 playing a tumour suppressor role in a range of lung cancers, with NSCLC being particularly well documented.
Nasopharyngeal carcinoma	In nasopharyngeal carcinoma, miR-379 plays a tumour suppressive role, and in patient tissues, there is a downregulation of the miRNA compared to healthy controls [176, 177]. Furthermore, overexpression of miR-379 *in vitro* led to a reduction in cell migration, invasion [177], and cellular proliferation [178].
Oral cancer	Tissue samples from patients with oral squamous cell carcinoma showed lower miR-379 expression compared to healthy adjacent tissues, suggesting that miR-379 plays a tumour suppressive role [179]. In addition to this, upregulation of miR-379 was shown to inhibit the colony formation ability of oral squamous cell carcinoma cell lines [179].

Despite an overwhelming number of cancers citing miR-379 as a tumour suppressor, there has been mention that miR-379 can also act as an oncogenic miRNA. A summary of all the cancers in the literature that have mentioned aberrant miR-379 in cancer is shown in Figure 10. Below this, there is also mention of the few studies with evidence of miR-379 as an oncogenic miRNA.

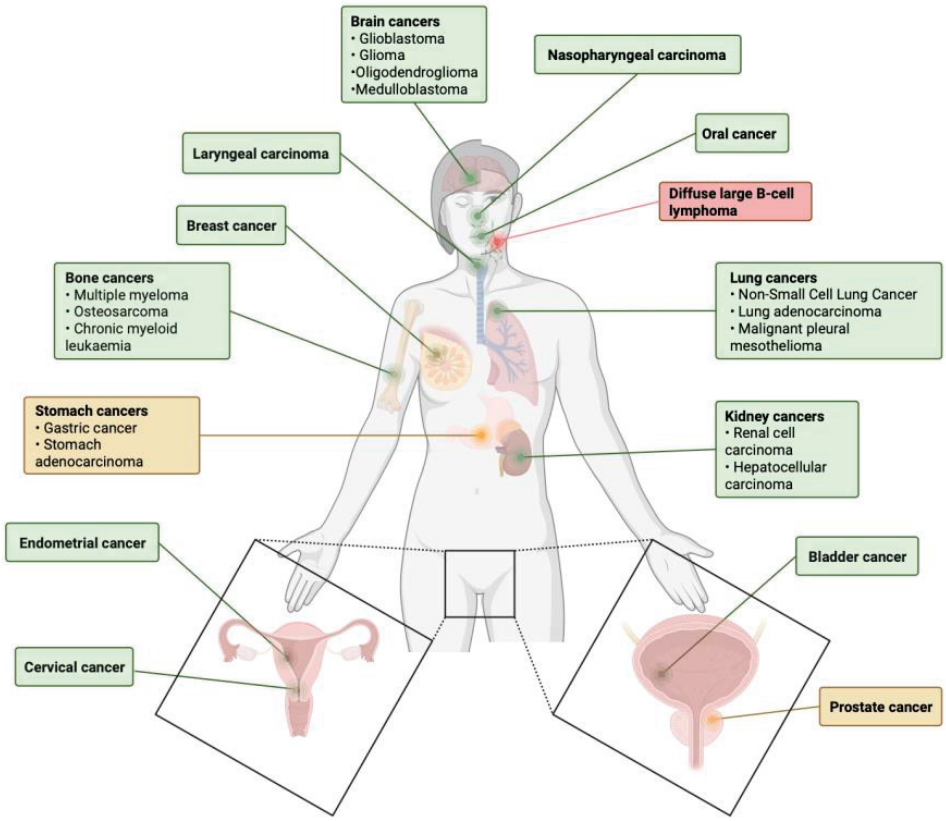

Figure 10.
Cancers affected by aberrant miR-379 expression. The cancers highlighted in green have a consensus for miR-379 as a tumour suppressor and the cancers for which there have been both tumour suppressive and oncogenic miRNA properties are highlighted in orange. Diffuse large B-cell lymphoma which has reported mir-379 as an oncogenic miRNA is highlighted in red. Figure generated using BioRender [43].

Diffuse large B-cell lymphoma. Unlike most cancer types, in diffuse large B-cell lymphoma, the role of miR-379 seems to be that of an oncogenic miRNA, with a study by Cao *et al*. reporting significantly higher miR-379 expression in patient tissues [180].

Stomach cancers. There have been multiple studies looking into the relationship between miR-379 and gastric cancer. Again, miR-379 is seen to be a tumour suppressor in this cancer [181-183]. There has also been *in vitro* data showing that miR-379 can inhibit proliferation and migration in gastric cancer cells [183], as well as epithelial-mesenchymal transition [184]. Interestingly, it has been suggested that the downregulation of miR-379 is also involved in the development of cisplatin resistance in gastric cancer [182]. As opposed to miR-379-5p, which we have detailed thoroughly during this thesis, there is a lot less research carried out on miR-379-3p. In patients with stomach adenocarcinoma, the -3p arm is implicated in disease prognosis, with high expression of the miRNA being associated with worse clinical outcomes and significantly reduced patient survival [185]. This and diffuse large B-cell lymphoma are the only cancers where miR-379, albeit the -3p form in stomach adenocarcinoma, is seen to play a role as an oncogenic miRNA.

Role of miR-379 in PCa

Prior to this thesis, our group had published two papers that would suggest miR-379 plays a tumour suppressive role. An *in vivo* functional screen identified that reduced expression of miR-379 drives the metastatic spread of PCa to the bone and can increase cell growth and colony formation *in vitro* [114]. The screen used PC3 cells transduced with a library of 123 anti-miRNA shRNA clones, and this was injected into the prostate. From these, primary prostate tumours and metastases were assessed. The metastatic sites investigated were the liver, lungs, and bone marrow. The enrichment of the anti-miRNA at each site could be compared to that of the primary tumour, with the following anti-miRNAs showing the highest fold increase at each site: anti-miRNA-135b was enriched 14-fold in liver metastasis, anti-miRNA-23b was enriched four-fold in lungs, and anti-miR-379 was enriched over 100-fold in the bone marrow. Furthermore, to validate that anti-miR-379-expressing cells could drive spread to the bone, another *in vivo* experiment was setup. This time the 22Rv1 cell line was used, which does not typically metastasise to the bone like PC3, and this was transduced with anti-miR-379 or a scrambled control sequence, as well as green fluorescent protein. Cells were injected intracardially, and the presence of bone metastasis was measured with immunohistochemical staining. Of the five mice injected with anti-miR-379, all developed bone metastasis, whereas none of the scrambled control mice

did. Further *in vitro* analysis of cells transduced with anti-miR-379 showed that cell growth and colony formation were increased upon downregulation of miR-379. Levels of miR-379 were also investigated in a patient cohort from Umeå. The expression levels of miR-379 in this cohort were significantly reduced in bone metastasis compared to primary tumours and non-cancerous prostatic tissue. In the paper by Voss *et al*., decreased miR-379 levels were also found to be associated with treatment resistance, metastasis, and shorter overall survival in a patient cohort from Malmö [114, 186]. Furthermore, in the Taylor dataset [187], miR-379 is reduced in PCa tumours and metastases compared to healthy prostates.

Independently to the research performed in our group, there has only been one previous reported study that has stated miR-379 may play an oncogenic miRNA role in PCa. In this study, they found increased expression is correlated with progression-free survival in PCa patients [188]. The reported findings by Gururajan *et al*. have some discrepancies however, as firstly, there is no mention of the *in vivo* results for miR-379. This seems odd since they discuss both miR-379 and miRNA-154* in the paper and perform many parallel experiments stating: 'Genetic manipulation of miRNA-154* and miR-379 was performed to determine their role in tumour growth, EMT and bone metastasis in mouse models' [188]. Since we know they performed intra-cardiac injections with luciferase-tagged ARCaPM control and ARCaPM-154*inhibitor cells and found reduced bone metastasis, it would be interesting to know if they also performed this in ARCaPM cells with miR-379 inhibition. They also state in the paper that miR-379 shows a positive correlation with progression-free survival in patients. However, they base this conclusion on data from the Taylor dataset [187], but they make no mention of comparing groups and just state that reduced miR-379 is associated with patient survival but do not compare miR-379 levels in tissues. In summary, despite conflicting reports on the role of miR-379, it is only as a tumour suppressor that we see strong *in vivo* data and patient data supporting this notion.

The present investigation

Paper I: Expression of microRNA-379 reduces metastatic spread of prostate cancer

Background

We have previously shown that miR-379 downregulation can drive the metastatic spread of PCa to the bone and that its relative expression is lower in bone metastasis compared to normal prostate tissue [114]. Patients sorted by miR-379 high expression also had a significantly better survival outcome compared to those with low miR-379 expression [186]. Furthermore, many cancer phenotypes were shown to be increased in PCa cells with downregulated miR-379, leading to increased proliferation and colony formation *in vitro* [114]. From a therapeutic standpoint, however, we are more interested in wheter miR-379 overexpression inhibits the spread of PCa to the bone.

Summary

PCa cell lines were successfully transduced to overexpress miR-379, and these were used for several functional experiments. In parallel with this, we also successfully differentiated human mesenchymal bone cells into osteoblasts, from which we could derive osteoblast-conditioned medium (OBCM), which was also used during the *in vitro* functional experiments. By assessing differences between both miR-379 and a scrambled control in normal media as well as OBCM, we could evaluate the impact of miR-379 upregulation and how the bone microenvironment can influence cell growth, migration, colony formation, and adhesion. The results show a significant inhibition of cell growth, migration, and colony formation upon miR-379 overexpression. The impact of OBCM compared to normal media was negligible for cell growth and migration; however, for colony formation, it was only in the OBCM that

visible colonies were seen in significant numbers, and this was inhibited by miR-379 upregulation. For assessment of cell adhesion, both miR-379 upregulation and downregulation were assessed since this was not tested in the miRNA screening paper [114]. We found that upon upregulation of miR-379 there was significantly less adhesion, between PC3 cells and osteoblasts, as well as the osteosarcoma cell line MG63, compared to the scrambled control. Furthermore, 22Rv1 cells with transduced anti-miR-379 showed increased adhesion to osteoblasts and MG63 cells.

PC3 cells were also successfully transduced with a luciferase vector to visualise metastatic spread *in vivo* using the IVIS Spectrum In Vivo Imaging System machine and assess if miR-379 upregulation can inhibit metastatic spread to the bone. To investigate this, mice were injected intracardially to imitate PCa metastasis in circulation. We found a significant reduction in the number of mice with bone metastasis in the miR-379 group compared to that of the scrambled control group, and furthermore, the overall number of metastatic sites and tumour burden were also reduced. Figure 11 shows one of the scrambled control mice with metastatic spread to the bone undergoing 3D X-ray imaging. After the imaging, mice were sacrificed and tissues were also dehydrated and embedded in paraffin, the bone metastases were also morphologically assessed, and a difference between the miR-379 bone metastasis and control metastases was seen, with those derived from miR-379 PC3 cells being more differentiated and less heterogeneous.

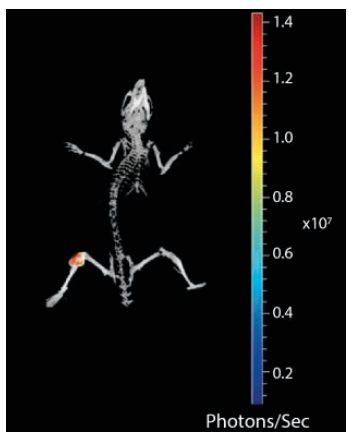

Figure 11.
X-ray imaging of a mouse with metastatic spread to the tibia. IVIS imaging was used, and a 3D X-ray image was taken of one of the scrambled control mice with bone metastasis. The cells were transduced with a luciferase vector and will emit photons after systemic injection of D-luciferin.

A cytokine array was also performed to better understand the signalling between the bone microenvironment and PCa cells with reduced miR-379 expression. Conditioned media taken from anti-379 PCa cells and control cells were added to osteoblasts to explore cytokines that are deregulated as a result. GDF-15 was selected as it was both highly expressed, and showed a substantial increase in the anti-379 setting compared to that of the scrambled control setting. In an external patient cohort, the expression of the GDF-15 receptor was also investigated and found to be higher in metastasis compared to normal prostate samples.

Limitations of study

One major limitation of our study is the fact that in both the *in vitro* and *in vivo* experiments, we almost solely looked at the effect of miR-379 upregulation in one PCa cell line. We know that AR signalling seems to be implicated in the action of miR-379, and so to primarily just use an androgen-unresponsive cell line may limit the generalisability of our results. There were several efforts to perform the *in vivo* experiments in other androgen-responsive cell lines; however, both the LNCaP AR-hi and C4-2B cell lines would not metastasise to the bone, so they were not suitable models for this project. MDA PCA-2b cells were also initially designed to be used as well; however, these cells were too sensitive to transduce with miR-379 in the first place, so they could not be assessed. When assessing the effects of miR-379 downregulation in the study by Ivkovic *et al.*[114] we did use 22Rv1 cells for *in vivo* and *in vitro* experiments and these also support the consensus of miR-379 as a tumour suppressor but it would be useful to also test the impact of upregulation here.

Our efforts to try and reconstitute a bone microenvironment *in vitro* can also be scrutinised since the conditioned media we used was derived purely from osteoblasts and so fails to account for the other cells present in this niche. Of course, *in vitro* experiments will never fully recapitulate the complexity of the bone; however, it would be interesting to try and incorporate signalling from other bone cells to assess miR-379 effects. There have been many efforts to use bone organoids to model metastasis, and a recent review by Chen *et al*. highlights some of the latest developments in this field [189]. However, there are still many limitations, such as the inability to recapitulate the immune system.

Discussion

In this study, we found further evidence of miR-379 playing a tumour suppressive role in PCa, supporting our previous findings [114, 186] but contradicting those of the study by Gururajan *et al*. [188]. The reported findings by Gururajan *et al*. have some discrepancies. Firstly, there is no mention of the *in vivo* results for miR-379 upregulation only for that of miRNA-154*, and their use of the Taylor dataset [187] only looked into patient survival and did not compare miR-379 levels in tissues [188]. Given that the study by Ivkovic *et al*. used the same publicly available dataset [187] and found that miR-379 was expressed at lower levels in bone metastases compared to benign tissue [114], this does not seem to make a great deal of sense. Nonetheless, the study used ARCaP cells for its experiments and found a more pronounced epithelial phenotype [188], and since we have not investigated the effect of this cell line on epithelial-mesenchymal transition, we cannot refute this claim.

There are many ways to further study the therapeutic potential of miR-379 in the lab. We could alter the *in vivo* experiments by tracking how miR-379 upregulation effects cells that have an intraprostatic injection into mice over time. I think from a therapeutic perspective, the most value gained would be from seeing how miR-379 effects PCa that has already spread to the bone. There have been techniques, such as the injection of cells directly into the tibia of mice, that allow us to reliably establish bone metastasis for further experimentation [190]. There has also been work by Martine *et al*. to create a bone cancer model in mice using a humanised tissue-engineered bone construct [191]. PC3 cells grow preferentially in this model relative to the mouse bone, suggesting that it has a stronger affinity for human PCa cell lines and may be a better representation of the disease [192]. By exploiting the latest research into modelling bone metastasis, we could test if miR-379 mimic could inhibit PCa progression after metastatic spread has occurred. This potential plan to use a miR-379 mimic, however, has its own caveats, namely, how to successfully deliver the mimic to the tumour site. We have discussed some of the developments in miRNA delivery systems earlier in the thesis, so we will not go into depth on this. There is a recent publication by Han *et al*. that successfully managed to use nanoparticles derived from miRNA-34a, polyethylenimine 25 k, and alendronate moieties to successfully target bone metastasis derived from breast cancer cell lines *in vivo* and reduce the tumour burden [193]. It is feasible that a similar delivery system could be used with

miR-379 and that this could be used to assess its clinical effectiveness in PCa bone metastasis.

Taken together, our results from this study show that miR-379 can be a promising inhibitor of metastatic spread however, there is still a long way to go before we can realistically see miR-379 used in a clinical setting. Future studies looking into not only the biological mechanisms and pathways involved with miR-379 but also how to successfully deliver the miRNA payload to the bone would be particularly interesting.

Paper II: MicroRNA-379 influences PSA expression in metastatic prostate cancer (manuscript)

Background

We have shown in Paper I that miR-379 can inhibit the spread of PCa to the bone in our *in vivo* model system. In the paper, there was also a cytokine array performed on osteoblast cells grown in anti-379-conditioned media and compared to those grown in scrambled control media. This cytokine array identified GDF-15, which is known to play a role in bone metastasis [194-196] and this was validated using ELISA assays. It was interesting to understand how the bone microenvironment responds to the presence of miR-379 dysregulation; however, we did not investigate the effect of the bone microenvironment on PCa cells with reduced miR-379 expression. Since there is reciprocal signalling between the two cell types, it would be particularly interesting to see what cytokines are released from these PCa cells. A basic schematic of this signalling feedback loop is shown in Figure 12.

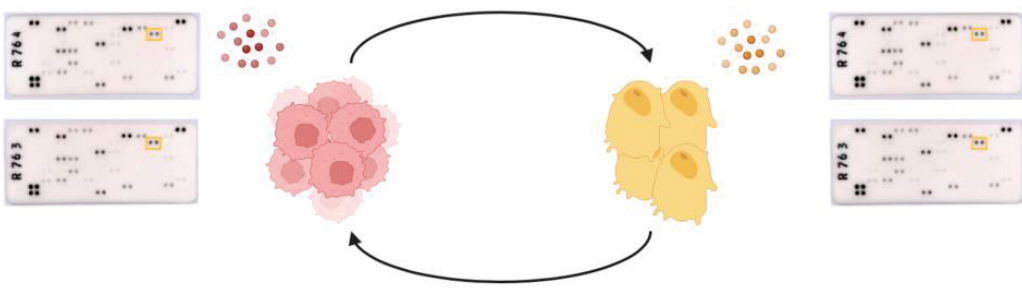

Figure 12.
Cytokine arrays were performed on PCa and bone cells grown in conditioned media. PCa cells transfected with anti-miR-379 or a scrambled control were grown in OBCM and compared (paper II) as well as osteoblasts grown in conditioned media from anti-miR-379 PCa cells or scrambled control cells (paper I). The figure was generated using BioRender [43].

Summary

We performed a cytokine array focusing on which proteins are secreted from 22Rv1 cells with reduced miR-379 expression in an osteoblast setting. This was carried out twice, and PSA was identified as the most highly enriched

cytokine for both repeats in the osteoblast setting. We validated this using DELFIA assays and found that secreted PSA was indeed higher in anti-379 22Rv1 cells compared to control cells for both fPSA and tPSA. This trend was also seen in the normal media setting and intracellularly. Furthermore, when miR-379 was overexpressed in 22Rv1 cell lines, fPSA and tPSA were reduced, both secreted and intracellularly. We did not know if the effect of miR-379 on PSA was through direct binding, so we performed an AGO2-immunoprecipitation to identify direct targets of the miR-379 RISC complex. PSA was not found; however, AR, which regulates PSA via binding in the promoter region, was identified in this assay. We therefore performed a luciferase reporter assay and found that increasing miR-379 did lead to binding in this region, suggesting that the effect of miR-379 on PSA was indirect and mediated through AR. To further investigate the impact of miR-379 on AR, we performed Western blots and found that increased miR-379 reduced AR protein expression, consistent with the proposed mechanism of miR-379 on AR and PSA. We also investigated the impact of miR-379 dysregulation on PSA and AR in patient cohorts. We found that miR-379 expression in prostatic tissue had a negative correlation with serum PSA levels and that tissues with higher AR staining had reduced miR-379 expression. The findings from the patient cohort are consistent with what we have found in our *in vitro* data. Since this paper is still only in manuscript form, we will continue our experiments and analysis to further understand this miR-379-regulated pathway.

Limitations of study

One limitation is the lack of cell lines used; so far, we have primarily focused on the 22Rv1 cell line. However, our most recent experiments using VCaP show the same trend, thus supporting our hypothesis that miR-379 indirectly downregulates PSA levels before secretion. However, this will have to be repeated before any conclusions can be drawn, and we will also perform experiments to test the effect of miR-379 overexpression in VCaPs on AR protein expression using Western blots. The use of OBCM to mimic the bone environment is also a limitation, as previously mentioned in the limitations of Paper I, but since this has been discussed, I will not expand on this in any more detail here.

This is still a work in progress, and so far, we have presented a case for the effect of miR-379 on PSA and AR. I would also like to further investigate the

effect of PSA and AR manipulation on miR-379 in PCa cells. If PSA does drive osteoblastic proliferation, as suggested in previous reports [58, 120, 197, 198], it would be interesting to see if we downregulate or remove PSA, we could inhibit metastatic spread. We could also look at the effect of miR-379 on other androgen regulated genes such as KLK2 and TMPRSS2 to validate the effect we see on AR. The study by Salehi *et al*. found that miR-379 was influenced by androgen signalling and that upon AR overexpression, the exosomal release of miR-379 was increased [141]. This shedding of miR-379 from the cells resulted in a net reduction of the miRNA, which in turn drove cellular proliferation. It would be interesting to see if this exosome response is also seen in PCa cell lines, and this could be setup *in vitro* using a range of different concentrations of the synthetic androgen r1881 [199].

Discussion

In this study, we found that miR-379 expression can downregulate both PSA and AR. Furthermore, we found a negative correlation between miR-379 and PSA/AR in patient cohorts. PSA is well known in the field of prostate research, with most people aware of its diagnostic use in the clinical setting and that when PSA is expressed at normal levels, it is part of the healthy male orgasm as it is a component of the prostatic fluid. Less known, however, is that PSA overexpression may be contributing to the osteoblastic nature of PCa bone metastasis [58, 120, 197, 198]. Since we know that miR-379-downregulated 22Rv1 cells show metastatic spread to the bone, it would also be interesting to see if reducing PSA expression could rescue this spread. Some of the mediators that have been listed, such as PTHrP, TGF-β, and Runx2, could also be explored to test if they could also impact miR-379-dysregulated PCa cell lines. We did find that miR-379 did not directly bind to PSA but could decrease the activity of the PSA promoter.

We proposed that the mechanism of action to be mediated through AR. The publication by Salehi *et al*. found that androgens could stimulate the exosomal release of miR-379 in granulosa cells. It would therefore be interesting to see if AR signalling could induce the same effects in PCa cell lines.

Since the topic of this thesis looks at the therapeutic potential of miR-379, it would be interesting to see if miR-379 overexpression could inhibit the invasive properties of cells with aberrant PSA expression. The paper by

Cumming *et al*. found that pro-PSA, the precursor to PSA, increased bone tumour burden *in vivo* [200]. By coupling this experiment with miR-379 overexpression and comparing it to a scrambled control, we could test if miR-379 alone is enough to mitigate this effect.

There can be many different approaches to researching the impact of miR-379 expression on both PSA and AR signalling in different PCa model systems. Future studies should first validate that the effect of miR-379 on PSA is in fact mediated through AR and, secondly, if there are actionable targets from a therapeutic standpoint within this signalling axis.

Paper III: Functional consequences of A-to-I editing of miR-379 in prostate cancer cells

Background

Now that we have established that miR-379 plays a tumour suppressor role in PCa and have started to get a better understanding of the pathways and targets that are affected by its dysregulation, we still need to be reminded that all our experiments have been conducted using the unedited form of the miRNA. We know A-to-I editing can have a huge consequence on miRNA function, so it is important to question whether this process can have a biological impact. Research in our group previously identified that miR-379 was edited more frequently in patients with PCa compared to those with BPH and that only the unedited form of miR-379 had a significant impact on the development of metastasis, resistance to ADT, and overall survival in patients [186]. Since we cannot stably transduce the edited isoform, we transfected a total of four PCa cell lines, two AR-dependent and two AR-independent, with mimics for unedited and edited miR-379 to investigate the differences in functional experiments. The mimics we used to transfect cells were based on both the unedited form and the edited form with the inosine nucleotide and a negative control sequence, and the sequences of these can be found in Table 4. After successful transfection, several functional experiments were performed, and the effects of miR-379 were assessed. We also investigated the protein expression of epithelial and mesenchymal markers, and predicted targets and pathways were also explored using bioinformatic tools to also try and determine the effects of miR-379 editing.

Table 4.
Mimics used in Paper III. Sequences of the sense and antisense strands are included. The negative control sequence is based on *Caenorhabditis elegans*-miR-67.

Mimic	Sense strand	Antisense strand
Negative Control	5'.U.A.C.U.C.U.U.U.C.U.A.G.G.A.G.G.U.U.G.U.G.A.U.U.3'	5'.U.C.A.C.A.A.C.C.U.C.C.U.A.G.A.A.A.G.A.G.U.A.G.A.3'
Unedited miR-379	5'.U.A.C.G.U.U.C.C.A.U.A.G.U.C.U.A.C.C.A.U.U.3'	5'.U.G.G.U.A.G.A.C.U.A.U.G.G.A.A.C.G.U.A.G.G.3'
Edited miR-379	5'.U.A.C.G.U.U.C.C.A.U.A.G.U.C.C.A.C.C.A.U.U.3'	5'.U.G.G.U.I.G.A.C.U.A.U.G.G.A.A.C.G.U.A.G.G.3'

Summary

We found that the AR status of cell lines plays a large role in how the editing of miR-379 affects functional assays. PC3 and DU145 cells, which are AR-independent, had increased cell growth in the unedited miR-379, yet for the AR-dependent 22Rv1 and LNCaP cells, the unedited form inhibited cell growth. In Paper I, transduction of miR-379 (unedited) in PC3 cells decreased cell growth, yet transfection of unedited miR-379 in this paper seems to enhance cell growth. The effect of unedited miR-379 on 22Rv1 cells seems to support our group's previous experiments on the anti-379 driving increased cell growth seen by Ivkovic *et al*. [114], with both sets of experiments suggesting that unedited miR-379 can suppress growth in the 22Rv1 cell line. The results from the SRB assay support and contradict some of previous findings and suggest that it is only the unedited isoform of miR-379 that affects cell growth.

As discussed previously in the thesis, EMT is known to play a role in cancer progression. Epithelial and mesenchymal markers were investigated with the expression of CDH1, EpCAM, and Vimentin measured using Western blotting. One particularly interesting observation from these experiments was that we found miR-379 editing seemed to affect glycosylation, with edited miR-379 showing significantly higher levels of the 35 kDa isoform of EpCAM. The levels of the 40 kDa isoform were also significantly higher in unedited and edited miR-379 for PC3 cells compared to the negative control. We wanted to determine whether the changes to EpCAM had a functional consequence, and in both the unedited and edited isoforms of miR-379, we saw increased cell-cell adhesion. This is consistent with what was seen in terms of increased expression of the 40 kDa EpCAM isoform.

We also incorporated *in silico* analysis to determine the effect of miR-379 editing on predicted targets. In total, there were 560 targets predicted for unedited and edited miR-379, with 260 of these being specific to the unedited isoform, 296 to the edited isoform, and only four targets shared between the two miR-379 isoforms. By investigating the general functions of the targets, we could determine the most common pathways associated with the targets. From this, we found that the highest percentage pathway in the unedited was positive regulation of cell proliferation, consistent with our SRB assay. The edited miR-379 pathways were mainly focused on the regulation of transcription by the RNA pol II promoter.

Limitations of study

A limitation of this paper is that all the experiments were performed *in vitro,* so it may not be an ideal model for testing the effect of editing on miR-379. There is, however, a major hurdle when contemplating an *in vivo* approach for this project. Since we were using mimics, which can only be expressed in a transient manner, then a similar setup to Paper I where we intracardially inject cells into mice would not be feasible since the mimics would have degraded after a few days. It is also not possible to stably transduce the edited miR-379 isoform since inosine is not encoded in the genome. As mentioned previously in this thesis, since inosine can base pair with cytidine, it is comparable to guanosine but with less affinity for cytidine since there are only two hydrogen bonds between the nucleosides [98]. The use of guanosine instead of inosine to replicate edited miR-379 for *in vivo* experimentation has been described previously [201]. Given the differences between inosine and guanosine and the huge impact that subtle structural differences can have on miRNA binding, this is not a reliable way to investigate the impact of A-to-I editing *in vivo*. One potential method for exploring this would be through the injection of miR-379 mimics in mouse models, either directly to the tumour site subcutaneously or systemically. Systemic delivery would be a better model since the tumour microenvironment could be better recapitulated but does present more challenges. With the increasing knowledge and research into miRNA delivery vectors, this could be a potential method for delivering an edited miR-379 molecule to a PCa bone metastasis in the future to assess its effect. We know that miR-379 is influenced by signalling from the bone from Paper I, and so it may also be worth repeating functional experiments using OBCM. I think colony formation, which we know to be strongly expedited by OBCM, would be particularly interesting since it may provide more clinically relevant information about the effects of editing. This would also be a much easier way of trying to recapitulate the microenvironment, but it is still far from ideal.

Discussion

In this paper, we sought to uncover the function of miR-379 editing. We had previously assigned miR-379 to play a tumour suppressor role in Paper I and prior research from the group [114, 186]. There was no particularly strong phenotypic trend for edited miR-379 in our functional experiments; however, there did seem to be an AR-dependent effect on cell growth with unedited miR-379. This seems to contradict our previous finding that miR-379 reduced cell growth in PC3 cells and supports the study of Gururajan *et al*., who found

decreased cell growth when miR-379 levels were reduced in ARCaP cell lines [188]. The ARCaP cell line is interesting since it was the first human PCa cell line found whose growth is suppressed by androgen signalling [202]. Future studies should try to determine the relationship between miR-379 and AR and how this impacts PCa progression before there could be any feasible use for miR-379 therapeutically.

We may also be partially blinded by our interest in AR, since this is a major component of both Paper II and PCa research in general. When assessing differences between the groups of PCa cell lines we used, AR dependence is the obvious discriminating factor, but that does not necessarily mean that there may be some other element that is mediating the observed effect. Since PC3 and DU145 cells don't express AR [203], one way of testing this would be to overexpress this and look at the functional effects. If we find that the PC3 or DU145 cells that now express AR behave like the 22Rv1 and LNCaP cells, then we could conclude with more certainty that it is AR responsible for these phenotypic changes.

The publication by Xu *et al*. found that edited miR-379 inhibited cell proliferation through induced apoptosis in a range of cancer cell lines [201]. We did see this effect in our 22Rv1 cells, but the remaining three cell lines showed no significant difference between the edited miR-379 isoform and a negative control. Furthermore, the study did not use PCa cell lines to assess proliferation, and so any comparison on miR-379 editing between these publications is tenuous.

There was an interesting discovery that in PC3 cells, an isoform shift could be seen in EpCAM in the edited miR-379, suggesting that A-to-I editing may drive post-translational modification of proteins. We postulated that this editing effect on glycosylation could have an impact since the fully glycosylated 40 kDa isoform is more stable and has a longer half-life at the plasma membrane [204]. When comparing the expression of the 40 kDa isoform in PC3 cells and relative adhesion in PC3 cells across unedited miR-379, edited miR-379, and the negative control, the trend looks identical. This is interesting since we know EMT plays a role in metastasis, with a more mesenchymal phenotype typically associated with increased migratory potential [52]. If we had measured adhesion to the bone, as we did in Paper I,

it would have been fascinating to see if the opposite trend would also be seen with less adhesion to bone in the edited and unedited miR-379 isoforms compared to the control.

Conclusions and future perspectives

The aim of this PhD was to try and determine the therapeutic potential of miR-379, and upon reflection while writing this thesis, it was an ambitious goal. I would not dare say we have gotten any closer to seeing miR-379 in a clinical setting, but I am confident in saying we have in some way started to better understand the role of miR-379 in PCa. As with most scientific work, we tend to come up with more questions than when we started, and I think this thesis hopefully provides some framework for others to be able to answer them. The reciprocal signalling between the tumour and the microenvironment it metastasises to has always been a fascinating subject.

During this PhD, trying to uncover how miR-379 and the bone niche interact has been both challenging and incredibly exciting. We have identified GDF-15 and PSA, indirectly through AR, to be some of the potential drivers of metastatic progression; however, there are far more molecules and pathways involved that contribute. Future research in this field will inevitably help us better understand these complex networks, and as a result, this will translate into better outcomes for patients suffering from PCa.

The clinical impact miR-379 will have is yet to be determined, but its repeated association with tumour suppressive roles in a multitude of cancers should warrant further investigation. Despite the lack of miRNAs being utilised in a clinical setting to date, we know that the field of biomarkers and therapeutics is continuously evolving, and these small RNA molecules can provide a new avenue to help improve the current medical protocols. Future studies should try to better understand the pathways regulated by miR-379 and establish with more certainty the function of its unedited and edited isoforms using *in vivo* studies.

The use of better models to help recapitulate PCa bone metastasis will also play a pivotal role, with many newer technologies, such as humanised tissue-

engineered bone constructs, being developed. Overall, our work has shown that miR-379 can inhibit metastatic spread to the bone, demonstrated that miR-379 targets can play a role in metastatic establishment, and demonstrated that A-to-I editing can play a dramatic role in miRNA target selection. Working in this field has been a privilege, and I look forward to following and hopefully contributing to its progression.

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Paper I

Paper II

Paper III