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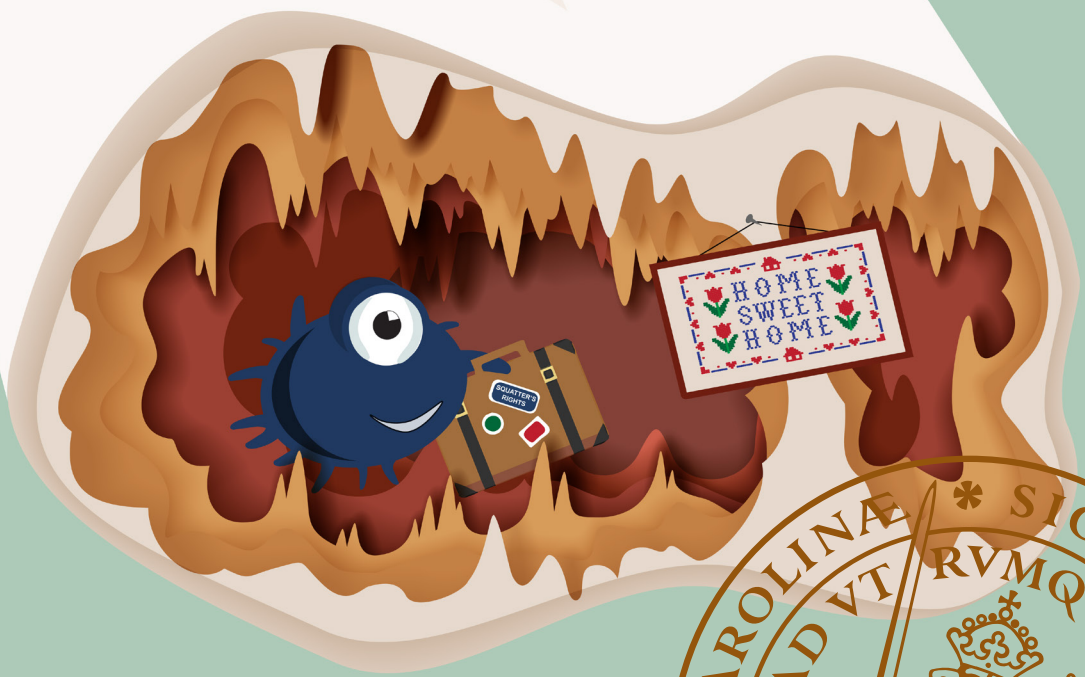

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Gjendine Voss

LUND UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

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In Paper I, we showed that miR-96 can upregulate the mRNA and protein expression of adhesion proteins E-Cadherin and EpCAM through direct interaction with target sites in the mRNA coding sequence. We also showed that miR-96-transfected cells had increased cell-cell adhesion to both each other and osteoblasts, and an increased colony formation potential.

In Paper II, we performed an *in vivo* anti-miRNA library screen, and identified miR-379 as a suppressor of prostate cancer bone metastasis. Downregulation of miR-379 enhanced incidence of bone metastasis in mice, increased colony formation potential in osteoblast-conditioned media, and increased cell growth. In bone metastasis samples from prostate cancer patients, miR-379 was frequently downregulated.

In Paper III, we developed a two-tailed RT-qPCR method for the sensitive and specific quantification of A-to-I-edited miRNAs. Using this method, we could reveal that unedited, but not edited, miR-379 was frequently downregulated in prostate cancer patients with metastasis, treatment resistance, and shorter overall survival. The editing frequency of miR-379 was higher in prostate cancer tissues compared to benign tissues.

In Paper IV, we compared the biological functions of unedited and edited miR 379 in prostate cancer cells. We found that unedited miR-379 increased cell growth in androgen-independent cell lines, but inhibited cell growth in androgen-sensitive cell lines. Both unedited and edited miR-379 also slightly enhanced colony formation and cell migration in all tested cell lines.

Overall, this thesis reports multiple findings and tools that can help us better understand the process of prostate cancer bone metastasis and the role that miRNAs play in this process.

Key words: prostate cancer, bone metastasis, microRNA, RNA editing, A-to-I editing, ADAR, miR-96, miR-379

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“Da steh ich nun, ich armer Tor!
Und bin so klug als wie zuvor.”

“And here, poor fool! with all my lore
I stand, no wiser than before.”

– Faust

in “Faust I” by Johann Wolfgang von Goethe

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Carcinogenesis (2020), 41(7):865-874.

Paper II

Functional *in vivo* screening identifies microRNAs regulating metastatic dissemination of prostate cancer cells to bone marrow
Tina Catela Ivkovic*, Helena Cornella*, Gjendine Voss*, Anson Ku, Margareta Persson, Robert Rigo, Sofia Gruvberger Saal, Lao H Saal, Yvonne Ceder
*equal contribution
Manuscript.

Paper III

Quantification of microRNA editing using two-tailed RT-qPCR for improved biomarker discovery
Gjendine Voss, Anders Edsjö, Anders Bjartell, Yvonne Ceder
RNA (2021), 27(11):1412-1424.

Paper IV

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

Abbreviations

ADAM	a disintegrin and metalloproteinase
ADAR	adenosine deaminase acting on RNA
ADT	androgen deprivation therapy
AGO	Argonaute
AGS	Aicardi-Goutières syndrome
AR	androgen receptor
ARE	androgen-responsive element
BCR	biochemical recurrence
BPH	benign prostatic hyperplasia
CAF	cancer-associated fibroblast
CDH1	E-Cadherin
CDS	coding sequence
ceRNA	competing endogenous RNA
CTC	circulating tumour cell
ctDNA	circulating tumour DNA
DGCR8	DiGeorge syndrome critical region gene 8
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ETS	E twenty-six
EV	extracellular vesicle
FDA	United States Food and Drug Administration

FFPE	formalin-fixed paraffin-embedded
FXR1	fragile-X-mental-retardation-related protein 1
HCV	hepatitis C virus
IP	immunoprecipitation
ISG	interferon-stimulated gene
LHRH	luteinising hormone release hormone
MET	mesenchymal-epithelial transition
miRNA	microRNA
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
PARP	poly(ADP-ribose) polymerase
PIN	prostatic intraepithelial neoplasm
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
PTHRP	parathyroid-hormone-related protein
qPCR	quantitative PCR
RANKL	receptor activator of nuclear factor kappa-B ligand
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RT	reverse transcription
shRNA	short hairpin RNA
TCGA	The Cancer Genome Atlas
TGF-β	transforming growth factor β
TMPRSS2	transmembrane serine protease 2

TRBP	TAR RNA-binding protein
UTR	untranslated region
VEGF	vascular epithelial growth factor
VIM	vimentin
ZEB1	zinc finger E-box binding homeobox 1

Abstract

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Introduction

What makes a cell function? Which pathways and players have to come together to ensure a cell's survival and continued adaptation to the challenges it faces throughout its lifetime? What role does an individual cell play in the context of a multicellular organism?

These are questions that we are only beginning to answer even after decades of research. One way in which we can learn more about cell biology is through the study of cells that fail to fulfil their role – cancer cells.

Cancer

Cells can become malignant in a variety of ways through the disruption of different growth control and differentiation programs, and throughout tumour evolution, they will continue to acquire more changes as they adapt to their changing environment. In fact, cancer cells are particularly good at surviving and adapting to changes, partly because of the loss of control mechanisms that are in place to maintain a cell's differentiation state and prevent mutations. As the cells lose these control mechanisms, they acquire changes rapidly – which is not always in the best interest of the organism. This ability to adapt is the reason that cancer therapies fail, and that cancers often come back stronger and harder to treat when patients relapse. It also helps the cells to metastasise and thrive in different organs, ultimately taking over the entire body of the patient.

In order to navigate the mechanisms that cancer cells use to adapt to their changing environments, and identify viable strategies to put a cure for cancer within reach, there have been attempts to categorise these mechanisms into broader terms. In the following sections, I will briefly summarise some of the key concepts that I will continue to refer to throughout the thesis.

The hallmarks of cancer

In an effort to understand the complexity of cancer biology, and break it down into simpler concepts, Hanahan and Weinberg published their much-cited review "The Hallmarks of Cancer" in the year 2000 [1]. The original six hallmarks include sustaining proliferative signalling, evading growth suppressors, enabling replicative

immortality, resisting cell death, activating invasion and metastasis, and inducing angiogenesis. While the first four hallmarks refer to different aspects of enabling uncontrolled tumour growth and inhibiting endogenous control mechanisms and checkpoints, the last two points allude to cell functions that are not limited to the tumour cells themselves, but rely on their interaction with the surrounding tissue.

It is therefore not surprising that in the update published a decade later [2], the authors added an entire section discussing the tumour microenvironment and the communication with other cell types therein. In this update, they also decided to include four more recent research directions, which they termed "enabling characteristics and emerging hallmarks": tumour-promoting inflammation, genome instability and mutation, deregulating cellular energetics, and avoiding immune destruction. This last hallmark has been especially highlighted recently, as the field of immunotherapy is exploding and has been brought to attention with the Nobel Prize in Physiology or Medicine to James Allison and Tasuku Honjo in 2018 [3].

Tumour heterogeneity, precision medicine & tumour evolution

One reason that "The Hallmarks of Cancer" has been so popular is that it was an effort to unify all tumour diseases and to describe them in very general terms, whereas in the day-to-day of cancer research and care, it is easy to get lost in all the details that differ between tumours.

Tumours are characterised by a remarkable heterogeneity. On one hand, there is a substantial amount of inter-tumour (or inter-patient) heterogeneity, which means that no two cancers are the same, and that among different patients' cancers with the same organ of origin, the tumours and prognoses can differ vastly. Tissue architecture and differentiation [4-6] or staining of protein markers such as the proliferation marker Ki-67 [7-10] are traditionally used both as a prognostic tool and to guide treatment decisions in the clinic.

With the advent of affordable microarray and high-throughput sequencing technologies, there have also been increasing efforts to define subtypes based on mutational signatures or gene expression profiles [11-14]. This development has taken place at the same time as targeted treatments have been established in the clinic, which can directly target the tumour cells that carry a certain mutation or overexpress a certain protein. Examples of this include the use of imatinib to target the Bcr-Abl gene fusion in chronic myeloid leukemia [15], trastuzumab targeting human epidermal growth factor receptor 2 (HER2) in HER2-positive breast cancer [16, 17], and vemurafenib targeting melanoma cells harbouring the BRAF(V600E) mutation [18].

The approaches complement one another – identifying commonly mutated cancer drivers has helped guide which targets could be promising candidates for therapy development, and the usefulness of targeted therapies depends on being able to identify exactly which patients carry the targeted alteration and would likely benefit from treatment. As both of these interconnected fields advance and become more

accurate, we are arriving in the so-called era of precision medicine, sometimes also referred to as personalised medicine, where the molecular profile of a tumour can help in deciding on the optimal treatment [19]. The goal here is not to find that one cure of cancer, but to do enough research into different cancer drivers and therapeutic targets so that there are enough different treatment options for each patient to be given a suitable treatment for their specific type of cancer.

Intertumoural heterogeneity should also be considered with regards to multifocal disease. Multifocal disease refers to the presence of multiple primary tumours in the same tissue. Two mechanisms can lead to multifocal disease: The tumours can be monoclonal, in that they are all derived from the same transformation event, and are essentially local metastases of one another. This is the predominant mechanism for cancers of the ovaries, endometrium, kidneys, and bladder [20-22]. Alternatively, the tumours can be polyclonal and derive from separate transformation events, and thereby differ as much from each other as they would in different organs or different patients, both in their genetic alterations and in their progression rates. This is frequently the case in prostate cancer [23-27], or in cancers resulting from genetic pre-disposition such as familial adenomatous polyposis [28]. The formation of multiple tumour lesions has also been suggested to result from pre-conditioning of a larger tissue area, referred to as field cancerisation [29]. This theory was first suggested in oral cancers, and has been used to explain common recurrences after initial excision of the primary tumour as the development of a new tumour from the same pre-exposed tissue [30].

To add another layer, there is also a remarkable amount of intra-tumour heterogeneity [26, 31]. This is brought on by genome instability, named an enabling characteristic by Hanahan and Weinberg [2]. Genetic instability can be brought on by multiple factors, such as exposure to carcinogens, deficiency in DNA repair mechanisms, and cancer treatment such as chemotherapy or radiation [32]. Furthermore, due to sustained proliferative signalling, DNA quality checkpoints and cell death signals are suppressed. Therefore, cells will continue to divide despite the accumulation of mutations that would normally halt further proliferation.

The acquisition of mutations can be beneficial for the cells, as it enables the tumour to adapt to its changing environment. As the tumour grows and each cell acquires different mutations, the cells best adapted will be more successful at expanding, and make up a larger part of the tumour [31, 32]. This tumour evolution is very dynamic and has garnered much interest in the research community. In a number of exciting studies, it has been possible to follow the expansion and extinction of different subclones over time and throughout disease progression [33-36]. The co-existence of hundreds of low-frequency subclones is a plausible mechanism for the development of treatment resistance, as it is enough for a few individual treatment-resistant cells to survive, which can then facilitate outgrowth of a resistant tumour [31, 32].

Both intratumoural heterogeneity and multifocal disease can also cause difficulties in accurately assessing a tumour in the clinic due to sampling bias: A

single biopsy may not necessarily be representative of the whole tumour, and a more aggressive clone may be present elsewhere in the tumour, or in separate foci [26, 32].

The tumour microenvironment

As briefly mentioned above, the historical view of a homogeneous tumour mass is further challenged by the recognition of multiple different cell types in the tumour microenvironment. The communication with these surrounding cells is crucial for tumour growth, and there is increasing evidence that tumour cells actively recruit and manipulate these cells to perform tumour-supportive functions (Figure 1a).

The tumour microenvironment is not only made up of cells, but also of extracellular matrix (ECM). The tumour ECM has been shown to differ in stiffness and composition from other tissues, and it is crucial for multiple steps of tumour development. For example, the ECM can sequester or store growth factors [37], regulate metastasis by supporting or preventing cell invasion, or prevent diffusion of tumour-targeting drugs [38]. The ECM consists of a variety of extracellular proteins such as collagens, proteoglycans and glycoproteins, which are produced and deposited by fibroblasts [39, 40]. Through the secretion of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase (ADAM) family members by tumour and stromal cells, the sequestered growth factors can be released and enhance tumour growth [38, 41].

Cancer-associated fibroblasts (CAFs) are important not only for the production of the ECM, but they can also produce signalling molecules that promote tumour growth and plasticity [39]. The precise origin of CAFs is still debated, but it seems that they are not only derived from converted tissue-resident fibroblasts, but that other cell types might also be converted to CAFs [39]. Single-cell sequencing studies found that there are different CAF subtypes that perform different functions in the tumour, and potentially have different origins [42].

As tumours grow and need to maintain access to nutrients and oxygen, they actively initiate angiogenesis to facilitate the sprouting of new blood vessels [43]. When the oxygen tension drops in the growing tumour, hypoxia-inducible factors transcriptionally activate the production of vascular endothelial growth factor (VEGF) [44]. VEGF is secreted into the tumour microenvironment and attracts endothelial cells to form new blood vessels [43, 45]. Tumour angiogenesis is poorly controlled and often results in faulty vasculature such as collapsed or leaky vessels. This is in part due to the fact that the continued production of VEGF in the tumour prevents the maturation of the blood vessels [45], and because different components of the ECM can affect blood vessel formation [40]. In addition to endothelial cells, there are additional cells that are essential for the integrity of the blood vessels. Pericytes are a still somewhat elusive cell type that is thought to support vessel stability and prevent leakiness [46]. In tumours, the frequency of pericytes is

reduced, and they are more loosely associated with blood vessels. This likely contributes to the leakiness of the tumour vasculature [46].

Lastly, the tumour microenvironment is rich in immune cells. These have a dual role: Inflammation is generally conducive of tumour growth, through the production of cytokines which can drive tumour proliferation and ECM remodelling [47, 48]. On the other hand, due to their high mutational burden, cancer cells produce a large number of neoantigens, which would lead to the destruction of the tumours by the adaptive immune system [49]. The tumour must therefore actively reprogram immune cells in its microenvironment, by supporting the growth of pro-inflammatory cells such as certain macrophage subtypes, and preventing recognition by the adaptive immune system [49].

In addition, depending on the tissue, there may be additional cell types present. This is especially relevant when it comes to metastasis. It is known that certain cancer types often metastasise to the same site. This organ tropism can be due to tissue-specific cell types producing growth factors or signalling molecules that promote the outgrowth of seeded cells. An example of this will be discussed in a later chapter focusing on the cell types and interactions involved in prostate cancer bone metastasis.

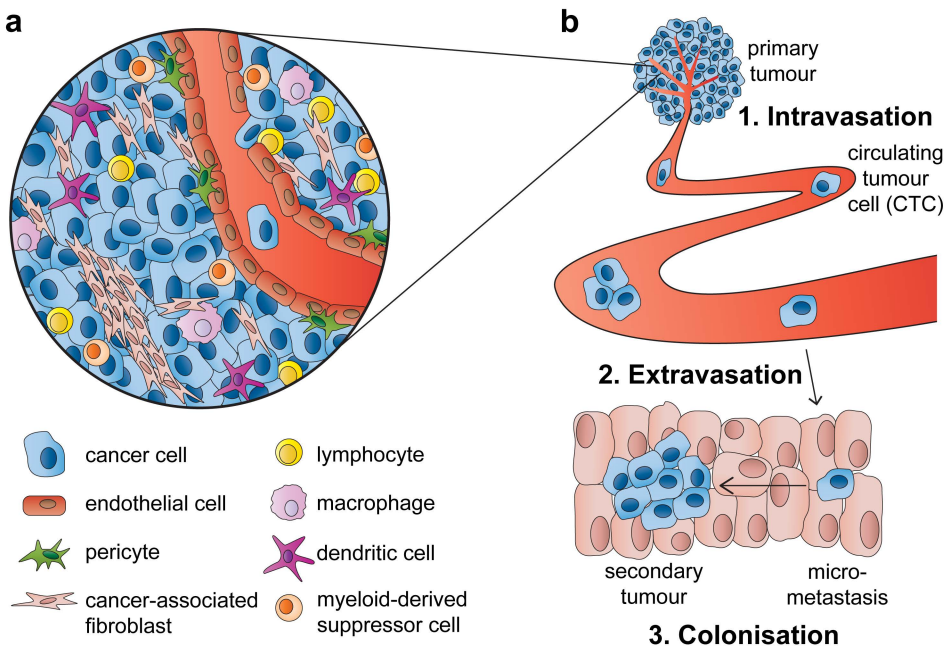

Figure 1.
a. The tumour microenvironment and its different cell types. **b.** The metastatic cascade. One or several tumour cell(s) leave the primary tumour, are transported through vessels, and enter a foreign tissue. In the case of successful colonisation, a secondary tumour can grow.

There are also reports of primary tumour cells "educating" the pre-metastatic niche through systemic signalling, for example through the secretion of extracellular vesicles (EVs) [50]. These EVs can contain information that allows recipient cells to remodel their tissue microenvironment to be more tumour-promoting [51], or, vice versa, EVs from the microenvironment can support the cancer cells [52]. It has been shown that based on the integrins they express, some tumour-derived EVs are taken up only by specific cell types [53]. The organ tropism of a cancer cell line could be changed by education of a different (pre-)metastatic niche with EVs from another cell line with a different preferred site of metastasis [53]. This suggests that what determines whether a specific cell type or tissue will support secondary tumour outgrowth depends not only on what signals it can send, but also on what messages it can receive.

Metastasis

The overall process of metastasis can be summarised in a few key steps [54], shown in Figure 1b:

1) Cancer cells leave the primary tumour mass and enter either lymphatic or blood vessels, a process termed intravasation.
2) The circulating tumour cells (CTCs) are transported through circulation and eventually enter tissues at a more distant site – extravasation.
3) Colonisation: At the metastatic site, the cancer cells divide and remodel their microenvironment to form macroscopic secondary tumours.

In order for the cancer cells to be able to leave the primary tumour, they need to be able to detach from their surrounding cells. This involves gaining migratory abilities to acquire cell motility as well as the ability to invade tissues and clear a way through the ECM. The secretion of proteases such as MMPs and ADAMs by both tumour and other cells in the stroma can actively degrade and remodel the ECM to clear the way for invasion and migration [38, 41]

Once the tumour cell is in circulation, many factors influence where in the body it will extravasate and enter a tissue. Firstly, there is a purely mechanical factor, in which the circulating tumour cells (CTCs) are more likely to "get stuck" in small capillary beds or organs through which the blood stream will almost certainly lead them, such as the liver [54]. Secondly, attaching to and crossing the vessel wall and migrating into the tissue may depend on adhesion molecules or chemoattractants that are tissue-specific. For example, CXCR4-expressing prostate cancer cells are attracted to the bone due to the expression of stromal-derived factor 1 (SDF-1) in the bone microenvironment [55].

Just because a cell has entered a distant tissue, it does not necessarily mean that it will also divide and form a secondary tumour. Many cells remain dormant for years before outgrowth. Triggers for outgrowth include microenvironmental

signals, or systemic changes that support of tumour growth, such as stress, hormonal changes, or ongoing inflammation [56]. Some models also assume that the loss of inhibitory signals from the primary tumour following treatment for primary disease can trigger metastasis [57-59].

Epithelial-mesenchymal transition

In most solid tumours, the malignant cells are epithelial, which means that they are quite immobile and maintain close cell-to-cell contacts with their neighbouring cells [60]. These are mediated through homotypic adhesion molecules like E-Cadherin, epithelial cell adhesion molecule (EpCAM), claudins, and occludins [61, 62].

While cell-cell adhesion is essential for epithelial tissue integrity, it would hinder migration and invasion. It is widely believed that in order to gain migratory features, the cancer cells undergo epithelial-mesenchymal transition (EMT). This mechanism was first described in the context of developmental biology, and commonly occurs during gastrulation [60]. EMT is primarily a transcriptional program, in which transcription factors like SNAIL, SLUG, TWIST and zinc finger E-box binding homeobox (ZEB) suppress the expression of epithelial adhesion molecules [60, 63, 64]. At the same time, the expression of proteins like the adhesion protein N-Cadherin, the microtubule regulator Vimentin (VIM), and MMPs is upregulated [60]. This makes mesenchymal cells more migratory, and more invasive. Furthermore, these cells have been described to be more resistant to treatment, and have certain stem-like features [65].

However, when it comes to the colonisation of the secondary site, mesenchymal cells are expected to be relatively unsuccessful at establishing a solid tumour mass [66]. Instead, it has been proposed that the cells undergo mesenchymal-epithelial transition (MET) to revert back to their original epithelial phenotype [67]. The benefit of cell-cell contacts and epithelial phenotypes for the formation of metastatic tumours has been demonstrated in multiple studies [66-69].

Alternative models

On the other hand, multiple alternative models have been proposed. For example, EMT may be incomplete, resulting in a hybrid expression pattern with both epithelial and mesenchymal markers [70-73]. These cells may be merely transitory and will switch to either an epithelial or a mesenchymal phenotype, or they may permanently coexist on a spectrum [71, 73].

Some models suggest that, rather than migrating as single cells, epithelial and mesenchymal cells migrate together in what is called collective invasion. In this model, the mesenchymal-like cells can clear the way and lead invasion, bringing with them a group of cells with more epithelial features [74-77].

There is also evidence that mesenchymal cells are not the only cells that can migrate, but that there are also other modes of migration, such as amoeboid cell migration [78].

Prostate cancer

Over one million men are diagnosed world-wide with prostate cancer every year, and over 300,000 die of the disease annually [79]. In Europe, the prostate is the most prevalent primary site for cancer in men [79, 80]. The incidence has risen over the past decades, which could be due to the fact that the life expectancy is generally increasing, and prostate cancer is an age-associated disease, so that its incidence would increase with an ageing population [81].

Another factor leading to rising numbers is an increase in diagnoses since the implementation of prostate-specific antigen (PSA) for early detection of prostate cancer [82-85]. In fact, autopsy studies have shown that many men above a certain age have neoplasms in their prostate, but that these tumours were not clinically relevant during the patient's lifetime [86, 87]. The use of PSA has therefore caused more of these clinically insignificant prostate tumours to be diagnosed, and likely resulted in overtreatment of patients that might have never shown symptoms [82-84, 88]. Furthermore, a rise in serum PSA can also be the result of non-malignant conditions of the prostate such as benign prostatic hyperplasia (BPH) or prostatitis [82, 85, 89]. Although there is an association between the incidence of BPH and prostate cancer, this link may not be causal, and BPH is not generally believed to be a pre-malignant stage of prostate cancer [90, 91]. Inflammatory conditions like prostatitis on the other hand can create an inflammatory environment that will drive both hyperplasia and tumorigenesis [29, 91].

Despite the likely overtreatment of many patients with indolent prostatic neoplasms, some patients do have aggressive prostate cancer that will require immediate medical attention and treatment. Aggressive prostate cancers will metastasise to other organs, most frequently nearby lymph nodes or the bone [92]. These bone metastases cause the patients severe pain, hypercalcaemia, anaemia, bone fractures, and spinal cord compressions leading to paralysis. In addition, visceral metastases in the brain, liver and lungs can impact organ function. It is therefore metastasis that leads to the patient's death, rather than the primary prostate tumour itself. As will be discussed below, advanced prostate cancer is also more difficult to treat, and more likely to develop treatment resistance.

The prostate is a glandular organ that produces fluids with components needed for sperm function. Both the urethra and the seminal vesicles pass through the human prostate, which is a spherical organ that is roughly divided into three zones (Figure 2a) [92, 93]. The central zone is the zone surrounding the ejaculatory duct, while the transitional zone lies in the anterior prostate directly below the bladder, close to the proximal transitional urethra. BPH occurs almost exclusively in the transitional zone; in contrast, most prostate adenocarcinomas arise from the peripheral zone [92], which surrounds the distal part of the urethra. In addition to this, the periurethral gland region directly surrounding the proximal urethra and the fibromuscular region adjacent to the transitional zone in the anterior part of the prostate are often distinguished.

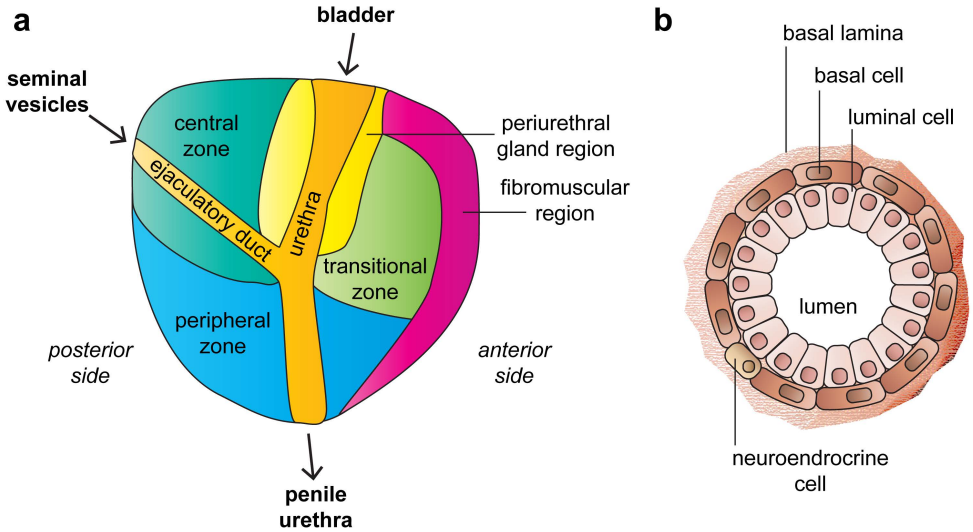

Figure 2.
The prostate. (**a**) Schematic anatomy of the prostate with three zones. BPH mostly arises from the transitional zone; most prostate tumours arise from the peripheral zone. (**b**) Schematic histology of the prostate. Luminal epithelial cells surround the lumen, surrounded by basal epithelial cells and a basal lamina.

Histologically, the prostate is organised into ducts and acini, consisting of luminal and basal epithelial cells, as well as a small population of neuroendocrine cells. (Figure 2b) [92]. Prostate adenocarcinomas typically arise from luminal or basal cells [94], but neuroendocrine prostate cancer has also been described. Neuroendocrine prostate cancer is however mostly formed through transdifferentiation at later stages of prostate cancer, rather than from transformed neuroendocrine cells [95]. I will not discuss prostate cancer with neuroendocrine differentiation in more detail in this thesis.

Prostate cancer biology

Genetic alterations in prostate cancer

DNA sequencing efforts to characterise the somatic mutations driving prostate cancer have revealed that, rather than point mutations, the most common driver mutations in prostate cancer are copy number alterations and gene fusions. The 5′ partner of these gene fusions is typically part of an androgen-responsive gene or its promoter, frequently transmembrane serine protease 2 (*TMPRSS2*), and the 3′ partner is a growth-promoting gene, often oncogenes from the E twenty-six (*ETS*) family of transcription factors [96]. In a TCGA large-scale analysis of primary prostate cancer, seven subtypes were defined based on their genetic alterations; more than half of all samples had fusion with either *ERG* or another *ETS* family gene [97]. Mutually exclusive with *ETS* fusions, 10% of tumours had *SPOP*

mutations. *SPOP* encodes an E3 ubiquitin ligase that functions as a tumour suppressor in prostate cancer by inducing the degradation of multiple growth-promoting proteins [98]. The remaining 30% of tumours had *FOXA1* or *IDH1* mutations, or none of the genetic alterations that would match any of the seven subtypes based on the TCGA study [97]. Other genes that are commonly mutated in primary prostate tumours throughout all subgroups include tumour protein 53 (*TP53*), phosphatase and tensin homolog (*PTEN*), and DNA-repair genes such as breast cancer 2 (*BRCA2*) and *ATM* [92, 97].

In advanced prostate cancer, more mutations occur that can further drive progression. The frequency of *PTEN*, *TP53* and retinoblastoma-1 (*RB1*) mutations increases drastically in advanced compared to localised prostate cancer, as well as *MYC* amplification [92, 97, 99]. DNA repair pathway mutations are also present in around 20% of metastatic cancers [99]. There is also a large number of different androgen receptor (AR) mutations in aggressive prostate cancer [99]. As will be discussed below, these mutations are mostly adaptations to the androgen pathway-targeting drugs that are frequently used in the clinic.

Prostate cancer development

Prostate cancer arises from precursors known as prostatic intraepithelial neoplasia (PIN) [92]. Typically, patients with a high risk of prostate cancer will have multiple high-grade PINs in their prostate, some of which eventually develop into a tumour. Therefore, prostate cancer is often multifocal. The independent origin of these foci has been confirmed in genomic studies [100, 101]. It has even been suggested that supposedly unifocal prostate cancer is merely the result of multiple foci "merging" into one heterogeneous tumour [24]. This is similar to hypotheses in bladder cancer suggesting that initially, there is oligoclonal development of multiple foci, before eventually one clone takes over, resulting in supposed monoclonality of late-stage lesions [102]. Due to this multifocality, one biopsy may not be representative, making it more difficult to make predictions based on biopsies alone. The phenomenon that Gleason scores are often upgraded after prostatectomy compared to the biopsy result [103-105] is supportive of the fact that biopsies cannot always identify the most aggressive lesions. Especially for low grades, the high proportion of upgraded tumours is likely due to sampling error [105].

On the other hand, for treatment decisions in more advanced disease, some studies have suggested that the major genetic drivers of metastatic prostate cancer can be found by assessing a single metastasis [23, 106] or liquid biopsy [34]. The alterations in this sample would represent the drivers of metastasis and tumour formation, and would thus be the ones that should guide a treatment decision [34].

Androgen signalling

Prostate epithelial cells naturally express AR and are responsive to androgens [92]. More so, these cells usually depend on androgens to sustain their growth signalling. While the entire set of AR-regulated genes contains both growth-promoting and

growth-suppressing genes, it has been suggested that with development from a slow-growing indolent prostate tumour to aggressive prostate cancer, the growth-suppressive portion of target genes is selectively downregulated [107]. Furthermore, as discussed above, activation of androgen-responsive promoters that are fused to growth-promoting genes can cause the cancer cells to grow in response to androgen signalling.

The general mechanism of androgen signalling is displayed in Figure 3a. Androgens are lipophilic steroid hormones that can pass through the plasma membrane. In the cytosol, they can bind to their receptor. As a result of androgen binding, AR will be translocated into the nucleus and form homodimers, which can then recognise and enhance transcription from androgen-responsive elements (ARE) in promoter sequences [108].

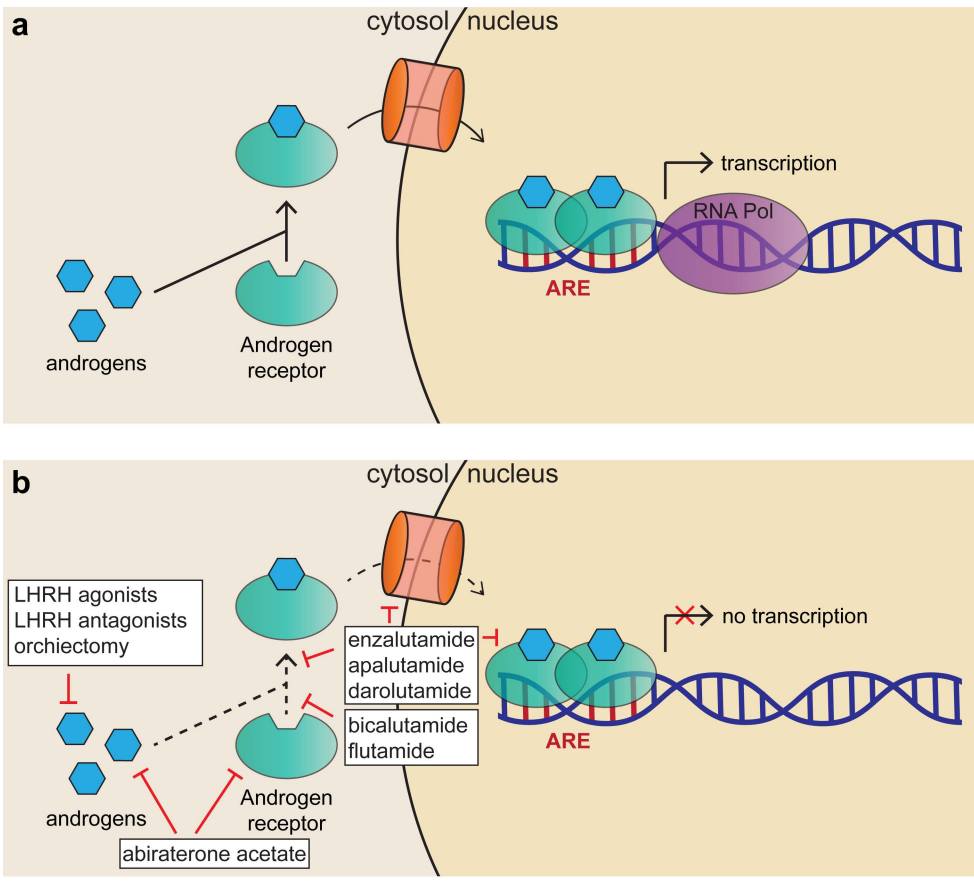

Figure 3.
Androgen signalling. (**a**) The androgen receptor (AR) pathway. Upon ligand binding, AR translocates to the nucleus, and homodimers bind to androgen-responsive elements (ARE) to induce transcription of target genes. (**b**) Hormone therapy in prostate cancer. Androgen deprivation therapy lowers systemic levels of available androgens, and anti-androgens prevent AR ligand binding and AR function.

Genes that are regulated by androgen signalling include those coding for proteases such as KLK2 (kallikrein-related peptidase 2; also known as human kallikrein-2, hK2), KLK3 (kallikrein-related peptidase 3; also known as PSA) and TMPRSS2. Proteases are essential for normal prostate function and thought to play a role in liquefying semen for optimal sperm function [109]. The loss of normal tissue architecture upon tumorigenesis triggers the release of PSA and hK2 into the bloodstream from the otherwise confining prostate, providing us with an easily accessible proxy for AR activity throughout disease development [82].

A cornerstone of prostate cancer therapy (which will be discussed in more detail below) is the use of drugs that disrupt androgen signalling in prostate cancer cells. The discovery of androgen deprivation therapy (ADT) by Charles Brenton Huggins was awarded with a Nobel prize in 1966 [110]. By decreasing androgen levels, the prostate cancer cells are deprived of most of their growth signals. Castration is achieved either by surgical orchiectomy, or, now more commonly, by using luteinising hormone releasing hormone (LHRH) agonists or antagonists that prevent testicular androgen production [111, 112].

Unfortunately, most patients develop resistance to androgen deprivation. This does not mean that the androgen pathway is no longer active; in fact, recurrence and resistance is often accompanied by a renewed rise in serum PSA, indicating that the androgen pathway is still active in the cancer cells [92]. Resistance to ADT can be due to residual androgens that result from the production of androgens by cytochrome P450 17A1 (CYP17A1) in the adrenal glands. AR signalling can also be maintained by AR amplification [113] or mutations in the ligand-binding domain that will allow AR to become activated by other steroid hormones as well [114, 115]. Alternative splicing of AR can produce splice variants such as AR-V7, which is constitutively active without ligand binding [116, 117].

Residual androgen production can be blocked by inhibition of CYP17A1 with abiraterone acetate. In addition to inhibiting CYP17A1, abiraterone acetate may also inhibit AR ligand binding and function [118]. Abiraterone acetate administered together with prednisone has been shown to prolong survival in patients with castration-resistant prostate cancer in clinical trials [119]. Another strategy that has been beneficial in clinical trials is combining ADT with an anti-androgen [88]. These AR antagonists include first generation agents bicalutamide and flutamide, and second generation agents enzalutamide, apalutamide and darolutamide [112]. Enzalutamide, apalutamide and darolutamide also have an additional function to prevent nuclear translocation and DNA binding of AR, inhibiting its function on multiple levels [120-122]. A summary of different ADT strategies and AR antagonists is depicted in Figure 3b.

However, eventually, resistance to anti-androgens develops as well. In addition to the mechanisms above, AR mutations in the ligand-binding domain can render AR responsive to the anti-androgens itself: Certain mutations allow AR to become activated by the anti-androgens that are meant to competitively inhibit it [115, 123, 124]. At the point of resistance to anti-androgens, the prostate cancer is very difficult

to halt further, and metastasis develops rapidly. Palliative treatment options that can be initiated at this stage are discussed below.

Bone metastasis

The most common distant site of metastasis in prostate cancer is the bone. Homing of prostate cancer cells to the bone depends on their interaction with cells of the bone niche. Multiple factors produced by the cancer cells induce osteoblast proliferation and differentiation, such as Wnt ligands, bone morphogenetic proteins (BMPs), VEGF, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), endothelin 1, and transforming growth factor beta (TGF-β) [125, 126]. In turn, the induced osteoblasts produce growth factors such as insulin-like growth factor (IGF-1) and interleukin-6 and -8 (IL-6/-8) [126, 127]. The activation of osteoblasts also leads to the production of receptor activator of nuclear factor kappa-B ligand (RANKL), which stimulates osteoclasts and enhances to osteoclastogenesis [126-128]. The resulting increase in bone resorption in turn leads to the release of growth factors that are embedded in the bone matrix such as TGF-β and IGF-1, stimulating tumour cell growth [126-128]. Osteoblasts are also able to limit bone resorption by production of osteoprotegerin, which binds and inhibits RANKL [125, 128].

Depending on whether the osteoblasts or the osteoclasts become predominant, an osteoblastic or an osteolytic lesion will form, or a mixed lesion with features of both. In osteoblastic lesions, the formation of bone is induced, but because it does not mature correctly and is deposited in an unorganised manner, the newly formed bone is prone to fractures [126]. In osteolytic lesions, the balance is shifted towards bone resorption, making the bone more fragile, and again more likely to fracture [126]. Most prostate cancer bone metastases are osteoblastic [125, 126]. One mechanism that enables this is the cleavage of parathyroid-hormone-related protein (PTHRP) by PSA secreted from prostate cancer cells. PTHRP normally enhances bone resorption; its degradation therefore skews the lesion towards an osteoblastic phenotype [125, 129-131].

The outgrowth of macrometastases seems to be most successful in areas of high bone turnover [126]. Bone remodelling is induced upon hormonal changes [126, 132], which offers an explanation as to why hormone-driven cancers like breast and prostate cancer metastasise to the bone. It also raises concerns about the use of ADT and anti-androgens in prostate cancer treatment – adverse effects on bone density may make the bone microenvironment more conducive to prostate cancer metastasis outgrowth.

Clinical management

Diagnosis and prognosis

Multiple different events can cause a physician to suspect prostate cancer in a patient. These could be the development of symptoms, an abnormal digital rectal examination, a high PSA value during screening, or the suspicion of a lesion based on imaging. Typically, the first step is to perform a digital rectal examination and a transrectal ultrasound, and to take biopsies. Here, one needs to differentiate between two kinds of biopsies: For systematic biopsies, 10–12 cores are taken bilaterally across the entire peripheral zone [133]. Alternatively, if the position of the suspected lesion is already known, it can be targeted directly. Taking a prostate biopsy is not without risk, as biopsies are frequently performed transrectally, so that contaminating faecal bacteria may cause inflammation and even sepsis [134]. Hence, there are suggestions to perform magnetic resonance imaging (MRI) first in order to avoid unnecessary biopsies [88, 135]. For example, a recent Swedish trial concluded that MRI-guided biopsy could identify clinically significant prostate cancers equally well as standard systematic biopsies, while diagnosing fewer clinically insignificant cancers and performing fewer biopsies overall [135]. Based on this and other recent studies, MRI prior to first-time biopsy is now generally recommended in patients with a low or moderately elevated PSA value in blood [133], whereas the question of performing systemic or targeted biopsies is still under debate.

The biopsy cores are then graded by a pathologist to determine the presence of tumour tissue or PINs. If a tumour is present, it is traditionally graded using the Gleason system [5]. In this system, the degree of differentiation is graded on a scale from 1 to 5, with 5 being the most de-differentiated. What sets the Gleason system apart from most other systems is that both the predominant pattern and the secondary pattern are reported. This means that if large parts of the tissue section are showing a moderate amount of dysplasia, but there is one area with highly dysplastic cells, this will be immediately evident from the Gleason score reported by the pathologist. Gleason scores were recently grouped together into five grade groups in the ISUP system [136].

Upon tumour grading, a decision is made whether and how the cancer should be treated. One needs to remember that killing cancer cells *per se* is not difficult; doing so in a manner that will not kill the patient is the challenge. Finding a tolerable treatment with sufficient effect is a particular concern in a disease like prostate cancer, which mostly affects elderly men, many of whom have comorbidities that render them vulnerable to treatment side effects.

As mentioned above, prostate cancer is often indolent and will not require treatment. Since treatments can have adverse effects and cost a substantial amount, it is not advisable to immediately treat low-grade tumours. Based on the tumour grade as well as tumour stage and PSA value, patients are classified as low risk, intermediate risk and high risk [133]. For high-risk and some intermediate-risk

patients, further diagnostic imaging procedures should be performed to determine whether the cancer has already metastasised [88]. Positron-emission tomography (PET) targeted to prostate-specific membrane antigen (PSMA) has been shown to be very sensitive and is used especially for the localisation of recurrent disease [137, 138].

For low-risk and some intermediate-risk tumours, active surveillance is the recommended course of action if the patient has an estimated life expectancy of at least ten years and is suitable for active treatment in the case of disease progression. During active surveillance, the patient will be regularly monitored by PSA, MRI and re-biopsy for signs that would indicate that active treatment with curative intent should be initiated. In patients that have a low life expectancy at diagnosis, and are therefore likely to die from another cause before a slow-growing prostate cancer would become life-threatening, watchful waiting is generally recommended. In contrast to active surveillance, patients under watchful waiting are monitored less frequently and offered palliative treatment in case of symptoms and disease progression [133]. Large clinical studies have found that the mortality with a watchful waiting approach was not higher than with immediate treatment [139], or that there was only a slight overall disadvantage of watchful waiting approach, with the difference being mostly evident in younger patients [140]. Especially for patients with many comorbidities, immediate intervention did not give a survival advantage compared to active surveillance in clinical trials [141]. It is however crucial to explain this rationale to patients, as many would prefer to be treated immediately in order to combat the anxiety associated with a cancer diagnosis [142-145].

Treatment

If there are indications that the prostate cancer is progressing or is at risk of progression, a localised cancer can be cured by surgery or radiation therapy, with or without neoadjuvant chemotherapy or ADT. In the case of surgical prostatectomy, the entire prostate is removed, commonly by robot-assisted laparoscopic surgery [133]. Radiation therapy may be performed either using an external beam and/or by brachytherapy [133]. According to a range of clinical studies, 50% of patients or more do not experience a biochemical recurrence (BCR), marked by increased PSA levels, after curative treatment [146]. Local treatment of the primary tumour with radiation therapy is beneficial even in patients that already present with metastases at the time of diagnosis, as long as the metastatic burden is limited [137]. Upon BCR, salvage radiotherapy and/or hormone therapy (see below) can prevent further progression in the majority of cases [88].

Even in cases that appear to be localised at diagnosis, there can be occult metastases or micrometastases already present, and relapse can occur [92]. In the case of metastasis, either at diagnosis or later on, surgical or pharmacological castration therapy or treatment with anti-androgens are the standard of care for hormone-naïve cancers [88]. Systemic ADT may be combined with local treatment

of individual lesions in the case of oligometastatic disease [137]. As discussed above, the androgen pathway is active even in most patients with castration-resistant disease, so that targeting the androgen pathway with abiraterone acetate or anti-androgens like bicalutamide, flutamide, enzalutamide, apalutamide or darolutamide is valuable. In the case of a high metastatic burden, ADT and/or AR inhibition combined with docetaxel has been shown to be beneficial to patients [147-150]. In most cases, aggressive prostate cancers eventually become resistant to castration and AR inhibition. In these cases, palliative treatment with chemotherapeutic agents like docetaxel or cabazitaxel is an option that can be used to reduce the disease burden and delay time to death [151, 152].

Other treatments include agents for direct targeting of bone metastases like zoledronic acid and denosumab, a monoclonal antibody targeting RANKL. Although neither of these drugs have shown a clear survival benefit in this group of patients, both drugs reduced the risk of and time to skeletal-related events [153-155]. In addition to preventing skeletal-related events by inhibiting osteoclast activity and combatting the bone-weakening adverse effects of androgen deprivation therapy, zoledronic acid has also been suggested to directly inhibit prostate cancer cell growth and to remodel the bone microenvironment towards an anti-cancer phenotype [156]. Radium-223 is a bone-targeting radioisotope that locally emits alpha particles with a 3.6 month survival advantage in a Phase III study on patients with metastatic castration-resistant prostate cancer [157]. Other studies have shown an overall survival benefit in patients with metastatic castration-resistant prostate cancer upon treatment with the beta particle-emitter Lutetium-177 conjugated to a PSMA ligand compared to standard of care of cabazitaxel [158, 159].

Some newer targeted treatments are also arising. In tumours with DNA damage repair deficiencies, such as *BRCA1* or *BRCA2* mutations, the use of poly(ADP-ribose) polymerase (PARP) inhibitors has shown promise in clinical trials [160, 161] and has been approved by the FDA. Alternatively, platinum-based chemotherapeutic agents like carboplatin may be used [137].

Some immunotherapies have also been tested in prostate cancer trials, such as checkpoint inhibitors and cancer vaccines. The FDA-approved prostate cancer vaccine Sipuleucel-T primes the patient's own leukocytes to target prostatic acid phosphatase (PAP), leading to a PAP-directed anti-tumour immune response [162]. As tumour cells are being lysed during this reaction, more tumour antigens are released, leading to antigen spread and an even larger immune response to a variety of tumour targets [163-165]. However, although clinical trials have shown some responses in patients with asymptomatic and minimally symptomatic metastatic prostate cancer, the overall effect on survival was modest, and there was no difference in the time to progression [166, 167]. There have also been trials using checkpoint inhibitors, with mild success in some patients, but no overt survival advantages over the entire patient population [168-170]. The response rate was slightly higher in patients with DNA damage repair deficiencies, likely due to the

higher mutational burden and therefore a higher immunogenic capacity in these patients [162, 171]. The only FDA-approved immunotherapy for prostate cancer so far is the use of pembrolizumab for solid tumours with high microsatellite instability [172]. Generally, prostate tumours are considered relatively immune-cold with a highly immunosuppressive tumour microenvironment, so that either selecting the correct patient population or altering the tumour microenvironment will be challenges that need to be tackled in the future [162]. Multiple trials combining immunotherapy with other treatments are currently ongoing [162].

Challenges and research gaps

Recently, the men's health funding organisation Movember conducted a landscape analysis to determine onto which areas of prostate cancer research they should focus their funding efforts [173]. A group of experts in the field issued a consensus statement after prioritising an extensive list of suggestions gathered from stakeholder interviews with patients, healthcare providers, researchers, and different foundations. The three highest-ranked research needs were the following [173]:

- Establish more sensitive and specific tests to improve disease screening and diagnosis
- Develop indicators to better stratify low-risk prostate cancer in determining which men should go on active surveillance
- Integrate companion diagnostics (for example, liquid and/or tissue biopsy and imaging modalities) into randomized clinical trials to predict treatment response

In my opinion, the three goals have one thing in common: They require the establishment of better biomarkers that are more sensitive, specific and predictive than what is currently used in clinical practice. As discussed in the beginning of the chapter, PSA is a relatively unspecific biomarker. While it can be useful for pointing towards an initial testing for diagnosis or in indicating a BCR, PSA can only predict so much when used on its own. Instead, being able to access predictive information without the need for a tissue biopsy or expensive imaging techniques would be advantageous. As mentioned in the report, biomarkers are needed both for screening, for prognosis and treatment decisions, and to monitor treatment response.

Non-coding RNAs

With recent technological advances and discoveries, there are now several new avenues for biomarker research that go beyond classical protein- or metabolite-based indicators. A new promising class of molecules includes non-coding RNAs. Only around 2% of the genome code for proteins, which was previously thought to be the main function of genes [174]. The rest are so-called non-coding RNAs. This term itself is the result of the protein-centric view that is still widespread in field – dubbing these RNAs non-coding simply because they are not protein-coding is

misleading. Of course, these RNAs do encode essential information, since many are evolutionarily conserved and mutations in non-coding regions do cause diseases like cancer [174].

Many different classes of non-coding RNAs have been associated with functions that play a role in cancer and prostate cancer. Table 1 lists some of these classes with examples of their use in the prostate cancer context, especially with regards to their potential as biomarkers. One of these classes, microRNAs (miRNAs), will be discussed in more detail in a later chapter.

Table 1.
Non-exhaustive list of non-coding RNA classes and their functions in general, and in the context of prostate cancer in particular.

Class	Function	Example in prostate cancer
tRNA (transfer RNA) & tRFs (tRNA-derived fragments)	codon recognition during protein translation	tRFs are differentially expressed in prostate cancer and associated with disease outcome [175]
rRNA (ribosomal RNA)	structure and catalysis in the ribosome	mutations in mitochondrial 16S rRNA in prostate cancer; detectable in liquid biopsies [176]
snoRNA (small nucleolar RNA) & sdRNA (snoRNA-derived RNA)	processing and modification of other RNAs such as rRNA	expression of SNORD78 and its derivative sdRNAs is associated with prostate cancer metastasis [177]
lncRNA (long non-coding RNA)	regulation of transcription and translation; structural scaffold; regulation of miRNA function	*PCA3* is an FDA-approved biomarker to aid in prostate cancer diagnosis [178]
miRNA (microRNA)	regulation of translation	miQ score based on the expression of four miRNAs (miR-96,-183,-145, -221) is a potential biomarker of aggressive prostate cancer [179]
circRNA (circular RNA)	regulation of miRNA function; regulation of protein function; protein/peptide production	Ccirc index based on five circRNAs contained in urine EVs can predict high grade prostate cancer at initial biopsy [180]

Liquid biopsies

Another field that has garnered much attention recently is that of liquid biopsies [181]. This refers to using blood samples and other bodily fluids instead of tissue samples to obtain information about the tumour(s). In the case of prostate cancer, the use of urine and semen samples is especially relevant, as these fluids pass through the prostate. Relevant disease markers for prostate cancer can be further enriched in urine by using first catch urine or by performing mild prostatic massage prior to sample collection [182]. The advantage of using urine or semen samples is that there is virtually no risk to the patients, and sample collection is even less invasive than for blood samples. Liquid biopsies can allow analysis of CTCs, EVs, and circulating tumour DNA (ctDNA).

The number of CTCs is thought to predict the likelihood of metastasis, and multiple technologies have been developed to isolate CTCs based on their physical properties, or based on markers that are considered cancer cell-specific [183]. For example, the FDA-approved CellSearch technology identifies CTCs based on

EpCAM expression [183, 184]. This has been criticised, as EpCAM expression can vary with different degrees of EMT, so that some cells with metastatic potential and low EpCAM expression may be missed [184, 185]. CTCs can be evaluated either simply based on cell number to predict metastasis, or by screening the cells for specific genes or transcripts such as the AR-V7 splicing variant that is associated with resistance to anti-androgen therapy [186, 187].

These AR splicing variants can also be found in plasma EVs [188, 189]. In addition to mRNAs, EVs contain a plethora of molecules that can be predictive of outcome, such as proteins, DNA, and non-coding RNAs. As EVs carry many of their parental cell's molecules, their contents give a good picture of the biological processes that are currently driving the tumour. For example, an early publication showed that *TMPRSS2:ERG* gene fusions can be detected in urine of some prostate cancer patients [182]. This same publication also demonstrated the presence of *PCA3*, a long non-coding RNA that has since been commercialised and FDA-approved [178, 190]. For small non-coding RNAs, a pipeline for the discovery of miRNA biomarkers for prostate cancer in urine was recently developed in our lab [191], showing that the deregulation of cancer-associated miRNAs could be assessed using EVs. Some groups have identified promising protein biomarkers based on cell lines and are now validating these in tissue patient samples [192, 193]. Other groups have opted to identify protein biomarkers in EVs directly in patient cohorts, but their candidate proteins are yet to be confirmed in larger studies [194].

Lastly, even tumour-derived nucleic acids that are not enclosed in cells or vesicles can be detected and quantified in blood and other bodily fluids. One study found that ctDNA correctly reflected the mutational signature of metastatic castration-resistant prostate cancer in matched samples, indicating that liquid biopsies can yield the same information as tissue biopsies [195]. In a different study taking into account tumour evolution, ctDNA indicated the dominant driving metastatic clone, sometimes along with other subclones present in the same patient [34]. Another study focused on methylation patterns in ctDNA, and identified prostate cancer-specific signatures [196]. Studies like these are encouraging with regards to using liquid biopsies to monitor prostate cancer status and disease progression in the future.

Diversity, equity and inclusion perspective

As with most cancers and diseases, patients with a lower socioeconomic status are more likely to die of prostate cancer [197-199]. Especially patients without access to healthcare or in underfunded and understaffed healthcare systems are more likely to be diagnosed too late in the disease progress, or to not receive treatment. But even in the Nordic countries, in which all residents have access to inexpensive healthcare, a lower socioeconomic status is associated with higher prostate cancer mortality rates [200-203]. Some not-for-profit organisations like Movember have therefore

suggested that projects take into account equity considerations, so that as many patients as possible may benefit [173].

Black men have significantly higher rates of early-onset aggressive prostate cancer and worse survival [197-199, 204]. Most research that implies that African-American and Black men have higher prostate cancer mortality was performed in the United States and other Western countries; it is therefore possible that the underlying causes are tied mostly to socioeconomic status and access to healthcare in this systematically disadvantaged group [197-199, 204-206]. Studies have shown that African-American men are less likely to participate in screening programs and clinical trials, and are also less likely to be offered definitive treatment upon diagnosis [206]. It is also possible that a potential underlying genetic predisposition plays a role [207]. To be able to differentiate between environmental and genetic factors, it has been suggested to conduct more high-quality research in the original source populations in Western Africa [208].

Due to the increased risk of aggressive prostate cancer in black people, it is often recommended that they are screened at younger ages than the rest of the population [88, 133]. Black patients on active surveillance may require reclassification and active treatment sooner, although there is a relative lack of clinical studies in these men [209]. It will also be important to include more Black patients in clinical trials in order to ensure that treatments work well and are tolerable for these high-risk patients. For this, it is essential that clinical trial facilitators are trained to address potential mistrust in the medical system in general and clinical trials in particular due to past crimes against Black communities [206].

Another group of patients that are commonly discriminated against or made feel uncomfortable are homo- and bisexual men. In the context of prostate cancer, while there is no effect of sexual orientation on the risk of diagnosis or advancement of prostate cancer, there are certain aspects that should be considered in patient interaction [210-212]. According to surveys, there are several concerns in gay and bisexual men that are related to consequences of prostate cancer treatments pertaining to a variety of sexual practices and self-identity [211, 213, 214]. Men who are sexually active with men may therefore have additional questions or concerns, and may not feel comfortable to bring these up with their doctor; or when they do, their doctors may not be equipped to answer [210-212, 215, 216]. Offering additional training for doctors and nurses can aid them in better addressing these needs in the future [210, 211, 216, 217].

Lastly, even for prostate cancer, a gender perspective needs to be considered. Transgender women, including those that have undergone gender-affirming surgery, usually retain their prostates [218, 219]. With increasing acceptance in society in recent decades, there are now more and more ageing transgender women at risk of prostate cancer [219]. Relatively little systematic research has been performed, but based on current data it can be concluded that the incidence of prostate cancer is lower in transgender women compared to cisgender men [219-221]. There are multiple case reports of prostate cancer in transgender women [222-

230], in many of whom the disease is already at a late stage and difficult to treat. As many transgender women choose to undergo hormone treatments, their prostates become atrophic and the formation of tumours is unlikely. It has therefore been suggested that prostate cancer in transgender women arises from lesions that existed before the commencement of hormone treatments [219, 221]. However, the prostate cancers that do develop do so in a testosterone-low environment and are therefore inherently castration-resistant, which makes them more aggressive and limits treatment options [230]. Other theories suggest that oestrogen may exacerbate prostate cancer growth [228].

Many transgender women are not reached by large-scale screening effort because they may not be aware of having a prostate and therefore being at risk for prostate cancer, or may not be invited if they are registered in the system as female. There are also certain anatomical aspects to consider during prostate cancer treatment, such as a significantly smaller prostate volume due to atrophy resulting from hormone treatments, or the presence of a neovagina between the prostate and the rectum in women who have elected gender-affirming surgery [230, 231]. It should not be neglected to provide information and check-ups to transgender women [219, 221, 229, 231-234] and to ensure that there is a place for them in the support system that is currently geared towards heterosexual cisgender men [216, 231]. There is also a point in making language more inclusive, both to make female prostate cancer patients feel more comfortable, and to enable their inclusion in screening programs and clinical trials that may help improve their care [230, 231, 235].

MicroRNAs

In 1993, two different groups in parallel published their discovery of a class of small non-coding RNAs, later termed miRNAs, that were able to regulate other RNAs through antisense complementarity [236, 237]. First only identified in worms, it soon became clear that miRNAs exist in all animals as well as in plants and some viruses, and that they fulfil a number of important functions.

Biogenesis of miRNAs

The canonical miRNA biogenesis pathway [238, 239] is shown in Figure 4. There are alternative pathways for the production of miRNAs, such as from miRNA-containing introns (termed "mirtrons"), the splicing of which produces pre-miRNAs without the need for the microprocessor complex [240]. These and other non-canonical miRNA biogenesis pathways will not be discussed further in this thesis.

Generally, initial transcription of a primary miRNA (pri-miRNA) is facilitated by RNA polymerase II or III. Most miRNAs are located in polycistronic transcripts harbouring multiple miRNAs. The pri-miRNA has a complex structure, which is

processed by the microprocessor complex, consisting of Drosha and DiGeorge syndrome critical region gene 8 (DGCR8). The resulting precursor miRNA (pre-miRNA) hairpins are exported from the nucleus *via* Exportin 5 (XPO5) in complex with Ran-GTP. In the cytosol, the pre-miRNA is further cleaved by Dicer, supported by TAR RNA binding protein (TRBP), producing two single-stranded mature strands with an average length of ~22 nt each. This last processing step by Dicer takes place in close coordination with Argonaute (AGO) [238].

In many cases, one of the two mature strands is degraded (passenger strand), and only one of them is biologically active (guide strand). However, there are exceptions in which both strands are active, or strand-switching is induced in certain tissues or upon specific stimuli [241, 242].

Importantly, the possible outputs of a miRNA gene are not limited to these two alternative strands: Throughout and after the processing of miRNAs, they can be post-transcriptionally modified [243]. One mechanism is through the modification of internal bases by A-to-I editing of pri-miRNAs. A chapter will be dedicated to this process later in this thesis. Apart from internal modifications, multiple modifications to the ends of miRNAs take place. These terminal isoforms, referred to as isomiRs, can be achieved through multiple mechanisms, such as alternative Drosha or Dicer processing, removal of terminal nucleotides, or addition of non-templated terminal nucleotides [239, 243]. If the difference occurs at the 5′ end, the seed sequence is altered, likely resulting in a different set of target genes [244]. Modifications on the 3′ end can have equally dramatic consequences. For example, uridylation of pre-miR-324 can lead to arm switching [242], and adenylation of mature miR-21 leads to its degradation [245].

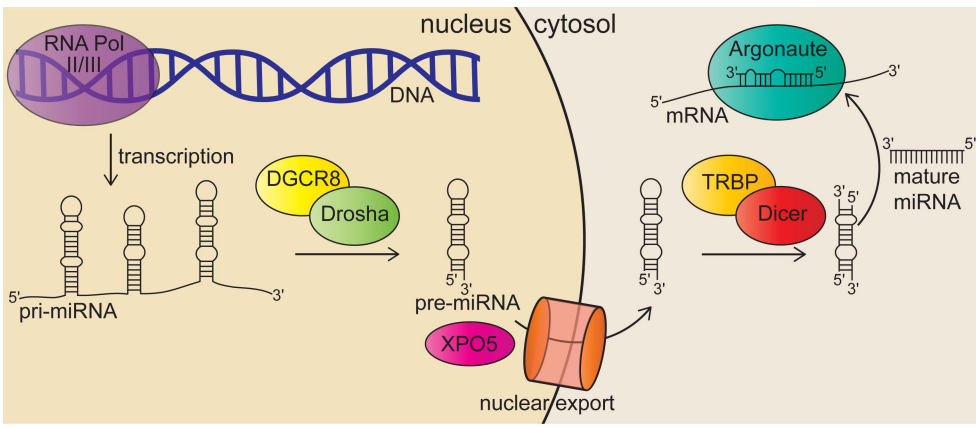

Figure 4.
Canonical miRNA biogenesis pathway. The primary miRNA (pri-miRNA) is processed by the microprocessor complex. The resulting pre-miRNA is exported to the cytosol, where it is further processed by Dicer. The mature single-stranded miRNA is incorporated into Argonaute (AGO) protein complexes.

Functions of miRNAs

The mature miRNA incorporated into an AGO protein complex binds mRNAs and can effect a number of regulatory events. In the case of perfect or near-perfect complementarity between the miRNA and mRNA, some AGO proteins can catalyse slicing of the mRNA, inducing its degradation [246]. While this mode of action is common in plants, it is rare in most animals [239]. Much more commonly, there is imperfect base pairing between the miRNA and the mRNA.

Target selection

In most cases, targets are selected based on a stretch of perfect complementarity in the so-called seed region encompassing nucleotides 2–8 in the 5′ part of the miRNA (Figure 5). The remaining bases can fine-tune the affinity between miRNA and mRNA through supplementary partial binding [243, 247]. Other miRNA targets contain a single nucleotide bulge in the seed-complementary region [248]. There are also rarer non-canonical modes of target binding, such as having only an impartial seed match and an additional region of compensatory binding [239, 247].

An often-heard generalised statement is the idea that miRNAs bind in the 3′ untranslated region (UTR) of mRNAs, leading to their repression. Although this statement can be used as a rule of thumb, the truth is much more complex, and expecting this mode of action may cause us to miss important pathways. For example, there are approximately as many miRNA targets sites in the coding sequence (CDS) as in the 3′ UTR; in addition, there are also some, albeit fewer, miRNA binding sites located in the 5′ UTR [249-252].

On average, each miRNA has several hundred targets in a human cell [239, 247]. These targets are distributed across many different mRNAs, which can have opposing or similar functions, or be part of the same pathway or of competing pathways. This ability to globally alter mRNA abundance and translation has led to the understanding that miRNAs are likely responsible for maintaining homeostasis and safely manoeuvring and fine-tuning any remodelling events [253].

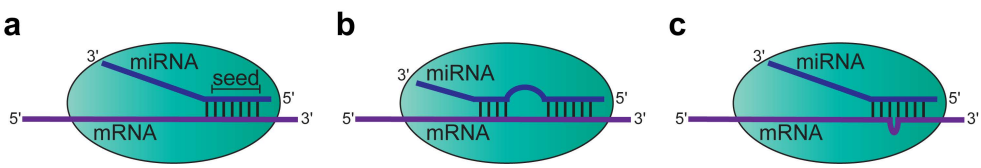

Figure 5.
Three common types of miRNA:mRNA target site interactions. **a**. Perfect complementarity in the seed region (nt 2–8 of the miRNA). **b**. Supplementary binding in nt 12–16 of the miRNA in addition to seed binding. **c**. Seed binding with a bulk in the mRNA target sequence between nt 5 and 6 of the miRNA.

The sheer number of potential targets means that many binding sites compete for the binding of a limited amount of miRNA. The titration hypothesis suggests that the purpose of several low-affinity targets that are only mildly repressed by miRNAs is not for these transcripts to be regulated, but rather for them to titrate how much miRNA is available to bind to a higher-affinity target that mediates the main effect of the miRNA [239, 243, 254]. Which transcripts are targeted by a specific miRNA is therefore not only based on whether or not the predicted target is present in the cell or not, but also on which other target mRNAs are present in the gene expression pool [254]. This is important to keep in mind when comparing miRNA regulation events in different species, different cell types and different conditions.

The competing endogenous RNA (ceRNA) hypothesis goes one step further and predicts that individual transcripts can competitively bind miRNAs so efficiently that other miRNA targets are de-repressed, but this theory is disputed in the field [239, 243, 255].

Target regulation

As mentioned above, miRNAs mostly lead to the repression of the targeted mRNA. This effect is mediated by GW182 proteins (TNRC6A/B/C in vertebrates). GW182 binds RISCs and recruits multiple downstream effector molecules. PAN2-PAN3, DCP1-DCP2, and CCR4-NOT complexes lead to deadenylation of the mRNA and its consequent degradation. The CCR4-NOT complex can further recruit DEAD box 6 (DDX6) and eIF4E transporter (4E-T), which lead to the repression of cap-dependent translation initiation [256].

However, there are alternative mechanisms which are understood less well. For example, there is evidence of upregulated translation from mRNAs under certain conditions. Upon cell cycle arrest, induced by stress such as starvation, miR-369-3 promotes translation of tumour necrosis factor (*TNF*) mRNA upon binding of an AU-rich motif in the 3′ UTR [257, 258]. Likewise, translation of *HMGA2* mRNA was activated by let-7 upon growth arrest [257]. The stimulation of translation in non-dividing cells was brought on by association of fragile-X-mental-retardation-related protein 1 (FXR1) with AGO2 [257, 258]. Another research group reported the upregulation of *TLR4* transcript translation by miR-511 in non-dividing cells [259]. A similar observation was made in Xenopus oocytes, which are also quiescent, for the upregulation of *Myt1* mRNA translation by miR-16 [260]. Other reports have found translation activation of mRNAs with short poly(A) trails by RISC in fly extracts [261], and stimulation of translation by miR-1 in the mitochondria, but not the cytoplasm, of muscle cells [262]. In both of these reports, GW182 was absent from the translation-activating AGO ribonucleoprotein complexes. The suggested mechanism is that FXR1 can initiate non-canonical translation in mTOR-low conditions during which canonical translation initiation is suppressed [263, 264].

Another distinct mechanism for miRNA-mediated upregulation of translation is through the inhibition of negative regulators. For example, under starvation

conditions, miR-10a targeting in the 5′ UTR could alleviate translational repression mediated through an adjacent 5′ TOP motif [265]. This provides a mechanism to enable production of ribosomal proteins even under starvation conditions.

In another study, *IL-10* mRNA was stabilised by miR-466l in Toll-like receptor (TLR)-triggered macrophages. Binding of miR-466l to its target site in the 3′ UTR of *IL-10* mRNA prevented binding of tristetraprolin, which would otherwise induce degradation of the mRNA [266].

Studies in the brain found that miR-346 could bind the 5′ UTR of a splicing isoform of receptor-interacting protein 140 (*RIP140*) mRNA in the brain and induce its translation [267]. Interestingly, this function did not seem to depend on the presence of AGO. The authors did not provide a more detailed mechanism, and there were no other known regulatory elements present in the 5′ UTR near the miR-346 binding site. A second study on miR-346 in the brain found AGO-dependent positive regulation of amyloid-beta precursor protein (*APP*) translation induced by miR-346 binding to the 5′ UTR in neurons with low iron levels [268]. The authors proposed that the RISC could displace the inhibitory iron response protein 1 (IRP1) from the 5′ UTR, disinhibiting translation. Another possibility is the active initiation of translation by AGO, a known function of AGO1 – in fact, AGO1 was originally termed eIF2c translation initiation factor [269, 270].

There are other examples of context-dependent switches towards translation activation. For example, it was found that miR-206 repressed Krüppel-like factor 4 (KLF4) expression in breast cancer cells, but enhanced translation in immortalised normal mammary epithelial cells [271]. Unfortunately, the authors did not provide a mechanism.

An interesting phenomenon has been observed in Hepatitis C Virus (HCV): HCV transcripts can bind cell-endogenous miR-122 in their 5′ UTR, leading to a stimulation of translation [272]. This depends on HCV-specific sequences like the IRES and the HCV 3′ UTR, and on the absence of a cap on the RNA [273]. The mechanism is therefore likely specific for viral RNAs. Interestingly, in addition to stimulating HCV mRNA translation, miR-122 also promotes replication of HCV RNA [274].

Lastly, even a role for miRNAs in the nucleus has been suggested, after multiple reports that mature miRNAs can be re-imported into the nucleus [246, 275]. Several examples of miRNAs with functions inside the nucleus have been described, but are beyond the scope of this review. Instead, more information is available in a recent review article [276]. Proposed functions of miRNAs in the nucleus include the regulation of RNA processing and abundance, and transcriptional silencing or activation [276, 277].

I do want to point out the first paper proposing transcriptional activation by a miRNA, which was published 2008 and claimed to provide evidence for a role of miR-373 in activating transcription from the E-Cadherin promoter. Concerns were raised about image manipulation in which parts of a Western blot had been mirrored and spliced together [278]. Due to the fact that false evidence was presented in parts

of the paper, I do not believe that any of the data should be trusted[1]. It should be noted that many of the other papers proposing transcriptional activation through miRNAs originate from the same lab, which has been accused of fraud on multiple occasions, so that this particular mechanism is doubtful in my eyes.

Roles of miRNAs in cancer

Owing to their ability to maintain homeostasis in changing conditions [253], it is not surprising that miRNAs are often deregulated in cancer, and that most miRNAs are expected to be tumour suppressors whose function it is to dampen any unusual pathways. This is also supported by the fact that Dicer downregulation commonly occurs in cancer, and that Dicer mutations predispose to certain types of cancer [279]. These tumour suppressors include miRNAs like miR-15/16 [280], miR-34 [281], and let-7 [282]. However, there are also oncogenic miRNAs that drive cancer-associated processes, such as miR-21 [283].

miR-96

The miR-183-96-182 family, forming its own cluster encoding for a polycistronic pre-miRNA, plays an important role in the development of hearing in the normal organism [284, 285]. Germline mutations of miR-96 lead to hearing loss [286, 287]. In addition to this function, the miR-183/-96/-182 family has also been shown to be upregulated across different cancer types in many studies [288, 289]. In prostate cancer, miR-96 upregulation has been found to be associated with worse clinical outcomes [179, 290-292]. It has also been proposed as a biomarker in combination with other miRNAs [179].

Multiple targets for miR-96 in prostate cancer have been found and confirmed. The tumour suppressor forkhead box O1 (FOXO1) is repressed by miR-96 in prostate cancer cells [291, 293, 294] as well as in other cancer cells [295-297], with miR-96 overexpression leading to increased proliferation. Other members of the same protein family are also suppressed by miR-96, such as FOXO3 [297, 298] and FOXF2 [299]. Another effect of miR-96 and other members of the miR-183-96-182 family is the repression of zinc transporter expression, leading to lower levels inside the cells [300]; lower intracellular zinc levels are a known feature of prostate cancer [301]. Especially in already treated patients, miR-96 inhibits retinoid acid receptor γ (RAR-γ) expression, leading to increased cell viability [302]. Other targets of miR-96 include metastasis suppressor protein 1 (*MTSS1*) [303], adherens junction-

[1] An investigation committee came to the conclusion that "the manipulation of the images […] could only have occurred intentionally, representing instances of scientific misconduct". It was not possible to identify the person who fabricated the images. It is puzzling to me that the paper was not retracted, but that the authors were allowed to post a correction. I have decided against citing the original paper here in order to not conflate the paper's metrics and further reward dishonesty. A link to the original reference can be found in the cited correction.

associated protein 1 (*AJAP1*) [304], and ETS variant gene 6 (*ETV6*) [305] mRNAs. In the case of *ETV6* suppression, the authors suggested that this was the result of increased miR-96 expression following epithelial growth factor receptor (EGFR) signalling [305].

One study found that hypoxia induced miR-96 expression, enhancing autophagy through miR-96-mediated mTOR repression. Interestingly, when miR-96 levels were raised even further, autophagy was inhibited due to downregulation of ATG7 by miR-96, resulting in a biphasic effect [306]. Another factor that has been reported to increase miR-96 expression is TGF-β. This led to the suppression of AKT substrate 1 (*AKT1S1*) mRNA by miR-96. AKT1S1 negatively regulates mTOR kinase, so that TGF-β and miR-96 effectively enhance mTOR function. This would provide a role for miR-96 in promoting bone metastasis, as its levels would be increased in the TGF-β-rich microenvironment of the bone [307].

It has been shown that miR-96 function in prostate cancer cells could be dampened by long non-coding RNAs ADAMTS9-AS1 [308] and FGF14-AS2 [304]. Another factor suppressing miR-96 is ZEB1. The EMT-promoting transcription factor can directly repress the expression of the miR-183/-96/-182 family in breast cancer cells [309, 310]. In turn, miR-96 repressed expression of ZEB1 and SLUG, promoting an epithelial phenotype [310]. To my knowledge, this negative feedback mechanism has not been investigated in prostate cancer cells.

miR-379

One of the largest miRNA cluster described is located on chromosome 14q. This miR-379-656 cluster encodes close to 50 miRNAs, and it is maternally imprinted [311]. The cluster is downregulated in multiple cancers [312], and its downregulation is associated with worse clinical outcome [313]. Mechanisms for the downregulation of the miR-379-656 cluster include hypermethylation [312, 314] and downregulation of the transcription factor MEF2, which drives expression from this locus [312, 314-316]. The cluster contains different miRNA families, including the miR-379 family and its members miR-379, miR-411, miR-758 and miR-1247.

Due to its deregulation, miR-379 has been suggested as a biomarker in EV-based liquid biopsies in lung cancer [317] and prostate cancer [318]. Interestingly, in a study investigating liquid biopsies in gastric cancer, the miR-379 content in EVs was higher in patients with metastasis. Conversely, miR-379 expression was lower in the tumour tissue itself, implying differential sorting of miR-379 into EVs. The authors suggested that this could provide a potential mechanism for the downregulation of miR-379 in the tumour cells [319].

Many targets for miR-379 have been described in cancer. For example, miR-379 repressed the expression of focal adhesion kinase (FAK) and inhibited EMT in gastric and non-small cell lung cancer [320, 321]. Other examples are the downregulation of phosphoinositide-dependent kinase-1 (PDK1) in osteosarcoma [322], and the downregulation of Cyclin B1 (CCNB1) and cyclooyggenase-2 (COX2) in breast cancer [323, 324]. In liver and non-small cell lung cancer, Insulin-

derived growth factor receptor 1 (IGFR1) expression was suppressed by miR-379 [325, 326]. In bone-metastatic breast cancer, it was shown that miR-379 repressed TGF-β-induced IL-11 expression, which normally drives bone metastasis. In addition, miR-379 also reduced other TGF-β-regulated genes, and dampened SMAD signalling in general [327].

Another miR-379 target that was recently described in chronic myeloid leukaemia is Aldo-keto reductase 1 family member C3 (AKR1C3) [328]. AKR1C3 can produce testosterone from androstenedione and has been shown to be upregulated in castration-resistant prostate cancer [329]. In addition, AKR1C3 was shown to be a transactivator of AR. Upon ligand binding – of either an androgen or androstenedione –, both AR and AKR1C3 translocated to the nucleus and induced PSA transcription [330]. Pharmacological inhibition of AKR1C3 has been suggested as a treatment option of castration-resistant prostate cancer [330-332], and miR-379 might exert the same effect.

In prostate cancer cells themselves, miR-379 has only been investigated in one study. Surprisingly, the publication described both miR-379 and miR-154*, which is also part of the miR-379-656 cluster, to have tumour-promoting functions. The authors found that inhibition of miR-379 and miR-154* in mesenchymal prostate cancer cells resulted in an increase in E-Cadherin expression and MET. They presented *in vivo* data for miR-154* showing decreased bone metastasis upon miR-154* inhibition, but did not show equivalent data for miR-379 [333].

Testing the potential of miR-379 in cancer therapy, it was shown that intratumoural injection of miR-379 mimics halted the growth of subcutaneous osteosarcoma xenografts [322]. In another study, systemic administration of EVs derived from miR-379-overexpressing mesenchymal stem cells (MSCs) was able to reduce the growth of subcutaneous breast cancer xenografts [324]. The authors in this last study also tried to achieve delivery of miR-379-containing EVs by injection of the MSCs themselves, but there was no effect on tumour growth in this setting.

Clinical applications for miRNAs

Given the large number of studies that have been performed studying the deregulation of miRNAs in cancer, it is not surprising that their clinical use has been suggested many times. There are two main applications for miRNAs – measuring their deregulation could be useful as a biomarker, or their function could be inhibited or enhanced in order to treat cancer.

Use of miRNAs as biomarkers

Due to their high stability in various biological fluids and tissues – even ones that are heavily treated such as formalin-fixed paraffin-embedded (FFPE) samples, or have been stored for a long time – miRNAs can be assessed in routine clinical samples without adapting the preservation protocols that are currently used for

DNA- or protein-based markers [334, 335]. Additionally, as they are present in bodily fluids and also in EVs, miRNAs biomarkers are good candidates for use in liquid biopsies [181, 336].

A number of miRNA-based biomarkers are marketed and available for clinical use. In most cases, these are panels of multiple miRNAs, rather than individual miRNAs. Some of these tests are now even covered by medical insurance companies, enabling their use in routine practice [337]. However, there are still no FDA-approved miRNA biomarkers, and therefore actual usage in the clinic is still limited.

One reason for the lack of clinically available biomarkers is certainly the fact that different studies commonly disagree on the deregulation of miRNAs. The reasons for this can lie in the usage of different isolation protocols, different assays, or different normalisation methods [338]. There is therefore a dire need to standardise protocols [339].

In addition, most biomarker studies do not take into account the full diversity of miRNAs. Many studies do not report on miRNA isoforms, either with regards to terminal isoforms or A-to-I-edited miRNAs. As discussed above, these isoforms are also differentially expressed in different contexts, and can have different functions. If miRNAs are not analysed in an isoform-specific manner, predictive power and associations with clinical parameters could be attributed to the wrong isoform, or clinically relevant deregulations could be overlooked. It will therefore be important that researchers are made aware of the existence and the importance of miRNA isoforms, and that they are given the tools to analyse them. For this, technologies like qPCR will need to be refined.

Use of miRNAs as therapeutic targets or agents

Similarly to the use of miRNAs as biomarkers, miRNA research has sparked interest in using miRNAs for therapies, either as targets (for tumour-promoting miRNAs) or as therapeutic agents (for tumour-suppressing miRNAs). In theory, miRNAs are well-suited for this purpose: With combination therapies on the rise, and given the fact that miRNAs have multiple targets, one could think of using miRNAs as the ultimate combination therapy, targeting and balancing multiple pathways at once and limiting the potential for resistance.

There are a few miRNA-based therapeutics that are currently being tested in clinical trials [340, 341]. For example, Miravirsen inhibits miR-122 in the liver, treating chronic HCV infections [342]. For cancer, only few clinical trials have been conducted, with mixed success [343]. For example, in the case or MRX34, which used a miR-34 mimic, the trials were halted due to safety concerns, and up until then, there were only few responders [344, 345].

Immune-related adverse effects are the major safety concerns, owing to the delivery of exogenous nucleic acids [341]. At least in the case of miRNA inhibition it may therefore be advantageous to use small molecule inhibitors rather than antisense oligonucleotides [346]. These could also be easier to be deliver – the

delivery of miRNAs and miRNAs inhibitors is another challenge to face [341, 343]. With the recent development of RNA-based vaccines and therapies, it is to be hoped that knowledge gained about the delivery and safety profiles can be used for therapies employing small RNAs as well [347].

RNA editing

A-to-I editing of RNAs refers to the deamination of adenosine residues to form inosine, which is mediated by adenosine deaminase acting on RNA (ADAR) enzymes. Whereas adenosine forms hydrogen bonds with uridine, inosine binds more strongly to cytidine, albeit at a lower affinity than that of guanosine and cytidine base pairs (Figure 6). A-to-I editing has several functions in the cell and the organism, including the suppression of autoimmunity and inflammatory responses, the recoding of certain mRNAs and protein diversification, and regulation of microRNA processing and function. The deregulation of ADAR expression and A-to-I editing has been observed in several diseases, including cancer.

ADAR enzymes

In mammals, there are three known ADAR proteins: ADAR1, ADAR2 and ADAR3. All three of these ADARs have a double-strand binding domain that will recognise and bind double-stranded RNA (dsRNA), but only ADAR1 and ADAR2 possess the catalytic deaminase domain [348-351]. This does however not render ADAR3 redundant [352]; it is thought to have an important function in regulating RNA editing by competitively binding ADAR substrates and preventing their editing [348]. Interestingly, ADAR3 can also bind single-stranded RNA [348]. All three ADAR proteins also have editing-independent functions that are mediated simply through the binding of certain RNA structures [353-355].

ADAR1 further comes in two isoforms: the smaller p110 isoform, which is constitutively expressed and mainly located in the nucleus, and the larger p150 isoform, which is expressed upon interferon stimulation and localises mainly to the cytoplasm [351]. Here, ADAR1 p150 contributes to suppressing aberrant activation of the innate immune system (see below).

ADAR2 is mostly located in the nucleus in mammals [351], and its main function is thought to be the recoding of mRNAs, leading to amino acid substitutions on the protein level (see below). Unlike ADAR1, which is expressed in most tissues, both ADAR2 and ADAR3 are primarily expressed in the nervous system [348].

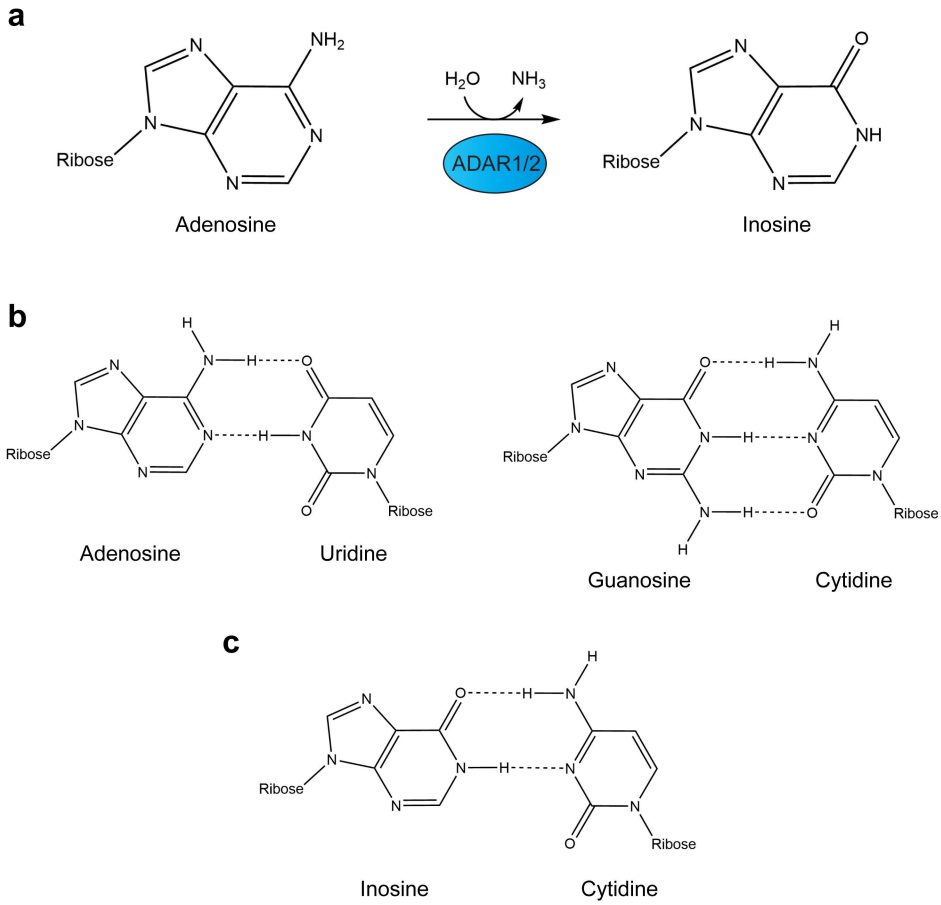

Figure 6.
a. Deamination of adenosine by ADAR enzymes forms inosine. **b.** Canonical Watson-Crick base pairs. Adenosine and uridine form two hydrogen bonds between one another, guanosine and cytidine form three hydrogen bonds. **c.** Inosine preferentially binds with cytidine, with which it forms two hydrogen bonds.

ADAR functions

Suppression of innate immune responses

ADAR1 is essential for mammals. In mice, ADAR1 knockout or mutation leads to embryonic lethality. ADAR1 knockout mice die around embryonic day 11–12.5, showing signs of apoptosis, hematopoietic defects and ultimately foetal liver disintegration [356, 357]. This effect is mainly editing-dependent, as knock-in of a catalytically inactive ADAR1 mutant results in embryonic lethality by embryonic day 13.5, barely delaying lethality. Both editing-deficient ADAR1 mutant and ADAR1 knockout mice had a strong interferon response signature [358, 359].

The embryonic lethal phenotype of ADAR1 mutant mice could be rescued by knockout of either melanoma differentiation-associated protein 5 (MDA5) or its downstream mediator mitochondrial antiviral-signalling protein (MAVS) [358, 359]. The main substrates of ADAR1 in human cells are Alu repeats and other endogenous repetitive elements [360, 361], which frequently form double-stranded RNA structures that can activate innate cytoplasmic nucleic acid sensors and initiate an interferon response [358, 359]. On the other hand, I:U dsRNA, as produced by ADAR1 upon editing, cannot be recognised by MDA5, and will therefore not trigger an interferon response; in fact, I:U dsRNA has even been shown to actively suppress the MDA5-mediated innate immune response [359, 362]. This implies that the main role of ADAR1 in mammals is to deaminate endogenous cytoplasmic dsRNAs in order to prevent an autoinflammatory response.

ADAR1 mutations in humans have a very similar effect to the phenotype in mice. They can cause different diseases such as dyschromatosis symmetrica hereditaria [363], bilateral striatal necrosis [364], spastic paraglegia [365], and Aicardi-Goutières syndrome (AGS) [366]. AGS is a rare severe neuroinflammatory disease, which clinical features such as encephalopathy, basal ganglia calcifications, white matter abnormalities, lymphocytosis, seizures, low-grade fevers, and chilblain lesions [367]. Other known genetic causes of AGS include mutations in other enzymes related to nucleic acids metabolism such as RNase H2 [368], TREX1 [369], and SAMHD1 [370]. All of these enzymes play pivotal roles in nucleic acids synthesis and maintenance, and recent studies have shown that their mutations converge onto the same phenotype: accumulation of endogenous nucleic acids, activation of the innate immune response, and excessive interferon signalling [371]. Interestingly, AGS can also be caused by gain-of-function mutations of the cytoplasmic nucleic acids sensor MDA5 [372]. Patients with AGS have elevated levels of type I interferons [373], and an increase in the expression of interferon-stimulated genes (ISGs) [374].

Of note, in a case study analysing families with a high occurrence of glioma and prostate cancer, it was found that the affected family members were heterozygous for mutations in the *ADAR* and *RNASEH2B* genes [375]. In the gliomas, some features of AGS such as calcifications and increased expression of ISGs were found [375].

The ADAR1 mutations associated with AGS affect the editing activity of ADAR1 p150 more than the activity of ADAR1 p110, suggesting that the cytoplasmic p150 isoform is the one whose dysfunction is associated with aberrant immune responses [359]. A role for the ADAR1 p150 isoform in dampening interferon responses is especially plausible in light of the fact that it is an ISG, thus providing an elegant feedback mechanism. Overall, these findings point towards a main role of ADAR1 p150 in protecting cells from inflammatory responses to endogenous dsRNAs by the immune system. This is mediated through so-called hyperediting of repetitive elements and other immunogenic dsRNAs, rather than site-specific recoding of certain transcripts.

Recoding of mRNAs

Unlike ADAR1 knockout, ADAR2 knockout in mice does not lead to an immune phenotype. It is, however, still lethal: Homozygous ADAR2 knockout mice are carried to term, but they die shortly after birth due to severe seizures [376]. This was due to lack of editing at one editing site in the *GRIA2* mRNA. This codon is nearly 100% edited in wild-type mice, which changes a glutamine residue to an arginine in the encoded protein, GluA2 [377, 378]. Recoding results from the fact that inosine will be recognised as a guanosine during translation. This amino acid change is essential for the function of GluA2 in limiting Ca2+ influx through AMPA receptors, of which it is a subunit [378]. Only edited GluA2 can limit the Ca2+ permeability of the receptor, and loss of GluA2 or loss of editing has been associated with excitotoxic cell death [378, 379]. A knock-in of a genetically encoded G at this site could fully rescue survival in the ADAR2 knockout mice [376]. It should however be noted that these mice were not without a phenotype; hearing problems and some behavioural differences were uncovered in a later study, showing that there is a certain level of nervous system dysfunction in these animals [380].

The main function of ADAR2 in mice and possibly other mammals therefore seems to be the recoding of a specific and very limited set of transcripts in the nervous system [360]. A later study crossed rescued ADAR2 knockout mice with rescued ADAR1 knockout mice, and found that even there, the complete loss of editing at all sites except *GRIA2* did not cause a deleterious phenotype [381]. This excludes the possibility that redundant editing by ADAR1 protected ADAR2 mice from a worse outcome.

To my knowledge, ADAR2 mutations have not been described in humans. This implies either that ADAR2 mutations are inconsequential and simply are not noted when they occur, or that ADAR2 loss would be lethal and lead to death *in utero*. It is possible that in the primate nervous system, which is more complex than that of mice, more functions are associated with RNA editing by ADAR2.

Despite the lack of documented ADAR2 mutations in humans, we can compare the role of GluA2 editing in humans to the role uncovered in the knockout mice: ADAR2 downregulation has been observed in several neurological diseases, such as mood disorders [382] and amyotrophic lateral sclerosis (ALS) [379, 383, 384]. Specifically, in ALS, neuronal death due to decreased ADAR2 activity and therefore loss of *GRIA2* editing has been observed [379, 383, 384]. This implies that ADAR2 does fulfil important functions in the human nervous system.

Nonetheless, the proportion of significantly edited mRNAs overall is relatively small in humans and other mammals. Less than 1% of transcripts are highly edited in humans and mice, and around 3% in invertebrates like flies [385].

There is one notable exception among the invertebrates: Cephalopods like squid and octopus have a remarkably high proportion of editing activity, with approximately 60% of transcripts in the nervous system being edited by ADAR2 [385]. Interestingly, ADAR2 is present and actively edits transcripts not only in the nucleus, but also in the cytoplasm of squid neurons [386]. Furthermore, RNA

editing in cephalopods seems to depend on temperature, suggesting that it may be a mechanism to adapt to different environmental stressors by optimising protein sequence and function [387]. This unique regulation and the high extent of ADAR editing activity in the cephalopod nervous system imply that A-to-I editing in cephalopods has evolved quite differently from any other species.

Editing in non-coding regions

RNA editing by ADARs is not restricted to coding RNAs. The double-stranded structure of pri-miRNAs makes them excellent targets for A-to-I editing. Especially large miRNA clusters with a complex pri-miRNA structure may have an advantage, as nearby stem loops may act as editing inducer elements, that can help to recruit ADAR enzymes for efficient editing [388].

Multiple pri-miRNAs and their resulting mature miRNAs have been observed to be edited. Figure 7 depicts editing of pri-miRNAs by ADAR in the nucleus and its consequences.

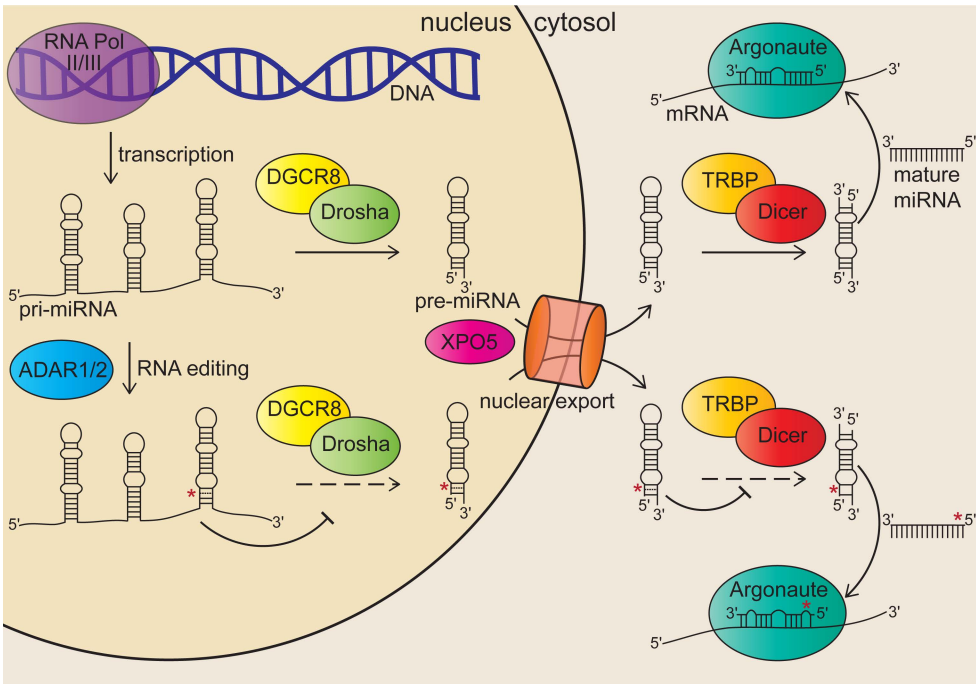

Figure 7.
Biogenesis and A-to-I editing of microRNAs. The miRNA biogenesis pathway is shown as in Figure 4. The pri-miRNA can be substrates for deamination by ADARs. In case of editing, subsequent maturation processes can be affected, altering levels of mature miRNAs; or the mature miRNA may bind a different set of targets.

Both ADAR1 p110 and ADAR2 can edit miRNAs, with somewhat different preferences, but nonetheless substantial overlap. Due to the different base pairing preferences of adenosine and inosine (Figure 6), A-to-I editing affects both the structure of the pri-miRNA as well as the function of the mature miRNA. As a result of the changed secondary structure of the pri-miRNA, editing can also result in a decreased processing efficiency by Drosha and/or Dicer [389]. Examples of this include the inhibition of Drosha cleavage by editing of miR-379, or the inhibition of Dicer processing by editing of pri-let7-g [389]. Some studies also describe an effect of editing on miRNA strand selection [390].

If a mature miRNA is produced, the altered sequence can potentially influence on target selection – especially when the seed region is affected by the editing site. In fact, it seems that, when comparing A-to-I editing sites across different positions along the miRNA, editing sites are enriched in the seed sequence [391].

For example, miR-376 editing redirects its target preference in the brain, with unedited miR-376 targeting threonine and tyrosine kinase (*TTK*) mRNA and edited miR-376 targeting phosphoribosyl pyrophosphate synthetase 1 (*PRPS1*) mRNA [392]. In melanoma, ADAR1 expression and therefore also miR-455 editing was reduced. While edited miR-455 would act like a tumour suppressor, the now predominant unedited miR-455 inhibited expression of the tumour suppressor cytoplasmic polyadenylation element binding protein 1 (CPEB1), and thereby promoted tumour growth [393]. Another example for which target redirection has been described is miR-379. Here, it was suggested that unedited miR-379 promotes tumour growth, whereas edited miR-379 had a tumour-suppressive effect. The authors observed that edited miR-379 targeted CD97, whereas unedited miR-379 targeted the FAK protein tyrosine kinase 2 (PTK2) [394].

Of note, editing the miRNA is not the only strategy to disrupt or enable miRNA regulation of mRNAs. Another possibility is the editing of the miRNA target site in the target mRNA [391]. In the *XIAP* mRNA, several miR-513a target sites are created upon editing in cancer [391, 395]. On the other hand, multiple miR-129 target sites are lost through editing in cancer [391].

A-to-I editing of other regulatory regions can also affect gene expression and protein sequence. Editing of pre-mRNAs can affect splicing by creating or destroying splice sites. For example, in rat *Adar2* mRNA, a more proximal 3′ splice acceptor site can be created by ADAR2 enzymes, changing AA to AI in order to mimic the AG of splice acceptor sites. This alternative splice site adds 47 nt and therefore causes a frameshift, demonstrating a fascinating feedback loop through which ADAR2 regulates its own expression [396]. A different example is alternative splicing of SH2 domain-containing tyrosine phosphatase PTPN6 (*SHP1*) mRNA. This putative tumour suppressor mRNA is subject to increased A-to-I editing in acute myeloid leukaemia, leading to the retention of an additional intron and thereby a nonsense transcript. The editing silences the A at the branch site 27 nt upstream of the 3′ splice acceptor site, thereby causing intron retention [397].

ADARs and A-to-I editing in cancer

Many of the examples in the previous section already allude to a larger phenomenon: A-to-I editing is deregulated on a global level in many cancers. In the majority of cancers, ADAR1 expression is increased [398]. The *ADAR1* gene is located on chromosome 1q, a region that is commonly amplified in cancer, leading to ADAR1 overexpression [399]. Furthermore, as ADAR1 p150 is induced by interferons, the inflammatory environment common to many cancers can contribute to elevated ADAR1 levels [400]. In contrast to ADAR1, ADAR2 is downregulated in most cancers. Overall, however, there is an increase in A-to-I editing in most cancers, except kidney cancers, for which there is decrease in editing [398, 401].

One consequence of increased ADAR1 expression and activity is of course an increase in editing of different editing sites in mRNAs and miRNAs. For example, some reports have suggested that alternative editing events of AR in prostate cancer may occur in castration conditions [402]. A good summary of several individual editing events that are known to play a role in cancer can be found in this review [403].

The strong increase in editing gives the tumour cells a welcome platform for diversification of their transcriptome and proteome beyond their DNA, and the option of multiple co-existing versions of the same protein [404]. All this is expected to support tumour heterogeneity and evolution even further. For example, it was shown that RNA editing could decrease drug sensitivity in a variety of cancer cell lines [401]. At the same time, extensive RNA editing and increased proteome diversity also bear the risk of increased immunogenicity, suggesting that tumours with high RNA editing levels would be good candidates for immunotherapy [405].

As previously mentioned, the majority of RNA editing sites are located in repetitive elements [360]; in addition to this, even in cancer, most cancer-specific editing events affected non-coding regions [401]. Hyperediting, mostly mediated by ADAR1, also occurs in cancer cells – and many cancer cell lines seem to depend on this effect. Especially cancer cell lines with relatively high expression of ISGs are dependent on ADAR1 activity for proliferation and survival [406-408]. ADAR1 may therefore be an interesting target for cancer therapy in the future.

Aims of the thesis

The initial aims of the thesis were to

1) identify and characterise miRNAs that can promote or suppress prostate cancer bone metastasis,

   and to

2) clarify their biological role in prostate cancer progression.

These aims are mainly addressed in papers I and II.

Upon identifying miR-379 as a potential tumour suppressor and learning that it can be subject to A-to-I editing, we further wanted to

3) develop a method for the reliable quantification of A-to-I-edited miRNA isoforms,

   and to

4) investigate whether and how A-to-I editing of miRNA-379 affects its biological function in prostate cancer cells.

These aims are the focus of papers III and IV.

The present investigation

Paper I: Regulation of cell-cell adhesion in prostate cancer cells by microRNA-96 through upregulation of E-Cadherin and EpCAM

Background

The aim of this project was to determine the role of miR-96 in prostate cancer bone metastasis. We wanted to identify relevant targets, and define the effect of miR-96 deregulation on the biological functions of prostate cancer cells. We knew that miR-96 was upregulated in prostate cancer, and based on an analysis of its expression levels in our own cohort of men with prostate cancer [291], we saw that miR-96 expression was associated with metastasis. This association was confirmed in a cohort of bone metastases that we received from collaborators in Umeå whereby miR-96 levels were increased in bone metastases compared to primary tumours. To elucidate the underlying mechanism, we screened for miR-96 targets in prostate cancer cells.

Summary

After miR-96 transfection of DU145 cells, AGO2 complexes were isolated by immunoprecipitation (IP) to see which mRNAs were bound to miR-96:RISCs. Microarray analysis was used to determine which transcripts were enriched. Upon pathway analysis, targets involved in cell-cell interactions were the most enriched. A look at the list of individual targets gave us both E-Cadherin and EpCAM among our top 25 hits. Using transfection with miR-96 mimics of prostate cancer cells and different downstream analyses, we could confirm that E-Cadherin and EpCAM protein and mRNA expression were indeed regulated in response to miR-96 in both DU145 and 22Rv1 cells. The nature of the effect was however surprising in that there was a positive correlation between miR-96 and its putative targets. A public cohort with miRNA and gene expression data confirmed the positive association between miR-96 and E-Cadherin and EpCAM expression also in patients.

To determine if these effects could really be mediated through direct interaction between miR-96 and the mRNA – as would be suggested by the fact that the targets

were identified in an AGO2-IP, based on physical interaction between the miRNA and the target – we searched for target sites along the mRNAs. For each mRNA, two potential target sites were predicted, and we used dual luciferase assays and target site blockers to narrow down the target site responsible for the regulation. For both mRNAs, we could identify one target site in the CDS that was responsible for the regulation. Blocking these sites with target site blockers could prevent the miR-96-mediuated upregulation of the proteins. I also tried using transient transfection of the prostate cancer cells with anti-miR-96. Interestingly, I did not observe the opposite, but rather what pointed towards a biphasic, dose-dependent effect. Blocking of miR-96 in DU145 cells, just like overexpression of miR-96, led to an increase in EpCAM protein. However, in the case of miR-96 inhibition, the effect was only seen on the protein level, and not on the mRNA level, implying a separate mechanism. It can be speculated that this effect is mediated through the other predicted target site on *EpCAM* mRNA, or through an indirect mechanism.

We also performed functional studies, finding that miR-96 increased the cells' potential to adhere to one another as well as to osteoblasts. Furthermore, miR-96-transfected cells formed more colonies in anchorage-independent assays.

High expression of E-Cadherin and EpCAM has previously been shown to promote prostate cancer bone metastasis [68, 69, 409]. We therefore propose that high levels of miR-96 promote the establishment of bone metastases by upregulating these two adhesion molecules.

Limitations of the study

In my opinion, the biggest limitation of the study is that fact that we did not perform any rescue experiments to prove that miR-96-based upregulation of E-Cadherin and EpCAM really is the cause for the observed increase in cell-cell adhesion and bone metastasis. There are also more potential targets that could contribute to the observed phenotype than just these two proteins. While we performed extensive literature research to build our mechanism for how the increase in E-Cadherin and EpCAM expression would contribute to metastasis, we did not perform any *in vivo* experiments to prove that it is the regulation of the cell:cell adhesion molecules that is responsible for the increased metastatic potential of miR-96-overexpessing cells.

Discussion

How can we explain the positive regulation of E-Cadherin and EpCAM by direct interaction of miR-96 with their mRNAs? As discussed, some mechanisms have been described, but they are mainly considered exceptions. On the other hand, it is possible that many more such "exceptions" exist, but due to the way gene expression analyses are often filtered when looking for miRNA targets – only considering repressed targets – many of these regulation events might remain undiscovered.

In the studies describing positive regulation of mRNA translation by miRNAs, the common factors are often the absence of GW182 proteins [257, 258, 260-262], which would normally mediate repression by miRNAs, and lack of mTOR activity to initiate canonical translation [263, 264]. Lower levels of GW182 are plausible, as we were able to show in Paper I that GW182 protein family transcripts were downregulated in metastatic prostate cancer. Furthermore, as miR-96 has been suggested to regulate mTOR activity [306], a change in canonical translation initiation upon miR-96 deregulation is possible as well.

However, these publications typically only observed an increased rate of translation rather than an effect on mRNA levels. In our study, on the other hand, we did observe an upregulation of mRNA also. This could be either through increased mRNA stability through an unknown mechanism, or an increase in transcription through another, additional mechanism. That there are likely two mechanisms at play – one through direct interaction of miR-96 with the target site and one additional mechanism that did not depend on the target site – is supported by the fact that target site blockers only partially alleviated the upregulation of E-Cadherin protein by miR-96.

A good candidate for a second mechanism has been extensively characterised in breast cancer cells: Together with its other family members, miR-96 can repress the transcription factor ZEB1 [309, 310, 410]. ZEB1 is normally responsible for the repression of E-Cadherin and EpCAM expression during EMT [411], so that its downregulation by miRNAs results in a re-expression of these epithelial adhesion molecules. ZEB1 also represses the expression of the miR-183-96-182 cluster to form a negative feedback loop [309, 310].

Increased E-Cadherin expression and promoter activity have also been observed in miR-96-transfected bladder and pancreatic cancer cells [412, 413]. Interestingly, similarly to our observations supporting an indirect mechanism of E-Cadherin upregulation in addition to the direct one we suggested, the authors of a study focusing on the expression of E-Cadherin in prostate cancer bone metastases noted evidence for a ZEB1-indedendent mechanism [69]. In Paper I, we may be describing that mechanism. A schematic of the two mechanisms that may govern miR-96-mediated epithelial adhesion molecule expression in parallel is found in Figure 8.

The observation of a biphasic effect of miR-96 on EpCAM expression is very interesting, and although it was just a side observation in this project, it is intriguing to speculate. As mentioned in the introduction, biphasic effects of miR-96 have been described for autophagy regulation, where both very low and very high levels of miR-96 inhibited autophagy, but it was enhanced at intermediate levels [306]. If EpCAM expression is lowest at an intermediate miR-96 concentration, then cells with continuously rising levels of miR-96 would first lose their EpCAM expression and later regain it. Seeing that EpCAM is frequently lost during EMT [61], but later needed to form prostate cancer bone metastases [409], the deregulation of a single miRNA could orchestrate for the cells to first invade and metastasise, and then to revert back to an epithelial phenotype to successfully colonise the secondary site.

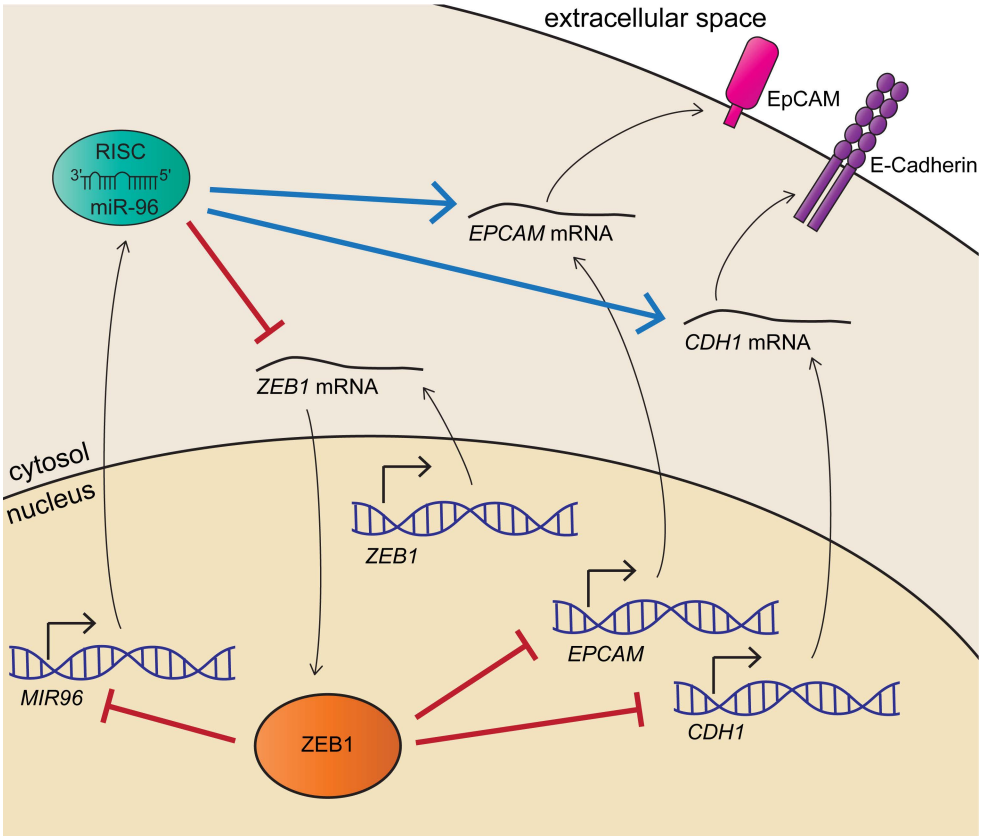

Figure 8.
Schematic depiction of the proposed mechanisms for upregulation of E-Cadherin and EpCAM by miR-96. The indirect ZEB1-dependent double-negative mechanism that has been described in the literature is drawn with red arrows. The direct mechanism mediated by direct interaction with the mRNA that was described by us in Paper I is drawn with blue arrows.

Since miR-96 has been described to be induced by TGF-β signalling [307, 413], we thought that this might provide an elegant mechanism for the microenvironmental control of this pathway. By that logic, the cancer cells that have just left the primary tumour and entered a new tissue site would encounter TGF-β in the bone matrix, leading to the upregulation of miR-96. This, in turn, would upregulate the adhesion molecules, and thereby initiate a program that would allow the cells to recover epithelial features and successfully colonise the bone. However, when I began to test this hypothesis *in vitro*, the cells did not upregulate miR-96 as a consequence of TGF-β treatment (data not shown). There was no increase in miR-96 expression upon treatment with osteoblast-conditioned media, which are predicted to contain a variety of other bone-secreted factors, either. This mechanism was therefore unlikely.

Instead, we now favour the hypothesis that out of all the cells that enter the bone marrow through the metastatic cascade, the cells that have inherently high miR-96 expression will be better at colonising the bone. This is supported by the fact that we found higher miR-96 expression in the primary tumours to be associated with successful metastasis, implying that the cells have acquired this high expression already in the primary tumour.

Paper II: Functional *in vivo* screening identifies microRNAs regulating metastatic dissemination of prostate cancer cells to bone marrow

Background

The goal of this project was to identify miRNAs that would normally suppress prostate cancer metastasis, with the aim of potentially finding novel therapeutic avenues. For this, we performed a screen with a library of anti-miRNAs that was transduced into the prostate cancer cell line PC3. In this pool of transduced cells, every cell would produce a different anti-miRNA. This cell pool was then injected orthotopically into mice (Figure 9a). We later identified the anti-miRNAs that were expressed in the cells that could expand to form a primary tumour and/or metastasise. The setup was designed with the clear goal of identifying tumour suppressor miRNAs, *i.e.*, the miRNAs whose loss would induce a phenotype.

Summary

Approximately one month after mice were injected with the cell pool, we harvested their prostates, lungs, livers, and femurs and performed DNA sequencing to identify any anti-miRNAs that were expressed. By analysing anti-miRNA expression, we could find clues as to which anti-miRNAs allowed the cells to expand well *in vivo* in the different microenvironments. The most abundant anti-miRNA in the primary tumours was anti-miR-493. Publications have described miR-493 as a tumour suppressor in prostate cancer [414]. In the lung metastases, anti-miR-23b was the most frequent anti-miRNA. Its target miR-23b has previously been described as a metastasis suppressor in prostate cancer [415, 416]. The most frequent anti-miRNA in liver metastases was anti-miR-135b. In prostate cancer, miR-135b has previously been described as a tumour suppressor that suppresses AR expression [417-419]. In bone metastases, anti-miR-379 was the most enriched anti-miRNA. Only one study on the function of miR-379 in prostate cancer has been carried out, and the authors concluded that it promoted metastasis [333].

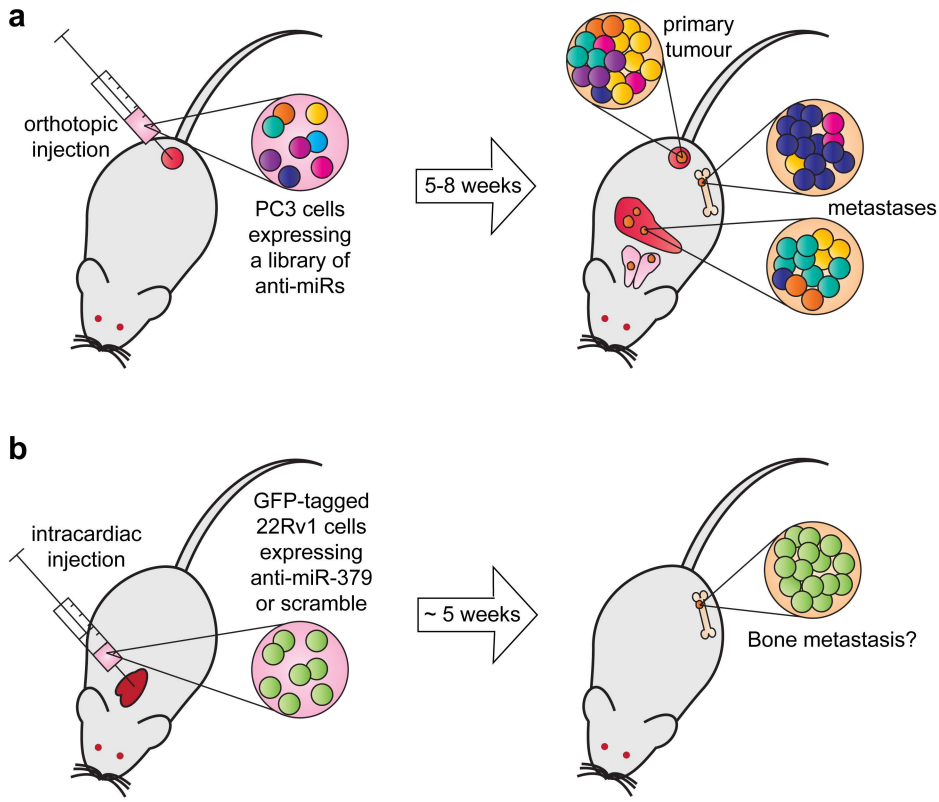

Figure 9.
Illustrations of the *in vivo* studies performed in Paper II. **a.** Orthotopic injection of PC3 cancer cells that were transduced with a library of anti-miRNAs. After 5-8 weeks, mice lost weight, and primary and metastatic tumours were harvested. DNA sequencing was performed on the tumours to determine which anti-miRNAs were enriched in the different lesions. **b.** Intracardiac injection of GFP-tagged 22Rv1 cells expressing either anti-miR-379 or a scrambled sequence. After approximately 5 weeks, mice lost weight, and organs were harvested. Bones were analysed for GFP-positive cells and metastases.

Apart from these analyses of anti-miRNA expression in the entire organ, we also analysed metastatic tumours individually in one liver to learn more about the intra- and intertumour heterogeneity in the metastases. Interestingly, the different metastatic tumours had different anti-miRNA profiles, and each individual metastasis contained multiple anti-miRNAs. This indicates that the metastatic tumours did not arise from single disseminated cells, but from multiple cells, which supports collective cell migration as a mechanism for metastasis [74-77].

As the bones are the most common site of distant metastases in prostate cancer patients, and the inhibition of miR-379 had not previously been described to inhibit prostate cancer bone metastasis, we focused on this miRNA. In a second *in vivo* experiment using the less metastatic 22Rv1 cell line, we performed intracardiac injections of GFP-tagged 22Rv1 cells that expressed either anti-miR-379 or

scramble (Figure 9b). All mice that received the anti-miR-379 cells developed bone metastases, whereas none of the mice injected with scramble cells had macroscopic bone tumours. The cells used for this experiment were clones isolated from 22Rv1 cells that had been transduced with the same anti-miRNA library. The clones were isolated in anchorage-independent colony formation assays, performed in normal or osteoblast-conditioned media. Strikingly, the number of clones of each anti-miRNA in these assays revealed that in osteoblast-conditioned media, anti-miR-379-expressing clones were the most enriched compared to the cell pool used for the experiment, paralleling the observation *in vivo*. Indeed, anti-miR-379-expressing 22Rv1 cells had a higher colony formation potential compared to scramble cells in bone-like but not in normal conditions. In subsequent functional *in vitro* experiments, anti-miR-379-expressing cells also displayed increased cell growth. No differences in their migratory potential were observed.

We also performed AGO2-IP experiments on anti-miR-379 and scramble 22Rv1 cells grown in normal or osteoblast-conditioned media. Sequencing revealed that the targets that were differentially associated with AGO2 differed vastly in normal and osteoblast-conditioned media. This underlines the influence of the mRNA pool present in a cell at a given time on which of these mRNAs can be targeted by a miRNA. In the bone-like conditions, the most enriched pathways included a variety of cell communication pathways such as Notch signalling, and responses to hormones and cytokines.

Lastly, in patient cohorts, we could show that miR-379 was expressed at lower levels in prostate cancer primary tumours and bone metastases.

Limitations of the study

One of the main issues with the use of the anti-miRNA library is that we cannot really evaluate how well the knockdowns work for each individual miRNA other than having to trust the manufacturer. Any studies on the library pool would be meaningless, and the isolation of individual clones for the purpose of evaluating the knockdown efficiency is simply not practical. It is therefore possible that, if some anti-miRNAs were inefficient, these miRNAs would not have been identified in the screen, because they were not efficiently inhibited in the first place.

In the second part of the paper, we did isolate individual clones of anti-miR-379-expressing 22Rv1 cells that would allow us to establish their knockdown efficiency. Simply measuring miR-379 levels would however not suffice; since anti-miRNAs often simply bind the miRNA instead of degrading it, the total levels of the miRNAs may remain the same. One would therefore have to assay the amount of active miRNA, for example by assessing how much miRNA is incorporated in RISCs (and therefore expected to be actively suppressing targets), or by using the expression of a known target as a proxy [420]. Another possibility to determine the amount of active miRNA in a cell would be to perform luciferase assays with one or multiple target sites for the miRNA of interest.

Another concern with anti-miRNAs is their specificity. We cannot know for sure that these anti-miRNAs really are specific. If an anti-miRNA binds another miRNA in addition to or instead of the miRNA it was designed for, it could cause us to attribute the effect to the wrong miRNA. This concern is especially valid for miRNAs that have other closely related family members, or that can exist in multiple isoforms. For example, miR-379 can be subject to A-to-I editing [389]; with the knowledge we currently have, we cannot know whether anti-miR-379 inhibits both of these isoforms with a similar efficiency, inhibits both at vastly different affinities, or is specific for one of the two isoforms.

Discussion

In Paper II, we found evidence that supports a role of miR-379 in the suppression of bone metastasis. This evidence was based on the finding that anti-miR-379 expression increased the capacity for bone metastasis in two cell lines *in vivo* and increased proliferation and colony formation *in vitro*. These results are seemingly at odds with a previous publication claiming that miR-379 and another miRNA from the same cluster, miR-154*, promoted bone metastasis [333]. In this publication, inhibition of miR-379 in mesenchymal ARCaP prostate cancer cells led to MET. In light of our findings and discussion for Paper I, anti-miR-379 inhibition-based MET could in fact drive the establishment of bone metastases due to the increased expression of epithelial markers, which have been shown to promote metastasis formation [68, 69]. Unfortunately, the authors performed *in vivo* evaluation only for miR-154*-inhibited ARCaP cells, but not with miR-379 inhibition [333]. The effect of the more epithelial phenotype on metastasis establishment in mice could therefore not be evaluated. As we, in turn, did not evaluate E-Cadherin expression and other epithelial features in our own miR-379-inhibited cells, we cannot compare these results either. The only thing at odds between the published study and our own study are therefore our interpretations of our different sets of evidence, rather than contradictions in the actual results.

Similarly, while we found that miR-379 was downregulated in prostate cancer and bone metastases, the authors of the published study did not show miR-379 levels in different tissue samples, but only showed survival data for patients based on miR-379 expression [333]. Gururajan *et al.* did not mention whether they also attempted to compare sample groups. But if they did, they would have reached the same conclusion as we did in our study: They, too, used the publicly available Taylor dataset [421] in which we found decreased expression of miR-379 in prostate tumours and their metastases compared to healthy prostates.

But what is the cause of this downregulation? There are multiple mechanisms through which miR-379 can be regulated. The miR-379-656 cluster has been shown to be regulated by the transcription factor MEF2 [312, 314-316], in addition to epigenetic mechanisms through promoter methylation [312, 314]. As briefly mentioned above, miR-379 is also subject to A-to-I editing. Editing of pri-miR-379

has been shown to inhibit miR-379 processing and maturation [389], so that it, too, may lead to a downregulation of mature miR-379 levels. The analyses performed here did not evaluate miR-379 isoform levels, and whether one or both of them were deregulated. Furthermore, the two editing isoforms of miR-379 may have different targets [394], so that understanding which isoform is involved will be important in order to pinpoint the underlying biological mechanisms.

Paper III: Quantification of microRNA editing using two-tailed RT-qPCR for improved biomarker discovery

Background

Now that we had understood that miR-379 downregulation might be a driving force behind prostate cancer development and metastasis, we wanted to understand how it is deregulated. As discussed, one potential mechanism is A-to-I editing of pri-miR-379 – therefore, we set out to investigate this possibility. Furthermore, measuring individual miR-379 editing isoforms and its editing frequency could give us information about which of the isoforms is deregulated in patients. This would allow us to speculate whether one or both isoforms are biologically relevant for prostate cancer bone metastasis.

However, the selection of a method to this end was difficult. RNA sequencing is expensive and cumbersome, and, as miR-379 is expressed at low levels, it is unlikely we would have been able to acquire enough high-quality reads for meaningful calculations and statistics. The alternative is RT-qPCR; but previous publications warned us that commercially available assays would not be able to resolve single nucleotide differences or distinguish between isoforms [422-424]. Indeed, when we tested the commercially available TaqMan assays, it became clear that these would not be suitable for our purposes (Figure 10a). We therefore set out to design a method that could distinguish between A-to-I-edited miRNA isoforms, based on highly specific two-tailed RT-qPCR assays that were described shortly before our study began [422].

Summary

The two-tailed RT-qPCR assays that I designed were highly specific, with relative detection of the non-target isoform below 1% (Figure 10b). The assays were also 1000-fold more sensitive than the commercially available reagents – this is very relevant for a miRNA like miR-379, which is expressed at low levels. In addition to the isoform-specific assays, I also designed assays for pan-miR-379, *i.e.*, assays that would recognise both isoforms equally well and quantify total miR-379 in a sample.

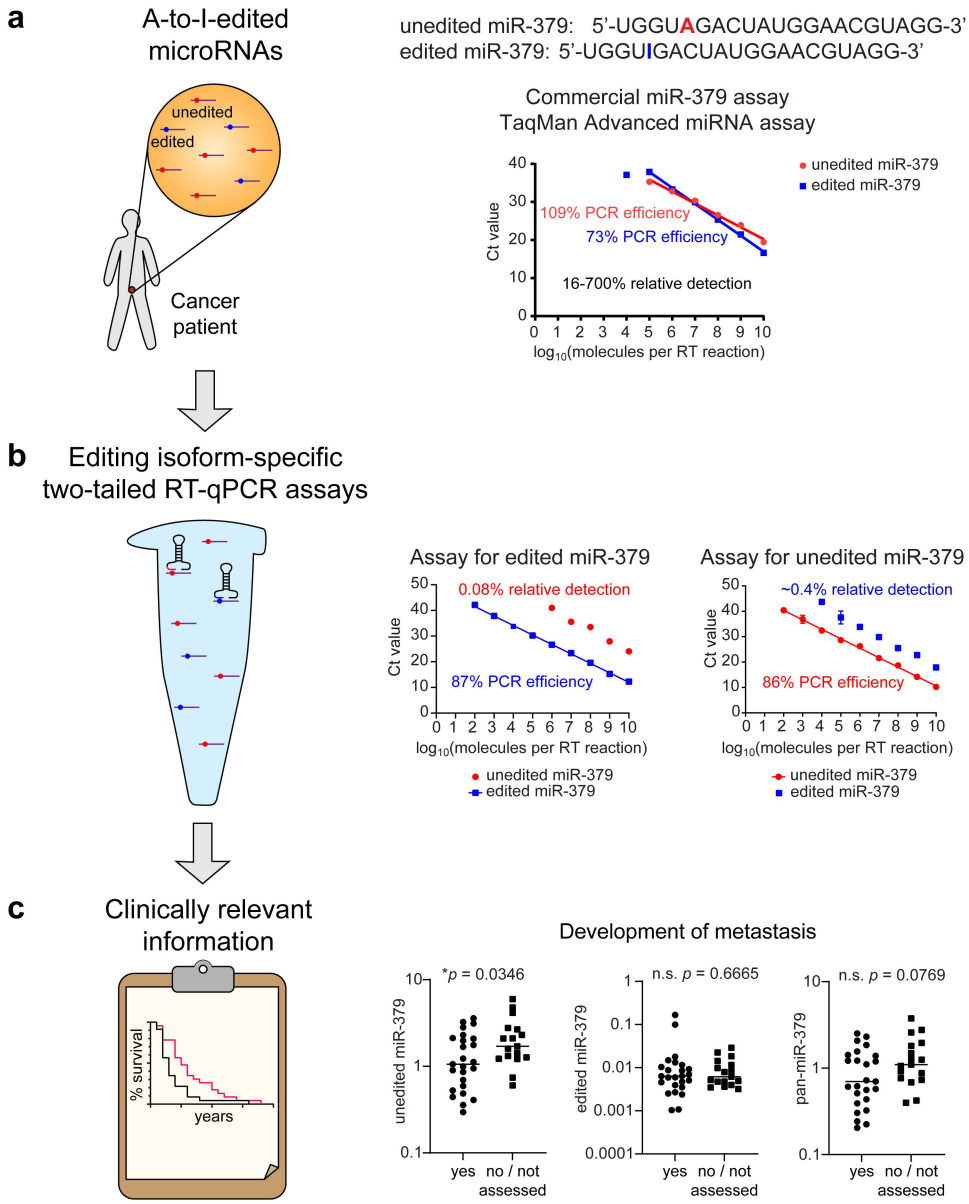

Figure 10.
Summary of Paper III. **a.** In patient samples, different miRNA isoforms co-exist. For example, miR-379 is edited at nt 5, which cannot be distinguished by commercially available miRNA assays. **b.** We have developed two-tailed RT-qPCR assays for miR-379 that can reliably distinguish between editing isoforms, with high specificity and sensitivity. **c.** In a patient cohort, miR-379 isoform expression was associated with clinically relevant parameters. Shown here are the data for prostate cancer metastasis, which was associated with lower expression of unedited miR-379, but not edited miR-379 or pan-miR-379.

I could confirm that these assays are indeed isoform-blind. Using artificial yeast RNA backgrounds and oligonucleotides of other miR-379 family members, I could also confirm that the presence of other RNA species did not confound the assay, and that the assays could distinguish miR-379 from its other close relatives.

The "original" protocol for two-tailed RT-qPCR assays is based on SYBR Green qPCR [422]. In order to open up the possibilities for other chemistries, which can be preferable in some contexts with a need for multiplexing or absolute quantification, I also adapted the assays for hydrolysis probe-based qPCR and digital PCR, albeit with a slight loss in sensitivity and specificity.

A possible application of the assays for *in vitro* research was demonstrated using ADAR-overexpressing PC3 cells. The cells were transduced with ADAR overexpression vectors, and miR-379 editing was measurably increased in the cells overexpressing catalytically active ADAR2.

In addition to the accurate quantification in cell lines, I could also measure miR-379 expression and editing levels in a variety of human tissue samples. In a comparison of the editing frequencies with published RNA sequencing-based data, the numbers matched the expected editing levels well. Both editing and expression of miR-379 were highest in brain tissues. The editing frequency in most non-brain tissues was below 3%.

Lastly, I tested the expression of each miR-379 isoform, total miR-379 and the miR-379 editing frequency in a patient cohort consisting of 23 patients with BPH and 47 patients with prostate cancer. Comparing BPH and prostate cancer, the editing frequency of miR-379 was statistically significantly higher in the cancer samples. Among the prostate cancer tissues, unedited miR-379 was downregulated in patients with metastasis (Figure 10c) and patients with castration-resistant prostate cancer. Edited miR-379 expression did not differ between these groups. Lower expression of unedited miR-379 was also associated with shorter overall survival. When assessing pan-miR-379, the differences between groups were sometimes statistically significant, sometimes not. The levels of pan-miR-379 are expected to differ, since levels of unedited miR-379 were changed, and this isoform made up the vast majority of total miR-379 in the samples. But since pan-miR-379 assays also measure edited miR-379, the presence of the other isoform can confound the analysis and potentially hide a true deregulation, as demonstrated by the lack of statistical significance in some of the comparisons.

Overall, our study demonstrated that the two-tailed RT-qPCR assays were suitable to distinguish and quantify miRNA isoforms, more specifically miR-379 editing isoforms, and that their usage can reveal information that would not have been resolved with promiscuous commercial assays.

Limitations of the study

In my view, the major limitation of this study lies in the patient data. Firstly, it would be good to confirm the findings in at least one more cohort, ideally a larger one. It

would also be interesting to study a cohort that includes bone metastasis samples, to determine if any further deregulation takes place in the metastases compared to the primary tumours. Furthermore, although the effect was statistically significant for many of the patient comparisons, the effect size was rather small, and there was a large overlap between the groups. This means that, on its own, miR-379 or its isoforms would not be a sufficient biomarker. Instead, it may be well-suited for usage in a larger panel of biomarkers.

It should be noted that there are probably many alternative and more suitable biomarkers than miR-379. We simply used it as a model to design assays that could quantify A-to-I-edited isoforms because we were already working on this miRNA, and because we hoped that the assays could help us answer questions in regards to the biological function of miR-379 in bone metastasis. The primary aim was to understand the deregulation of miR-379 and to study which of its isoforms is likely involved in prostate cancer bone metastasis – not to identify the ideal biomarker. We are however confident that we have developed a tool that could be used for this purpose in future biomarker discovery studies. As is discussed in more detail in Paper III, the two-tailed assays are easily adaptable for different miRNAs, and should therefore be applicable to a large range of miRNAs and diseases.

As for the question of studying the deregulation of miR-379, we only have the data for mature miR-379 editing. As pri-miRNAs are expressed at very low levels [390], and our RNA extraction method selected for small RNAs [425], it was not possible to study editing of pri-miR-379. This means that we cannot compare the editing levels of pri-miR-379 to those of mature miR-379, or determine how much less efficiently edited pri-miR-379 might be processed [389]. We also could not study alternative mechanisms for miR-379 deregulation, such as promoter methylation or deregulation of its transcription factor MEF2. An accessible proxy for the regulation of the entire cluster would be to quantify the other miRNAs that are contained in this cluster. If these are also deregulated and correlated with one another and miR-379, transcriptional regulation is likely.

Discussion

To summarise the biological findings, we could confirm that, just as it has been reported for other tissues [389], ADAR2 is the main editor of miR-379 in prostate cancer cells. In the patient cohort, we found that the miR-379 editing frequency was higher in prostate cancer tissues compared to BPH tissues. This is in line with TCGA-based analyses finding higher miR-379 editing levels in prostate cancer compared to normal tissues [391], and higher expression of ADAR2 in these samples [398].

In the prostate cancer samples, we found that unedited miR-379 was the isoform that was deregulated in aggressive prostate cancer, and not edited miR-379. This implies that loss of unedited miR-379 may be the more biologically relevant event, although of course this would have to be confirmed experimentally. It is possible

that the lower expression of unedited miR-379 is the result of higher ADAR2 editing activity, as the editing frequency and pan-miR-379 levels were negatively correlated with one another. This mechanism would be plausible based on the fact that pri-miR-379 editing inhibits its maturation and processing [389], which would effectively downregulate it.

Without any functional data, based only on the findings in the patient cohort in this study, it is also possible that the increased editing frequency in prostate cancer and the downregulation of unedited miR-379 in aggressive prostate cancer do not have a biological function. They could merely be the result of increased ADAR2 activity or a loss of miR-379-656 cluster transcription.

Based on these findings, we can now ask the following questions: Is there a biological function for miR-379 editing? If yes, is its function to change the expression of one isoform, or to switch between two functionally distinct isoforms?

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

Background

Starting from the research questions "Is there a biological function for miR-379 editing? If yes, is its function to change the expression of one isoform, or to switch between two functionally distinct isoforms?", we decided to design a setup that would scan a range of macroscopic biological functions. We had mimics of edited and unedited miR-379 custom-made to mimic the correct sequences – including the inosine nucleotide and its unique binding preferences – and transiently transfected four different prostate cancer cell lines with these. We then performed a range of functional *in vitro* assays. The four cell lines that we used and their defining characteristics, which will be discussed in the context of the findings, are listed in Table 2.

Table 2.
Four commonly used prostate cancer cell lines that were used in Project IV. AR status and androgen response are stated based on a systematic analysis by van Bokhoven *et al.* in 2003 [426].

Cell line	Patient	Origin	AR status	Androgen response	Reference
PC3	62-year old Caucasian male	bone metastasis	−	independent	[427]
DU145	69-year old Caucasian male	brain metastasis	−	independent	[428]
22Rv1	Serial transplantation in castrated mice of the CWR22 xenograft (originally derived from a primary prostate tumour)		+	sensitive	[429]
LNCaP	50-year old Caucasian male	lymph node metastasis	+	sensitive/dependent	[430]

Summary

Transfection of prostate cancer cells with miR-379 did show an effect on different biological functions, but to varying degrees. Interestingly, the effect differed based on which cell line was used: Androgen-independent PC3 and DU145 cells reacted to unedited miR-379 with increased cell growth, whereas androgen-sensitive 22Rv1 and LNCaP cells showed the opposite effect. Edited miR-379 showed similar trends as unedited miR-379, but the trends were not always statistically significant. The growth-suppressive effect of miR-379 transfection on 22Rv1 cells is in line with our findings in Paper II, which observed that miR-379 inhibition led to an increase in cell growth in this cell line.

In colony formation and migration assays, both miR-379 showed a trend towards increased migration compared to negative control cells. This was the case in PC3, DU145 and 22Rv1 cells. LNCaP cells had not been able to grow in colony formation assays, and we have not yet performed the migration assays with LNCaP cells.

We also assessed mRNA expression of different EMT and stemness markers. Multiple studies have suggested a role for miR-379 in the regulation of EMT and MET programs [320, 321, 333, 431-433]. We found no strong evidence for this, at least on the mRNA level. While there were effects of miR-379 transfection on the expression of many of the tested mRNAs (*CDH1*, *OCLN*, *VIM*), we did not find a general up- or downregulation of EMT or stemness markers.

Limitations of the study

The major limitation of this study is quite clearly the fact that we were relying on the use of transient mimics, which are only in the cell temporarily, and are also brought into the cell in a rather artificial manner. To understand the biological role of a miRNA, it would be best to study its function inside a biological organism, where the cell would encounter all the cell types and factors that it would normally interact with. The transient nature of mimics limits this possibility, as *in vivo* experiments typically take several weeks, and the mimics are diluted and degraded within days after transfection. If we wanted to study the effect of miR-379 in mice using mimics, we would have to re-administer the mimic at least weekly, either by direct injections or by systemic administration. An injectable tumour would have to be subcutaneous, so we would not be able to use our preferred models such as intracardiac injections or orthotopic models which more closely resemble the natural microenvironment of tumours. Furthermore, xenograft take rates for subcutaneous injections are relatively low [434]. Systemic miRNA delivery on the other hand – as exciting as it would be from a therapeutic standpoint – comes with its own pitfalls and weaknesses. These include challenges in delivering the miRNA at all, ensuring that the delivery method is specific enough to only reach the target cell, and the potential immunogenicity of miRNA therapies [341].

Stable overexpression would therefore be more suitable, and is done using short hairpin RNA (shRNA) constructs [435]. This works well for most miRNAs – as long as their sequence can be genomically encoded. In Paper IV, we briefly discuss that inosine cannot be genomically encoded, and that a substitution of the edited nucleotide by guanosine simply cannot recapitulate the precise affinities with which an inosine-containing miRNA would bind its targets. Here, I would like to take the opportunity to expand further on my thoughts about developing a system that could achieve isoform-specific stable overexpression of A-to-I-edited miRNAs.

Due to the double-stranded nature of shRNA precursors, it is in theory plausible that ADAR could bind and edit stably overexpressed miRNAs, although this has not been tested to my knowledge. As it has been shown that surrounding RNA structures like those present in polycistronic pri-miRNAs can support editing by acting as editing inducer elements [388], a single shRNA hairpin might be inefficient at recruiting ADAR.

It should be explored whether fusing one or multiple editing inducer elements to the shRNA could potentiate ADAR recruitment and induce editing of the shRNA. Alternatively, one could include an RNA aptamer in the shRNA construct that could recruit a co-expressed genetically modified ADAR with a matching domain or tag to induce a high level of editing. The adaptation of site-directed RNA editing technologies [436, 437] to recruit endogenous ADARs to edit miR-379 would also be of interest, either to edit an overexpressed miR-379 and thereby achieve overexpression of edited miR-379, or to edit endogenous miR-379 to change the miR-379 editing frequency while maintaining biological levels of the miRNA.

However, if this research direction were to prove that shRNA precursors really can be edited, this means that the overexpression of unedited miRNAs cannot be a given. In this case, it would be relevant to develop a system that is editing-resistant. Only if there is no editing of the shRNA, this expression system can be used for isoform-specific analysis of unedited miRNA.

While any of the above approaches would be time-consuming and require much optimisation, I do think that the miRNA field would benefit tremendously from having the option to stably overexpress miRNA editing isoforms.

Discussion

In this study, when we were looking for editing isoform-specific effects, we ended up uncovering AR status-specific effects. I want to briefly discuss each of these findings and put them into context.

One of the main aims of the project was to test whether the two miR-379 editing isoforms had different functions, as has been suggested by others before [394], and to build an explanation for the role of miR-379 editing in the prostate cancer cells. Xu *et al.* suggested that unedited miR-379 promoted cell growth in a range of different cancer cell lines – prostate cancer cells were not among them – and that, instead, edited miR-379 suppressed tumour growth [394]. Our findings only

partially align with this. We did find a growth-enhancing effect of unedited miR-379 in androgen-independent cell lines, which would match the findings in non-prostate cancer cell lines, as these are not expected to respond to androgens either. However, we did not see a strong tumour-suppressive effect of edited miR-379. In androgen-sensitive 22Rv1 cells, there was a reduction of cell growth upon edited miR-379 transfection, but this was the same effect as was seen for unedited miR-379.

In general, edited miR-379 seemed to have only mild effects. In most of the functional assays, edited miR-379-treated cells showed an effect that lay somewhere in between the effect of unedited miR-379 and negative control mimics. Relative to the negative control, edited miR-379 cells usually displayed a trend towards the same phenotype as unedited miR-379, or no phenotype. Based on the functional assays alone, I would therefore lean towards hypothesis 1 that we formulated in Paper IV, postulating that edited miR-379 either has no function or the same one as unedited miR-379. This suggests that the main purpose of miR-379 editing would be to lower the overall levels of miR-379 rather than to create edited miR-379.

When taking the gene expression panel into account, however, cells transfected with unedited or edited miR-379 differed more from one another. Especially in androgen-independent cells, edited miR-379 caused rather strong up- and downregulation of different transcripts. There is therefore likely some specific effect of edited miR-379, but we have not been able to pinpoint it in this study.

The finding that the effect of miR-379 overexpression on cell growth seemed to depend on the AR status of the cells also sparks a lot of new questions. It is certainly an aspect that needs to be considered if miR-379 should be even explored therapeutically, as it would very clearly point towards the need of selecting the correct patient population both to achieve benefits and to avoid detrimental effects in other patients. The potential impact of AR status also cautions us to blindly compare our findings with the previous study of miR-379 in prostate cancer metastasis by Gururajan *et al. [333]*: This study used ARCaP cells, which are inhibited by AR signalling [438]. If AR signalling does play a role in how miR-379 functions, as is implied in Paper IV, then ARCaP cells would be expected to react to miR-379 very differently. Importantly, we have not yet proven that it really is the AR status that determined the effect of miR-379 in the cells. It is a potential explanation that felt rather intuitive based on how we usually classify prostate cancer cell lines (see Table 2), but it could also be purely coincidental. This would therefore have to be supported either by adding more cell lines to the study, or by providing functional evidence.

In my opinion, studying the pathways that are deregulated by miR-379 and its isoforms in prostate cancer cells would be a good first step. Any transcripts that are present in one group of cell lines, but not the other, and deregulated upon miR-379 transfection, could be of interest. This could then be further tested by genetically manipulating the cell lines to mimic one another, for example by deleting or overexpressing AR, or any transcripts and regulators that emerged on the basis of the transcriptomic profile.

Conclusions & Future perspectives

The attentive reader will have noticed that the ”The present investigation” section ended just like it started: with a list of experiments that should be done. To quote Goethe’s Faust, it can indeed feel like we are “no wiser than before”. But really, what has happened is that our research questions can grow more and more refined, and we are asking different questions now than we were a few years ago. Just like one cell does not make a cancer, but relies on many other cells in its microenvironment, one discovery will not give us all the answers. This research was built on centuries of scientific and medical research, and will in turn only form a small contribution to our collective knowledge.

Simply put, we have shown experimental evidence for the involvement of two miRNAs, miR-96 and miR-379, in prostate cancer metastasis. We found that miR-96 enhanced bone metastasis formation and suggested that this might be due to an increase in cell-cell adhesion in these cells. We identified miR-379 as a suppressor of prostate cancer bone metastasis based on an *in vivo* screen. After developing a qPCR method that could distinguish between A-to-I-edited miRNA isoforms, we identified unedited miR-379 as the isoform that is deregulated in patients with metastatic prostate cancer. In functional studies in cell lines, we found that this isoform had a different effect on cell growth in different cell lines, presumably based on AR status.

All of these rather tangible results open up many new research avenues. The biggest interest for translational research will lie in investigating these miRNAs for therapeutic options. For miR-379, this will require quite a lot of effort to establish which factors and expression profiles determine the outcome of miR-379 addition. Furthermore, we will need to identify more specific pathways that are regulated by this miRNA. In addition, the work on identifying the functions of the different editing isoforms must continue so that the right function is attributed to the right molecule. I believe that for miR-96, the goal would be a lot closer. Extensive research has identified many of its targets and regulated pathways in a multitude of tissues and cancers. Moreover, a small molecule inhibitor for miR-96 has already been described, which might avoid some of the difficulties that the field of miRNA therapeutics is still faced with. However, potential biphasic effects must be clarified in order to ensure that we know where the therapeutic window lies, and find a way to not go beyond this concentration range

But also in regard to biomarker research, this thesis provides some future avenues. Both miR-96 and miR-379, as well as their family members, have already been

described to be deregulated in cancer by many studies and suggested as biomarkers. Our findings in patient cohorts confirm this notion. Furthermore, with the RT-qPCR assays that we describe in Paper III, we hope that biomarker research and discovery can become more refined. Using editing isoform-specific assays should both give more specific information about which molecule is being assayed, thereby contributing to better reproducibility, and help in identifying biomarkers that have been overlooked so far because they may have been masked by other isoforms that were erroneously quantified.

For me, personally, this understanding that miRNA isoforms can make a difference was the biggest paradigm shift. It has been eye-opening to be able to actively contribute to research on RNA editing isoforms and to see for myself that considering these isoforms in a cohort analysis can give us more information than we would have gotten before.

But other aspects of the thesis also challenge established paradigms: In Paper I, we found that miR-96 upregulated two of its target mRNAs, and we provided evidence that this was at least in part mediated through a direct effect. The mechanism behind this will need to be further studied and defined, but the findings do remind us of the fact that miRNAs can act through more than one mechanism, and we are possibly underappreciating some of these mechanisms. It is also important to consider the possibility of positive regulation by miRNAs when performing large-scale studies or generating hypotheses based on transcriptomic data.

Overall, the work described in this thesis has contributed to our understanding of miRNA function in prostate cancer bone metastasis. Beyond that, we can take away lessons for how we think about miRNAs and use this knowledge to further improve the research quality in this field in the future.

Popular scientific summary

Prostate cancer affects mostly older men, and receiving a diagnosis does not at all have to be a death sentence. The cancer is often slow-growing, and if it is detected early, most patients will either be cured, or might not even need treatment at all. However, some patients do have aggressive disease, and the cancer can spread through their bodies in a process called metastasis. Therefore, when diagnosing a patient and deciding on how to manage the cancer, the clinician needs two tools:

1) a way to know whether the cancer is likely to become aggressive

2) treatment options that can better prevent and treat metastatic cancer

The work in this thesis is focused on a group of molecules that can do both – microRNAs. These molecules are short chains of RNA letters that all of our cells normally produce, and that help the cell decide which genetic plans should be executed, and which best remain silent. In the cookbook of life, we can imagine them as the little post-it notes that we might stick onto the recipes we liked a lot, want to try in the future, or didn't like at all – in fact, microRNAs are picky eaters, and in most cases, they will tell us which recipes we shouldn't cook. Just like having our post-it notes and recipes in order helps us keep a balanced diet (and maybe whip up something fancy in case of unexpected guests), microRNAs are important for our cells to function correctly. They help them grow at exactly the right rate, produce exactly the right compounds, and maintain a healthy relationship with their cellular neighbours. In cancer, we often find an imbalance of these microRNAs, which can cause our cells to grow uncontrollably and leave their tissue of origin.

In the first two papers of the thesis, we studied two such microRNAs that are produced at the wrong levels in cancer. In paper I, our focus was on miR-96: Previous work in our lab and other labs has found that miR-96 can suppress the signals that tell our cells to stop growing. When the cells have too much miR-96 – which is commonly the case in prostate cancer – they will therefore grow much faster than the normal rate, and form a tumour. In this paper, we studied the fact that miR-96 can also help the cancer spread. In patient samples, the levels of miR-96 were even higher in tumours that had spread to the bone. Then we performed an experiment that let us look at all the messages that miR-96 bound to – all the recipes that had a miR-96 post-it stuck to them. Among these, there were two messages that code for sticky cell surface proteins, E-Cadherin and EpCAM, that help the cancer cells stick together with their neighbour cells. We found that in this case, miR-96 encouraged the cells to make more of these proteins. Cells that had higher levels of miR-96 were indeed especially good at sticking both to one another and to bone

cells. They were also better at forming small tumours ("colonies"). We therefore came to the conclusion that miR-96 supports the formation of metastatic tumours in the bone by increasing the production of sticky E-Cadherin and EpCAM proteins in the cell.

In Paper II, we instead looked for microRNAs that would normally suppress prostate cancer metastasis. For this, we performed a screening in mice: We took a mixture of cancer cells, and in each cell, a different microRNA was inactivated. These cells were then injected into the prostates of mice, and after a few weeks, we isolated the tumours that had formed. We looked at metastatic tumours in the lungs, liver and bone, as well as the "original" tumours in the prostate, and determined which microRNAs were inactivated in each one. We found that in cells that had metastasised to the bone, miR-379 had been inactivated. This suggests that miR-379 normally prevents bone metastasis, but when it is removed, the cell is able to metastasise. When we performed the same experiment in a lab dish, the cells in which miR-379 had been inactivated were once more the ones that formed the most colonies in conditions that mimicked the bone environment. We also found that blocking miR-379 caused the cells to grow faster. In patient samples, the levels of miR-379 were lower in tumours that had spread to the bone compared to tumours that were growing in the prostate.

Around this time, we became aware that miR-379 exists in more than one version. There are enzymes in the cell that can chemically modify microRNAs and other genetic messages, changing the RNA letters that define them. In the case of miR-379, one letter is changed, and this could potentially make a difference in whether or not it can recognise and police certain messages, or whether it is produced at all. Unfortunately, there are not many methods that allow us to quickly check which version of the post-it we are looking at. So, when we wanted to ask which miR-379 version is lacking from the metastatic cancer cells, we didn't have the tools to do so. Therefore, in Paper III, we developed a method that was precise enough to recognise the subtle differences between the different versions of miR-379. Using the new method, we found that a higher proportion of miR-379 molecules was modified ("edited") in cancer cells compared to benign prostate cells, and that in patients that had metastatic or treatment-resistant disease, the "original" unedited miR-379 was lacking, whereas the levels of edited miR-379 were the same. Patients with lower levels of unedited miR-379 also died sooner than men with higher levels. We therefore concluded that unedited miR-379 is important to prevent prostate cancer metastasis to the bones.

This, however, could not answer our question whether unedited and edited miR-379 had different functions. Would these two have the same taste in food and stick to the same recipes? In Paper IV, we wanted to answer that question by adding either unedited or edited miR-379 to prostate cancer cells in a lab dish. We did find some differences, but the function also depended on which type of prostate cancer cells we used. How well a meal is received always depends on who eats it after all. In cells that are sensitive to the stimulation by hormones – this is often the case in

early-stage prostate cancer – unedited miR-379 could reduce the cell growth. Instead, in cells that do not react to hormones – these are thought to be a model for more aggressive, treatment-resistant prostate cancer – unedited miR-379 instead promoted cell growth. Edited miR-379 wasn't as consistent in producing an effect, and no clear pattern was visible. The different effect in different cell lines is both interesting and concerning – if the function of miR-379 can be the opposite in different stages of prostate cancer or in different patients, it is important to understand what determines this difference before moving the findings towards the clinic. More studies on this research question will therefore be needed in the future.

Overall, this thesis shows new avenues for prostate cancer research. We characterised two different microRNAs and their effects on prostate cancer cells and the metastatic process, and developed a new method that can distinguish between highly similar microRNAs. Understanding the role of microRNAs in prostate cancer may eventually lead to the development of new treatments, such as blocking miR-96. Furthermore, as many microRNAs are edited in our cells, the method from Paper III could be useful for many other researchers wanting to study microRNAs in cancer or in other diseases.

Populärvetenskaplig sammanfattning

Prostatacancer drabbar för det mesta äldre män, och att få diagnosen behöver inte alls leda till en säker död. Cancern växer oftast långsamt, och om den hittas tidigt, kan de flesta patienter antingen läkas, eller slippa behandling helt. Men några av patienterna har ändå en aggressiv cancer, och cancern kan då spridas igenom kroppen, så kallad metastasering. När en patient diagnostiseras och det ska bestämmas en plan för hur cancern ska hanteras är det därför viktigt att läkaren har två verktyg:

1) möjligheten att veta om cancern är aggressiv
2) terapier som kan förhindra eller bota metastatisk cancer

Arbetet som presenteras i denna avhandling fokuserar på en grupp molekyler som kan göra både och – mikroRNA. Dessa molekyler består av korta kedjor RNA-bokstäver som alla våra celler producerar, och som hjälper cellen att bestämma vilka genetiska planer som ska utföras, och vilka som ska tystas. I livets kokbok kan vi föreställa oss mikroRNA som de små klistermärken som vi kanske klistrar på de recepten som vi tycker om, vill prova i framtiden, eller inte gillar alls… MikroRNA är faktiskt rätt så kräsna, och för det mesta så säger de bara till oss vilka recept vi inte ska laga. Att ha våra post-it lappar och recept i ordning hjälper oss att äta en balanserad kost (och kanske att piska ihop något gott till spontana besökare), och precis på samma sätt så ser mikroRNA till att våra celler fungerar rätt. De hjälper cellerna att växa med precis rätt hastighet, att producera precis rätta ämnen, och att behålla en bra relation till granncellerna. När det gäller cancer, så är mikroRNA oftast obalanserade, vilket kan få våra celler att växa på ett okontrollerbart sätt och att lämna vävnaden de växer i.

I de första två delarbeten av avhandlingen undersökte vi två mikroRNAs som produceras i fel mängder i cancerceller. Delarbete I fokuserar på miR-96: Tidigare arbete i vårt labb har visat att miR-96 kan blockera signaler som säger till våra celler att växa mer. Om cellerna har för mycket miR-96 – vilket vanligtvis är fallet i prostatacancer – kommer cellerna att växa mycket snabbare än normalt, och en tumör bildas. I delarbete I undersökte vi hur miR-96 kan hjälpa cancern at sprida sig i kroppen. Mängden av miR-96 var mycket högre i prover från patienter med tumörer som hade spridit sig till skelettet. Sedan utförde vi ett experiment där vi tittade på alla genetiska budskap som miR-96 band till – alla recept där det klistrats en miR-96 lapp. Bland dem fanns det två genetiska budskap som kodar för klistriga proteiner på cellytan, E-Cadherin och EpCAM, som hjälper cellerna att fästa vid granncellerna. Det visade sig i det här fallet att miR-96 sade till cellerna att

producera mer av dessa proteiner. Celler som hade stora mängder av miR-96 var därför särskilt bra på att fästa både vid varandra och vid benceller. De var också bättre på att bilda små tumörväxter (”kolonier”). Därför kom vi till slutsatsen att miR-96 stödjer bildningen av metastatiska tumörer i benen genom att höja cellernas produktion av klistriga E-Cadherin och EpCAM proteiner.

I delarbete II tittade vi istället på mikroRNA som normalt skulle hindra metastaseringen av prostatacancer. Till detta ändamål utförde vi en screening i möss: Vi tog en blandning av celler där ett mikroRNA hade inaktiverats i varje cell. Cellerna injicerades i prostatan på mössen, och efter några veckor isolerade vi de tumörer som hade bildats. Vi tittade på metastaser i lungorna, levern och benen, och ”ursprungliga” tumören, och konstaterade vilka mikroRNAs som hade inaktiverats. Vi såg att miR-379 hade inaktiverats i celler som hade metastaserat till ben. Det tyder på att miR-379 normalt sett hindrar benmetastasering, men om den tas bort, kan cellerna metastasera. När vi utförde samma experiment i petriskål insåg vi att det var cellerna med inaktiverad miR-379 som bildade fler kolonier i omgivningar som liknade benmiljön. Vi såg också att miR-379 inaktivering fick cellerna att växa snabbare. I patientprover var mängden av miR-379 lägre i tumörer som spridits till ben i jämförelse med de tumörer som växte i prostatan.

Sedan blev vi uppmärksamma på att det finns mer än en version av miR-379. Det finns enzymer som kemiskt kan modifiera mikroRNA och andra genetiska budskap och på så sätt förändrar RNA-bokstäverna som definierar dem. I fallet för miR-379 förändras en bokstav, vilket kan göra skillnad för vilka genetiska budskap den känner igen och dirigerar, och det kan även leda till att mikroRNAt inte produceras alls. Tyvärr finns det inte många metoder som låter oss kolla på vilken version av post-it lappen vi ser. När vi ville undersöka vilken av miR-379 versionerna det är som saknades hade vi därför inte de verktyg vi behövde. Därför utvecklade vi en metod i delarbete III som var exakt nog för att känna igen subtila skillnader mellan de olika versionerna av miR-379. Vi använde sedan den nya metoden och såg att en högre andel av miR-379 var modifierad (”editerad”) i prostatacancer jämfört med en normal prostata, och att oeditерad miR-379 saknades i patienter med metastatisk eller behandlingsresistent cancer. Patienter med låga miR-379-mängder dog också tidigare än män med större mängder. Vi kom därför till slutsatsen att oeditерad miR-379 är viktig för att förhindra benmetastasering av prostatacancer.

Det kunde dock inte besvara frågan om oeditерad eller editerad miR-379 kunde ha olika funktioner. Skulle de två ha samma smak i mat och fästa till samma recept? I delarbete IV ville vi svara på denna fråga genom att tillsätta oeditерad eller editerad miR-379 till prostatacancerceller i en petriskål. Vi såg några skillnader, men effekten berodde också på vilken typ av prostatacancerceller vi använde. Om det smakar gott beror ju ändå lite på vem det är som äter. I celler som är känsliga för stimulering med hormoner – det är oftast fallet i tidiga stadier av prostatacancer – ledde miR-379 till en reduktion i celltillväxt. Men i celler som inte reagerar på hormoner – dessa anses vara en modell för aggressiv behandlingsresistent prostatacancer – ledde oeditерad miR-379 istället till ökad celltillväxt. Editerad

miR-379 visade ingen enhetlig effekt, och det fanns inget tydligt mönster. Att vi såg olika effekter i olika cellinjer är både intressant och oroväckande – om funktionen av miR-379 kan vara motsatt i olika cancerstadier eller olika patienter är det viktigt att vi förstår vad skillnaden beror på innan vi använder miR-379 i kliniska sammanhang. Det kommer därför behövas flera studier om detta i framtiden.

Sammanfattningsvis visar denna avhandling på nya vägar i prostatacancerforskningen. Vi karakteriserade två olika mikroRNA och deras effekter på prostatacancerceller och den metastatiska processen, och utvecklade en ny metod som kan skilja mellan snarlika mikroRNAn. Att förstå rollen av mikroRNA i prostatacancer kan leda till utvecklingen av nya terapier, såsom att blockera miR-96. Utöver det så vet vi att många mikroRNA är editerade i våra celler, och metoden i delarbete III kan därför vara till nytta för många andra forskare som väljer att utforska mikroRNA i cancer eller andra sjukdomar.

Populärwissenschaftliche Zusammenfassung

Prostatakrebs betrifft vor allem ältere Männer; dennoch ist die Diagnose noch keineswegs ein Todesurteil. Der Krebs wächst oft langsam, und solange er früh erkannt wird, können die meisten Patienten entweder geheilt werden, oder sie müssen noch nicht einmal behandelt werden. Allerdings haben einige Patienten dennoch einen aggressiven Krankheitsverlauf, und der Krebs kann sich durch so genannte Metastasierung im Körper ausbreiten. Daher müssen bei der Diagnose und Entscheidung über weitere Behandlungen zwei Werkzeuge zur Verfügung stehen:

1) Methoden, um einen aggressiven Krankheitsverlauf schon früh zu erkennen
2) Therapien, die metastasierten Krebs besser verhindern und behandeln können.

Die Arbeit in dieser Dissertationsschrift handelt vor allem von einer Gruppe von Molekülen, die beides kann – microRNAs. Diese Moleküle sind kurze Ketten aus RNA-Buchstaben, die unsere Zellen auch unter normalen Umständen herstellen, und die der Zelle bei der Entscheidung helfen, welche genetischen Pläne ausgeführt werden sollen, und welcher besser stillliegen sollen. Im Kochbuch des Lebens können wir sie uns wie die kleinen Klebezettel vorstellen, mit denen wir vielleicht die Rezepte markieren, die uns gut geschmeckt haben, die wir mal probieren wollen, die uns gar nicht gefallen haben… Tatsächlich sind microRNAs mit dem Essen ziemlich mäkelig, und sagen uns in den meisten Fällen nur, dass wir ein Rezept besser nicht kochen sollten. Und genau wie uns die Klebezettel und Rezepte dabei helfen, uns ausgewogen zu ernähren (und vielleicht was Schnelles zu zaubern, wenn unangemeldeter Besuch vor der Tür steht), helfen microRNAs der Zelle dabei, richtig zu funktionieren. Sie sorgen dafür, dass die Zelle genau im richtigen Tempo wächst, dass sie die richtigen Stoffe herstellt, und dass sie eine gute Beziehung mit ihrer Zellnachbarschaft pflegt. In Krebszellen sind diese microRNAs oft unausgewogen, was dazu führen kann, dass die Zellen unkontrolliert wachsen und das heimische Gewebe verlassen können.

In den ersten beiden Projekten dieser Arbeit haben wir zwei microRNAs untersucht, die in Krebszellen in den falschen Mengen produziert werden. In Projekt I lag der Fokus auf miR-96: Vorherige Studien in unserem und anderen Laboren haben herausgefunden, dass miR-96 die Signale unterdrücken kann, die unseren Zellen sonst sagen, dass sie nicht mehr wachsen sollen. Wenn die Zellen zu viel miR-96 enthalten – was bei Prostatakrebs häufig der Fall ist – wachsen sie daher viel schneller als sonst und bilden ein Geschwür. In diesem Projekt haben wir uns

mit der Tatsache beschäftigt, dass miR-96 auch die Metastasenbildung verstärken kann. In Patientenproben konnten wir nachweisen, dass die miR-96-Level höher waren in Tumoren, die in die Knochen gestreut haben. Dann haben wir ein Experiment durchgeführt, um herauszufinden, welche genetischen Botschaften von miR-96 erkannt werden – alle Rezepte mit einem miR-96-Zettel. Darunter waren unter anderem zwei Botschaften, die für klebrige Proteine auf der Zelloberfläche kodieren, E-Cadherin und EpCAM, welche den Krebszellen dabei helfen, sich an ihren Nachbarzellen festzuhalten. In diesem Fall konnten wir feststellen, dass miR-96 die Zellen dabei unterstützt hat, noch mehr von diesen klebrigen Proteinen herzustellen. Zellen mit hohem miR-96-Level konnten daher besonders gut aneinander und auch an Knochenzellen festkleben. Außerdem konnten sie besser kleine Geschwüre formen („Kolonien"). Wir sind daher zu dem Schluss gekommen, dass miR-96 die Bildung von Knochenmetastasen dadurch unterstützt, dass die Herstellung von klebrigen E-Cadherin- und EpCAM-Proteinen angetrieben wird.

In Projekt II haben wir uns stattdessen mit microRNAs beschäftigt, die Prostatakrebsmetastasen normalerweise unterdrücken würden. Dafür haben wir ein *Screening* in Mäusen durchgeführt: Wir haben eine Mischung von Krebszellen genommen, und in jeder Zelle eine andere microRNA inaktiviert. Diese Zellen haben wir dann in die Vorsteherdrüsen von Mäusen verpflanzt und nach ein paar Wochen die Tumore entnommen, die in der Zwischenzeit entstanden waren. Wir haben die Metastasen in der Lunge, der Leber und in den Knochen untersucht, und außerdem das „ursprüngliche" Geschwür in der Prostata. Wir konnten feststellen, dass in den Zellen, die Knochenmetastasen geformt haben, miR-379 inaktiviert worden war. Das legt nahe, dass miR-379 normalerweise Knochenmetastasen verhindern würde; wird miR-379 jedoch entfernt, kann die Zelle metastasieren. Als wir das gleiche Experiment in der Petrischale durchgeführt haben, konnten wir ebenfalls beobachten, dass Zellen mit inaktivierter miR-379 in knochenähnlichen Bedingungen mehr Kolonien geformt haben. Ferner haben wir festgestellt, dass das Blockieren von miR-379 auch zu einem schnelleren Zellwachstum geführt hat. In Patientenproben waren die miR-379-Level in Knochenmetastasen niedriger als in Prostatatumoren.

Ungefähr um diese Zeit herum wurden wir darauf aufmerksam, dass miR-379 in unterschiedlichen Versionen vorkommt. Es gibt Enzyme in unseren Zellen, die microRNAs und andere genetische Nachrichten chemisch modifizieren und dadurch die RNA-Buchstaben der microRNA verändern können. Im Falle von miR-379 wird nur ein einziger Buchstabe verändert, aber schon das kann möglicherweise einen Unterschied machen und dazu führen, dass einige Botschaften nicht erkannt oder kontrolliert werden; oder, dass die microRNA überhaupt nicht produziert wird. Leider gibt es nicht viele Methoden, mit denen wir schnell im Detail nachschauen könnten, welches Klebezettelchen da nun gerade klebt. Als wir also herausfinden wollten, welche miR-379-Version denn nun im streuenden Krebs eigentlich fehlt, fehlten uns die nötigen Werkzeuge dafür. Daher haben wir in Projekt III eine Methode entwickelt, die präzise genug ist, um subtile Unterschiede zwischen den

beiden Versionen von miR-379 zu erkennen. Mit der neuen Methode konnten wir herausfinden, dass in Krebszellen ein größerer Anteil an miR-379 verändert („editiert“) war im Vergleich zu gesunden Prostatazellen, und dass bei Patienten mit metastasiertem oder behandlungsresistentem Krebs die uneditierte Form von miR-379 fehlte, aber die Level der editierten Form unverändert blieben. Daraus folgerten wir, dass uneditierte miR-379 eine wichtige Rolle dabei spielt, Knochenmetastasen zu verhindern.

All dies kann jedoch nicht erläutern, ob uneditierte und editierte miR-379 unterschiedliche Funktionen haben könnten. Haben die beiden wohl den gleichen Geschmack und würden sich die gleichen Rezepte aussuchen? In Projekt IV wollten wir diese Frage beantworten, indem wir Prostatakrebszellen in einer Petrischale entweder mit uneditierter oder editierter miR-379 behandelt haben. Wir haben einige Unterschiede bemerkt, die Funktion hing jedoch auch davon ab, welche Sorte Prostatakrebszellen beobachtet wurde. Wie gut eine Mahlzeit schmeckt, hängt nun mal auch immer davon ab, wer da gerade isst. In Zellen, die für Hormonbehandlung empfindlich waren – dies sind oft Zellen in einem frühen Prostatakrebsstadium – konnte uneditierte miR-379 das Zellwachstum beschränken. In Zellen, die auf eine Hormonbehandlung nicht reagieren – diese Zellen werden meist als Modell für aggressive, behandlungsresistenten Prostatakrebs verwendet – führte uneditiere miR-379 hingegen zu einem Wachstumsanstieg. Die editierte Version von miR-379 zeigte relativ unbeständige, unklare Ergebnisse. Die unterschiedlichen Effekte in verschiedenen Zelllinien sind sowohl interessant als auch bedenklich – falls miR-379 in unterschiedlichen Krebsstadien oder in unterschiedlichen Patienten die entgegengesetzte Funktion haben kann, ist es wichtig zu verstehen, wie es zu diesem Unterschied kommt, bevor es zu einem Einsatz in der Klinik kommen kann. Mehr Forschung wird dieser Frage in der Zukunft nachgehen müssen.

Insgesamt zeigt diese Dissertation mehrere neue Wege für die Prostatakrebsforschung auf. Wir haben zwei verschiedene microRNAs und deren Effekte auf Prostatakrebszellen und die Metastasierungskette beschrieben, und eine neue Methode entwickelt, die zwischen zwei sehr ähnlichen Versionen der gleichen microRNA unterscheidet. Das Verständnis der Rolle von microRNAs in Prostatakrebs könnte irgendwann zur Entwicklung neuer Therapien führen, z.B. durch Blockieren von miR-96. Des Weiteren werden in unseren Zellen zahlreiche microRNAs editiert, sodass die Methode aus Projekt III auch für andere Forscher*innen von Nutzen sein könnte, die microRNAs in Krebs oder in anderen Krankheiten untersuchen möchten.

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