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To my family and friends

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ABSTRACT

A significant proportion of men develop prostate cancer (PrCa) during their lifetime, which causes challenges for public health. PSA (prostate-specific antigen) screening studies have shown that testing all men at specific ages causes overdiagnosis of PrCa and unnecessary treatments for individuals who have indolent disease. Additional methods are required alongside PSA testing to be able to prognosticate the disease outcome and to focus the treatments on aggressive PrCa cases. The chromosomal location 2q37.3, where the transmembrane protein coding gene anoctamin 7 (*ANO7)* resides, is associated with PrCa susceptibility in linkage analyses and genome-wide association studies (GWAS). In addition, *ANO7* has been considered to be expressed specifically in prostate tissue, and it has been suggested as a target for PrCa immunotherapy.

This thesis work concentrates on investigating the role of ANO7 in PrCa. *ANO7* expression was significantly higher in prostate tissue than in any other tissue type, and *ANO7* expression was elevated in PrCa compared to benign tissue. Moreover, elevated expression was associated with poor survival among patients. Next-generation sequencing (NGS) analysis of the *ANO7* gene revealed possible truncating rare germline variants, which were found only in PrCa patients and not in controls in the initial screening. These variants were genotyped from several different PrCa patient cohorts and sets of unaffected males. The stop-gain variant in exon 1 was associated with poor survival, and the variant in intron/exon 4 was associated with aggressive disease. Because the intron/exon 4 variant was related to aggressive cancer but not to poor survival, we investigated whether the variant carriers would have a good response to docetaxel treatment in castration-resistant PrCa. As was hypothesized, the variant carriers had a better response to docetaxel than the non-carriers. When investigating ANO7 protein interactions, we observed an enrichment specifically in proteins related to vesicle trafficking. This study indicates that ANO7 has a role in PrCa development and that truncating mutations in the gene predispose patients to aggressive PrCa. The variants reported in this study could facilitate precision medicine for PrCa patient care.

KEY WORDS: ANO7, biomarker, castration-resistant prostate cancer, prostate cancer, protein-protein interaction

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Eturauhassyöpä on merkittävä kansanterveydellinen haaste, koska suuri osa miehistä saa taudin vanhetessaan. PSA-seulontatutkimukset ovat osoittaneet, että tietyn ikäisten miesten testaaminen aiheuttaa ylidiagnosointia ja turhia hoitoja niille, joiden syöpä ei etene aggressiiviseksi. Sen lisäksi, että hoitojen kustannukset terveydenhuoltojärjestelmälle ovat suuret, ylidiagnosointi aiheuttaa haittaa ja huolta myös itse potilaille. PSA-testauksen rinnalle tarvitaankin menetelmiä, joiden avulla potilaiden taudin ennustetta voidaan paremmin määrittää. Kromosomaalinen alue 2q37.3, jossa solumembraaniproteiinia koodaava anoktamiini 7 (*ANO7*) sijaitsee, on yhdistetty eturauhassyöpään genominlaajuisissa assosiaatioanalyyseissa ja kytkentäanalyyseissa. *ANO7*-geenin on ajateltu ilmentyvän vain eturauhasessa ja sitä onkin ehdotettu eturauhassyövän immunoterapian kohteeksi.

Tässä väitöskirjassa selvitettiin ANO7:n roolia eturauhassyövässä. Tutkimuksessa selvisi, että *ANO7* ilmentyy enemmän eturauhasessa kuin muissa kudoksissa ja enemmän syövässä kuin normaalikudoksessa. Voimakas ilmentyminen oli lisäksi yhteydessä lyhentyneeseen elossaoloaikaan. *ANO7*-geenistä löytyi uuden sukupolven sekvensointimenetelmällä proteiinille todennäköisesti haitallisia harvinaisia ituratavariantteja. Variantti eksonissa 1 oli yhteydessä lyhentyneeseen elinaikaan ja intronissa/eksonissa 4 oleva variantti assosioitui sairastumisriskiin ja aggressiiviseen tautiin. Koska intronin/eksonin 4 variantti oli yhteydessä aggressiiviseen tautiin, mutta ei lyhentyneeseen elinaikaan, tutkimme, hyötyvätkö variantin kantajat erityisen hyvin kastraatioresistentin syövän hoidossa käytettävästä docetaxel-lääkkeestä. Variantin kantajat saivat oletuksen mukaisesti paremman lääkevasteen. Kun tutkimme ANO7:n kanssa vuorovaikuttavia proteiineja, havaitsimme, että solujen vesikkeliliikenteeseen osallistuvat proteiinit olivat yliedustettuina. Tämä tutkimus osoittaa, että ANO7 on osallisena eturauhassyövän kehityksessä ja haitalliset geenivariantit altistavat aggressiiviselle eturauhassyövälle. Tutkimuksessa löydettyjä variantteja voidaan mahdollisesti hyödyntää tulevaisuudessa eturauhassyövän yksilöllistetyssä hoidossa.

AVAINSANAT: ANO7, biomarkkeri, eturauhassyöpä, kastraatioresistentti eturauhassyöpä, proteiini-proteiini -interaktio

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Abbreviations

22Rv1	22 Human Xenograft Prostate
3'UTR	Three prime untranslated region
ADT	Androgen deprivation therapy
AKT	AKT serine/threonine kinase 1
ANO	Anoctamin
AP2B1	AP-2 complex subunit beta
APC	APC regulator of WNT signalling pathway
AR	Androgen receptor
Arp2/3	Actin-related protein 2/3
AR-V7	Androgen receptor splice variant 7
ATM	ATM serine/threonine kinase
BioID	Proximity-dependent biotin identification
BirA	*Escherichia coli* biotin ligase
BRCA1/2	BRCA1/2 DNA repair associated
Ca2+	Calcium ion
CADD	Combined annotation-dependent depletion
CI	Confidence interval
Cl-	Chloride ion
COPG2	COPI coat complex subunit gamma 2
CRPC	Castration-resistant prostate cancer
C-terminal	Carboxy-terminal
DNA	Deoxyribonucleic acid
DU145	Dura mater 145
DuCaP	Dura mater cancer of the prostate
EP156T	Immortalized prostate primary epithelial
eQTL	Expression quantitative trait loci
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded

FOXA1	Forkhead box A1
G2/M	Mitotic gap2 phase/mitotic phase
GO	Gene Ontology
GS	Gleason score
GWAS	Genome-wide association study
HPLC	High-performance liquid chromatography
HR	Hazard ratio
HRP	Horseradish peroxidase
HSPA1A	Heat shock 70 kDa protein 1A
IgG	Immunoglobulin G
IQR	Interquartile range
kDa	Kilodalton
LaPC	Los Angeles Prostate Cancer-4
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization tandem mass spectrometry
LNCaP	Lymph node carcinoma of the prostate
mCRPC	Metastatic castration-resistant prostate cancer
mRNA	Messenger ribonucleic acid
N	Number
NMD	Nonsense-mediated decay
OR	Odds ratio
OS	Overall survival
PARP	Poly (ADP-ribose) polymerase
PC3	Prostate cancer 3
PCR	Polymerase chain reaction
PFS	Progression-free survival
PIN	Prostatic intraepithelial neoplasia
Pir	Prostate cancer samples from Tampere University Hospital, Tampere, Finland
PrCa	Prostate cancer
PrEC	Prostate epithelial cell
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
p-value	Probability value
RALP	Robotic-assisted laparoscopic prostatectomy (samples)
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SND1	Staphylococcal nuclease domain-containing protein 1
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
STED	Stimulated emission depletion
T cell	T lymphocyte
TCGA	The Cancer Genome Atlas
TMPRSS2-ERG	Transmembrane protease serine 2:v-ets erythroblastosis virus E26 oncogene homolog
TNM	Tumour, node, and metastasis
TP53	Tumour protein p53
TPCC	Turku Prostate Cancer Consortium
VCaP	Vertebral cancer of the prostate

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Kaikkonen E, Rantapero T, Zhang Q, Taimen P, Laitinen V, Kallajoki M, Jambulingam D, Ettala O, Knaapila J, Boström PJ, Wahlström G, Sipeky C, Pursiheimo JP, Tammela T, Kellokumpu-Lehtinen PL; PRACTICAL Consortium, Fey V, Maehle L, Wiklund F, Wei GH, Schleutker J. *ANO7 is associated with aggressive prostate cancer.* Int J Cancer. 2018 Nov 15;143(10):2479-2487. Doi: 10.1002/ijc.31746. Epub 2018 Sep 22.

II Kaikkonen E, Ettala O, Nikulainen I, Taimen P, Lehtinen I, Boström P, Kellokumpu-Lehtinen PL, Schleutker J. *ANO7 rs77559646 Is Associated With First-line Docetaxel Treatment Response in Metastatic Castration-resistant Prostate Cancer.* Anticancer Research. 2019 Oct;39(10)5353-5359. Doi:10.21873/anticanres.13728.

III Kaikkonen E, Takala A, Pursiheimo J-P, Wahlström G, Schleutker J. *The interactome of a prostate specific Anoctamin 7*. (In press.)

1 Introduction

Prostate cancer (PrCa) is one of the most commonly diagnosed cancer types, but due to effective diagnostic methods and treatments, only a small proportion of cases are lethal. However, every year, more than 900 patients die of PrCa in Finland. To avoid these deaths, more effective tools are required to differentiate aggressive cases from indolent cases at the early stage of cancer. Clinical determinations i.e., tumour staging and grading, and prostate-specific antigen (PSA) measurement can confirm that the already advanced cases are aggressive, but they are not optimal for predicting aggressiveness at earlier stages of the disease.

Another central challenge in the field of PrCa is to decide how the advanced cancer type should be treated. Some locally treated PrCa cases continue progressing, and the patients are often treated hormonally to prevent cancer growth. At some point, however, the cancer transforms into a castration-resistant form (castration-resistant prostate cancer (CRPC)) and grows despite treatment. Currently, there are several treatment options for CRPC, but they are not durable. Different drugs are administered sequentially to prolong the survival time, and the treatment selection depends more on the patient condition and age than on tumour characteristics. On the other hand, it is known that the response to the first CRPC treatment is the strongest, which highlights the importance of selecting the most effective treatment option for each individual.

Biomarkers, such as germline variants, are ideal for predicting cancer aggressiveness and treatment selection since they are easily and reliably detected in blood samples and remain static for the whole lifetime.

ANO7 was selected for a more detailed analysis since it has been linked to PrCa susceptibility and has been reported to have prostate-specific expression. The aim of my thesis was to investigate the role of ANO7 in more detail and to identify germline variants that were associated with disease outcome and thus could predict cancer aggressiveness and possibly treatment effectiveness. In addition, the goal of this thesis was to reveal ANO7 protein-protein interactions to obtain information about the unknown function of the protein.

2 Review of the Literature

2.1 Prostate cancer

2.1.1 PrCa epidemiology

Prostate cancer (PrCa) is the most common cancer type among men in Finland, and the disease affects over 5,000 men annually (The Finnish Cancer Registry, 2016 report). Although the mortality rate is relatively low (5-year survival rate of 92%), PrCa is still the second leading cause of cancer death among males, accounting for 900 deaths per year (The Finnish Cancer Registry, https://cancerregistry.fi/statistics/cancer-statistics, 2016 report).

2.1.2 Risk factors

The risk of having PrCa increases with age. Early-onset cases are usually more aggressive than late-onset cases (Grönberg and others 1994).

According to the International Agency for Research on Cancer (World Health Organization), PrCa incidence is the highest in Oceania, followed by North America and Europe. By contrast, Asia and Africa have the lowest incidence rates. African American background is related to the highest PrCa prevalence. The reason why PrCa is more common in developed countries may depend not only on the genetic background but also on lifestyle factors and the more effective diagnostics in these countries.

Lifestyle-related factors that predispose to PrCa include low physical activity and obesity (Kaaks and Stattin 2010; Keogh and MacLeod 2012), diets containing, for example, milk, fat and grilled meat (Chan and others 2005), and exposure to carcinogens such as cigarette smoke (Huncharek and others 2010). In addition, infections and prostate inflammation can predispose patients to PrCa (De Marzo and others 2003; Sutcliffe 2010).

PrCa is one of the most heritable so-called common cancers, and genetic factors explain 58% of the risk (Hjelmborg and others 2014). At the greatest genetic risk are men who have an affected brother or father (Zeegers and others 2003). The risk is also higher if a man has several affected relatives. Additionally, Chen and others

reported that familial PrCa was associated with a more aggressive phenotype than non-familial PrCa and an early-onset of disease (Chen and others 2008).

2.1.3 Prostate anatomy, function and tumorigenesis

The prostate surrounds the urethra and is located below the urinary bladder. The prostate consists of three parts: the peripheral zone, where the majority of prostate carcinomas originate from, the central zone, and the transitional zone, where hyperplasia commonly develops (McNeal 1988). Histologically, it consists of stromal tissue and glands. The prostatic ducts are formed by a double row of epithelial cells that consists of luminal and basal cell layers, and neuroendocrine cells (van Leenders and Schalken 2003) (Figure 1). The secretory luminal cells of the glands produce mucus, which is secreted via the duct lumen into the prostatic utricle (Figure 1). The mucus, produced by the prostate, forms most of the semen and its role is to make the semen more alkaline and protect sperm cells from the acidic vaginal environment. The prostate-specific antigen (PSA) is produced by the prostate to liquefy the content of the semen. PrCa can arise from any of these cell types, but most commonly, it starts from luminal or basal cells (adenocarcinomas). The neuroendocrine type of PrCa is much rarer and originates from the neuroendocrine cells. In this doctoral thesis, the focus is on prostate adenocarcinoma.

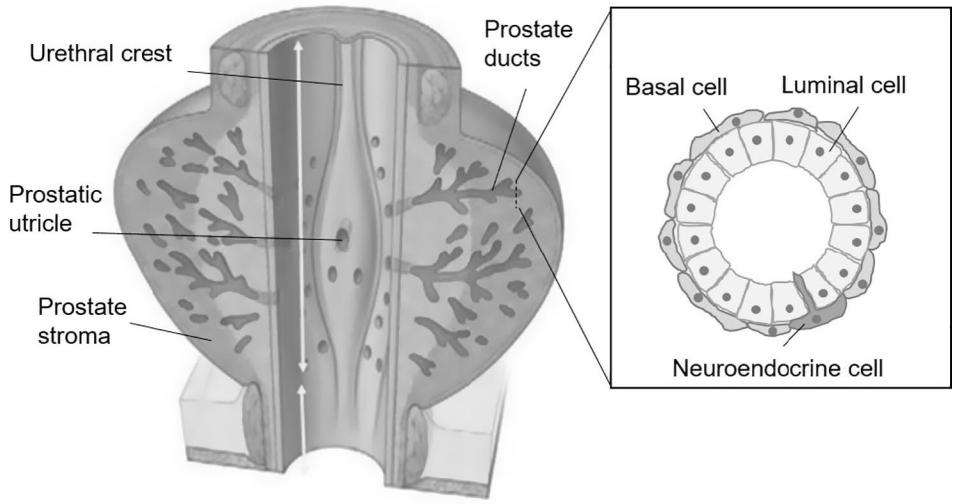

Figure 1. Prostate anatomy. The figure is modified from the illustration of https://www.earthslab.com/anatomy/urethra/.

PrCa development is a multi-step process. First, the cells start to divide inside the prostate gland and form a pre-cancerous prostatic intraepithelial neoplasia (PIN)

lesion. In the next step, the cells invade out of the gland but are still localized only inside the prostate. If the cancer continues progressing, the cells can penetrate through the prostate capsule and spread into the nearby organs, i.e., seminal vesicles and the bladder, and eventually into lymph nodes and other organs (Shen and Abate-Shen 2010).

2.2 PrCa diagnostics

PrCa does not necessarily cause any symptoms; alternatively, the symptoms are similar to other urinary problems, such as the symptoms of benign prostatic hyperplasia, including urinary problems, blood in the urine, pain, and erectile dysfunction. In cases in which the cancer has already metastasized to the bone, a pathological bone fracture or pain in bones can be the first symptoms. If there is a suspicion of cancer, the prostate is first palpated, and any anomalies in shape or size are examined. PSA is usually measured to estimate the probability of PrCa. An increased level is an indication for prostate imaging and possibly for fine needle biopsy.

2.2.1 PSA measurement and prostate imaging

PSA is used in PrCa screening. It is measured from blood serum, and it helps in deciding whether there is a need for core needle biopsies. PSA is a protein that is specifically produced in prostate tissue from the kallikrein 3 (*KLK3*) gene. The normal concentration for men between 40 and 49 years is <2.5 ng/ml, and for men over 70 years, it is <6.5 ng/ml. An elevated level can indicate prostate carcinoma, but an increase is not PrCa-specific (Stamey and others 1987). Earlier, when the 12-core biopsies were taken systematically from the prostate, only 25% of patients with concentrations above normal but under 10 ng/mL had PrCa based on biopsy analyses (Greene and others 2013). Currently, imaging techniques such as multiparametric magnetic resonance imaging (MRI) and ultrasound are commonly used in order to target the biopsies to the suspected cancer sites. MRI imaging before the biopsy has been shown to increase the number of positive cancer findings to 38% (Kasivisvanathan and others 2018). The MRI results can be utilized for both targeting the biopsies and estimating the probability of clinically relevant PrCa. MRI imaging results are interpreted according to the Prostate Imaging – Reporting and Data System Version 2 (PI-RADS TM v2) (Weinreb and others 2016). The PI-RADS category 1 corresponds to very low risk, PI-RADS 2 low, PI-RADS 3 intermediate, PI-RADS 4 high, and PI-RADS 5 very high risk of having clinically significant cancer. PSA level generally increases during the ageing process. Determination of the portion of free PSA (unbound to proteins) can be utilized for PSA values below 10 ng/mL to obtain more information about the likelihood of cancer.

Systematic PSA measurement is recommended only for men who are at high risk of developing PrCa due to a family history of PrCa (Mottet and others 2017). An elevated PSA level alone cannot be used for diagnosing PrCa; prostate biopsy is also required.

2.2.2 Tumour evaluation

Most commonly, biopsies are taken from the visible lesions based on the imaging results. If there is no visible tumor, 12 biopsy sample cores are systematically taken from different parts of the prostate. The level of cell differentiation for adenocarcinomas is histologically evaluated using the Gleason grading system. The cancer grade can reach a value of 3, 4 or 5: a Gleason grade of 3 represents moderately differentiated adenocarcinoma and a grade of 5 represents poorly differentiated adenocarcinoma. Poor differentiation is, in turn, related to more aggressive PrCa. The Gleason score (GS) is determined by summing the most common and the second most common Gleason grades, and the score can therefore vary between six and ten. The GSs are further divided into five grade groups by 'The International Society of Urological Pathology' (ISUP) according to the risk for biochemical relapse after radical prostatectomy. GS 3+3=6 corresponds to the ISUP grade group 1, GS 3+4=7 to the ISUP grade group 2, GS 4+3=7 to ISUP 3, GS 8 to the ISUP 4 and GS 9 and 10 to ISUP 5 (Epstein and others 2016).

2.2.3 Risk evaluation

According to European Association of Urology (EAU) guidelines (Mottet and others 2017), cancers are classified into three groups according to their risk for biochemical recurrence after primary treatment: low-risk, intermediate-risk, and high-risk. In addition to the Gleason score and the ISUP grade grouping, the classification is dependent on the tumour stage (T), the presence of nodal or distal metastases (N and M) and the PSA level. Localized tumours with PSA below 10ng/ml and a Gleason grade under seven (ISUP 1) belong to the low-risk group, whereas localized tumours with a Gleason grade of 7 (ISUP 2 or 3) or PSA over 10 but under 20 ng/ml belong to the intermediate-risk group. If the cancer is locally advanced, the Gleason grade is greater than 7 (ISUP 4 or 5), or PSA is greater than 20 ng/ml, the tumour is classified into the high-risk group. This classification is utilized in predicting cancer aggressiveness and treatment selection. Furthermore, other factors influencing the decision include age and life expectancy, other diseases, and the patient's own opinions.

A recent study by Zelic and others compared the performance of different PrCa mortality prediction systems. They found that 'The Memorial Sloan Kettering Cancer Center nomogram with the number of positive and negative cores as the additional

predictors in the model' (MSKCC_cores) that takes into account the information about PSA, primary and secondary Gleason grades (<3 or ≥4), clinical tumour stage (T1/T2/T3+) and the number of the positive and negative cores, gives the best estimation of PrCa mortality. The other well-performing prediction systems in the study were 'The UCSF Cancer of the Prostate Risk Assessment' (CAPRA) and 'Cambridge Prognostic Groups' (CPG) risk group systems.(Zelic and others 2019).

2.3 PrCa treatment

Figure 2 shows the stages and treatment options of PrCa. Low-risk patients are usually surveyed to avoid unnecessary treatments, whereas intermediate- and high-risk patients are treated with local therapy that can be combined with adjuvant therapy.

2.3.1 Surveillance

PrCa patients classified as low risk are recommended to be monitored rather than treated to avoid possible adverse effects (Mottet and others 2017). During active surveillance, PSA can be measured regularly, new biopsies can be taken, and the cancer can be treated if it progresses during the follow-up. If the life expectancy of the patient is relatively short, watchful waiting with fewer PSA measurements is also an option.

2.3.2 Local therapy

Cancers that are localized inside the prostate can be treated with prostatectomy (radical removal of the prostate) or radiotherapy, which can be combined with neoadjuvant therapies. Prostatectomy is recommended for intermediate- and high-risk patients if the life expectancy is >10 years (Gillessen and others 2018). Additional adjuvant androgen deprivation therapy (ADT) therapy is given in the presence of nodal metastases. Radiotherapy is usually recommended for those patients who have shorter life expectancy or if the patient is not suitable for prostatectomy.

Other treatment options for localized PrCa include cryotherapy and the high-intensity focused ultrasound HIFU method.

2.3.3 Hormonal treatment of advanced PrCa

The PSA level is measured postoperatively after local treatment(s) to determine whether the PrCa progresses despite treatment. An increased PSA after the nadir (the lowest PSA value after the treatment) is a strong indicator of advancing cancer. Huggins and Hodges reported in 1941 that prostate tumours were hormone-

dependent and that castration reduced the size of PrCa tumours (Huggins and Hodges 1941). Because both normal and cancerous prostate cells are androgen-dependent, castration or ADT is utilized to decelerate the growth of cancer cells.

2.3.4 Treatment of CRPC and mCRPC

Almost all hormonally treated PrCa patients develop castration resistance at some point, meaning that the cancer can grow independent from androgen ablation. The progression can be seen as either biochemical or radiological recurrence. The treatments aim at relieving symptoms and prolonging survival time rather than curing the cancer. Cytostatic drugs and second-generation androgen receptor (AR)-targeted therapies are often used sequentially when the disease continues progressing despite medication. The decision of which drugs are given and in which order is currently based on the patient's clinical characteristics (Halabi and others 2014) – not on tumour characteristics or biomarkers. On the other hand, it is known that first-line CRPC therapy provides the best response, highlighting the importance of treatment selection.

Taxanes (docetaxel and cabazitaxel) were approved for treating metastatic castration-resistant prostate cancer (mCRPC) in 2004 and 2010, respectively, by the U.S. Food and Drug Administration (FDA). Docetaxel was shown to improve the median survival time by over two months compared to mitoxantrone treatment (Tannock and others 2004), and after FDA approval, it has been widely used as first-line chemotherapy. Cabazitaxel, in turn, is a second-line drug designed for CRPC and is used after docetaxel treatment (de Bono and others 2010). Taxanes prevent microtubule polymerization. Due to improper microtubule function, the cell is arrested at the G2/M checkpoint, which causes apoptotic cell death (Schutz and others 2014). The other possible effect of taxanes on PrCa cells is that they may disturb AR translocation into the nucleus (Zhu and others 2010), which further decreases the transcription of AR-targeted genes. However, taxanes cannot always be used for treating patients since they have many adverse effects, such as lowered blood cell counts, nausea and allergic reactions.

The FDA first approved the second-generation antiandrogens enzalutamide and abiraterone acetate as second-line treatments for metastatic CRPC in 2011 and 2012, respectively. Later, they were approved for use as first-line CRPC treatments for asymptomatic or mildly symptomatic patients. Enzalutamide prevents AR translocation into the nucleus and inhibits AR-mediated transcription, and abiraterone inhibits intratumoural androgen biosynthesis and blocks AR activation (Helsen and others 2014). While taxanes have been the gold standard in mCRPC treatment, second-generation antiandrogens are currently preferred over taxanes as first-line therapy because they have fewer side effects. However, they are also much more expensive than taxane therapies.

Radium-223 is an approved therapy for patients with bone metastases who are not suitable for chemotherapy.

If PrCa is diagnosed at the metastasized phase, it is castration-naïve, and it should be treated hormonally and preferably in combination with docetaxel therapy (Mottet and others 2017). Abiraterone and enzalutamide or other second-generation antiandrogens are not approved as compensated drugs for castration-naïve disease by the Social Insurance Institution of Finland (KELA).

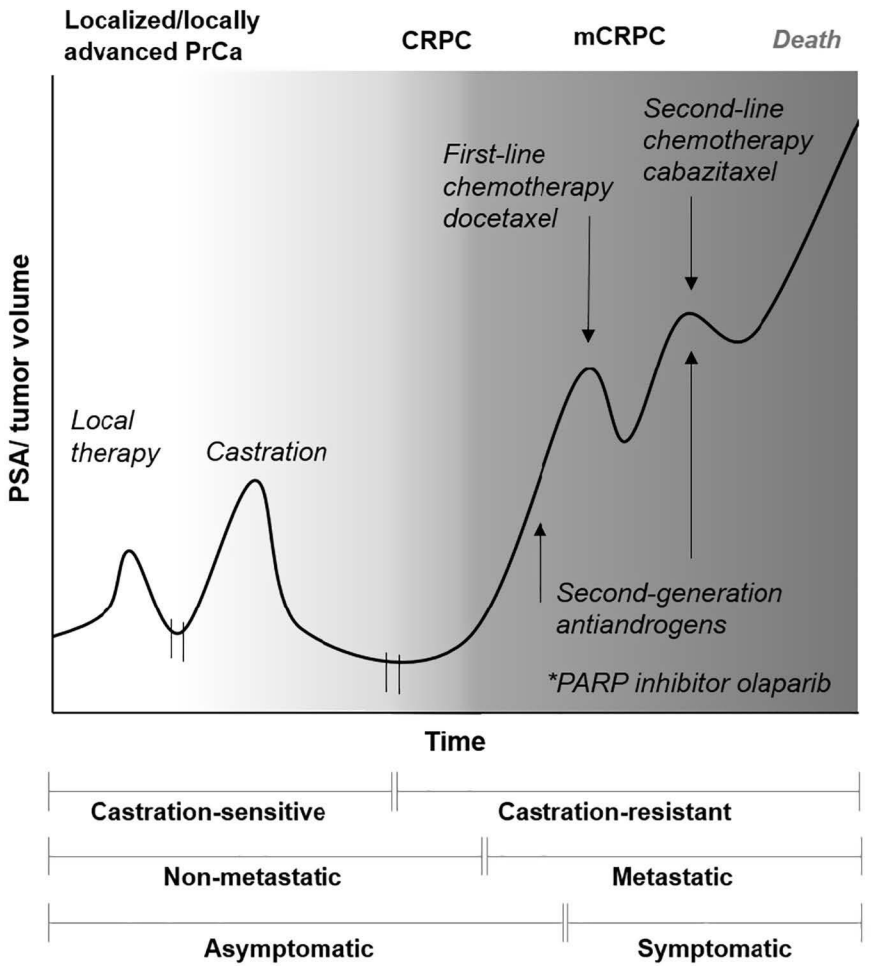

Figure 2. Prostate cancer progression and treatment of patients diagnosed with localized PrCa. In addition, PrCa can be diagnosed as de novo metastatic, hormone-sensitive stage (not shown in the plot), which can be treated with docetaxel. The second-generation antiandrogens can be administrated either before taxanes, after docetaxel or as the only CRPC treatment. PARP inhibitor olaparib has proven effective for mCRPC patients with genetic alterations in *BRCA1*, *BRCA2* or *ATM* DNA repair genes. All treatments are not necessarily given to the same patient.

2.4 Diagnostic PrCa tests and prognostic and predictive biomarkers

PSA is the only biomarker commonly used in PrCa detection and follow-up. Due to the limitations of the PSA analysis, it has been of great interest to find cancer-specific biomarkers that would also help in predicting cancer aggressiveness and the most suitable treatments. Hugosson and others investigated whether all males should be PSA screened at a certain age to diagnose cancer cases at early stages and to prevent PrCa deaths. They reported that 570 men must be PSA screened to avoid one PrCa-caused death, which may lead to overdiagnosis. In addition, the cost effectiveness of this kind of screening is rather weak. Thus, PSA is not recommended for screening alone (Hugosson and others 2019).

Modern medicine is increasingly turning towards personalized patient care. Biomarkers can be roughly divided into metabolic biomarkers and proteins, tumour RNA-based markers, somatic DNA alterations detection from tumours, and germline DNA alteration investigation. At tissue level Alpha-methylacyl-CoA racemase (AMACR), tumor protein 63 (p63/p40) or cytokeratin 5/6 are utilized in order to differentiate the neoplastic changes from the benign ones.

Some biomarkers, for example, the PCA3 test (Bussemakers and others 1999), the four kallikreins test (Bryant and others 2015) and STHML3 (Grönberg and others 2015) combine information about clinical variables and measurements and additionally over 200 single nucleotide polymorphisms (SNPs). The latter two promise to help predict cancer aggressiveness. Other biomarkers predicting cancer aggressiveness include the fusion gene of the transmembrane protease serine 2:v and ets erythroblastosis virus E26 oncogene homolog (*TMPRSS2-ERG*) (Kulda and others 2016; Nam and others 2007) and the androgen receptor splice variant 7 (AR-V7) (Antonarakis and others 2014; Guo and others 2009). *TMPRSS2-ERG* fusion has been reported to serve as a prognostic marker for taxane resistance (Reig and others 2016), and AR-V7 has been reported as a marker of resistance to abiraterone and enzalutamide in CRPC (Antonarakis and others 2014).

Among the known germline biomarkers, mutations in the BRCA2 DNA repair associated *(BRCA2)* gene seem to be the best predictive markers for cancer aggressiveness (Castro and others 2013). However, *BRCA2* mutations are rare, and therefore, all mutations in the *BRCA2* gene or even all mutations in the DNA repair pathway are often considered together as one score. Additionally, studies have indicated that *BRCA2* mutations are even rarer in Scandinavians than in other populations (Ikonen and others 2003; Mayrhofer and others 2018). Recently, the carriers of mutations in the DNA repair pathway have been shown to benefit from abiraterone and enzalutamide (Antonarakis and others 2018) and poly (ADP-ribose) polymerase (PARP) inhibitors (Mateo and others 2018). Thus far, the only predictive genetic markers that have been confirmed in clinical trial are alterations

in homologous recombination repair genes ATM serine/threonine kinase (*ATM)*, BRCA1 DNA repair associated (*BRCA1)* and *BRCA2* (https://clinicaltrials.gov/ct2/show/NCT02987543). The carriers of these alterations clearly benefit from PARP inhibitor olaparib.

2.5 PrCa genetics

2.5.1 Tumour genome

New technologies, such as high-throughput sequencing, have emerged as a new method for the genetic investigation of cancers. These methods are able to analyse DNA and RNA molecules simultaneously. Not only are they fast and accurate, but the costs are currently reasonable and allow the usage of these methods in both research and diagnostic settings. Sequencing can be conducted for germline DNA to screen for cancer susceptibility or inherited genetic changes that predispose to aggressive cancer, or alternatively, the tumour DNA and RNA can be sequenced. Large projects such as 'the International Cancer Genome Consortium' (ICGC) and 'The Cancer Genome Atlas' (TCGA) have collected sequencing data from various cancer types to make the data available for researchers and clinicians.

For cancerous transformation, the cell needs to change genetically. According to the Hallmarks of Cancer theory by Hanahan and others (Figure 3) for transformation from a normal cell to a cancer cell, the cell needs to be capable of escaping growth suppressors and cell death, retaining proliferative signalling, inducing angiogenesis, invading and metastasizing, promoting inflammation, modifying cellular energy systems and increasing genomic instability and mutation rates (Hanahan and Weinberg 2011).

There are several types of genetic alterations. Chromosomal rearrangements include insertions (addition of chromosomal parts), deletions (removal of chromosomal parts), duplications (parts of the chromosome are duplicated), inversions (parts of the chromosomes are turned around), and translocations (parts of the chromosomes move into another position). Substitutions of one nucleotide are called single nucleotide variants (SNVs). They can occur in the non-coding or coding region of the gene. The nucleotide changes in coding regions can be synonymous (do not change the amino acid), missense (change the amino acid into another), or nonsense (change the amino acid into a premature stop codon) variants. The changes can be inherited in the germline, or alternatively, they can be somatic.

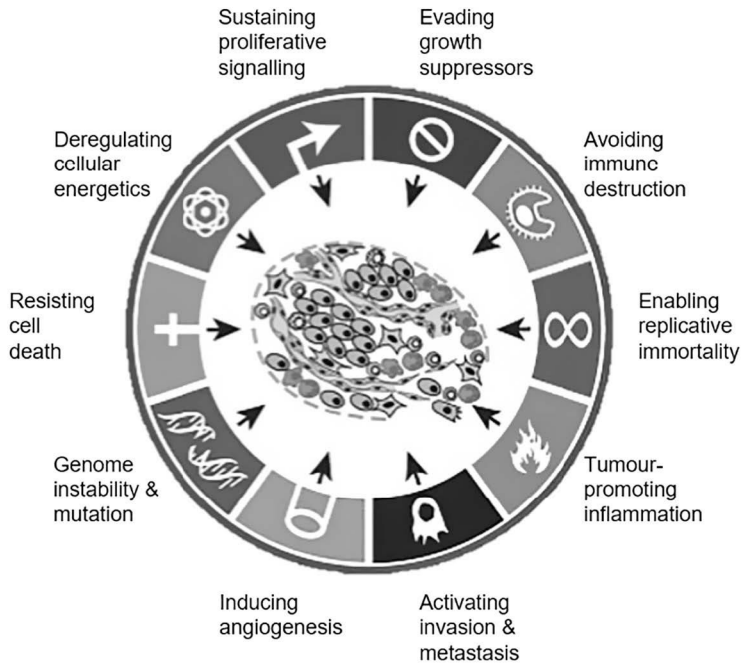

Figure 3. Modified from Hallmarks of Cancer: The Next Generation (Hanahan and Weinberg 2011).

Genes related to cancer formation are traditionally divided into tumour suppressor genes or proto-oncogenes. The tumour suppressor genes are responsible for decelerating cell proliferation and division. Usually, one functioning copy of the gene is enough to protect the cells, and only a lack of both copies causes problems. Inherited mutations in tumour suppressor genes are much more common than mutations in proto-oncogenes, and they are – with some exceptions – inherited recessively.

In hereditary cancers, a person has only one functional copy of the gene and is therefore more susceptible to cancer development. Knudson has described this phenomenon as the 'two-hit hypothesis' (Knudson 1971) (Figure 4). The heritable cancer types that develop in adulthood are commonly a result of recessive changes in tumour suppressor genes.

Mutations in proto-oncogenes usually dominantly affect the growth and survival of mutated cells. In contrast to mutations in tumour suppressor genes, hereditary mutations in proto-oncogenes are rare. Somatic mutations in oncogenes, in turn, are common in cancers.

DNA repair genes are classified as a separate group from tumour suppressor genes, and they play a central role in many hereditary cancers, including PrCa. Their responsibility is to correct DNA damage. Mutations in DNA repair pathway genes

are typically recessive. It is noteworthy that the majority of the identified PrCa susceptibility genes are involved in DNA repair.

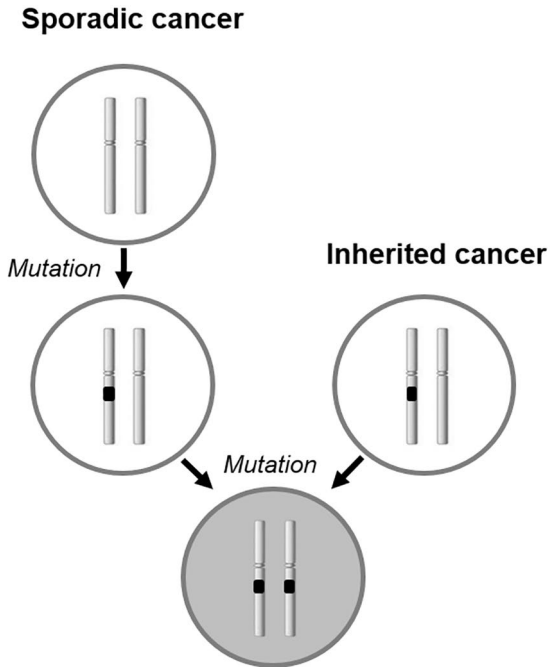

Figure 4. Knudson's two-hit hypothesis. A patient with inherited cancer already has one nonfunctional copy of the gene and needs only one more mutation in order to develop cancer.

Epigenetic changes do not alter the DNA sequence, but gene activity can be regulated by, for example, epigenetic DNA methylation and histone protein modifications. Hypermethylation (i.e., silencing of gene expression) is very common in the early stages of PrCa, whereas hypomethylation (i.e., improper gene activation) occurs in metastatic PrCa (Yegnasubramanian and others 2008). Another type of epigenetic regulation of gene expression can occur via histone modifications. Histone modification patterns have been suggested to correlate with PrCa outcome and contribute to intertumoural heterogeneity (Seligson and others 2005).

2.5.2 Inherited PrCa susceptibility

The traditional methods for detecting new germline risk genes for PrCa include genome-wide association studies (GWAS) and linkage analysis. Both analyses are used for identifying genomic locations related to PrCa. The main difference is that while linkage analysis is used in affected families to define which loci are inherited

with the disease, GWAS is used for case-control or case-case analyses for non-related patients. More than 160 SNPs are related to PrCa susceptibility (Dadaev and others 2018). In Finland, the most relevant PrCa susceptibility genes are reported to be *RNASEL* (Carpten and others 2002), which encodes the apoptosis-inducing protein ribonuclease L, *CHEK2* (Seppälä and others 2003), which encodes a DNA damage checkpoint kinase, and *HOXB13* (Laitinen and others 2013), which encodes a prostate-specific transcription factor. The G84 variant in *HOXB13* has the strongest impact on PrCa susceptibility, which has been continuously reported (odds ratio (OR) of 8.8) in Finnish PrCa patients (Laitinen and others 2013). Some of the most frequently reported PrCa susceptibility genes are listed in Table 1. The vast majority of these are DNA repair genes. Although the significance of the germline *BRCA2* gene has been reported several times, mutations are relatively rare among PrCa patients (Edwards and others 2003). Notably, in the Finnish population, *BRCA2* gene alterations do not appear to play an important role in PrCa susceptibility (Ikonen and others 2003).

Table 1. Genes related to PrCa susceptibility and aggressive progression.

GENE NAME	MECHANISM OF ACTION	PRCA SUSCEPTIBILITY	RISK FOR AGGRESSIVE PRCA
BRCA1	DNA repair	Leongamornlert and others 2012; Struewing and others 1997	
BRCA2	DNA repair	Kote-Jarai and others 2011; Struewing and others 1997	Castro and others 2013; Pritchard and others 2016; Robinson and others 2015
HOXB13	Dual function as tumour suppressor and proto-oncogene	Ewing and others 2012; Kote-Jarai and others 2015; Laitinen and others 2013; Xu and others 2013	
CHEK2	DNA repair and tumour suppressor	Dong and others 2003; Pritchard and others 2016; Seppälä and others 2003	Pritchard and others 2016
RNASEL	Tumour suppressor	Carpten and others 2002; Rökman and others 2002; Schaid 2004	
ATM	DNA repair	Angèle and others 2004	
MLH1	DNA repair	Grindedal and others 2009	
MSR1	DNA repair	Xu and others 2002	
MSH2	DNA repair	Grindedal and others 2009	
ELAC2	Tumour suppressor	Rökman and others 2001; Tavtigian and others 2001	
MSH6	DNA repair	Grindedal and others 2009	
NBN	DNA repair	Cybulski and others 2004	
TP53	Tumour suppressor	Stacey and others 2011	

2.5.3 Germline mutations predisposing to aggressive PrCa

Only a few of the variants that predispose to PrCa are associated with aggressive disease (Helfand and others 2015) (Table 1). According to the study by Pritchard and others, germline mutations in *BRCA2* and *CHEK2* are statistically significantly enriched in metastatic PrCa cases compared with nonmetastatic PrCa cases (5.3% vs. 0.3% and 1.9% vs. 0.6% of the cases, respectively) (Pritchard and others 2016). They also found that deleterious germline mutations in DNA repair genes are enriched among metastatic cases when compared to cases of localized disease (11.8% vs. 4.6%) (Pritchard and others 2016). Robinson and others, in turn, investigated germline variants from mCRPC patients and found that both *BRCA2* and *BRCA1* were drivers in the development of castration resistance (Robinson and others 2015). Inherited *BRCA2* mutations have also been associated with poor survival among PrCa patients (Castro and others 2013).

2.5.4 Somatic mutations driving PrCa progression and the development of mCRPC

Somatic mutations can be caused by radiation or chemicals, or they can arise spontaneously. The most central somatic mutations driving CRPC development are located in the genes tumour protein P53 (*TP53), AR,* phosphatase and tensin homolog *(PTEN),* RB transcriptional corepressor 1 (*RB1),* forkhead box A1 (*FOXA1),* APC regulator of WNT signalling pathway (*APC)* and *BRCA2* (Armenia and others 2018). Robinson and others similarly reported that the most frequently mutated genes in CRPC are *AR, TP53, PTEN, APC* and *BRCA2,* but they also found some additional drivers, such as alterations in the DNA repair genes *BRCA1*, *ATM*, phosphatidylinositol-4,5-bisphosphate 3-kinase (*PIK3CA/B)*, and B-raf proto-oncogene, serine/threonine kinase/ Raf-1 proto-oncogene, serine/threonine kinase (*BRAF/RAF1)* (Robinson and others 2015).

AR mutations are very common in PrCa tumours and have a central role in the development of CRPC. To activate AR, a normally functioning AR needs testosterone or dihydrotestosterone ligand binding. After activation, AR moves to the nucleus, forms a homodimer and binds to androgen-responsive elements in the DNA. In CRPC, the AR signalling pathway can be activated despite castration, but in most cases, the tumour remains dependent on androgens (Attard and others 2008). *AR* can be amplified (Visakorpi and others 1995), or androgens can be more effectively produced from cholesterol and weak adrenal androgens (Stanbrough and others 2006; Twiddy and others 2011). Alternatively, AR can be activated by ligands other than testosterone and DHT (Coutinho and others 2016). In addition, alterations in AR coactivators can affect the transcriptional activation of the receptor (Taylor and others 2010). However, some somatic *AR* mutations and AR

splice variants are known to cause androgen-independent AR activation and can thus prevent a treatment response to second-generation antiandrogens (Joseph and others 2013; Romanel and others 2015). AR signalling can additionally be activated by other signalling pathways, such as the phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT serine/threonine kinase 1 (PI3K/AKT) pathway (Wen and others 2000).

Because cancer cells lack normal cell cycle control, they are genetically unstable. In addition to the substitutions of single nucleotides or structural changes, even the number of chromosomes can be altered. In PrCa, a well-characterized fusion of two genes is *TMPRSS2-ERG* fusion, which brings together an androgen-regulated *TMPRSS2* gene promoter and the proto-oncogene *ERG*, which further leads to elevated expression of *ERG* (Tomlins and others 2005).

A phenomenon called chromoplexy, which was first described in whole genome sequencing of PrCa cancer samples by Baca and others, is a common source of PrCa related chromosomal rearrangements. Such rearrangements involve several chromosomes at the same time and can simultaneously cause multiple deletions and translocations of DNA (Baca and others 2013).

2.6 ANO7 and PrCa

2.6.1 Anoctamin protein family and their roles in cancer

The anoctamin protein family has ten members (ANO1-10) that are Ca2+-activated membrane proteins. Although the name anoctamin refers to eight transmembrane domains, recent studies have demonstrated that most anoctamins have ten membrane-penetrating domains (Bushell and others 2019; Feng and others 2019; Paulino and others 2017).

Anoctamins have been reported to have various roles in cells. They are calcium-activating chloride ion channels, phospholipid scramblases, proteins that tether calcium repositories near the membrane, or non-selective cation channels. Suzuki and others reported that consistent with earlier studies (Schroeder and others 2008; Yang and others 2008), ANO1 and ANO2 have Cl- channel functions, whereas the other anoctamins do not have Cl- channel functions (Suzuki and others 2013). Instead, they found that anoctamins 3, 4, 6, 7 and 9 are able to scramble phospholipids, whereas anoctamins 5, 8 and 10 lack these capabilities. However, other studies have suggest that ANO5 (Gyobu and others 2016) and ANO10 (Bushell and others 2019) have phospholipid scramblase activity. ANO3 (Huang and others 2013), ANO4 (Reichhart and others 2019), ANO5 (Whitlock and others 2018), ANO6 (Scudieri and others 2015), ANO9 (Kim and others 2018) and ANO10 (Bushell and others 2019) have been suggested to have an additional nonselective

cation channel function. Moreover, at least ANO4 and ANO8 can bind to the endoplasmic reticulum near the plasma membrane (Jha and others 2019; Kunzelmann and others 2019), and this binding can activate cellular signalling by regulating membrane receptors and bringing calcium depositories close to the plasma membrane (Manford and others 2012).

The most extensively characterized members of the family are the Cl^- ion channel ANO1 and the phospholipid scramblase ANO6. *ANO1* is expressed in many secretory epithelial cells (Jang and Oh 2014), whereas a specific mutation in the *ANO6* gene causes the abnormal blood clotting disorder Scott syndrome (Suzuki and others 2010). *ANO5* mutations, in turn, are associated with muscular dystrophy (Liewluck and others 2013). Anoctamin proteins 1, 6, 7, 8, 9 and 10 are present in various epithelial cells, and they commonly localize on the apical side of polarized epithelial cells (Schreiber and others 2010). ANO2-5, in turn, are expressed in other cell types, such as neurons and muscle cells (Schreiber and others 2010). Anoctamins have varying localization between tissue types, but they also have different cellular localizations that can change depending on the cell stage or the type of cell (Kunzelmann and others 2019).

Kunzelmann and others suggest that anoctamins have various roles in the plasma membrane: releasing exosomes (ANO1 and 6) and mucus (ANO1), regulating membrane protein expression (ANO1 and 6), modifying membrane curvature (ANO6), and promoting cell migration and metastasis formation (Kunzelmann and others 2019). ANO1 has been associated with several cancers, such as gastrointestinal stromal tumours (Miettinen and others 2009), head and neck cancer (Reddy and others 2016) and breast cancer (Britschgi and others 2013). As would be expected due to the fluctuating expression profiles and cellular localizations, the role of anoctamins in cancers is not straightforward, and it does not seem to have only one mechanism. Elevated *ANO1* expression has been shown in many cancers (Liu and others 2012; Zeng and others 2019). Consistently, ANO1 inhibition has been shown to reduce cell proliferation (Guan and others 2016; Seo and others 2018). Other cancer mechanisms related to low *ANO1* expression levels are the induction of apoptosis (Song and others 2018) and retarded cell migration (Jacobsen and others 2013; Ruiz and others 2012). ANO6 was also proposed to increase migration (Ruiz and others 2012) and invasion abilities by inducing extracellular signal-regulated kinase (ERK) localization in the nucleus (Xuan and others 2019). However, ANO6 has also been shown to promote apoptosis instead of reducing it (Forschbach and others 2015; van Kruchten and others 2013).

2.6.2 ANO7

The genomic location of *ANO7* 2q37.3 has been associated with PrCa susceptibility in linkage analysis (Cropp and others 2011) and GWAS (genome-wide association) studies (Schumacher and others 2011).

The anoctamin 7 (*ANO7*) gene was reported for the first time in 2004 by Bera and others as 'a new gene expressed in prostate' (*NGEP*) (Bera and others 2004). Other names for the gene are transmembrane protein 16G (*TMEM16G*), *PCANAP5*, *IPCA-5* and Dresden-transmembrane protein of the prostate (*D-TMPP*). *ANO7* gene expression is also suggested to be regulated by androgens (Kiessling and others 2005), and the expression seems to be lower in PrCa metastases than in primary tumours (Chandran and others 2007).

ANO7 encodes two mRNA isoforms that are translated into proteins: ANO7L (long form) is a polytopic membrane protein that contains all 25 exons, and ANO7S (short form) is a small cytoplasmic protein that contains only the first four exons (Bera and others 2004; Katoh 2004) (Figure 5).

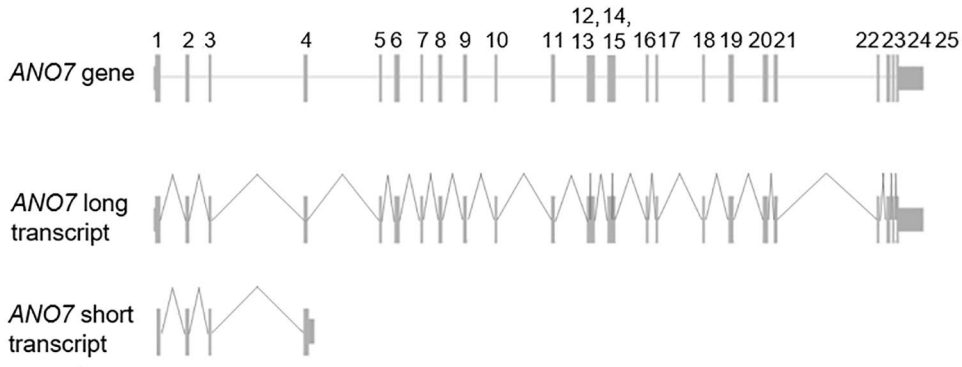

Figure 5. Structure of the *ANO7* gene and the two transcripts. The grey boxes represent its 25 exons. Modified from http://atlasgeneticsoncology.org/Genes/GC_ANO7.html.

ANO7L has been reported to be located in the plasma membrane (Bera and others 2004; Das and others 2007), endoplasmic reticulum (ER) (Duran and others 2012) and Golgi apparatus (Das and others 2007). The protein is found in lipid rafts, which are structures of the cell plasma membrane (Dubois and others 2015).

Very little is known about the function of ANO7. While the studies by Tian and Schreiber demonstrated that ANO7 creates chloride ion currents (Schreiber and others 2010; Tian and others 2012), Duran and Suzuki (Duran and others 2012; Suzuki and others 2013) showed that ANO7 does not have an ion channel function. Instead, Suzuki and others showed that ANO7 works as a phospholipid scramblase,

which flips the phospholipids of the plasma membranes from inner leaflet to the outer leaflet (Suzuki and others 2013).

At the protein level, low ANO7 has been reported to be associated with high-grade PrCa (Mohsenzadegan and others 2013). However, another study by Das and others did not observe any difference in protein expression between high- and low-grade tumours but demonstrated decreased expression in tumours compared to benign prostate tissue (Das and others 2007).

Interestingly, ANO7 has been suggested as a suitable target for PrCa T cell therapy. Cereda and others found that ANO7 could act as a target for a PrCa vaccine (Cereda and others 2010).

3 Aims

Currently, there are many unmet needs in PrCa diagnostics and in deciding the proper treatment for each patient. Although the clinical nomograms combined with PSA testing are considerably good in identifying PrCa patients who have already progressed, additional predictive biomarkers are still needed to discriminate indolent and aggressive cases at earlier stages of PrCa. PrCa is known to have a strong genetic background, but the genetic changes identified so far do not explain the susceptibility to aggressive PrCa.

The specific aims of the thesis were as follows:

1. To study the *ANO7* gene, which is located in the chromosomal region 2q37.3, a region that has been associated with predisposition to PrCa in GWAS and linkage analyses
2. To find specific germline variants in *ANO7* that predispose to prostate cancer
3. To survey whether the detected *ANO7* germline variants are associated with the aggressive type of PrCa
4. To examine whether *ANO7* variants could be useful in prostate cancer diagnostics or in planning the most suitable treatment for prostate cancer patients
5. To investigate ANO7 protein-protein interactions to learn more about the mechanisms of ANO7 in prostate cancer

4 Materials and Methods

Numbers I, II and III after each heading indicate the article in which the sample material or method was used.

4.1 Study material

4.1.1 Ethical approval and informed consent (I, II)

Informed consent was obtained from all the participants in the studies. The research was approved by the Institutional Review Boards of the Turku and Tampere University hospitals.

4.1.2 PrCa patient material

The patient characteristics are described in detail in Table 2.

4.1.2.1 PSA screening cases (I)

The 559 blood-derived DNA samples were obtained from affected patients who were diagnosed with PrCa in a Finnish PSA screening study FinRSPC (Finne and others 2003).

4.1.2.2 Clinical PrCa cases (I)

The unselected PrCa cases were collected from Tampere and Turku University Hospitals. In total, 844 diagnostic blood DNA samples were received from Tampere, and 200 PrCa blood samples originated from the Turku Prostate Cancer Consortium (TPCC) study. The unselected cases were diagnosed similarly as any new PrCa, but not in a predefined screening program. All TPCC patients additionally underwent radical prostatectomy.

Table 2. Patient characteristics in the articles I and II. N=number, IQR=interquartile range. In the PROSTY (I, II) and the Auria (II) cohorts PSA levels have been measured before docetaxel treatments

		ARTICLE I						**ARTICLE II**	
		PROSTY study (treatment cohort), n=22	Auria Biobank, Turku, Finland (docetaxel-treated patients), n=61	Familial cases, index patients, n=142	Turku Prostate Cancer Consortium (TPCC) (treatment cohort), n=200	Tampere University Hospital PrCa samples (diagnostic samples), n=844	FinRSPC screening samples n=559	PROSTY study (treatment cohort) n=98	Auria Biobank, Turku, Finland (docetaxel as the first-line therapy for CRPC) n=110
Age at diagnosis, years	Median (IQR)	64 (59-70)	64 (61-68)	66 (60-72)	63 (59-68)	59 (56-63)	67 (63-69)	65 (61-71)	67 (62-72)
Gleason, n(%)	≤7	3 (18%)	6 (22%)	72 (81%)	162 (81%)	625(81%)	384 (69%)	21 (24%)	42 (47%)
	≥8	14 (82%)	21 (78%)	17 (19%)	37 (19%)	146 (19%)	169 (31%)	65 (76%)	47 (53%)
	Missing data	5	34	53	1	73	6	12	21
PSA at diagnosis (ng/mL)	Median (IQR)	60 (30-120)	3.1 (0.47-13)	13 (6.9-38)	8.5 (5.7-14)	7.9 (5.2-14)	6.6 (4.6-11)	92 (38 - 230)	18 (8.6-81)

4.1.2.3 Familial cases (I)

The affected familial cases included 142 index patients who had the most severe PrCa within the family (one patient per family). This cohort was separate from the screening cases and TPCC cohort. Germline DNA was isolated from blood.

4.1.2.4 CRPC cases (I, II)

CRPC cases were analysed in articles I and II. All CRPC cases originated either from the randomized, registered, prospective trial PROSTY (NCT00255606) (Kellokumpu-Lehtinen and others 2013) or from Auria Biobank (Turku, Finland).

In article I, the PROSTY samples (n=22) that were used in targeted *ANO7* sequencing were formalin-fixed paraffin-embedded (FFPE) prostatectomy samples. The Auria samples used in genotyping were matched blood and prostate tissue pairs from 61 CRPC patients, who had been treated with docetaxel (not necessarily as the first-line therapy for CRPC). The prostate tissue samples were primary PrCa, which later developed CRPC.

In article II, in addition to the 22 samples that were used in the first article, we also included all 76 PROSTY prostate biopsies that had enough material for DNA extraction. The total number of PROSTY DNA samples was 98. The samples were taken from the primary cancer that later developed CRPC. The Auria samples were selected from the Turku University Hospital patient registry (between 2007 and 2018) so that they had similar inclusion and exclusion criteria as those in the PROSTY study (Kellokumpu-Lehtinen and others 2013). The Auria samples were selected retrospectively from the patient records, and the sample was requested from the Auria biobank if the patient received first-line docetaxel treatment for mCRPC, the patient had at least two cycles of docetaxel, and there was a sample available in Auria biobank. Early chemohormonally treated patients who had less than 90 days between the initiation of ADT and docetaxel administration were excluded. Thirty-one Auria samples overlapped with those used in article I. The additional 79 cases were blood samples or FFPE material from tissues other than prostate (n=62) and FFPE prostate tissue (n=17), making the total number of patients 110. The FFPE DNA samples originating from tissues other than the prostate were sourced from lymph node (n=19), skin (n=13), seminal vesicle (n=2), appendix (n=2), colon (n=2), liver (n=2), duodenum (n=2), gastric antrum (n=1), spinal canal (n=1), vertebral column (n=1), tongue (n=1), upper jaw (n=1), synovium, (n=1), lip (n=1), urinary bladder (n=1), and penis (n=1).

4.1.2.5 Non-Finnish validation cohorts (I)

The Swedish cohorts STHM2 (Nordström and others 2014) and PROCAP (Szulkin and others 2012), having 4,561 individuals (3,132 cases and 1,429 controls) and 669 patients, respectively and the Norwegian CONOR cohort (Naess and others 2008; Szulkin and others 2015) of 1,455 PrCa patients who were genotyped as part of the ONCOARRAY study were used for validation. The number of the patients who died of PrCa were 96 in STHM2, 225 in PROCAP and 764 in the CONOR cohort, respectively. DNA originated from blood in all cases and controls.

4.1.3 Control samples (I)

The set of non-affected control males consisted of 12 samples from the Finnish Red Cross and 122 from the Department of Medical Genetics (Genomics, Laboratory Division, Turku University Hospital, Turku, Finland) and 1,577 samples from the PSA screening study (Finne and others 2003). The males from the PSA screening study were found to have a normal PSA level (<1.4 ng/mL), whereas the other samples were chosen by age and sex among the population, and some of them may have had undiagnosed PrCa. Moreover, 522 family unaffected members from 43 families (relatives of the 'Familial cases') were used to study the segregation of the variants and PrCa.

4.1.4 PrCa cell lines (I, III)

In article I, the whole *ANO7* gene was sequenced from seven PrCa cell lines, DuCaP, LaPC, LNCaP, PC3, VCaP, DU145, and 22Rv1, as well as from the EP156T and PrEC cell lines, which were derived from normal prostate (Table 3.). The source of these cell lines was The American Type Culture Collection (ATCC).

Table 3. Prostate cell lines used in the articles.

CELL LINE	SOURCE	ANDROGEN DEPENDENCE/INDEPENDENCE
DUCAP	Dura mater metastasis	Dependent
LAPC	Xenograft from lymph node metastasis	Dependent
LNCAP	Lymph node	Dependent
VCAP	Vertebral metastasis	Dependent
PC3	Vertebral metastasis	Independent
DU145	Brain metastasis	Independent
22RV1	Xenograft	Dependent
EP156T	Normal prostate epithelium	Dependent
PREC	Normal prostate epithelium	Dependent

The LNCaP cell line was used in functional studies in article III because it expresses endogenous *ANO7* mRNA (Bera and others 2004; Mohsenzadegan and others 2015).

4.1.5 Databases (I)

RNAseq data were acquired from TCGA research network, cBio Cancer Genomics Portal (Cerami and others 2012) and microarray data from the Oncomine database (Rhodes and others 2004).

4.2 Methods

4.2.1 DNA and RNA isolation (I, II)

Genomic DNA from blood samples was extracted with a Nucleon BACC3 kit (GE Healthcare Life Sciences) according to the protocol of the manufacturer. Isolated DNA was eluted into TE buffer and stored at -80°C.

DNA and RNA from fresh prostate tissue were isolated with a Nucleospin kit (Macherey Nagel) according to the protocol of the manufacturer. Both DNA and RNA were extracted from the same piece of tissue.

For FFPE tissue DNA isolation, a QIAamp DNA FFPE Tissue Kit (Qiagen) was used, and the extraction was performed according to the kit's instructions.

4.2.2 Targeted NGS (I)

An Illumina Truseq Custom Amplicon library (Illumina) was designed to cover all 25 *ANO7* exons (from the hg19 assembly) with 97% gene coverage. The amplicon size was 175 bp, and 250 ng of DNA was used for sequencing (targeting and library preparation). The samples were prepared for MiSeq sequencing machine (Illumina). Sequencing was conducted on 22 mCRPC samples (tumour DNA) and 50 TPCC PrCa samples (blood DNA) as well as on 14 unaffected male controls (blood DNA).

The sequencing results were analysed with an in-house pipeline that included quality control with FastQC and adapter sequence trimming with Cutadapt, and the sequences were aligned to the hg19 reference genome using the Burrows-Wheeler Aligner. The files were then converted from SAM files to BAM files with the SAMtools package, BAM files from the same sample were merged, and duplicate reads were annotated with the Picard tools software suite. The variant call step was conducted with GATK using HaplotypeCaller, which produced gVCF files, which were further annotated using ANNOVAR.

The *ANO7* variants that had a deleterious impact according to the '*in silico'* analysis tool CADD, were rare globally (minor allele frequency <0.05) and

overlapped in previous familial PrCa studies were selected for validation in larger sample cohorts.

4.2.3 Genotyping (I, II)

For genotyping, ready-made TaqMan® SNP Genotyping Assay primers and probes (Bio-Rad) were used. The reactions were prepared according to the manufacturer's protocol using 10 ng of DNA, and the total reaction volume was 25 µl. The reactions were analysed with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the PCR programme recommended by the TaqMan protocol. In addition to the samples, all runs included a non-template control, positive control (heterozygous SNP carrier verified with Sanger sequencing) and negative control (SNP non-carrier verified with Sanger sequencing). The results were analysed with CFX Manager 3.1 software (Bio-Rad). Ten percent of the samples were randomly selected for Sanger validation. To investigate whether the *ANO7* rs77559646 and rs148609049 variants were mutated in tumours or if the variants are always in the germline, we analysed matching blood and prostate tissue samples from Auria cohort samples.

4.2.4 Statistical analyses (I, II)

In article I, all the analyses were performed with R version 3.2.2. Case-case analyses were performed to study the possible variant impact on PrCa susceptibility. To investigate the variant association with aggressiveness, the patients were divided into aggressive and non-aggressive groups for case-case analysis. In our studies, aggressive PrCa was defined according to the International Consortium for Prostate Cancer Genetics (ICPCG) criteria (Hunter 2017; Schaid and others 2006). The disease was classified as aggressive if PSA was ≥ 20 ng/ml, the Gleason grade was ≥ 8, the patient had metastatic disease, or PrCa was the cause of death. Differential expression was analysed the publicly available datasets from TCGA and Oncomine. In the analysis, we compared *ANO7* expression in normal and cancerous prostatic tissues. Expression quantitative trait loci (eQTL) analysis was performed for the rs77559646 variant in the Camcap and Stockholm datasets and for RNAseq results that originated from Finnish PrCa tumour RNAseq experiments (TPCC RALP samples). Kaplan-Meier and multivariate analyses were performed to investigate the association between patient survival and *ANO7* variants or *ANO7* expression level. The missing endpoints were censored from the survival analyses.

In article II, the statistical analyses were performed with SAS JMP Pro 14 version (SAS Institute). Kaplan-Meier analyses were performed for progression-free survival (PFS) and overall survival (OS). The PFS was defined from the date of initiation of docetaxel to the date of biochemical or radiological progression or

patient death. The radiographic response and progression were assessed following the RECIST 1.1 criteria (Eisenhauer and others 2009), whereas the criteria for biochemical response and progression were the same as those in the PROSTY study (Kellokumpu-Lehtinen and others 2013). OS was calculated from the date of docetaxel treatment initiation to the date of death.

4.2.5 BioID (III)

The principle of the Proximity-dependent biotin identification (BioID) method, which was originally created by Roux and others (Roux and others 2013), is visualized in Figure 6. In the method, the gene of interest is fused together with the biotin ligase BirA*. The fusion protein is expressed in cells, and BirA* labels all proteins that are in close proximity with the protein of interest. The biotin labelled proteins can then be purified with streptavidin beads and identified with mass spectrometry. The method allows the detection of both stable and transient interactions.

4.2.5.1 Cloning (III)

The BioID plasmid used was the pcDNA3.1 MCS-BirA(R118G)-HA plasmid (Addgene plasmid #36047; http://n2t.net/addgene:36047; RRID:Addgene_36047) (Addgene). The ANO7L insert was PCR-amplified from the pNGEP-L plasmid (insert size 2,799 bp). DH5 alpha competent cells were transformed with ANO7L and the control construct (the same plasmid without the ANO7L insert). The ANO7 insert was Sanger sequenced to verify the correct sequence.

An ANO7 construct that carried carboxy-terminal V5 and His-tags was used as a control for immunohistochemical protein-protein interaction validation staining.

4.2.5.2 Cell culturing, transfection and addition of biotin

Lymph node carcinoma of the prostate (LNCaP) cells were maintained in RPMI-1640 medium (Lonza) with inactivated 10% foetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM ultraglutamine in 5% CO_2 at 37°C. The cells were plated on Petri dishes and sterile glass cover slips. They were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) one day after plating with ANO7L-BirA*-HA and BirA*-HA constructs. In parallel, non-transfected LNCaP cells were grown. The transfection conditions were identical to those in Thermo Fisher Scientific's optimized protocol for the LNCaP cell line. The cells were incubated for 24 hours, and 59 µM biotin was added to the transfected and non-transfected cells. The cells were rinsed with PBS and lysed in lysis buffer after 24 hours. The samples were sonicated and rotated at 4°C for 15 minutes. The

supernatants were collected after centrifugation, and MyOne Streptavidin (MyOne Streptavidin C1 Dynabeads, 10 mg/ml, Invitrogen) beads were added to the lysates. After rotating the samples with the beads overnight, the beads were washed with BioID wash buffer and stored. Western blot samples were collected from the cell debris and purified beads and mixed with Laemmli buffer. The whole protocol was repeated three times to obtain a final number of replicates of six.

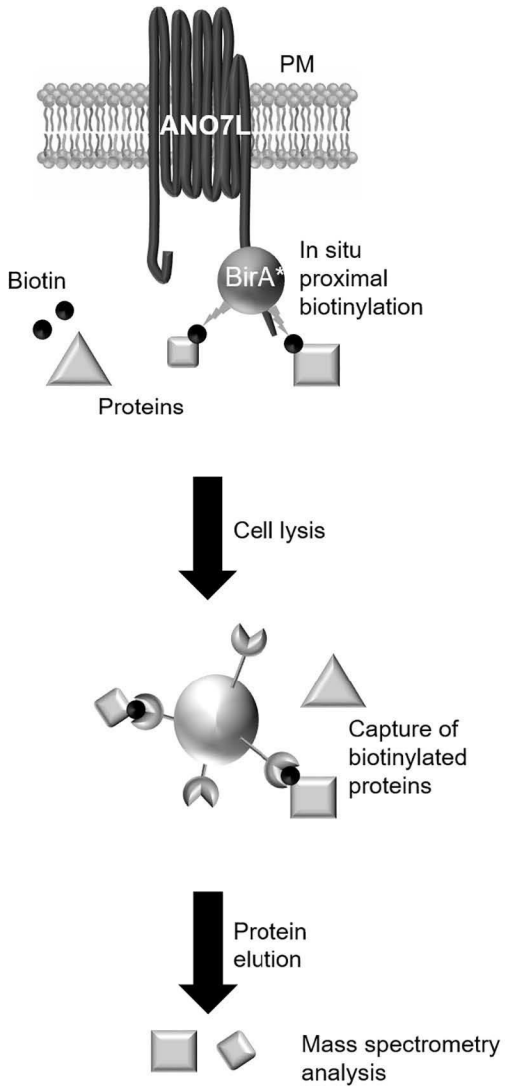

Figure 6. The principle of BioID purification. The transfected ANO7L-BirA*-HA fusion protein localizes on the plasma membrane (PM), and BirA* ligates biotin to all proteins interacting with ANO7L. The cells are then lysed, and streptavidin beads are used in collecting the biotinylated proteins. The proteins are then eluted and analysed with mass spectrometry.

4.2.5.3 Transfection of the dual-staining validation samples

For validation with dual staining, LNCaP cells were transfected with ANO7L-BirA*HA, BirA*-HA and ANO7L-His constructs. The cells were not treated with biotin.

4.2.5.4 Protein identification and filtering

The purified BioID samples were analysed at the Turku Proteomics Facility (Turku Bioscience, Turku, Finland) with an LC-ESI-MS/MS nanoflow HPLC system (Easy-nLC 1200, Thermo Fisher Scientific). The proteins were identified with Proteome Discoverer 2.2 software (Thermo Fisher Scientific). The required number of peptide-spectrum matches was two when filtering the data by peptide spectrum matches per protein. The list of proteins was compared to the common background of the BioID method (Roux and others 2013) and CRAPome database (https://www.crapome.org).

4.2.5.5 Enrichment analysis

The enrichment analysis for the identified proteins was performed with the Gene Ontology (GO) 'cellular component' enrichment tool. All proteins that passed the filtering described above were used in the analysis. The cut-off value for the false discovery rate (FDR) was <0.0001 with a fold enrichment >2.

4.2.5.6 Western blot

Western blot analysis was performed to validate that the fusion protein was the correct size and not degraded in the cells. Protein samples that were collected during the BioID protein collection protocol were run on 10% SDS-PAGE gels, and the fusion proteins as well as the biotinylated proteins were detected according to the protocol by Roux and others (Roux and others 2013). The biotinylated proteins were detected with Pierce™ High Sensitivity Streptavidin-HRP (21130, Thermo Fisher Scientific). Primary antibodies against the HA tag (H6908, Sigma-Aldrich) and HRP-conjugated anti-rabbit IgG (sc-2054, Santa Cruz Biotechnology) secondary antibodies were used in the detection of ANO7L-BirA*-HA and control BirA*-HA fusion proteins.

4.2.5.7 Immunofluorescence staining and imaging

The transfected LNCaP cells that were grown on sterile glass coverslips rinsed with PBS, fixed with PFA-PBS and permeabilized with Triton X-100. ANO7L-BirA*-HA was first verified to localize correctly near the plasma membrane. Additionally,

the biotinylation was verified to localize near ANO7L. For this purpose, antibodies against the HA tag and streptavidin-conjugated antibodies were used. After choosing the proteins for the dual staining colocalization test, ANO7L-BirA*-HA and ANO7L-His (and the BirA*-HA control) were stained in parallel with the endogenous interacting proteins. All antibodies used are listed in Table 4. The fusion proteins were verified with a Nikon Eclipse Ni-U upright fluorescence microscope (Nikon Instruments, Inc.) and the dual staining was visualized with a STED microscope (Abberior Instruments). ImageJ (NIH, version 18.0) was used for analysing the figures and generating fluorescence intensity profiles.

Table 4. Antibodies used in the BioID validation.

ANTIBODY	Source
Streptavidin-Alexa Fluor™ 488 conjugate	S32354 (Thermo Fisher Scientific)
Anti-HA tag antibody	H6908 (Sigma-Aldrich)
Anti-6X His tag® antibody	ab18184 (Abcam)
Mouse monoclonal [16B12] to HA tag	ab130275 (Abcam)
Anti-HSPA1A	ab79852 (Abcam)
Anti-AP2B1	ab220778 (Abcam)
Anti-COPG2	NBP2-55178 (Novus Biologicals)
Anti-SND1	ab65078 (Abcam)
STAR RED, goat anti-rabbit IgG	2-0012-011-9 (Abberior Instruments GmbH)
STAR 580 anti-mouse IgG	2-0002-005-1 (Abberior Instruments GmbH)

5 Results

5.1 PrCa patients frequently have *ANO7* mutations, and the types of variants vary between sample cohorts (I)

To identify the potential pathogenic *ANO7* variants, the whole gene from 14 unaffected males, 50 unselected RALP PrCa patients, 22 CRPC patients and 9 PrCa cell lines was sequenced. A total of 215 variants were found in the *ANO7* gene in the targeted whole gene sequencing. One was annotated as stop-gain mutation, seven were frameshift mutations, 16 were missense mutations, seven were mutations of the splice region, 64 were intronic mutations, 23 were non-coding mutations, four were synonymous mutations, and six were 3'UTR mutations. In addition, the variant call reported 44 variants downstream and 43 upstream of the *ANO7* gene. Control males did not have any splice site variants, unlike the PrCa cell lines, RALP patients and CRPC patients. Stop-gain variants were observed in CRPC and RALP patients but not in the controls or the PrCa cell lines. The CRPC patients more frequently had missense variants than the other groups. The PrCa cell lines, in turn, more frequent had frameshift variants than any other group (Figure 7).

Among the variants, we selected the three SNPs that were not observed in the controls but were seen in at least two CRPC patients, had a low minor allele frequency in the Finnish population (<5% in the ExAC database, http://exac.broadinstitute.org) and were deleterious according to mutation prediction programmes (combined annotation-dependent depletion [CADD] score >10*)*. The variants were a stop-gain variant, rs148609049 (C>T), in exon 1, a splice site/missense variant, rs77559646 (G>A), in intron/exon 4 and a missense variant, rs181722382 (T>C), in exon 25. The rs181722382 variant was so rare in genotyping results (<1%) that it was excluded from the more detailed analyses.

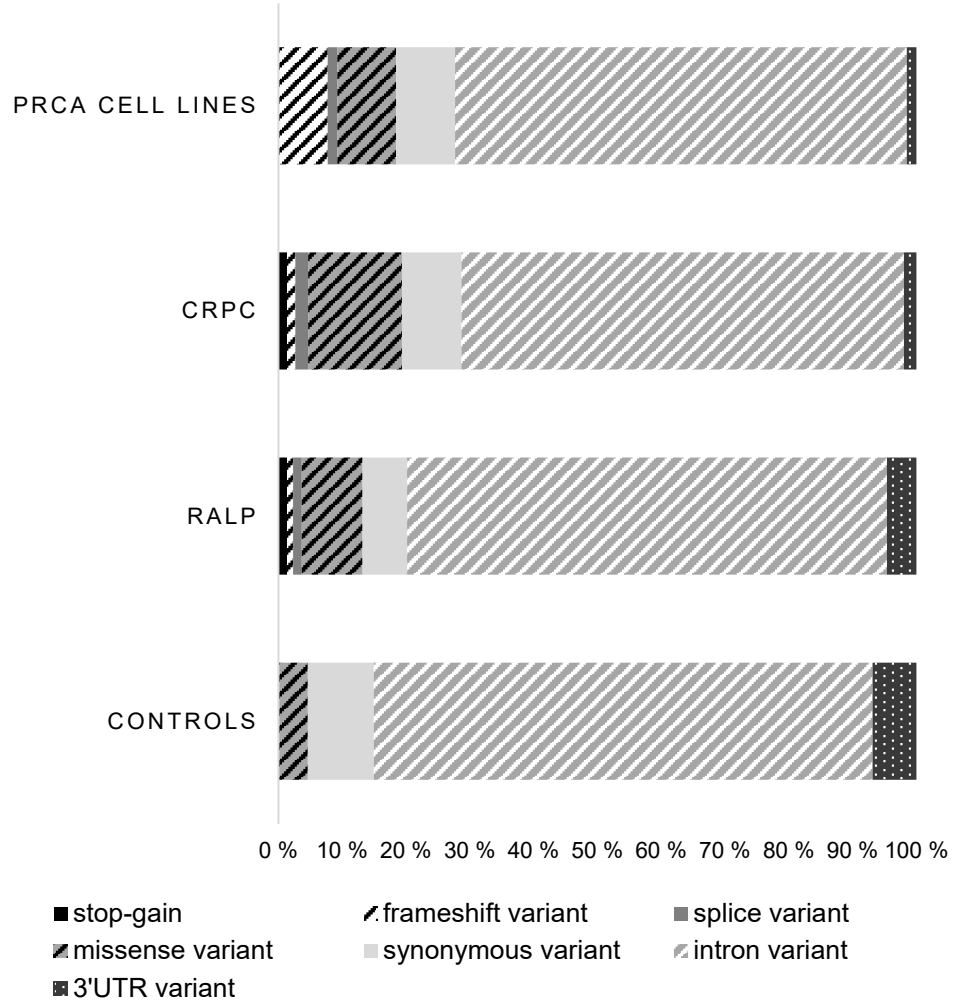

Figure 7. Variant types in targeted *ANO7* sequencing.

The variants rs148609049 and rs77559646 were genotyped in 1,679 PrCa patients and 1,711 controls. When observing different PrCa patient cohorts and unaffected control males, the rs77559646 frequency was the lowest in unaffected males (7.4%), followed by patients who were diagnosed in the screening trial (9.9%), clinical cases (10.5%), and familial cases (11.9%), and the greatest in docetaxel-treated CRPC patients (13.7%) (Figure 8.). The frequency of rs148609049 was relatively similar in all cohorts (7.2%, 5.9%, 7.6% and 7.6%, respectively) except in docetaxel-treated patients, in whom there were slightly more variant carriers (9.2%) (Figure 8.). The rs77559646 variant was also detected in the 22Rv1 PrCa cell line.

With matching blood and prostate tissue samples from the same patient, we showed that the variants already existed in the germline. Furthermore, the fact that the variant distribution in the sample was always approximately 0, 50 or 100 % suggested that the variants were not somatic mutations.

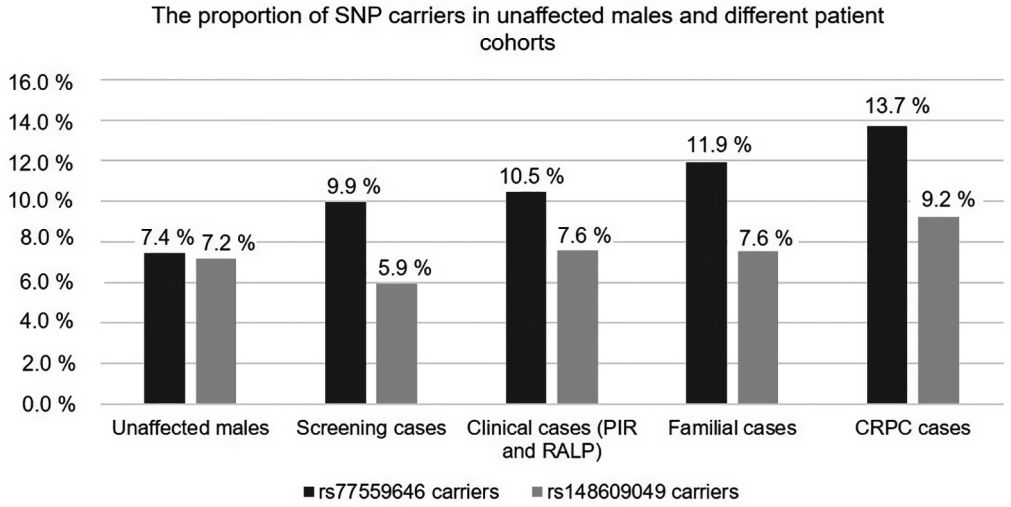

Figure 8. Rs77559646 and rs148609049 variant frequencies in different patient cohorts and unaffected males. The dark grey columns show rs77559646 carriers and the light grey columns rs148609049, respectively.

5.2 *ANO7* variants are associated with PrCa susceptibility and aggressiveness (I)

5.2.1 rs77559646 predisposes to PrCa in Finnish and Swedish males

According to the gnomAD (Genome Aggregation Database, https://gnomad.broadinstitute.org), the global minor allele frequencies for the variants rs148609049 and rs77559646 are 0.006 and 0.01, respectively, and for the Finnish population, they are 0.036 and 0.026, respectively. When transforming these numbers into the number of variant carriers, our results corresponded well to these proportions: 6.8% for rs148609049 and 6.9% for rs77559646.

We observed a statistically significant difference between the rs77559646 variant frequency in PrCa patients and that in unaffected males (OR 1.39; 95% CI 1.09-1.78), indicating that the variant predisposes to PrCa. A similar effect was observed in the Swedish cohort STHM2 (Nordström and others 2014) (OR 1.36;

95% CI 1.06-1.75). Rs148609049, in turn, was not associated with PrCa risk in any cohort.

5.2.2 rs148609049 and rs77559646 are enriched in PrCa families

The variants rs148609049 and rs77559646 were analysed in 142 PrCa families (664 family members in total). Both variants were frequently present in the investigated families (21/142 [14.8%] families carried the rs148609049 variant, and 25/142 [17.6%] carried the rs77559646 variant), showing an enrichment in the familial cases. However, in some cases, the index patient did not carry the variant, and some other family member(s) did. Eight families (5.6%) carried both variants. The variants were inherited separately in these families, which indicates that they are not in the same haplotype. Neither of the variants had complete segregation with the disease.

5.2.3 rs77559646 is associated with an aggressive PrCa phenotype

In case-case analyses, we compared the variant frequencies of non-aggressive PrCa cases and aggressive cases to investigate whether the variants were associated with an aggressive phenotype. The disease was classified as aggressive if one of the following criteria was met: PSA ≥20 ng/ml, Gleason score ≥8, presence of metastases, or PrCa was lethal (Schaid and others 2006). Rs77559646 was more common in the aggressive cases than in nonaggressive cases (genotype test p=0.04), whereas rs148609049 was not (genotype test p=0.68). Among the individual clinical variables, rs148609049 carriers had more other cancers than the non-carriers, and their cause of death was more frequently PrCa than other reasons (not statistically significant). In turn, there was a trend between rs77559646 and early-onset disease (not statistically significant). An enrichment in more aggressive cases was also seen when observing the frequency of the variant carriers in the different sample sets (Figure 8).

5.2.4 rs148609049 is related to decreased survival time among PrCa patients

Although the variant rs148609049 was not more common in aggressive PrCa cases than in nonaggressive PrCa cases, it correlated with poor survival in Kaplan-Meier log-rank analysis (p=0.049). In contrast, rs77559646, which was associated with aggressiveness, did not affect the survival time. The variants' effects on survival

were also assessed together with age, PSA and Gleason score. A Cox regression model revealed that rs148609049 had an independent unfavourable influence on patient survival (HR 1.56, 95% CI 1.03-2.37). Other significant factors in the model were age at diagnosis (HR 1.06, 95% CI 1.034-1.08) and Gleason score (HR 4.7, 95% CI 3.61-6.12).

5.3 rs77559646 is associated with elevated *ANO7* mRNA expression (I)

eQTL analysis for rs77559646 variant carriers and non-carriers indicated that the mutation was associated with an increased *ANO7* expression level in prostate tumour tissues. The association between the variant and mRNA expression was studied in three PrCa datasets. The first dataset included whole transcriptome sequencing data from the Finnish RALP samples (Turku Prostate Cancer Consortium), and the p-value for the eQTL test was 0.0086. The other two sample sets were from data from the Camcap and Stockholm cohorts that originated from Expression BeadChip-based transcriptional profiling analysis (Whitington and others 2016), and the p-values for their eQTL were $2.53*10^{-6}$ and $1.53*10^{-13}$, respectively.

5.4 rs77559646 is a prognostic marker for good docetaxel response (II)

Because the rs77559646 variant was observed to be associated with aggressive PrCa but there was no association with worse survival, we investigated whether docetaxel therapy would provide a benefit to variant carriers. Of the rs77559646 SNP carriers, 77.7% had a biochemical response to docetaxel treatment, whereas only 58.4% of the non-carriers responded (Figure 9). However, the difference between the groups was not statistically significant (OR=2.50; 95% CI 0.791–7.87; p=0.12).

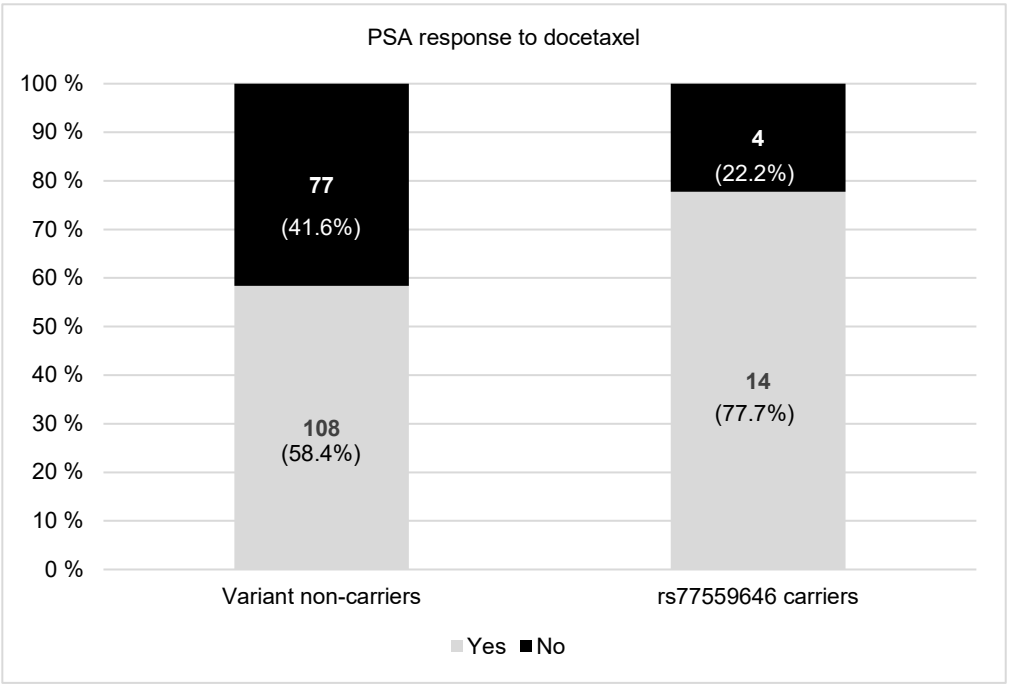

Figure 9. Biochemical response (PSA response) to docetaxel treatment among the rs77669646 carriers and non-carriers.

The docetaxel treatment-associated PFS times and the impact of rs77559646 on PFS were analysed in the PROSTY and the Auria mCRPC patient cohorts. As shown in Figure 10, the patients who did not have the rs77559646 variant had quite similarly shaped PFS curves in both cohorts. The PFS curves of the carriers, in turn, differed from those of the non-carriers. The PROSTY patients had a statistically significant difference between the carriers and non-carriers (p=0.005), but the Auria patients did not have significantly different PFS times when comparing the carriers and the non-carriers (p=0.09).

In the OS analysis, the only group that differed in survival time was the PROSTY rs77559646 variant carriers. The difference between the PROSTY carriers and non-carriers was statistically significant (p=0.003), whereas it was not significant for the Auria cohort (p=0.9). Notably, the non-carriers in the Auria cohort survived longer than the non-carriers in the PROSTY cohort.

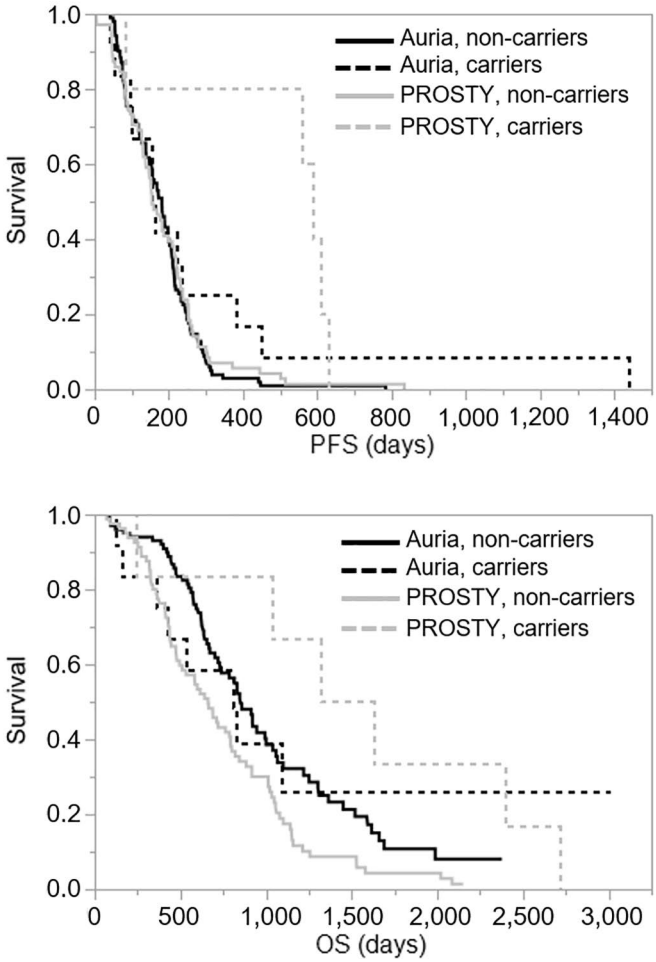

Figure 10. Kaplan-Meier survival plots for PFS and OS in the Auria (black lines) and PROSTY (grey lines) patient cohorts.

Cox regression analysis (Figure 11) was performed to model the impact of the rs77559646 variant and important clinical variables: Gleason score, PSA value at diagnosis and the time from diagnosis to docetaxel treatment initiation. The whole model was statistically significant for the PROSTY cohort ($p<0.0001$) but not for the Auria cohort ($p=0.057$). However, in both models, rs77559646 was a statistically significant variable that was able to predict PFS (PROSTY HR 3.9 [95% CI 1.5–12], $p=0.0027$; Auria HR 2.4 [95% CI 1.1-6.1], $p=0.024$). There were no other statistically significant variables in the Auria cohort. In the PROSTY cohort, the Gleason score was also a statistically significant variable in the Cox regression model (HR 1.8 [95% CI 1.0–3.1], $p=0.04$).

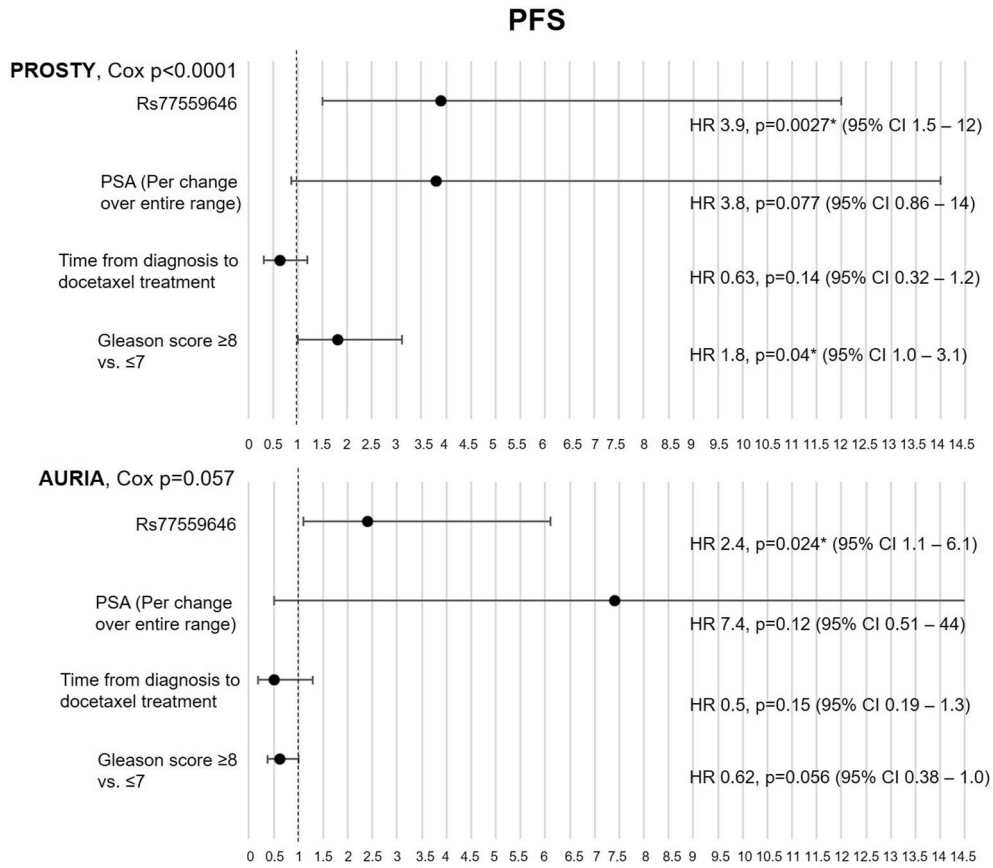

Figure 11. Cox regression analysis for progression-free survival time (PFS) after docetaxel treatment in PROSTY and Auria patient cohorts. The variables in the models are the rs77559646 variant, PSA, time from diagnosis to docetaxel treatment and Gleason score ≥8. PFS= progression-free survival; HR=hazard ratio; 95% CI= 95% confidence interval; PSA= prostate-specific antigen.

5.5 *ANO7* is highly expressed in PrCa, and the increased level predicts poor prognosis (I)

5.5.1 *ANO7* is highly expressed in prostate tissue, and its expression is elevated in PrCa vs. benign prostate tissues

ANO7 expression was monitored across normal and cancer tissues in the Oncomine database (Rhodes and others 2004). *ANO7* mRNA levels were higher in the prostate than in other tissues in both comparisons. Moreover, *ANO7* expression was elevated in PrCa versus benign prostate tissues in the TCGA prostate (Cerami and others 2012) and Arredouani prostate (Arredouani and others 2009) cohorts (p=0.00004).

5.5.2 Elevated *ANO7* expression is associated with shortened survival time among PrCa patients

To study whether *ANO7* mRNA expression has an impact on PrCa patient survival, Kaplan-Meier and Cox regression analyses were conducted. The TCGA database prostate cancer cohort (Cerami and others 2012) was used in survival analysis, and the patients were divided into 'higher' and 'lower' expression groups. The higher and lower levels were defined according to the mean expression value of the whole data set. The endpoint in this study was the death of the patient. High *ANO7* expression was strongly associated with poor survival among PrCa patients (hazard ratio 18.41; Cox p= 0.0255).

5.6 ANO7 interacts with proteins involved in cellular vesicle trafficking, the nuclear lumen, chromosomes, sites of DNA damage and the Arp2/3 complex (III)

The long form of ANO7 physically interacted with 64 proteins. The proteins were included in the list of interacting proteins if they were specific for the ANO7 construct and not found to interact with a control construct that lacked the ANO7 insert. These proteins were compared with the GO 'cellular component' enrichment tool, and the analysis revealed six partly overlapping components: 'Vesicle' (fold enrichment 2.54, FDR 9.26×10^{-6}), 'Extracellular exosome' (fold enrichment 3.77, FDR 2.57×10^{-7}), 'Nuclear lumen' (fold enrichment 2.37, FDR 5.51×10^{-5}), 'chromosome' (fold enrichment 5.50, FDR 2.67×10^{-7}), 'site of DNA damage' (fold enrichment 28.78, FDR 1.15×10^{-6}) and 'Arp2/3 complex' (fold enrichment >100, FDR 1.72×10^{-5}) (Figure 12).

ANO7 was validated to colocalize with four proteins identified with mass spectrometry: Heat shock 70 kDa protein 1A (HSPA1A), COPI coat complex subunit gamma 1 (COPG2), AP-2 complex subunit beta (AP2B1) and Staphylococcal nuclease domain-containing protein 1 (SND1) (Figure 13).

In Western blot analysis, ANO7L-BirA*-HA and the BirA*-HA control were confirmed to be the correct size (141 kDa and 36 kDa, respectively), and biotinylation of proteins was detected when the biotin ligase was present in the cells.

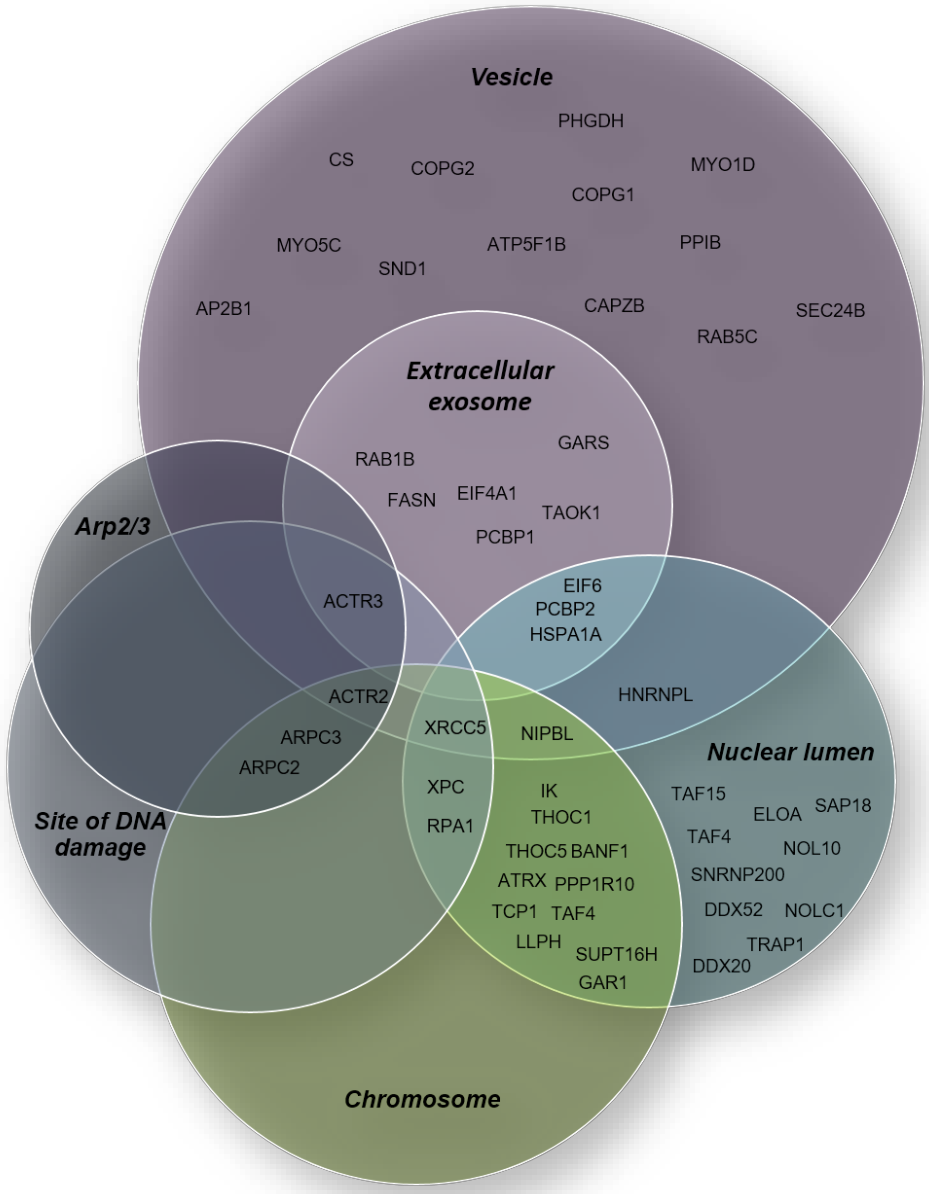

Figure 12. ANO7- interacting proteins divided into categories according to the GO cellular localization enrichment results. In total, 64 proteins were found to interact with ANO7, and they clustered into six cellular localizations, which were partly overlapping.

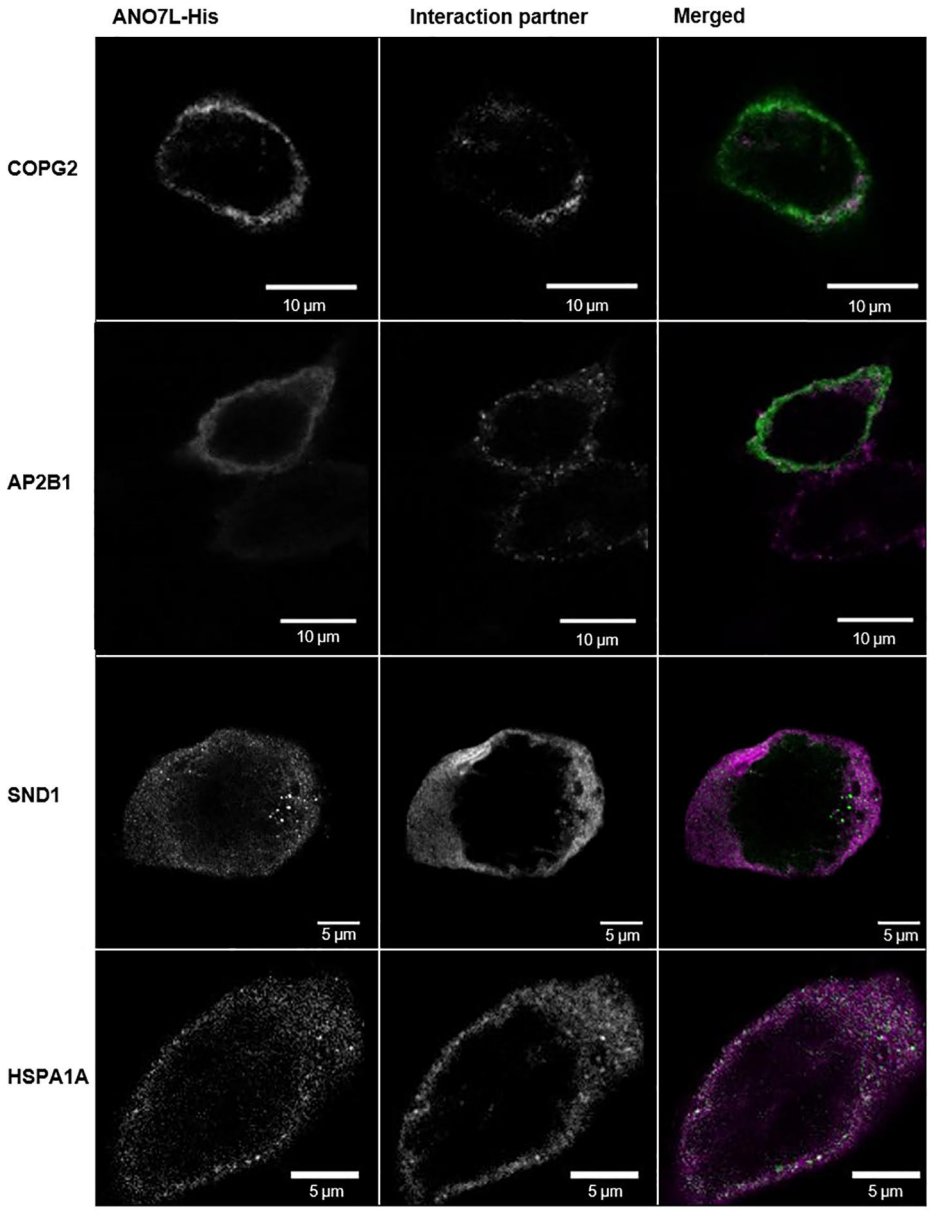

Figure 13. Double staining for overexpressed ANO7 and the identified interaction partners. The green colour shows the localization of transfected ANO7L-His, and the interacting proteins are shown in purple.

6 Discussion

6.1 ANO7 as a biomarker in PrCa

The *ANO7* variant rs148609049 was associated with poor PrCa patient survival in this study. Since the variant resides in the very first exon and creates a premature stop codon, it would be expected that no ANO7 protein is produced from the variant allele because the transcript is degraded by the nonsense-mediated decay (NMD) mechanism, which mainly destroys all transcripts that have a premature stop codon far from the real translation end site (Amrani and others 2006). We did not observe any impact of the variant on the mRNA expression level, but it still may have an impact on the protein level.

The variant rs77559646, in turn, was related to elevated *ANO7* mRNA expression, and it was demonstrated to be associated with PrCa susceptibility and aggressive disease. The '*in silico*' prediction suggested that rs77559646 would disrupt the splicing of the *ANO7* long form or alternatively cause an amino acid change in the *ANO7* short transcript. As the function of the soluble short form is unknown, it is difficult to predict its impact on protein function. However, the long form is likely degraded by the NMD mechanism. In normal cells, the level of functional protein would then be decreased. However, the NMD mechanism is known to be dysfunctional in cancer cells (Hu and others 2017), which can lead to the accumulation of truncated protein products. If the NMD mechanism is not working in the cancer cells, it could mean that the variant carriers would have a truncated version of ANO7L.

Both the rs77559646 and rs148609049 variants, which were found in this thesis, predict aggressive PrCa and could be used to separate indolent and aggressive cases. Resources could then be better focused on the aggressive cases. At the same time, indolent cases could avoid overdiagnosis and treatments that can cause severe side effects. Approximately one of ten Finnish PrCa patients carries the rs77559646 mutation, and one of fifteen carries the rs148609049 mutation, which would mean that there are approximately 500 and 300 PrCa affected variant carriers diagnosed each year, respectively. *ANO7* germline mutation detection can provide new tools for PrCa diagnostics and treatment selection. This test would add value to the already existing PrCa cancer panels.

The finding that the rs77559646 variant did not affect patient survival, although it is related to aggressive disease, led us to investigate the efficacy of CRPC treatment among carriers and non-carriers. It was shown that variant carriers indeed benefit from docetaxel medication more than non-carriers. However, before recommending docetaxel instead of other CRPC treatment options, extensive prospective studies in which docetaxel, abiraterone and enzalutamide are compared are required. Since abiraterone and enzalutamide have been in clinical usage for a relatively short time period, performing a similar retrospective study for the benefit of second-generation AR-targeted therapies is challenging.

Germline variants can be tested non-invasively from a blood draw. Compared to other available biomarkers, germline variant detection is sensitive, reliable, and immutable over time, and the signature does not vary between distinct cancers. Other detection methods are still useful in monitoring treatment effectiveness. Thus far, the only germline mutations that have been reported to have prognostic value in separating aggressive cases from indolent cases are located in DNA repair genes. They have been additionally demonstrated to affect CRPC treatment efficacy. PrCa patients with germline mutations in DNA repair genes were shown to benefit more from PARP inhibitors than from other mCRPC medications, but standard therapies have been shown to be as effective for variant carriers and non-carriers in retrospective studies (Mateo and others 2018). Although DNA repair genes are very relevant in PrCa progression, and the *BRCA2* gene is also related to aggressive PrCa (Castro and others 2013), DNA repair genes account for only 15% or less of the PrCa cases (Lindström and others 2007). Therefore, there is still a need for additional germline biomarkers, such as *ANO7* variants.

6.2 Elevated *ANO7* expression is linked to poor PrCa patient survival

This thesis work showed that the *ANO7* mRNA expression level was higher in PrCa than in benign prostate tissues. Moreover, the increased expression correlated with shortened survival time. In a study by Chandran and others ANO7 mRNA expression was demonstrated to be downregulated in PrCa metastases compared to the level seen in the primary tumour (Chandran and others 2007; Kiessling and others 2005). Possibly due to the different detection methods used, the existing literature conflicts with our results. Our analyses were performed on RNAseq data, while Kiessling and Chandran used probe-based detection that was designed for the last exon of *ANO7*. Therefore, the short *ANO7* transcript was not detected in their studies.

As we have detected all *ANO7* transcripts, including those that may not be translated into a functional protein, we cannot conclude that high amounts of ANO7

protein are seen in PrCa. However, ANO7 clearly has a role in PrCa development, which needs to be further investigated.

6.3 Possible functions of ANO7 in PrCa

The association studies indicated that ANO7 is biologically relevant in PrCa development. However, as so few experiments have been performed on the ANO7 protein, the mechanism of ANO7 and the effects of the identified variants could only be speculated. In particular, the question of why taxanes are effective in treating rs77559646 variant carriers has arisen. Is it because taxanes affect rapidly dividing and aggressively growing cells? Alternatively, is docetaxel affecting the pathways in which ANO7 functions? Since no pathway studies exist for ANO7, we wanted to capture ANO7 protein interactions to obtain a better understanding of ANO7 function. The results indicated that the enriched ANO7 localizations in the GO enrichment analysis were vesicles and extracellular exosomes, the nuclear part of the cell, and the actin-related protein 2/3 (Arp2/3) complex, which is involved in the actin nucleation process.

Four proteins that were found to interact with ANO7 according to the BioID analysis were associated with vesicle trafficking. These proteins were validated to localize with ANO7. Two of these proteins, COPG2 and AP2B1, are involved in intracellular vesicle trafficking. The two other proteins, HSPA1A and SND1, have been found in extracellular vesicles (Bijnsdorp and others 2013; Hosseini-Beheshti and others 2012; Soekmadji and others 2017). The colocalization of these proteins with ANO7 supports the suggested involvement of ANO7 in vesicle trafficking.

As many anoctamins, including ANO7, have been shown to have phospholipid-scrambling activity (Suzuki and others 2013), the vesicle formation process could be associated with their scramblase function. The phosphatidylserine phospholipids are unevenly distributed in the plasma membrane such that the majority of the phosphatidylserines are facing the cytosol. The phospholipid scrambling proteins can equalize the distribution between the inner and outer plasma membrane leaflets and cause bending in the plasma membrane and initiate cellular vesicle formation (Bevers and Williamson 2016). Other possible consequences of scrambling include the activation of the cell signalling system (Bevers and Williamson 2016). The ANO7 protein has been detected in patient-derived prostate-specific types of extracellular vesicles, i.e., prostasomes (Dubois and others 2015; Poliakov and others 2009), which again supports our finding that ANO7 interacts with other proteins involved in vesicles. As ANO7 has been detected on the apical side of epithelial cells (Schreiber and others 2010), it may have a function in producing and releasing extracellular vesicles, especially prostasomes, into seminal plasma. In cancerous tissues, where the cells lose their polarization, the released prostasomes

and prostate-derived exosomes can cause immunological suppression (Lundholm and others 2014), promotion of angiogenesis (Ronquist and others 2010) and even metastasis formation (Azmi and others 2013). Exosomes are interestingly shown to spread docetaxel resistance between PrCa cells (Corcoran and others 2012). This would be a possible explanation for the longer docetaxel treatment responses seen in *ANO7* rs77559646 variant carriers than in non-carriers. The theory warrants further investigation, but our finding that ANO7 is involved in vesicle transport is consistent with a recent study by Kunzelmann and others, which suggested that anoctamins are involved exosome production and membrane curvature modification (Kunzelmann and others 2019).

The Arp2/3 complex, which was among the enriched cellular localizations, could be related to intracellular trafficking since the vesicles are transported along the actin filaments and microtubules, and it has been demonstrated that the disturbance of the complex inhibits vesicle trafficking (Whitlock and Hartzell 2017). The innate interaction with nuclear proteins is not as clear since ANO7 has been shown to be located in membrane structures such as the plasma membrane (Bera and others 2004; Das and others 2007; Dubois and others 2015), ER (Duran and others 2012) and Golgi apparatus (Das and others 2007). Prostasomes as well as some other types of extracellular vesicles contain chromosomal DNA (Ronquist and others 2012), which could explain this association.

6.4 Study limitations

In article I, the patient material was limited to Caucasian patients, and in article II, all patients were Finnish. Therefore, the observations should be validated additionally in other populations.

In article II, as the variant rs77559646 is relatively rare at the population level, only a few carriers were present in our study cohorts. The small number of carriers weakens the reliability of the survival analysis results. Additionally, the DNA partly originated from FFPE tissues, which are not optimal for germline variant detection because, in theory, it is possible that ANO7 mutations could be somatic. However, we demonstrated by using blood and tumour tissue pairs from the patients that rs77559646 was always detected in the blood if it was present in the tumour sample. Moreover, even in prostate tissue, the frequency of the variant was always approximately 0%, 50% or 100%. Somatic mutation frequencies do not follow this distribution, and this fact is commonly utilized in the computational separation of somatic mutations from those in the germline (Sun and others 2018). Furthermore, while in the prospective PROSTY cohort the patients were followed on a regular basis, the retrospectively collected information was not as accurate because the data were collected afterwards, and the follow-up was more irregular. Additionally, the

treatment failure time points in the Auria cohort were not as reliable as those in the PROSTY cohort. This could also be the reason why the difference in PFS was not statistically significant in the Auria cohort. The patients in the PROSTY cohort were not treated with second-line abiraterone or enzalutamide, but most of the patients in the Auria cohort received one of these therapies. This can mask the effect of docetaxel on OS. When comparing the survival plots of the non-carriers in the Auria and PROSTY cohorts, the Auria patients had better survival than the PROSTY patients.

In article III, we used transient transfection and only one cell line to show the protein-protein interactions. The results represent the ANO7 interactions in PrCa but not necessarily the function of ANO7 in normal prostate cells. The reason for testing the interactions only in LNCaP cells was that it is the only PrCa cell line that endogenously expresses *ANO7,* and the interacting proteins were thus predicted to be present in the cells. In addition, the weakness of the BioID method is that it does not require the binding of the protein of interest to the interacting proteins, as the biotin ligase labels all proteins that are in close proximity in the cells. To prove that the proteins bind with each other, an additional method (for instance, co-immunoprecipitation) should be implemented. However, the detergents that are strong enough to remove the hydrophobic ANO7 from the membrane can also break the interaction between the proteins.

6.5 Future prospects

There are several additional issues that need to be addressed. What kind of transcripts do the normal and mutated *ANO7* genes produce? Are all the transcripts translated into a protein? Is the produced protein functional?

To further study the impact of the *ANO*7 rs77559646 variant on the mRNA and protein products, functional studies are still necessary. Because the SNP is predicted to weaken normal splicing of ANO7L, a splicing minigene assay (Steffensen and others 2014) would be useful in confirming this process. If normal splicing is disrupted, the protein product is likely to be destroyed by the nonsense-mediated decay mechanism, and no functional product would be formed. In ANO7S, rs77559646 leads to an amino acid change, which can change the functionality of the protein. By overexpressing the altered ANO7S tagged with a fluorescent label, it would be possible to detect whether the protein localization is from that of the normal short form. Overexpression studies could also be used to observe the effect of this protein product on, for example, cell viability and motility.

The function of the stop-gain mutation rs148609049 could be tested by silencing the whole *ANO7* gene. Accordingly, the impact of ANO7 on cancer cells can be tested by overexpressing the gene in cell lines. After silencing and overexpressing

ANO7, RNAseq could be performed to reveal the pathways that ANO7 influences. Furthermore, the impact of overexpression and silencing could be observed with invasion, proliferation, migration, and apoptosis assays. After receiving more information about ANO7 function, docetaxel (and possibly other CRPC drugs) could be added to the cell cultures to investigate how they affect cell growth.

The impact of ANO7 on vesicle formation could be determined by overexpressing and silencing ANO7 and then observing the number of vesicles that the cells produce.

This thesis has pinpointed that ANO7 has potential as a biomarker of PrCa, but before utilizing the test in clinical practice, additional functional studies are warranted. Some of these studies are already ongoing in our research group, and we expect to determine more about ANO7 in PrCa in the near future.

7 Conclusions

The *ANO7* variants that were found in this thesis work could be used as biomarkers for PrCa diagnostics for predicting disease progression. Additionally, the variant rs77559646 predicts a good response to first-line docetaxel chemotherapy and would therefore be a useful tool in personalized medicine and treatment planning. Before the test can be utilized in clinical practice, more extensive prospective studies are warranted. However, in combination with PSA, clinical information and other germline and somatic gene markers, testing of *ANO7* variants could prove useful.

Although this study revealed that ANO7 could function in vesicle trafficking, more information about the mechanisms of action is still needed.

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