Tampereen yliopisto

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CIRCULATING FREE DNA AS A BIOMARKER IN PROSTATE CANCER

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ABSTRACT

Lauri Ryyppö: Circulating free DNA as a biomarker in prostate cancer
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In this thesis, scientific articles on circulating free DNA and circulating tumor DNA will be reviewed to gain a revised understanding of how they are used in diagnosing and prognosing prostate cancer in its different stages.

Prostate cancer is the most common cancer for men. In recent years, the use of circulating free DNA has been studied as a prominent prognostic and diagnostic marker for prostate cancer. As analysis methods in investigating DNA sequences have evolved, so have the possibilities in using this novel approach to guide healthcare decisions for cancer patients.

In this thesis, research articles will be discussed to gain an understanding of how the use of cfDNA can be used to understand prostate cancer in all its stages. Initially, the concentration of ctDNA from cfDNA was used to predict the course of the disease; however, as sequencing methods have evolved, ctDNA's sequence itself has become the focus of studies. Nowadays, specific mutations on the nucleotide level can be analyzed using next-generation sequencing. In addition, chromosomal changes, epigenetic methylation, and DNA integrity can be analyzed to gain a more holistic view of changes in cancerous tissue. Using ctDNA, biopsies can be taken serially as a means of liquid biopsy. Liquid biopsies are without risk to the patient and cause less discomfort, unlike tissue biopsies. Liquid biopsies also give more information on cancer genetic heterogeneity.

While the mutations are the focal point of this thesis, the most prominent wet-lab applications are also covered on a fundamental level. Understanding the aberrations in prostate cancer will also enable more informed decision-making for the patient. This thesis aims to provide an overview of the most common mutations prostate cancers harbor and the current practice of treating them.

The use of ctDNA and cfDNA broadens the scope for treating prostate cancer; however, it cannot encompass all of the disease's complexity. Still, new approaches bring many exciting new methods to the prognosis and diagnosis of prostate cancer.

Keywords: circulating tumor DNA, circulating free DNA, prostate cancer, metastatic castration-resistant prostate cancer

The originality of this thesis has been checked using the Turnitin OriginalityCheck –program.

TIIVISTELMÄ

Lauri Ryyppö: Soluvapaa DNA eturauhassyövän biomarkkerina
Kandidaatintutkielma
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Tässä opinnäytetyössä soluvapaan DNA:n ja kasvaimesta olevan kasvain-DNA:n käyttösovelluksia arvioidaan eturauhassyövän yhteydessä.

Eturauhassyöpä on yleisin syöpä miehillä maailmanlaajuisesti. Viime vuosina soluvapaan DNA:n käyttöä on tutkittu mahdollisena ennusteellisena ja diagnostisena tekijänä eturauhassyövässä. Analyysimenetelmien kehittyessä myös soluvapaan DNA:n sekvenssien tutkiminen on kehittynyt, mikä mahdollistaa näiden menetelmien käyttämisen myös yksilöllistetyssä terveydenhuollossa eturauhassyöpäpotilailla.

Tässä työssä käydään läpi tieteellisiä artikkeleita, joiden kautta muodostetaan ymmärrys, miten soluvapaata DNA:ta voidaan käyttää eturauhassyövän tutkimuksessa sen eri vaiheiden aikana. Alun perin soluvapaan DNA:n ja kasvain-DNA:n osamäärää käytettiin taudin etenemisen arvioinnissa. Kuitenkin sekvensointimenetelmien kehittyessä kasvain-DNA:n sekvenssistä on tullut tutkimusten keskipiste. Nykyään tiettyjä mutaatioita voidaan analysoida nukleotiditasolla uuden sukupolven sekvensointitekniikoilla. Tutkimuksissa voidaan myös keskittyä kromosomitason muutoksiin, epigeneettisiin metylaatioihin tai DNA:n kuntoon, jotta saadaan tarkempi kokonaiskuva syöpäkudoksen muutoksista. Kasvain-DNA:ta voidaan myös eristää potilaasta nestebiopsiana, mikä aiheuttaa vähemmän riskejä ja haittoja potilaalle verrattuna kudoskoepalan ottoon sekä antaa paremman käsityksen syövän geneettisestä monimuotoisuudesta.

Mutaatiot ja niiden kehittyminen ovat tämän työn pääaiheena. Muutosten ymmärtäminen mahdollistaa myös parempaa päätöksentekoa potilaan hoitoon liittyen ja mahdollistaa yksilöidyn lääkehoidon identifioimalla mahdollisia hoidon kohteita genomissa. Tässä työssä käydään läpi erilaisia laboratoriomenetelmiä yleisellä tasolla. Tämä opinnäytetyö pyrkii myös kuvaamaan, miten eturauhassyöpä kehittyy ja mitä hoitoja siihen on tarjolla.

Soluvapaan DNA:n ja kasvain-DNA:n käyttö laajentaa mahdollisuuksia eturauhassyövän hoidossa, mutta se ei pysty kattamaan syövän kaikkea monimuotoisuutta. Kuitenkin uudet lähestymistavat tuovat uusia ja mielenkiintoisia tapoja ennustaa ja diagnosoida eturauhassyöpää.

Avainsanat: kasvain-DNA, soluvapaa DNA, eturauhassyöpä, metastaattinen kastraatioresistentti eturauhassyöpä

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CONTENTS

1. INTRODUCTION

Globally, prostate cancer is the second most frequent cancer and also the fifth leading cause of death caused by cancer in men (Bray et al. 2018). Early detection is crucial in prostate cancer; at a localized stage, prostate cancer can be treated curatively with local therapy, radical prostatectomy, or radiotherapy targeted at the prostate gland (Tan et al. 2017). If the disease progresses after local therapy, the prostate gland can be deprived of androgens, such as testosterone and dihydrotestosterone (DHT). The treatment is called androgen deprivation therapy (ADT). This treatment inhibits androgen signaling, which is crucial for prostate cancer progression. The hormone deprivation inhibits the prostate's growth and is often used as a first-line treatment for advanced prostate cancer. (Perlmutter, Lepor 2015)

Over time, ADT stops working for the patient, and the activity of the androgen receptor (AR) signaling route resumes. There are several ways how the prostate gland and the cancerous tissue can enable the ARs to continue the stimulation: by duplicating the AR-associated genes, which in turn produce more receptors for the declined number of androgens still in circulation, or by mutations to the prostate gland to start producing androgens of its own. The AR-associated gene expression might also activate independently, without the activation of the ARs. (Chen et al. 2004, Kellokumpu-Lehtinen and Tammela 2013)

In prostate cancer and other cancers as well, the primary tumor disseminates cancer cells by expanding to nearby tissues via the lymphatic system or blood vessels. This process is called metastatic dissemination, and it can cause the spread of cancer. At first, the cancer cells can create micrometastases in the patient's body. These micrometastases can remain inactive at first in their new location but can be activated when new mutations change the cell behavior. This mechanism creates clonal expansion from the primary tumor to other parts of the body. (Hanahan, Weinberg 2011)

When the efficacy of ADT lessens and metastases are present, the disease has progressed to metastatic castration-resistant prostate cancer (mCRPC). At this stage, the disease is incurable, and the need to understand the mechanisms behind the disease's progression is essential to enable efficient treatment for preventing disease progression. Median survival for mCRPC is estimated to be between 19-30 months. (He et al. 2020) The progression of prostate cancer, examples of mutations, and relative tumor activity is illustrated in Figure 1.

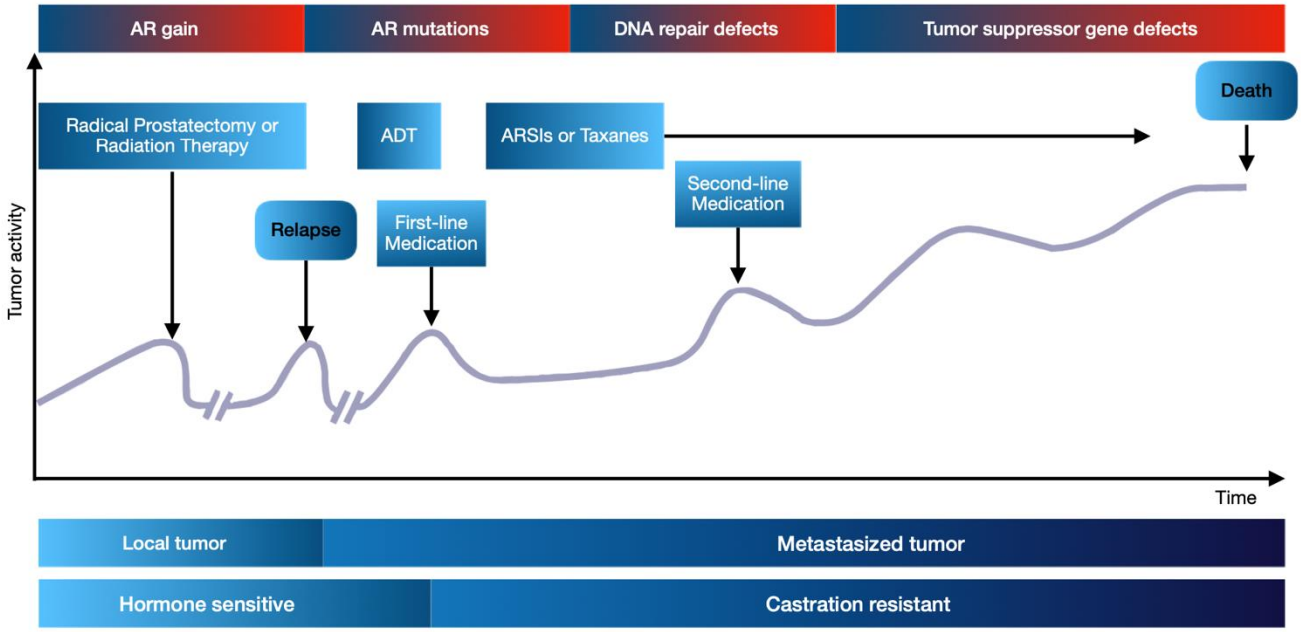

Figure 1. The development of prostate cancer with mutation and treatment examples. Over time cancer metastasizes and becomes castration-resistant. ADT: androgen deprivation therapy; AR: androgen receptor; ARSI: androgen receptor signaling inhibitors; Taxanes: docetaxel or cabazitaxel. Relative tumor activity from (González-Billalabeitia et al. 2019).

Circulating free DNA (cfDNA) and circulating tumor DNA (ctDNA) are discussed in this thesis as they offer a novel approach to tissue biopsies. cfDNA can be found circulating in the bloodstream under normal circumstances, and people do not have to have a cancer burden to have quantifiable amounts of cfDNA. This nonmalignant DNA can be found in the blood after heavy physical training, myocardial infarction, or during pregnancy as fetal DNA. ctDNA, on the other hand, can be detected in the bloodstream in 180 – 200 base pairs in size. These DNA fragments are derived from the circulating tumor cells (CTCs); this is the difference between ctDNA and CTCs. The ctDNA in a patient's blood can be derived from necrotic tumor cells or tumor cells undergoing apoptosis. In addition, ctDNA can be secreted to the bloodstream in an extracellular vesicle as a means of tumorigenesis communication. (Moreno, Gomella 2019)

The ctDNA can be acquired from a blood sample of prostate cancer patients as a means of a liquid biopsy. This method provides the ability to assess the genomic changes serially as blood can be drawn at various time points during treatment. The method also provides an overview of genetic changes of heterogeneous metastatic lesions within the patient. The use of ctDNA has also been proposed to be used as a biomarker to investigate relapse by identifying minimal residual disease (MRD) (Igna Us Ou et al. 2018). In addition, the method is less invasive, logistically simpler, and more cost-effective compared to tissue biopsies. (Sonpavde et al. 2019)

Advances in laboratory techniques and the field of bioinformatics have enabled more precise measurements to be conducted on nucleic strands in general, and these methods can be adapted to the

research of ctDNA as well. For example, digital polymerase chain reaction (dPCR) can be used to precisely measure the amount of DNA found in the original blood sample of the patient (Baker 2012). In bioinformatics, the methods have improved in detecting various alterations in the genome as the price of whole-genome sequencing has reduced, which means that the methods can be applied in a clinical diagnostic setting (Carla G Van El et al. 2013).

The acquired sequential data from the metastasized cancer can be analyzed as cancer treatment continues. Various treatments for mCRPC exist, but these treatments' effectiveness may differ significantly from one patient to another. Therefore, the need for personalized healthcare increases. Identifying ineffective treatments would result in fewer patients being exposed to unnecessary side-effects that the medications cause. (Adalsteinsson et al. 2018)

It is also worth noting that as castration-resistant prostate cancer (CRPC) research has evolved, so have the terms that describe the disease. CRPC, androgen-independent prostate cancer (AIPC), and hormone-refractory prostate cancer (HRPC) have all been used synonymously, while CRPC is the preferred term. (Bellmunt, Oh 2010). In addition, mCRPC refers to CRPC when cancer has metastasized.

Aims of the Study

This thesis discusses alterations in the cfDNA or ctDNA that have been reported in prostate cancer and mCRPC. Different mutations will be discussed and how they have been analyzed. In the last section of this thesis, the clinical significance of these studies will be summarized with a focus on personalized healthcare. This review aims to provide an introduction to the use of cfDNA analysis and how it could be used to improve diagnoses and treatment of prostate cancer in its different stages.

Electronic and manual search strategy

Two electronic databases, Andor and the National Institute of Health (NIH) were used to search for publications for this paper. In addition, many publications were added to this project from the references from the bibliographies of the articles that were preliminarily discovered. Dates from 2010 through 2021 were used in the searches. Articles were selected for this study based on their title and abstract. A flowchart of the search strategy can be seen in Figure 2.

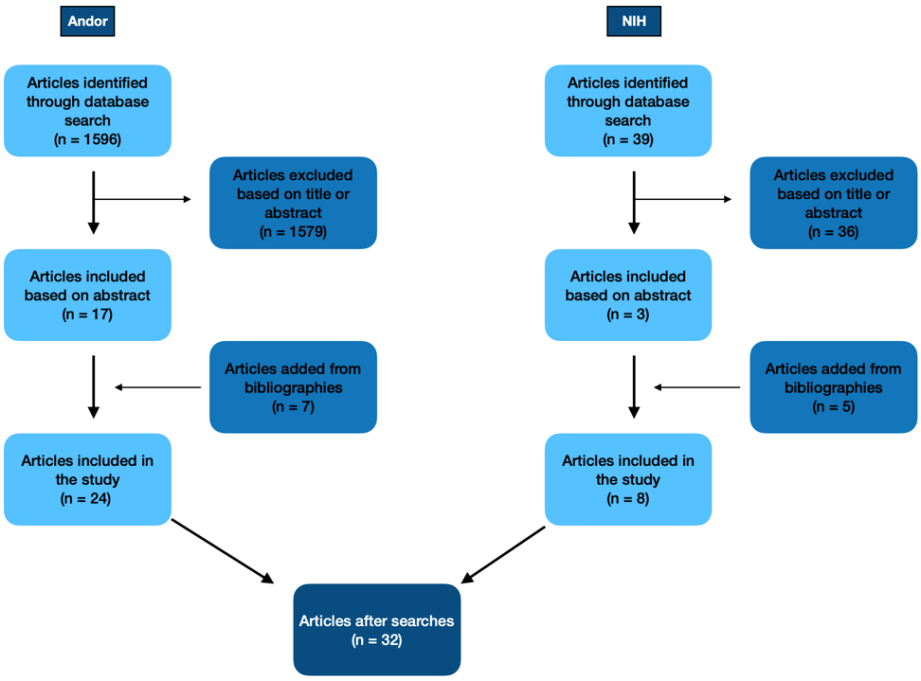

Figure 2. A flowchart of searches for this review

2. GENETIC ABERRATIONS IN PROSTATE CANCER

The main driver for cancers is mutations in the cancer cells and the neovascularization it can create. The mutations can be in the DNA sequence itself or affect by epigenetic means. Many cancer-specific mutations are recognized; however, some mutations are known to play a part in many different types of cancer. Prostate cancer-specific mutations are also known. These mutations can, for example, alter the expression of ARs. (Alberts et al. 2014)

Understanding these changes enables researchers to analyze how prostate cancer becomes malignant and differentiate them from benign prostate hyperplasia (BPH). Also, when prostate cancer evolves from a localized stage to mCRPC, the tumor mutation burden can predict metastasis and the disease's overall prognosis. The subsequent chapters address what cfDNA may uncover and what researchers have discovered so far. In Table 1. the studies used in this section are summarized.

Table 1. Studies with a focus on prostate cancer mutations and their significance. BPH: benign prostate hyperplasia; PCa: prostate cancer; PCR: polymerase chain reaction; PSA: prostate-specific antigen; NGS next-generation sequencing; OS: overall survival.

Reference	N	Population Characteristics	Analysis Technique	Significance
Jung et al. 2004	BPH: 34 & PCa: 91	No treatment for BPH. Hormonal therapy, radical prostatectomy, or radiotherapy for PCa.	Fluorometric microplate assay	cfDNA is of limited validity as a metastasis marker in PCa but could be used in combination with other methods in follow-up studies.
Kwee et al. 2012	8 CRPC	Docetaxel	Methylation-specific PCR	cfDNA concentration can be used to measure therapeutic responses.
Feng et al. 2013	96 PC & 112 BPH	Not mentioned	Real-time PCR	cfDNA integrity could be used to differentiate between BPH and PCa for patients with PSA ≥ 4 ng/ml.
Robinson et al. 2015	150 mCRPC	Various	Sequence data from whole-exome, germline, and transcriptomes.	Assessing genomic differences between primary PCa and mCRPC to determine these alterations' relevance to a clinical setting.
Azad et al. 2015	62 mCRPC patients	Undergoing abiraterone or enzalutamide	aCGH for chromosome copy number analysis. NGS of AR exon 8	*AR* gene aberrations in cfDNA are associated with resistance to enzalutamide and abiraterone in mCRPC.
Belic et al. 2018	148 mCRPC	Abiraterone or enzalutamide	Whole-genome sequencing and 31-gene deep sequencing.	Elevated ctDNA levels are strongly correlated with bone metastasis. AR amplifications are inconsistently associated with poor outcomes in AR-targeting drugs.
Haldrup et al. 2018	PC:169, mPC: 15, CRPC:7	Various	Methylation-specific PCR	Methylation of specific genes can be used for prognosis in PCa.
Mehra et al. 2018	517 mCRPC	Docetaxel or cabazitaxel	Commercial kits and spectrophotometer.	cfDNA concentrations can be used as an independent prognostic biomarker for mCRPC. A high baseline concentration predicts poor outcomes for taxane use.

Annala et al. 2018	202 mCRPC	Abiraterone or enzalutamide	Whole-exome sequencing & 72-gene deep sequencing.	Liquid biopsies can be used to guide the selection of AR-targeted therapies.
Hendriks et al. 2018	47 CRPC	Before chemotherapy or AR inhibitors	Methylation-specific PCR	Methylated areas of DNA can be used to make treatment decisions for patients with CRPC. Also, methylation markers can be used to predict OS.
Sonpavde et al. 2019	163 mCRPC	Various treatments	Guardant360 genomic profiler	*AR* & *MYC* alterations compromise clinical outcomes. Immune checkpoint, *AR*, and *MYC* inhibitors could be used for treatment.

2.1 Mutations in prostate cancer

Mutations in the AR pathway, DNA repair, cell cycle, PI3K, and WNT pathway are among the most essential genes and pathways that have been associated with the development and progression of prostate cancer. (Robinson et al. 2015)

These mutations, discussed in this chapter, are only a part of the mutations linked to prostate cancer. However, they are the most common and clinically most important ones. The mutations can be somatic, meaning that they only affect one individual and will not be passed on to future generations. Mutations can also be germline-related, meaning that the mutations will be passed on to the next generation, increasing the risk of prostate cancer if specific mutations run in the family.

2.1.1 Androgen receptor-related genes

ARs have an important role in the treatment of metastatic prostate cancer as primary care is ADT, where testosterone concentration is decreased to castration level. Arun A. Azad et al., (2015) discovered that using cfDNA AR's aberrations can be analyzed. The AR gene's aberrations were associated with treatment resistance to enzalutamide and abiraterone in mCRPC.

By performing copy number analysis on exon 8 of the *AR*, it was discovered that out of the 62 plasma samples of patients with mCRPC, over half of them had aberrations in the *AR* gene (Azad et al. 2015). These alterations were copy number increases and mutations or both. Furthermore, amplification of *AR* is linked to enzalutamide resistance.

It was also concluded that *AR* amplification was noticeably higher in patients on enzalutamide treatment (53 %) than on abiraterone (17 %) or other agents (21 %). When the samples were analyzed by deep sequencing, the *AR* mutations in cfDNA were found. The mutations were present on the 8. exon of the *AR*, such as H874Y, T877A, and F876L. The mutations are annotated to protein-coding domains and, in this instance, to the ligand-binding domain (LBD), abnormalities that enable hyperactivity of the ARs. (Azad et al. 2015)

2.1.2 Tumor suppressor genes

Sonpavde et al. (2019) studied alterations in cfDNA with patients diagnosed with mCRPC. The study comprised 514 patients in which 482 demonstrated at least more than one somatic mutation (94 %). In this study, *AR* mutations were common, with 22 % of the 514 patients harbored this type of mutation. Still, the most common mutation was related to a tumor suppressor gene, *TP53*. This mutation was found in 36 % of the patients (Sonpavde et al. 2019). It has also been suggested that defects in the *TP53* gene reduce prostate cancer's dependency on perhaps its main driver, the *AR* mutations. (Annala et al. 2018)

TP53 is a tumor suppressor gene, and its inactivation is a way for cancerous cells to evade apoptosis. The lack of function in the gene and protein of the *TP53* also debilitates a cell's option to halt cell-cycle progression (Hanahan, Weinberg 2011). In general, the loss of *TP53* function is the most common way for a tumor cell to evade apoptosis and is an example of the mutations common in many different malignancies.

A study by Annala et al., (2018) included 202 patients with mCRPC, which was treatment-naïve and then treated with abiraterone or enzalutamide. Somatic mutations in *TP53* were a sign of resistance to the medication received. Other most commonly mutated genes were *AR* at 11 % and *APC* and *PTEN*, both at 10 % in the patients. (Annala et al. 2018) *APC* and *PTEN* are also known to be tumor suppressor genes (Cordon-Cardo et al. 2005).

The phosphoinositide 3-kinase (PI3K) pathway or phosphatidylinositol-4,5-bisphosphate 3-kinase pathway is often linked to the PI3K/Akt/TOR pathway which is frequently mutated in cancer cells. The mutation enables the cancer cells to grow and divide uncontrolled. The signaling pathway works at different levels. A survival signal from the extracellular matrix activates a receptor tyrosine kinase (RTK) in a typical setting, which in turn activates PI3-kinase. This activation leads to a cascade in cell function, where Akt is activated by phosphorylation, and TOR, in turn, facilitates cell function. Without normal cell inhibition in TOR (mTOR in mammalian cells) can malfunction and act in tumor proliferation. (Alberts et al. 2014)

An alteration in PI3K pathway was found in 73 out of the 150 patients (49 %) with mCRPC. Also, a previously undetected alteration in the subunit of PIK3CB, which has catalytic activity in the pathway, was found. In addition, a fusion of PIK3CA and PIK3CB led to an overexpression of the gene product. This fusion, in turn, accelerated tumor development. (Robinson et al. 2015)

Wnt proteins are a class of signal molecules secreted into the extracellular matrix, where they work as local mediators. There are 19 individual Wnt proteins in humans, and they can be activated in different ways. Therefore, it is no surprise that they are known to play a part in cancers as well. In the study by Robinson et al. (2015), 27 out of the 150 (18 %) cases of mCRPC had alterations in the Wnt pathway. Abnormal regulation concerning the Wnt pathway may induce cell proliferation, which in turn leads to tumor development. As the cancerous cells proliferate, they may also metastasize to form other lesions within the patient. Furthermore, aberrations in such genes as *RNF43* and *ZNRF3* in Wnt signaling genes were found, which are also known to play a part in adrenocortical cancers, such as prostate cancer. (Robinson et al. 2015)

2.1.3 DNA repair genes

DNA repair pathway is a key for cancer cells to survive in their microenvironment. In a study by Robinson et al. (2015), the DNA repair gene *BRCA2* was altered in 12.7 % of cases of mCRPC (19/150). Out of 19 patients, 90 % had a biallelic loss, meaning that the gene had been lost on both the paternal and maternal chromosomes. (Robinson et al. 2015) Altogether alterations in DNA repair genes were found in 34 patients out of the 150 participants (22.7 %). The genes included *ATM*, which is also known to play a part in controlling cell growth and mitosis (https://medlineplus.gov/genetics/gene/atm/; Accessed February 23, 2021). Other genes included in the expanded research encompassed genes such as *BRCA1*, *CDK12*, *FANCA*, *RAD51B*, and *RAD51C*. (Robinson et al. 2015)

Cyclin-dependent kinases (CDKs) also may play a part in prostate cancer progression; however, their total effect is still unknown. In a study by Annala et al. (2018), the ctDNA genomics was observed to determine if it correlates with abiraterone or enzalutamide resistance when treating prostate cancer. The study found that the gene *CDK12*, a subtype of the cyclin-dependent kinases, affects the homologous recombination repair. This dysfunction of homologous repair might cause poor outcomes when using AR therapies. (Annala et al. 2018)

2.2 Epigenetic changes in prostate cancer

Next, the discussion will progress to some possibilities in epigenetic changes that might lead to the onset of prostate cancer or its malignancy. Epigenetic changes can also be passed on to the off-spring.

2.2.1 Hypermethylation of genes

Methylation, in general, is the act of incorporating a methyl group to a base in the DNA (Alberts et al. 2014). This methylation is passed on to progeny cells by a specific enzyme that duplicates the methylation. This way, the same genes can be suppressed from one generation to the other. Hypermethylation simply means that the methylation is done at an abnormally high level. (Kushwaha et al. 2016).

Hendriks et al., (2018) investigated the patterns in hypermethylation of two genes, *GSTP1* and *APC*, to see if epigenetic changes might play a role in prostate cancer. The study had 47 patients with castration-resistance prostate cancer and 30 healthy individuals for a control group (Kushwaha et al. 2016). The patients were enrolled before starting chemotherapy with docetaxel or cabazitaxel or medication that inhibits the AR-receptor signaling with abiraterone or enzalutamide. The study proposed that the epigenetic markers in *GSTP1* and *APC* could have prognostic value for CRPC patients as median levels of *GSTP1* and *APC* copies in the baseline were higher when compared to the control group. Furthermore, the patients with a lower baseline level of methylation had significantly fewer prostate cancer-related deaths. The concentration of cfDNA was also used as one of the biomarkers in this study to see if it had a prognostic value. (Hendriks et al. 2018) The use of cfDNA concentration will be addressed later in this thesis.

The gene *GSTP1* was matched with another gene called the *RARB2* in a study by Kwee et al., (2012). In the study by Kwee et al., the relation between cfDNA and 18F-Fluorocholine PET/CT imaging was done by whole-body measurement to detect tumor activity. In addition, the cfDNA concentration was assessed in this study. The measurements were performed on a relatively small group of only eight patients with CRPC. In this study, the promoter methylation of these two genes was studied from cfDNA samples while the CRPC patients were on chemotherapy using docetaxel. As a result, methylation prior to chemotherapy of both *GSTP1* and *RARB2* was found on two patients. *RARB2* was methylated in one and *GSTP1* in five patients. Furthermore, in the patient's leucocytes, the DNA was unmethylated in both of the genes under study. This lack of methylation would, according to the research, suggest that the ctDNA was, in fact, tumor originated. After chemotherapy, the methylation of cfDNA was lost in both genes by four out of the eight patients. This

loss of methylation was not associated with any significant difference in PSA levels. This study concluded that there is a change in the methylation patterns in genes associated with prostate cancer when the patient is under chemotherapy. Still, the study concluded that future studies must investigate this issue before clinical applications may be constructed. (Kwee et al. 2012)

2.2.2 Hypermethylation of promoters

Promoters are parts of the genome that contribute to the initialization of transcription. Their methylation may cause specific genes to be expressed in abnormal amounts. (Alberts et al. 2014) This abnormality may cause the progression of cancer. Haldrup et al., (2017) studied *ST6GALNAC3* and *ZNF660* promoter methylation from prostate tissue and *ST6GALNAC3*, *ZNF660*, *CCDC181*, and *HAPLN3* from liquid biopsies by analyzing ctDNA. (Haldrup et al. 2018)

The study was comprised of 110 nonmalignant and 705 prostate cancer samples. It was concluded that the hypermethylation of the genes *ST6GALNAC3* and *ZNF660* was specific to cancer. Furthermore, ctDNA samples were used to assess the difference between prostate cancer and BPH. They used 27 patients with prostate cancer and 10 patients with BPH. With droplet digital methylation-specific PCR analysis, they were able to use a three-gene combination which was able to tell the difference between BPH and prostate cancer with a 100 % specificity and 67 % sensitivity. This sort of diagnostic test could be used in the future to differentiate diagnoses, especially if PSA-values are vague in meaning. (Haldrup et al. 2018)

2.3 cfDNA integrity and concentration

Mutations and epigenetic changes have been on specific areas of the chromosomes to pinpoint different mutations or methylation patterns, which have been able to predict the disease's course in prostate cancer or BPH. These predictions have been possible due to different analysis methods that have improved over the years. Before the aberrations of genes or their transcription were researched, the abnormalities in cfDNA were observed.

2.3.1 The concentration of ctDNA

In a study by Jung et al., (2004) the plasma DNA concentrations of prostate cancer patients (n = 91), patients with BPH (n = 34), and healthy individuals (n = 59) were evaluated. The researchers found out that primary cancer patients had the DNA plasma levels in the reference interval. Counterintuitively, patients with BPH and patients with metastasized prostate cancer both had an increased cfDNA level. However, the patients in these two groups did not have a noticeable age difference, nor did the BPH patients have noticeable infections during the study. Also, PSA-values and plasma DNA were both equally able to predict survival time.

The study also noted that wet-lab protocols might significantly alter concentrations of cfDNA. Still, this study also stated that it was: "As far as we know the present study is the first systematic investigation on the quantitative changes of circulating DNA in PCa patients." In future studies, the differentiation between healthy BPH and prostate cancer can be noticed. (Jung et al. 2004)

A study by Kienel et al., (2015) used cfDNA to see how patients responded to chemotherapy when treated with taxanes. Assessing the correlation between cfDNA concentration and PSA response as a predictor of survival under taxane chemotherapy, the study concluded that cfDNA might be a cost-effective biomarker for people with CRPC. (Kienel et al. 2015)

Mehra et al., (2018) conducted a study with 571 mCRPC patients to assess the possibility of using cfDNA concentration as a biomarker in taxane-based chemotherapy. The study found out that patients with a higher baseline cfDNA concentration had shorter overall survival (OS). It was also found that in line with OS, the radiological progression-free survival (rPFS) was shorter for these patients on taxane therapy. (Mehra et al. 2018) The study also proposed that the decline in cfDNA within the first nine weeks of treatment was linked to a positive response to taxane chemotherapy. This study was done fifteen years after the first systematic investigation into cfDNA possibilities, and within that time, the usability of cfDNA has increased significantly. cfDNA concentration is one step towards better prognosis and diagnoses, but other means have improved as well. The levels of cfDNA and the fraction of ctDNA in cfDNA are found to have potential biomarker potential. Belic et al., (2018) found out that the high ctDNA levels also contribute to metastases found in the patient's bone. (Belic et al. 2018) This study is another exciting result using cfDNA as a biomarker and diagnostic tool.

2.3.2 The integrity of ctDNA

Finally, the integrity of cfDNA might also be used to differentiate between BPH and prostate cancer. Feng et al., (2013) studied if this differentiation could be done for patients with a PSA level of less than 4 ng/ml. The study had 112 patients with BPH and 96 with prostate cancer. The study used quantitative real-time PCR with two different primers, one amplifying a shorter fragment (115 bp) and another amplifying a longer fragment (247 bp), to multiply an *ALU* gene. The ratio of this was used to assess the integrity of cfDNA. As a result, the level of cfDNA was higher in patients with prostate cancer. Also, the integrity of cfDNA was poorer in patients with prostate cancer, referring that in this particular study, the ratio between the different *ALU* genes was altered. (Feng et al. 2013)

To summarize the experiment, the integrity of cfDNA had the best performing sensitivity and specificity when compared to serum's total PSA or cfDNA itself. The study concluded that cfDNA in

plasma with its integrity could be used to differentiate prostate cancer from BPH when the PSA value was lower than 4 ng/ml. The application of this discovery might also lead to fewer biopsies taken from the prostate itself. (Feng et al. 2013) This discovery could be a step towards better diagnoses of prostate cancer, which will be discussed later.

3. ANALYSIS OF cfDNA

After a patient undergoes a liquid biopsy, the cfDNA has to be extracted from the whole blood. First, for example, the plasma has to be separated from the blood, and then the cfDNA can be extracted. Also, the cfDNA has to be differentiated from the ctDNA. The aforementioned fraction of ctDNA and cfDNA can be used by itself as a biomarker to some extent, as some studies have suggested. In addition, the concentration of cfDNA can be used to differentiate BPH and prostate cancer.

As analysis methods have evolved and liquid biopsies using cfDNA have become more common new methods have been used to discover mutations from the ctDNA. Some methods explore new sites, and some focus on known mutations, for example, in the *AR* gene.

In this thesis, it is not feasible to go into detail about how whole genome sequencing or analytical tools work. Instead, this paper aims at covering major trends in cfDNA analysis in accordance with prostate cancer. Also, the implications that referred studies have found will be discussed.

3.1 Sequencing cfDNA

3.1.1 Whole-genome sequencing

Whole-genome sequencing can be applied to cfDNA. With this approach, it is possible to have a comprehensive look at how cancer evolves. In essence, whole-genome sequencing lacks filters to specific DNA regions, which provides a more holistic view of the genome. This method creates a vast amount of data and requires bioinformation professionals to make sense of it. (Carla G Van El et al. 2013)

Adalsteinsson et al., (2017) investigated patients who underwent sparse whole-genome sequencing where the tumor fraction (TFx) was estimated using a computational tool called ichorCNA. In essence, when performing whole-genome sequencing of cfDNA, a technique called ultra-low pass WGS (ULP-WGS) where the patient's genome coverage is only about 0,1x. This technique enabled

the researchers to evaluate major copy number alterations (CNAs) or abnormal chromosome counts, also called aneuploidy in mCRPC patients. This technique was first applied in a study with healthy donors, metastatic prostate, and breast cancer patients. (Adalsteinsson et al. 2017)

In a later study, Adalsteinsson et al., (2018) had multiple samples (663) from 140 different CRPC patients undergo a similar study where ichorCNA was used. In this study, the clinical responses were studied and how patients TFx was prognostic to their OS. As a result, TFx decline, meaning that the ctDNA load in cfDNA decreases, was a promising sign of treatment response. Also, the TFx value predicted overall survival in CRPC. (Adalsteinsson et al. 2018)

Another technique to scan an entire chromosome for copy number variations (CNV) can be done with array comparative genomic hybridization (aCGH), a developed version of CGH with microarrays with small DNA strands hybridize with patient DNA. Abnormalities can be detected this way to see if a patient has an abnormal CNV count. Azad et al., (2015) investigated the resistance to abiraterone and enzalutamide treatments in patients with mCRPC. The research was performed using aCGH on 62 samples and detected that over half of the samples had at least one CNV associated with cancer. The outcome of this study was that AR amplifications were more common in patients taking enzalutamide than abiraterone or other medication. Other mutations were also discovered, and the conclusion was that the resistance might be built up whether using either medication. (Annala et al. 2018)

3.1.2 Next-generation sequencing

As noted, specific mutations, for example, in the *AR* gene, might be valuable to know. With this knowledge, certain medications can be prescribed while others can be ruled out. In a study by Romanel et al., (2015) ctDNA was analyzed with next-generation sequencing (NGS) focusing on AR coding bases and some other regions in the genome that are of value in prostate cancer. They sequenced all AR exons from patients with CRPC at three different time points during the study, which were timed before, during, and after starting medication on abiraterone. This way, it was able to investigate the changes in copy numbers and the point mutations that might turn up. The study analyzed 217 from 80 different patients. The study was able to track how the copy number for the *AR* changed during the study. They were also able to observe that T878A or L702H point mutations were associated with resistance to the abiraterone treatment. (Romanel et al. 2015)

3.1.3 Sanger sequencing

While NGS and different methods derived from it are used today, other means of sequencing have also been used. For example, Sanger DNA sequencing, or dideoxy sequencing, has been used. In

this technique, chain-terminating dideoxynucleotides are used to end the elongation of the DNA strand. After the termination, the strand is read with a laser-detector module to see which dideoxynucleotides are attached to the strand. This way, the individual nucleotides are read one-by-one, and the underlying code is discovered. (https://www.atdbio.com/content/20/Sequencing-forensic-analysis-and-genetic-analysis; Accessed March 3, 2021)

This method was used, for example, in a relatively recent study by Annala et al., (2018) where abiraterone and enzalutamide resistance was researched. They found an *AR* rearrangement in a patient's genome. In this particular case, the AR transcript RNA had been shortened due to a stop codon being transcribed at the wrong location. The STOP codon occurred before the LBD, resulting in the patient not having an AR receptor that worked. The real-life result was that the patient did not respond to abiraterone. (Annala et al. 2018) In this particular case, sanger sequencing was used after PCR amplification of the mutated gene and only for this one patient. When studying large cohorts, this application is not very feasible when more time-conserving methods are available.

3.2 Other methods

Polymerase chain reaction (PCR) is a technique required to amplify the amount of DNA that has been discovered. In short, the DNA that is wanted for amplification is first pried apart by denaturation the two strands apart. The process is done at a high temperature at around 95 °C. Second, the primers are attached to the single strands of DNA. The temperature for this varies for many reasons but can be 55 °C, for example. The third phase is elongation, where the nucleotides pair up with the original strand's nucleotides. This three-stage operation is performed multiple times, and at the end, the original DNA sequence has been multiplied many times over. (https://www.atdbio.com/content/20/Sequencing-forensic-analysis-and-genetic-analysis; Accessed March 3, 2021)

When studying sequences in the genome, as is done when researching ctDNA, copy number variations and point mutations are of interest. The use of digital PCR or dPCR is based on PCR, as its name suggests. In dPCR, the original sample is diluted massively and then partitioned to millions of chambers where the PCR reaction happens. When the sample is diluted, the possible DNA sequence that is being searched for is present as only one copy of the sequence. When the PCR reaction is performed, the DNA is either multiplied heavily, which can be detected by fluorescent measurements, or not multiplied at all. This method gives a precise quantity of the sequence in question. (Baker 2012)

Finally, in real-time PCR, or quantitative PCR (qPCR), the amount of DNA can be measured during the PCR cycles; thus, the quantity of a DNA sequence is studied. This method is mainly in use today and considered the golden standard. For example, qPCR was used in a study by Speicher et al., (2018) when they studied genomic alterations with mCRPC patients receiving abiraterone or enzalutamide. qPCR was used in this study to confirm that patient's ctDNA actually had an amplification factor affecting the AR. The study concluded that amplifications concerning the AR do not always correlate to a poor response to abiraterone. (Kienel et al. 2015)

4. POSSIBILITIES IN PERSONALIZED HEALTHCARE

4.1 Commercial cfDNA assays

As mentioned, NGS is a valuable tool to inspect genes of interest to find patterns in prostate cancer cell evolution. Today, academic research groups can sequence genomes, but they can also be done using commercial kits such as Guardant360. The kit tests for alterations in known prostate cancer genes and the information can be used to guide the treatment the patient is receiving. For example, Sonpavde et al., (2019) used the Guardant360 to profile cfDNA. The research was a large cohort with 514 men diagnosed with mCRPC, and 94 % of them had one or more ctDNA alterations. The alterations were mutations that have been assessed in this paper as well, such as *TP53* in 36 % of the cases, AR in 22 % of the cases. The extensive study was in line with previously reported studies, and so it would suggest that the Guardant360 genomic profiler can be used to study patients successfully. (Sonpavde et al. 2019)

As the Guardant360 is a commercial product and not an academic group's protocol, the test's reliability has been analyzed in various studies. One such study was performed by Wyatt et al., (2019) where they compared the Guardant 360 test against an academic sequencing approach. The study had 24 patients with mCRPC. The Guardant360 had a research panel with 73 genes that were previously associated with prostate cancer. As a result, the commercial product did not find all mutations in DNA repair genes. It was concluded that ctDNA tests used by clinicians today should use them with care when going through their results and deciding on a treatment course.

In conclusion, the study found that the Guardant360 was in line with the academic sequencing approach in mutations with a high allele fraction. Downsides to the commercial test were that it was limited in detecting genomic rearrangements, insertions, and deletions of sequences in mCRPC patient's ctDNA samples. Thus, the accuracy and specificity of commercially available tests for

liquid biopsies need to be tested before implementation to clinical practice. (Taavitsainen et al. 2019)

4.2 Used treatments and patient response

Commercial products, such as Guardant360, aim at resolving what sort of mutations occur in prostate cancer. Also, this thesis has covered the evolution of prostate cancer. The real-life goal is to improve the treatment of prostate cancer. Also, prostate cancer is over-diagnosed; thus, it is important to select those patients who might not need extensive medical care from those who do. Besides, no one treatment suits all prostate cancer patients. It is also worth pointing that prostate cancer treatments, such as ADT, may cause more harm than good for the person undergoing the treatments. Finally, the financial burden can be significant and financial resources should be better allocated in the future.

To conclude, as personalized healthcare improves, the systematic development of cancer's driver mutations should guide the selection of treatments that better suit patients' needs. Some of these have been discussed in this thesis, and it has been the focus of many of the studies that this thesis has referred to as well. Inconveniently, although there might be a strong correlation between mutations of one gene and the use of one specific drug to medicate this combination, it is very difficult or impossible to make a combination that works with all patients.

An example of a more personalized healthcare approach might be used on treatment response of abiraterone or enzalutamide, which are AR signaling inhibitors (Hendriks et al. 2018). These drugs were first created to hinder the AR's activity after ADT had stopped working, after the formation of castration-resistance. Annala et al., (2018) aimed at providing clinically relevant information that might have a real-life application to the patient's treatments. In the study, they found that cross-resistance might be developed from first-line therapy from abiraterone or enzalutamide, and it can be detected from AR evolution mutations. (Annala et al. 2018)

Docetaxel and cabazitaxel are two taxanes that are used in chemotherapy. They target microtubules in cancer cells and cause stabilization or aggregation, which disrupts cancer cell proliferation (Hagiwara, Sunada 2004). cfDNA analysis has been used to investigate taxanes treatment responses (Kwee et al. 2012). Thus, cfDNA markers could be used to predict the therapeutic response to taxane-based chemotherapy in the future. (Kwee et al. 2012) The drugs used to treat metastatic castration-resistance prostate cancer are collected in Table 2.

ARSIs and Taxanes are only a part of all medications that can be given to prostate cancer patients. For example, radium-223 can also be given to mCRPC patients. Radium-223 emits alpha particles that target metastases of cancer. Parker et al., (2013) shown that the use of radium-223 improved

overall survival in a placebo-controlled study. (Parker et al. 2013) It is also worth pointing that PARP inhibitors can be used for prostate cancer patients. They act by inhibiting the PARP enzyme, which is active in repairing DNA. When the DNA has repaired the cell, in this case, a cancer cell, may evade apoptosis and progress cancer. Rucaparib and olaparib are used as PARP inhibitors. Genomic testing can be used to determine if a prostate cancer patient should use this sort of medication to hinder cancer development (https://news.cancerconnect.com/prostate-cancer/parp-inhibitors-effective-in-men-with-brca-mutant-prostate-cancer-Nc3UbXLM6kuqX7jPGn4V5Q; Accessed March 4, 2021).

Table 2. Summary of some of the medications used against mCRPC (J.-L. Tan et al. 2017, https://pubchem.ncbi.nlm.nih.gov; Accessed March 8, 2021).

Medication	Drug class	Administration	Action mechanism
Abiraterone acetate	Antineoplastic	Oral administration	Inhibits critical enzymatic activity in androgen synthesis
Enzalutamide	Non-steroidal anti-androgen	Oral administration	Inhibits AR in various ways
Docetaxel	Antineoplastic	Intravenous	Binding to beta-tubulin, hindering disassembly and causing apoptosis
Cabazitaxel	Antineoplastic	Intravenous	Inhibition of microtubule depolymerization in mitosis
Olaparib	PARP inhibitor	Oral administration	Inhibits PARP performance to repair DNA and enhances cytotoxicity
Radium-223	Alpha emitter	Intravenous	Emits alpha particles that destroy cancer cells, mainly in bone tissue

5. DISCUSSION

This thesis has covered how prostate cancer develops from benign prostate hyperplasia to cancer and incurable mCRPC. The progression happens due to the aberrations in the genome of the patient's cancer cells. These aberrations can be studied by using cfDNA or ctDNA.
The use of cfDNA is a relatively new method in cancer research, yet there is research on this topic from over a decade already. At first, the focus was on the fraction size of ctDNA in cfDNA. As different analysis methods to study ctDNA have evolved, the focus of the studies has also shifted from concentration to mutations and their different types. In the future, whole-genome sequencing will be a more routinely used method, which will increase the amount of data in cancer research, which will, in theory, trickle down to all cancer patients and generally improve the treatment of cancers.

Understandably, no method to diagnose or prognose the course of prostate cancer is without flaws. One of the most prominent problems when using cfDNA to investigate prostate cancer is that the DNA itself does not determine what quantity the DNA's transcript RNAs will be expressed or how much protein will be expressed ultimately. In addition, before ctDNA is used in clinical settings, the physician's analysis tools should provide valuable information on which aberrations would be considered to help in the treatment decision-making. For example, the use of PSA-levels does not always tell the entire truth of the stage of cancer, and the use of ctDNA and cfDNA might offer an additional level of reassurance to decision-making.

The use of ctDNA is a fascinating approach in guiding personalized healthcare. The use of whole-genome sequencing and other sequencing tactics in collaboration with methods in bioinformation opens a new way to understand not only cancer but its progression as well.

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