JÖNKÖPING UNIVERSITY
School of Health and Welfare

Doctoral Thesis

Exploring vitamin D and steroid hormone receptors

– from healthy elderly to prostate cancer cells

Maria Araceli Diaz Cruz

Jönköping University
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Dissertation Series No. 111 • 2022

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"***Be thankful for what you are now, and keep fighting for what you want to be tomorrow***"

- Anonymous

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Abstract

The genetic background together with environmental factors and lifestyle are key contributors to the health of an individual. Genetic background is inherited and irreversible unless mutations occur. However, lifestyle habits (i.e., diet, stress, physical activity, smoking, and alcohol consumption) are modifiable factors that contribute to health or disease by affecting methylation of DNA, which regulates transcription of genes.

One of the most relevant lifestyle habits for health is maintaining adequate vitamin D levels in the body as vitamin D promotes calcium and phosphate absorption, supports the nervous and immune system function, and protects bone and muscle structure. Extreme low levels of vitamin D, vitamin D deficiency, has become a global public health concern, especially in the elderly population as vitamin D deficiency can lead to several health problems such as bone fracture, decreased muscle strength, cardiovascular and autoimmune diseases, depression, and breast, pancreatic, and prostate cancer.

Prostate cancer is an uncontrolled growth of cells within the prostate gland in the male reproductive system. Human prostate carcinomas are sensitive to androgens, and hormonal ablation therapy gives a temporary remission, followed by a relapse to an androgen-insensitive state. This indicates that steroid hormones, especially androgens, play a significant role in human prostatic carcinogenesis. The molecular effect of vitamin D as a steroid hormone and which steroid hormone receptor (SHR) mediates this effect are not fully understood.

This research project aims to increase our knowledge about SHRs, primarily the vitamin D receptors, in both health and disease, focusing on genomic, epigenomic, and transcriptomic perspectives in healthy elderly individuals and prostate cancer cells.

The results from the studies in this thesis could help us understand the importance of a healthy lifestyle, which includes vitamin D for health, where we found specific methylation markers involved in the down-regulation of cancer pathways that are associated with high physical activity and vitamin D supplementation. We have further confirmed that SHRs rarely work in isolation but rather as a crosstalk at the genomic level to regulate their transcription. Hopefully, this will help clarify the modulation of transcriptional responses in SHRs and explain the development of steroid

hormone-dependent cancers such as prostate cancer. Last, but not least, we revealed that genetic and transcriptional markers are associated with the putative vitamin D receptor the protein disulfide isomerase family A member 3 (PDIA3). The genetic markers were detected in a healthy elderly population under vitamin D supplementation. The transcriptional markers, PDIA3, and a novel discovered isoform of PDIA3 (PDIA3N) were related to the androgen and cancer stage of prostate cancer cells and therefore are proposed as candidate markers for clinical diagnosis of prostate cancer.

Altogether, these findings support the relevance of studying vitamin D and steroid hormone receptors, especially the PDIA3 receptor, to understand some of the factors related to healthy aging and the etiology and progression of prostate cancer.

Original papers

Paper I

Diaz Cruz MA, Ulfenborg B, Blomstrand P, Faresjö M, Ståhl F and Karlsson, S.
Characterization of methylation patterns associated with lifestyle factors and vitamin D supplementation in a healthy elderly cohort from Southwest Sweden. *Submitted to BMC Clinical Epigenetics*

Paper II

Diaz Cruz MA, Ulfenborg B, Faresjö M, Ståhl F and Karlsson, S. Vitamin D supplementation is correlated with PDIA3 gene variants in an elderly healthy cohort from Southwest Sweden. *Manuscript*

Paper III

Diaz Cruz MA, Lund D, Szekeres F, Karlsson S, Faresjö M and Larsson D. Cis-regulatory elements in conserved non-coding sequences of nuclear receptor genes indicate for crosstalk between endocrine systems. *Open Med (Wars). 2021 Apr 12;16(1):640-650. doi: 10.1515/med-2021-0264.*

Paper IV

Diaz Cruz MA, Karlsson S, Szekeres F, Faresjö M, Lund D and Larsson D. Differential expression of protein disulfide-Isomerase A3 isoforms, PDIA3 and PDIA3N, in human prostate cancer cell lines representing different stages of prostate cancer. *Mol Biol Rep. 2021 Mar;48(3):2429-2436. doi: 10.1007/s11033-021-06277-1.*

Abbreviations

AD: Androgen dependent

ADT: Androgen deprivation therapy

AI: Androgen independent

AR: Androgen receptor

BCSDm: Basic conserved sequence detection method

ChIP: Chromatin immunoprecipitation

CRE: Cis-regulatory element

CVD: Cardiovascular disease

CYP2R1: Calciol-25-hydroxylase

CYP24A1: 24-hydroxylase

CYP27A1: Sterol 27-hydroxylase

CYP27B1: Calcidiol-1α-hydroxylase

DBD: DNA binding domain

DBP: Vitamin D binding protein

DMEM: Dulbecco's modified Eagle's medium

DMP: Differentially methylated probe/position

DMR: Differentially methylated region

ddPCR: Droplet digital PCR

EMEM: Eagle's minimum essential medium

ER: Endoplasmatic reticulum

ESR: Estrogen receptor

FDR: False discovery rate

FPKM: Fragments per kilobase of transcript per million fragments mapped

GR: Glucocorticoid receptor

GO: Gene ontology

HRE: Hormone response element

LBD: Ligand binding domain

LD: Linkage disequilibrium

MAF: Minor allele frequency

MAPK: Mitogen-activated protein kinase

NR: Nuclear receptor

NR1D1: Nuclear receptor subfamily 1 group D member 1

NR2F: Nuclear receptor subfamily 2 group F

NR4A3: Nuclear receptor subfamily 2 group F

NRS: Nuclear receptor superfamily

NTD: N-terminal domain

PCA: Principal component analysis

PEST: Penicillin-streptomycin solution

PGR: Progesterone receptor

PKC: Protein kinase C

PDIA3: Protein disulfide isomerase family A member 3

PDIA3N: Novel protein disulfide isomerase family A member 3

PSA: Prostate-specific antigen

QC: Quality control

RARA: Retinoic acid receptor alpha

RARB: Retinoic acid receptor beta

RORA: Retinoid-related orphan receptor alpha

RORB: Retinoid-related orphan receptor-beta

RORC: Retinoid-related orphan receptor gamma

RXRA: Retinoid X receptor alpha

RXRB: Retinoid X receptor beta

RXRG: Retinoid X receptor gamma

SHR: Steroid hormone receptor

SNP: Single nucleotide polymorphism

SRHS: Self-reported health status

SS: Splicing site

TFBDS: Transcription factor binding domains

TFBS: Transcription factor binding sites

TIS: Translation initiation site

TRPV6: Transient receptor potential vanilloid subfamily member 6

VDR: Vitamin D receptor

VDRE: Vitamin D response element

WHO: World Health Organization

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1. Introduction

People's health status is influenced by their genetics, lifestyle habits, and environmental factors [1]. Genetic background is inherited and irreversible unless mutations occur. For example, genetic polymorphisms may predetermine the aging process and are associated with chronic diseases [2]. However, lifestyle habits, (e.g., diet, vitamin supplementation, behavior, stress, physical activity, smoking, and alcohol consumption) contribute to the development of health or disease by repressing or activating specific genes through changes in the methylation of DNA [1, 3].

Global methylation of DNA is affected by vitamin D supplementation and vitamin D levels in the blood [4, 5]. Vitamin D belongs to a group of fat-soluble steroids that act as hormones in the human body and can be taken up through diet and supplements or be synthesized in the skin through sunlight exposure [6]. Historically, vitamin D is well known for curing rickets or bone deformities as it increases the calcium reabsorption and therefore bone mineralization [7]. Vitamin D deficiency can occur as the result of several factors, including dietary intake, endogenous synthesis, vitamin D absorption, and sunlight exposure. Vitamin D deficiency has become a global public health concern as about one billion people worldwide have vitamin D deficiency and 50% of the population has vitamin D insufficiency. The prevalence of vitamin D deficiency is common in highly pigmented individuals, obese people, hospitalized patients, and especially the elderly. In Europe, vitamin D deficiency is as high as 80% in institutionalized elderly but only 2–30% in adults [8]. Vitamin D deficiency in the elderly can lead to several health problems such as cardiovascular and autoimmune diseases, osteoporosis, depression, and cancer diseases such as breast, pancreatic, and prostate cancer [8-10].

Prostate cancer, the uncontrolled growth of cells within the prostate gland [11], is the most frequently diagnosed malignancy and the second leading cause of death among men in the United States and Europe. Risk determinants include environmental factors, of which diet and nutrition are the most significant. Furthermore, emerging evidence suggests that the development of

prostate cancer also depends on genetic factors [12]. As human prostate carcinomas are sensitive to androgens, hormonal therapy provides temporary remission, although this is followed by a relapse to an androgen-insensitive state [13]. This evidence indicates that steroid hormones, especially androgens, play a significant role in human prostatic carcinogenesis. Vitamin D also exerts several antitumor effects, such as decreasing cell proliferation, in *in vitro* and *in vivo* prostate cancer independent of the androgen stage [12, 14, 15]. However, the effect of vitamin D as a steroid hormone on a molecular level and which steroid hormone receptors mediate its effect are not completely understood.

Steroid hormone receptors (SHR) are typically intracellular receptors that initiate signal transduction for steroid hormones. The androgen receptor (AR) is responsible for maintaining normal conditions in the epithelia of the prostate. However, an abnormal overexpression of the AR stimulates cancer cell proliferation in prostate cancer patients, even if androgens are not present [16, 17]. The vitamin D nuclear receptor (VDR) is the main receptor for vitamin D. When vitamin D enters a cell, it binds to this receptor in the cytoplasm and secondarily to the retinoid X receptor alpha (RXRA). This complex translocates into the nucleus, promoting the activation of the genomic effects of vitamin D, such as its antitumoral effects [18-20]. PDIA3, a receptor that can be considered an SHR, is a putative receptor for vitamin D in the endoplasmic reticulum (ER). PDIA3 may eventually bind vitamin D in the plasma membrane, activating the rapid responses or non-genomic effects of vitamin D such as protein kinases pathways and calcium release within the cell [21, 22].

Although the role of AR in healthy prostate tissue, prostate cancer etiology, and progression is well understood, the role of the vitamin D receptors, VDR, RXRA and PDIA3 is less clear. Moreover, genetic and epigenetic markers of vitamin D receptors and other genes associated with a healthy lifestyle, including vitamin D supplementation, have not been investigated in detail. Increased knowledge of the impact of these receptors might benefit the elderly population, which usually is at risk of vitamin D deficiency and associated chronic diseases such as prostate cancer.

2. Background

2.1. Epistemological assumptions

Epistemology is the philosophical study of the nature, origin, and limits of human knowledge, specifically the rationality behind acquiring that knowledge [23]. Science is the pursuit and application of knowledge and understanding of the world and the scientific method follows a systematic methodology based on evidence [24].

Critical realism is based on the ontological belief that the world is divided into three layers, the real (structures and mechanisms, which cannot be seen), the actual (the consequences of these structures and mechanisms), and the empirical (experiences) [25]. Scientific realism is the view that the world described by science is accurate, independent of how it may be interpreted, and aims to produce proper descriptions of the events occurring in the world [26].

Positivism states that knowledge is the experience of natural phenomena and their properties and relations. Logical positivism or logical empiricism is a movement of positivism that uses science as a rational explanation of the phenomena through the verification process, which aims to acquire justified knowledge closer to the truth or reality [27, 28]. The scientific method starts from a series of problems that need to be solved by formulating theories or hypotheses about their solution. These hypotheses might be rejected through error elimination that consists of statistical approaches that evaluate the significance of the result. In the last term, this allows the conclusion, which is the rejection or not rejection of the null hypothesis [28].

Both logical positivism/empiricism and scientific realism are oriented to the empiricist approach, which means that experiments justify theories [29]. Our study will part from the scientific realism view and try to increase the knowledge about specific genetic and epigenetic variations of steroid hormone receptors connected to health and cancer by conducting experiments and interpreting empirical data. Logical positivism/logical empiricism will be used

as an epistemological approach to verify the results through the error elimination process and statistical analysis.

According to the holistic paradigm, the maintenance of health or development of a disease cannot be explained by its parts but only by considering it as a "whole." According to the reductionistic paradigm, everything can be scaled down to lower mechanistic levels and be explained by them [30, 31].

In an empiricist-reductionistic study, the methods selected, such as the sampling and analysis methods, define what results can be obtained from the study. The function of higher organization levels depends on the smaller parts at the lower organizational levels [31]. In an empiricist-holistic study, the perspective is about the whole system. In this study, we adopted the two paradigms since we not only investigated the regulation of steroid hormone receptors in cell models (reductionism), but we also investigated this regulation in the "whole"–i.e., the whole individual (holism).

The development of cancer depends on the combination of systems in the body, such as genomic replication and variation, metabolism, the immune system, and the endocrine system [32]. In reductionism, the whole functioning may be wrongly explained by its parts, leading the researcher to misinterpret the relationship and interactions between the essential functions [31]. As we are only investigating a small part of the equation (i.e., the study of the regulation of steroid hormone receptors at the molecular level in cell models), the reductionistic approach is sufficient to cover this isolated part. However, the reductionistic approach is not enough to explain the development and complexity of a disease, including prostate cancer.

When it comes to maintaining health, such as the individuals in our healthy elderly cohort, several factors play a role, including aging, genetic background, lifestyle habits, and psychological status. In holism, the whole has emerging characteristics that cannot be explained just by the essential functions. We wanted to investigate the regulation of steroid hormone receptors by considering the whole individual, where we could interpret the influence of those factors. For example, the effects of vitamin D supplementation on the regulation of steroid hormone receptors are not the same when isolated and when other factors are included, such as genetic

background and other lifestyle habits. The problem with holism is that it is difficult to determine causes in a complex system [31]. In this part of the study, it will become difficult to pinpoint the specific causes of health maintenance without considering all the factors.

2.2. The interdisciplinary perspective

Interdisciplinary research is crucial to understanding the complexity of health and cancer at different levels [33]. Interdisciplinary research is an approach carried out by an individual or a team that integrates theories, perspectives, concepts, information, techniques, and tools of two or more disciplines of specialized knowledge [33]. Interdisciplinarity aims to solve a common research problem whose solutions are beyond the scope of a single area of research practice [34].

The context of this project is healthy individuals and models of prostate cancer *in vitro* to investigate the involvement of steroid hormone receptors–i.e., vitamin D receptors–in health and prostate cancer. Thus, this investigation is translational since it will cover steroid hormone receptors regulation at different levels the cell, and the complex organism (i.e., the individual).

This project includes two cross-sectional studies and two studies based on reference models (reference genome and prostate cancer cell lines). In healthy individuals, the two cross-sectional studies will provide knowledge about the genomic and epigenomic regulation of the steroid hormone receptors related to vitamin D signaling and metabolic enzymes (Study I and II). The study of the reference genome will analyze the crosstalk of steroid hormone receptors involved in prostate cancer (Study III). Finally, the vitamin D receptors will be evaluated as candidate markers of prostate cancer staging (Study IV).

Study I, II, and IV combine techniques based on experimental science and bioinformatics, which links to clinical science, biomedicine, and informatics. Study III exclusively uses bioinformatics tools, methods, and software in the analyses. Thus, this project embraces an interdisciplinary approach mainly by integrating experimental science and bioinformatics. Furthermore, the results obtained in the different studies are discussed through the lens of biomedical

disciplines such as molecular and cell biology, biochemistry, and genomics and are considered from the perspective of health and well-being.

2.3. The perspective of health and well-being

2.3.1. In healthy elderly

Worldwide, the percentage of older adults above 60 years old is growing faster than expected [35, 36]. As people age, they become more susceptible to disease and disability. Thus, promoting health and well-being becomes precedence for aging well [37].

The World Health Organization (WHO) defines health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity." From this definition, elderly people in good health are not necessarily free from any disease, but they experience satisfactory physical, mental, and social health.

Well-being and physical and mental health are closely connected, and this connection may become more important at older ages in terms of life satisfaction, feelings of happiness, having a sense of purpose, and attaching meaning to life [37]. However, growing old often involves health and well-being problems, leading to physical and psychological impairments, isolation, and loneliness [35]. Thus, older adults may encounter more challenges in pursuing aging well. These consequences may lead to a sedentary lifestyle that can affect quality of life. Moreover, meaningful life has been related to a sense of health and well-being and increased physical activity [35, 37].

Maintaining physical health and functioning facilitates mobility and enables older adults to perform more integrated assignments, including daily living activities, fulfillment of social roles, and recreational activities [38]. In addition, good physical functioning reduces fall incidence and prevents the negative impact after a fall, such as activity restriction and isolation [37].

Other factors such as vitamin D levels in the body play a role in mental and physical health. Vitamin D preserves skeletal health, regulates sleep, participates in brain development and cognitive performance, and improves

cardiovascular and immune functioning [39, 40]. Sun exposure, which is involved directly in vitamin D synthesis, increases mental alertness, energy, positive mood, and confidence and can reduce pain, stress, and anxiety [41, 42]. Low vitamin D levels have been linked to various health problems, including cognitive decline, depression, osteoporosis, cardiovascular disease, hypertension, diabetes, and cancer in the elderly [39]. Showing clearly that lifestyle choices (e.g., physical activity, vitamin D levels and sun exposure) can impact the physical and mental health of the elderly and their well-being.

Aging has been investigated from the disease perspective using environmental factors, lifestyles, and genetics. However, few studies evaluate genetic backgrounds, lifestyles, and self-estimation of health in healthy elderly populations. Investigating genetic background and epigenetic changes associated with differences in lifestyle and other factors may offer clues about living a long life in good health, as this study will discuss.

2.3.2. *In cancer*

In people 70 years and older, 80% are affected at least by one of the following chronic diseases: arthritis, hypertension, heart disease, diabetes, respiratory diseases, stroke, and cancer [36]. The incidence of particular cancers in men over 65, such as prostate cancer and colorectal, is expected to increase 4-fold between 2000 and 2050 [43].

Health is a dynamic state that can be altered by cancer as it affects a diverse population, requires expensive treatments and resources, and defines the remainder of a person's life, from early disease to chronic disease to death. Cancer can be divided into two categories: health after early cancer detection and health with advanced cancer [44].

Health after early cancer means that the patient survived or was diagnosed but is obliged to face many challenges to recover health and well-being. Cancer survivors experience highly prevalent physical and mental symptoms such as chronic pain, fatigue, depression, anxiety, and sexual dysfunction. Furthermore, they share a lack of knowledge and information about well-being guidelines after the disease as well as most of them lack financial security [45].

Health with advanced cancer becomes limited since the patient's well-being depends on effective prognosis, treatment interventions, and in the end, the palliative care. The physical symptoms of advanced cancer often lead to a diminished quality of life: fatigue, weakness, pain, dysphagia, weight loss, and anorexia. Psychological symptoms include depression, anxiety, agitation, confusion, sleeplessness, isolation, and feelings of insignificance [46].

As patients with advanced, irreversible cancer have a limited life and suffer acute symptoms, research should focus on efficiently diagnosing and predicting the different stages of cancer. Moreover, treatments should be personalized and adapted to the different phases of the disease. Therefore, it is relevant to provide information about how cancer disease, specifically prostate cancer, can be regulated and predicted. This information could increase the knowledge about critical regulators in prostate cancer development and decrease patient uncertainty about the disease, perhaps the most significant outcome.

2.4. Factors influencing an individual's health

The health of a person or a population is assessed by considering aspects such as morbidity, impairments, anthropological measurements and indicators of functional status, and quality of life [47]. Factors influencing the health status are the settings, individual characteristics, environmental toxicants, and lifestyle habits (Figure 1) [1].

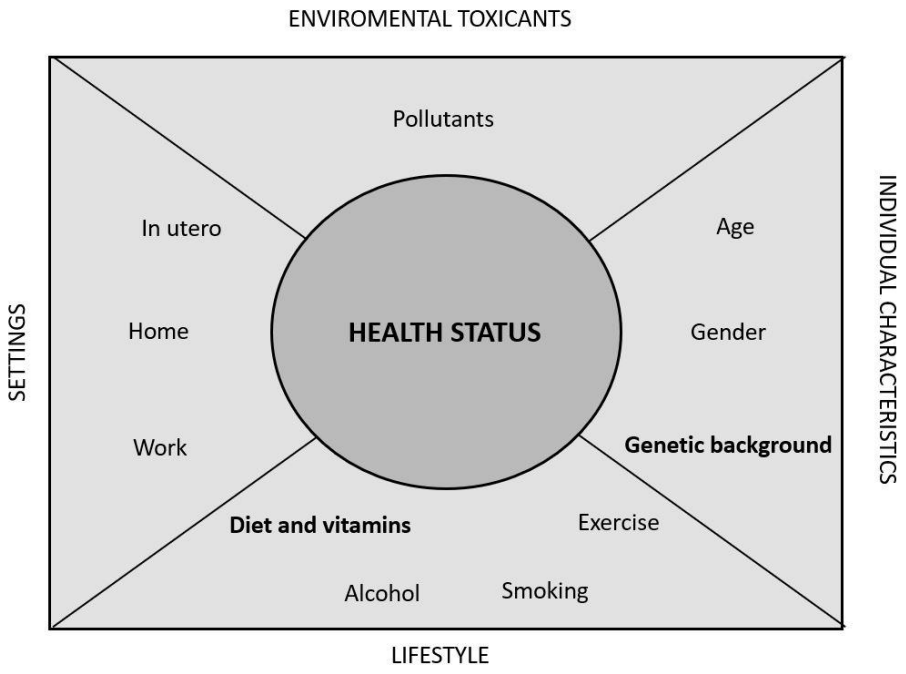

Figure 1. Factors involved in the health status of an individual.

The genetic background of an individual is inherited and irreversible unless mutations occur. Genetic polymorphisms, such as single nucleotide variations in a gene, characterize an individual's genetic background compared to another individual or the whole population. Genetic polymorphisms predetermine the aging process and are associated with chronic diseases [48, 49] –i.e., specific single nucleotide polymorphisms (SNPs) associated with lifespan [50], breast density, and risk of breast cancer [51].

SNPs may also be linked to several pathological conditions such as the alteration in vitamin D levels–i.e., SNPs in the vitamin D receptor (VDR) and in the vitamin D binding protein (DBP) associated to extreme low levels of vitamin D in postmenopausal women [52]. Further studies assessing SNPs in other genes and receptors related to the vitamin D pathway and metabolism could provide more clues about the inequality of vitamin D levels between different individuals and populations.

Lifestyle habits (e.g., diet and supplementation of vitamins, behavior, stress, physical activity, smoking, and alcohol consumption) are variable factors that contribute to the individual's development of health or disease by repressing or activating specific genes through changes in the methylation of DNA or through epigenetics [1].

Modulation of gene expression has been reported for the consumption of vitamins and minerals through dietary supplementation and fortification at the epigenome level [53]. Some studies show the impact of vitamin D supplementation and status on global DNA methylation [4, 5], although other studies found weak or no associations [54, 55]. So far, the results of the analysis of methylation of vitamin D receptors in response to vitamin D supplementation and associated metabolic enzymes are also inconclusive [56, 57]. Therefore, more studies are needed that assess the influence of vitamin D in global DNA methylation, the methylation of receptors involved in the vitamin D pathway, and the impact of these methylations on health.

2.5. Cancer as a complex disease

2.5.1. The hallmarks of cancer

As cancer often is influenced by genetic, epigenetic, and environmental factors, it is difficult to identify the cause of the disease. Furthermore, cancer is a complex disease because it is hard to study one isolated factor without considering other linked factors or pathways. Through the evolution of the disease, new properties can emerge from a holistic perspective. However, there are biological capabilities that all the cancer forms share during the multistep tumorigenesis, which are called The Hallmarks of Cancer: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative mortality, inducing angiogenesis, activating invasion, and metastasis [58, 59]. Emerging hallmarks are deregulation of cancer metabolism and avoiding immune destruction.

Two enabling characteristics promote the cell's acquisition of these hallmarks: genomic instability and tumor-promoting inflammation. These hallmarks and the enabling characteristics allow the cancer cells to survive, proliferate, and disseminate and constitute the basis for all the investigations with cancer [21,

22]. As epigenetic mechanisms regulate and contribute to each hallmark, it has been proposed as the hallmark of hallmarks in tumorigenesis.

This study focuses on three hallmarks: genomic instability and mutations (Study II and IV), sustaining proliferative signaling (Study III), and their signal's regulation by epigenetic mechanisms (Study I). However, all the hallmarks and enabling characteristics are inherent or indirectly related to the pathways investigated here.

2.5.2. Prostate cancer

Prostate cancer is the fifth leading cause of death among men worldwide [13, 43, 60, 61]. In the United States, prostate cancer is most frequently diagnosed non-skin cancer and the second most common cause of cancer-related death in men [62]. In Sweden, the disease is one of the most common causes of cancer death among men and has a ten-year survival rate of approximately 70% [63, 64].

The increasing incidence of prostate cancer, together with the concomitant decrease in mortality rates, has led to increasing numbers of men living with a previous prostate cancer diagnosis. Established risk factors for the disease are age, race, genetic predisposition, and diet [65, 68, 69]. By far, the two most important factors for prostate cancer are age and race/ethnicity [65, 66]. Prostate cancer is rare before 40 years old, but with aging, the risk of the disease is higher than any other cancer [67]. The incidence of prostate cancer is highest among African American men: 50–70% higher than in Caucasian Americans. The lowest rate of prostate cancer in the world is observed in Asian populations [66].

Prostate cancer occurs in the prostate, a small walnut-shaped gland that produces seminal fluids. The earlier stage symptoms include frequent urination, loss of bladder control, and blood in the seminal fluid and the urine. The later stage symptoms include swelling in the legs or pelvic area, numbness, bone pain, and fractures. There are five types of prostate cancer [68]:

- **Acinar adenocarcinoma**: This cancer develops in the prostate gland cells. This carcinoma is the most frequent type of prostate cancer.

- **Ductal adenocarcinoma**: This cancer develops in the duct cells of the prostate gland.

- **Transitional/Urothelial cancer**: This cancer develops in urothelial cells. It starts in the bladder and spreads into the prostate.

- **Squamous cell cancer**: This cancer develops from squamous cells that cover the prostate. These tumors grow faster and are aggressive.

- **Small cell prostate cancer**: This cancer is a neuroendocrine cancer that develops in the small endocrine rounded cells in the prostate.

Aside from the type of tumor, prostate cancer can be divided into two phases: androgen dependent (AD) and castrate or androgen-independent (AI). AD tumors respond to androgen deprivation therapy. They are easy to treat, but when the tumor progresses to AI, it does not react to castration surgery or ADT, and the only solution is prostatectomy [13].

The screening of prostate cancer is controversial because there is a lack of efficient diagnostic and prognostic tests that distinguish between benign and aggressive tumors [60, 69]. Standard approaches used in clinical decision-making are tissue pathology, Gleason score, imaging, and prostate-specific antigen (PSA) serum levels [13, 43, 60, 61].

Gleason score classification relies on pathology analysis, and the score is a sum of the scores obtained for the most observed two grades in the tumor. Usual Gleason scores range from 4 to 10. The higher the score, the more aggressive the cancer. This method is reliable but requires invasive techniques to extract the tumor biopsies. Imaging is not used if there is no suspicion of tumor growth, and it usually misses small tumors. In the case of PSA screening, it has been used for more than 20 years as the most effective non-invasive method [69].

Prostate-specific antigen (PSA), a protein exclusively produced in prostate tissue, is the most widely used biomarker to diagnose prostate cancer. PSA levels in the normal prostate are very high compared to the serum, and during

prostate cancer progression, serum PSA levels are reported to increase dramatically. This has allowed using serum PSA as a diagnostic tool for the detection of early prostate cancer. It has been reported that the primary regulator of PSA expression is the AR [67]. However, it is not clear whether AR amplification influences PSA expression levels.

Moreover, PSA measurements lack sensitivity to detect early and late stage tumors, and its non-specificity often gives false positives associated with other uropathies [69]. In conclusion, there is still a need for validated novel biomarkers for prostate cancer disease progression and aggressiveness.

2.6. Vitamin D

2.6.1. Vitamin D physiology

Vitamin D is a group of fat-soluble secosteroids that are ingested in the diet as vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) [6]. Few foods naturally contain vitamin D, primarily in the form of vitamin D3 and its metabolite 25(OH)D. These foods include fatty fish (such as trout, salmon, tuna, and mackerel), fish liver oils, beef liver, egg yolks, and dairy products. Mushrooms can also provide variable amounts of vitamin D2 [70].

Vitamin D3 can also be synthesized in the skin from 7-dehydrocholesterol upon exposure to UVB (290–320 nm). Vitamin D2 is derived from plants and yeast sources and produced exogenously by UV irradiation of ergosterol [71]. Once in the circulation, vitamin D3 and vitamin D2 is transported to the liver and hydroxylated by the calciol-25-hydroxylase (CYP2R1) to produce 25(OH)D or calcidiol [6]. Then 25(OH)D is further hydroxylated in the kidney by the calcidiol-1α-hydroxylase (CYP27B1) to produce 1,25(OH)D or calcitriol [6, 72]. The majority of circulating calcidiol and calcitriol in the blood is bound to DBP (80–90%) and albumin (10–20%), and only a small fraction of both is free [73]. At a later stage, the vitamin D–DBP complex is proteolytically degraded, releasing vitamin D metabolites for physiological action or metabolism [73, 74]. The half-lives of vitamin D, calcidiol, and calcitriol are approximately 24 h, 3 weeks, and 4 h, respectively [73]. In addition, liver production of calcidiol only depends on the availability of vitamin D and is not significantly regulated [73]. Moreover, calcidiol has a

higher serum concentration than calcitriol. Therefore, measuring the total serum calcidiol levels is considered the best estimate of vitamin D nutritional status. Calcitriol is the active form of vitamin D since it acts as a pre-hormone in the body, has the highest affinity with the VDR, and can bind with PDIA3 or a responsive gene, such as the calcium-binding protein [72, 75]. Alternatively, both calcidiol and calcitriol can undergo 24-hydroxylation by the 24-hydroxylase (CYP24A1) to generate 24,25(OH)D or dihydroxycholecalciferol, which is a degradation product with reduced physiologic activity [6, 72].

2.6.2. Epidemiology of vitamin D in health and disease

Vitamin D was initially described in the early 20th century as a substance that could cure rickets or bone deformities. Before this, the only recognized cure for rickets was cod-liver oil and sunshine exposure, with no explanation about the responsible molecules or physiological mechanisms behind it [76].

Vitamin D nutritional status has been associated with many pathophysiological conditions. Vitamin D deficiency–serum levels of 25(OH)D below 25nmol/l–is responsible for diseases such as osteomalacia and rickets. At the same time, vitamin D insufficiency–serum levels of 25(OH)D below 75nmol/l–is related to alterations in the parathyroid hormone concentration, which contributes to bone loss and fractures [7, 77]. Vitamin D insufficiency is an epidemic problem, especially in higher latitudes or low sunlight areas, individuals with darker skin, and the elderly [8]. Worldwide, 40–90% of the elderly have vitamin D insufficiency [77].

Sunshine exposure and vitamin D supplementation are the most effective methods to prevent vitamin D deficiency since UV light exposure and consuming vitamin D have a positive impact on increasing serum concentrations of 25(OH)D. Individuals can tolerate vitamin D at diary doses above 2000 IU without developing vitamin D intoxication. There is a fast conversion of vitamin D3 to 25(OH)D at low D3 concentrations and a much slower conversion rate at higher concentrations [78]. Thus, the increase of serum levels of 25(OH)D depends on the vitamin D3 levels [78]. However, because individual responses to the same therapy are different, there is no appropriate single dose of vitamin D that works equally in all individuals [79].

The serum levels of 25(OH)D < 250 nmol/l are still significantly below the toxicity level, and vitamin D intoxication is only observed when serum 25(OH)D > 374 nmol/l [80].

Both vitamin D2 and D3 have been used as supplementation. However, vitamin D2 is less efficient than vitamin D3 for increasing serum 25(OH)D. This could be explained because vitamin D2 has a lower affinity for DBP, resulting in a shorter half-life than D3, and because human liver enzymes convert vitamin D3 to 25(OH)D faster than vitamin D2 [81].

Epidemiological and case-control studies have shown that preserving enough vitamin D is vital for bone health, muscle strength, cancer, autoimmunity, and cardiovascular disease (CVD) [7, 10, 80]. A large prospective study involving 13,331 participants followed for nine years demonstrated that 25(OH)D levels < 44.5 nmol/l were associated with a 26% increased mortality compared with 25(OH)D levels >80 nmol/l. CVD and cancer mortalities were higher but not significant in the lowest 25(OH)D levels. Moreover, the risk of mortality was inversely associated with the supplementation with oral calcitriol [10].

Vitamin D in bone and muscle

Vitamin D status influences bone health. Rickets may occur in children with serum levels of 25(OH)D < 25 nmol/l, and osteoporosis may occur in adults with 25(OH)D levels < 80 nmol/l [82]. In a meta-analysis with oral vitamin D3 supplementation in older individuals (≥ 60 years), a vitamin D dose of 700–800 IU/d reduced the risk of hip fracture by 26 % and of non-vertebral fracture by 23%. However, a prospective study in central Sweden involving 60,689 women aged 40–74 years found no association between dietary calcium or vitamin D baseline levels and fracture risk [83]. In conclusion, adequate vitamin D levels need to be maintained for bone health, and doses higher than the current recommendation of dietary vitamin D are required.

Serum vitamin D levels are also associated with muscle size and strength [84]. In a retrospective cross-sectional study of hemodialysis patients receiving vitamin D analogs, patients in the vitamin D group had a larger thigh-muscle cross-sectional area and had an increase in strength. Moreover, a low 25(OH)D level was related to a high prevalence of falls in Japanese older women in a community-based survey of 2957 subjects (65–92 years) [85]. In

a retrospective study of 110 community-dwelling women with fractures, 96% had 25(OH)D < 80 nmol/l and 38% had ≤ 22.5 nmol/l [86]. Those with 25(OH)D ≤ 22.5 nmol/l had less low extremity function and higher falling rates [87].

Vitamin D and cancer

Vitamin D and its metabolites have been suggested as potential candidates for the prevention and therapy of several cancer forms, including prostate cancer [12, 14, 88]. Numerous reports demonstrate that vitamin D has an antitumoral effect since it stimulates differentiation and increases apoptosis and inhibits proliferation, invasiveness, and metastasis of cancer cells [12, 14, 15, 88]. 1,25(OH)D has been shown to mediate the repression of proto-oncogenes and activation of tumor-suppressor genes in normal and tumor tissues [89]. The metabolite 25(OH)D has also shown antitumoral effects by decreasing prostate cancer cell proliferation. This effect was enhanced when the metabolism of 25(OH)D to 1,25(OH)D and 24,25(OH)D was inhibited [88]

Sunlight exposure is inversely associated with incidence and mortality of prostate, breast, ovary, and colon cancer [9]. UVB radiation is associated with reduced risks of prostate, colon, breast, and non-lymphoma cancer. In a large cohort study of 416,134 skin cancer cases and 3,776,501 non-skin cancer participants, the risk of developing secondary cancer after skin melanoma was lower in the sunny countries. A multiple regression model including the variables diet, vitamin D, skin pigmentation, adiposity, geography, and physical activity for 47,800 men for up to 14 years could predict serum levels of 25(OH)D. An increase of 25 nmol/l in predicted serum 25(OH)D was associated with a 17% reduction in cancer incidence and a 29% reduction in cancer mortality [9]. Serum levels of vitamin D are associated with reduced risks of colorectal cancer and prostate cancer [90].

There are disconcerting data about the association of vitamin D and prostate cancer risk [91]. In an analysis of a cohort of 3414 white men (153 developed prostate cancer), residential sunlight exposure was associated with considerable reductions in prostate cancer risk [92]. An inverse correlation between sun levels and prostate cancer incidence and mortality was observed for white men but not for black men. However, higher latitude and low UVB

radiation were associated with a higher risk of prostate cancer mortality rates in the USA, 1970–94 and 1950–69 [93].

Other ingredients in the food can influence the effects of dietary vitamin D on prostate cancer. In a case-control study of 526 cases in Sweden, dietary vitamin D was not correlated with prostate cancer risk whereas calcium intake was correlated [94]. In a prospective study of 3612 men followed for 16 years, dietary vitamin D was not significantly associated with prostate cancer risk but calcium intake was [95]. No association of either dietary vitamin D or calcium intake with prostate cancer risk was found for 82,483 men followed for eight years in the Multiethnic Cohort Study [96]. In most of these studies, the vitamin D dietary intake was deficient vitamin D and therefore the actual effects of vitamin D on prostate cancer might not be reflected by these analyses.

When using serum or plasma levels of vitamin D metabolites, some case-control studies showed no significant connection between vitamin D and prostate cancer. In a nested case-control study of 232 cases followed for ten years, no significant association between 25(OH)D or 1,25(OH)D and prostate cancer risk was observed [97]. However, some studies show a meaningful relationship between serum or plasma vitamin D status and prostate cancer risk. In a nested case-control study involving 149 prostate cancer cases based on a 13-year follow-up of about 19,000 middle-aged men, prostate cancer risk was inversely associated with baseline serum 25(OH)D compared with those above the median [98]. In a prospective case-control study with 1066 men with incident prostate cancer followed for 18 years, those individuals with serum levels of both 25(OH)D and 1,25(OH)D below the medians had a significantly increased risk of aggressive prostate cancer [99]. In addition, there was a significant interaction between circulating 25(OH)D and specific vitamin D receptor polymorphisms for prostate cancer risk [99].

In the long run, the relationship between prostate cancer risk and vitamin D nutritional status is not conclusive. Genetic polymorphisms seem to play an important role, but more studies evaluating vitamin D supplementation and genetic variations in vitamin D receptors on prostate cancer risk are needed.

2.7. Steroid hormone receptors

Steroid hormones affect the development and growth of several human cancers [100, 101]. Therefore, understanding the mechanisms behind hormone-dependency of cancer may allow for the development of endocrine therapies to manage these pathological conditions [102].

Steroid hormones exert most biological effects through their receptors at the gene regulatory level [103-105]. Steroid hormones act mainly by binding with steroid hormone receptors (SHRs) but can also work by binding with other receptors such as the G protein-coupled receptors. The steroid SHRs can be found in the cytosol, in the nucleus, and the plasma membrane of target cells. The binding of the steroid hormones to SHRs can be at the cytoplasmic or nuclear level. Steroid hormone receptors that are predominantly cytoplasmic include receptors for mineralocorticoids, glucocorticoids, and androgen hormones. Steroid hormone receptors, usually nuclear, include the estrogen, thyroid, vitamin D, and retinoic acid receptors.

The nuclear receptor superfamily (NRS), the most studied SHR family, regulates gene expression by binding to specific DNA sequences and comprises 49 ligand-activated transcription factors that control essential developmental and physiological processes [83]. The relevance that several members of the NRS have in the regulation of cancer has increased the interest to target them therapeutically [83, 106]. Several NRs are involved in tumor pathogenesis, such as the estrogen receptor (ESR) in breast cancer and the AR in prostate cancer.

NRs are ideal therapeutic targets because their natural ligands are small molecules easy to design and mimic. This is especially true for the endocrine receptors VDR, ESR, thyroid hormone receptor (THR), AR, and glucocorticoid receptor (GR) [107]. Two well-known, established therapies targeting NR activity are androgen deprivation and selective ER modulators for prostate and breast cancer. These therapies strongly support the feasibility of NRs as drug targets to increase cancer treatment survival [83, 106, 107].

Generally, NRs are ligand-dependent intracellular transcription factors that activate or repress the expression of genes involved in critical physiological

processes such as organ development, cell growth, metabolite homeostasis, and response to external stimuli [101, 108-111]. NRs manage transmitting signals from a steroid/hormone to the target genes by interacting with specific response element DNA sequences and various coregulatory proteins [112-114]. Coregulatory proteins (coactivators and corepressors) exist in the same complex as the NRs, allowing for efficient transcriptional control mechanisms. These coregulator complexes work either by stabilizing the basal transcription machinery or by epigenetic changes [115-117]. Any dysregulation of NRs-coregulator complexes may affect normal homeostasis leading to the development of malignant phenotypes [115-117].

Available data show that signaling pathways elicited by NRs are complex and require a comprehensive understanding of their mechanism of action. An understanding of their molecular action will provide how these transcription factors function under physiological conditions and how their actions can be applied to clinical uses.

NRs are detectable due to similarities of certain protein domains, such as the N-terminal (NTD)-, the DNA binding domain (DBD), and the C-terminal ligand-binding (LBD)-domain [118]. After the ligand binds, the receptor experiences a conformational rearrangement and translocates to the nucleus, where it binds to specific DNA sequences known as hormone response elements (HREs) and assembles with the coregulator proteins [119].

The NTD structure of the NRs is the most difficult to predict since the NTDs are the most poorly conserved regions within the family even though they are responsible for transcriptional activity for the transcriptional function (AF1) regions [118-120].

DBDs are the most conserved regions responsible for the NR binding to specific HREs [118, 119]. Each DBD contains two zinc fingers and two helices perpendicular to each other, forming a hydrophobic core base. The current model of NR-HRE binding assumes that the high-affinity binding of NR to its specific HRE fastens the receptor at relevant sites on the genome [118, 119].

The LBD, the second most conserved domain of the NRs, contains the site for ligand binding and is involved in the conformational changes of the receptor

through dimerization. LBD consists of about 12 helices folded into a globular structure [118, 119]. Near the C-terminus of the LBD resides another activation function sub-domain (AF2) [118, 119]. The coregulatory proteins interact with NRs through these activation domains, AF1 and AF2, affecting the target gene transcription [120].

Recently, increasing evidence implicating NRs and their coregulators in several pathophysiological conditions has evoked immense interest in understanding the function of NRs. This helped open new strategies for developing novel steroid-based targeted therapies for cancer [115-117]. Moreover, clinical studies have investigated the potential NRs as prognosis or diagnostic markers for several cancers [121].

The disruption of cell- and tissue-specific functions of NRs leads to several types of malignancies such as breast cancer, leukemia, lymphoma, prostate cancer, ovarian cancer, and lung cancer [102, 113, 122]. Therefore, as steroids and hormones play essential roles in cell proliferation and tumor development, knowing functional relationships between the regulation of cell proliferation and NRs is a relevant issue. Hormonal therapy has contributed majorly to treating several cancers by reducing recurrence rates and increasing survival rates. However, most conventional hormonal treatments result in the last term in steroid resistance over time.

2.7.1. The androgen receptor (AR)

An example of a steroid hormone regulating cancer is androgen with prostate cancer. Normal prostate development depends on androgens, principally on dihydrotestosterone (DHT) but also on testosterone (T) [99, 100]. The biological and physiological effects of androgens are modulated through the nuclear AR [100].

AR is the nuclear receptor activated upon binding with DHT and T. This binding occurs in the cytoplasm and stimulates the migration of AR into the nucleus. When in the nucleus, AR binds to its correspondent HREs, regulating gene expression (Figure 2).

Androgen activation of AR is responsible for initiating the prostate's development and maintaining prostate epithelia under normal conditions.

However, an abnormal overexpression of the AR receptor observed in prostate cancer patients stimulates prostate cancer cell proliferation [17].

Most prostate cancers depend on androgen at initial phases, and endocrine-based therapies, such as ADT, are based on lowering the serum androgens and inhibiting AR activity [16, 123]. The abnormal increase of AR expression and subsequent permanent activation of the AR signaling may continue even when castration surgery is performed or ADT is applied [32]. Although androgen deprivation increases apoptosis of prostate cancer cells, they may develop resistance. However, the mechanism of this resistance is unknown.

Exon 1 of the AR gene contains a polymorphic CAG repeat sequence that encodes a polyglutamine (polyQ) chain in the AF1 region [81]. The length of the polyQ chain correlates inversely with AR transcription [124]. Thus, it is proposed that men with shorter repeat lengths have a higher risk of prostate cancer [17]. This relates to Mexican and African-American men having a shorter polyQ chain length in the AR [17, 125]. However, the molecular mechanisms that underlie changes in the AR transactivation activity due to polyQ chain length remain largely unknown. This may be due to the lack of information about the AR's transactivation domain, AF1 located in the N-terminal domain [124].

2.7.2. The vitamin D receptors

Three SHRs are involved in the activation and mediated effects of the vitamin D signaling pathway: the previously mentioned NR, the vitamin D receptor (VDR), and the protein disulfide isomerase family A, member 3 (PDIA3) receptor [22] (Figure 2).

VDR is localized in the cytosol and is activated upon binding with vitamin D (VD), which initiates its heterodimerization with the retinoid X receptor alpha (RXRA) receptor (Figure 2). This complex migrates to the nucleus where it modulates gene transcription after binding with the vitamin D response element (VDRE) in the genome [18, 107] (Figure 2). Thus, VDR is responsible for the "genomic or long-term actions," activating gene transcription by chromatin remodeling and regulating vitamin D biosynthesis, which leads to the attributed antitumoral effects of vitamin D [18].

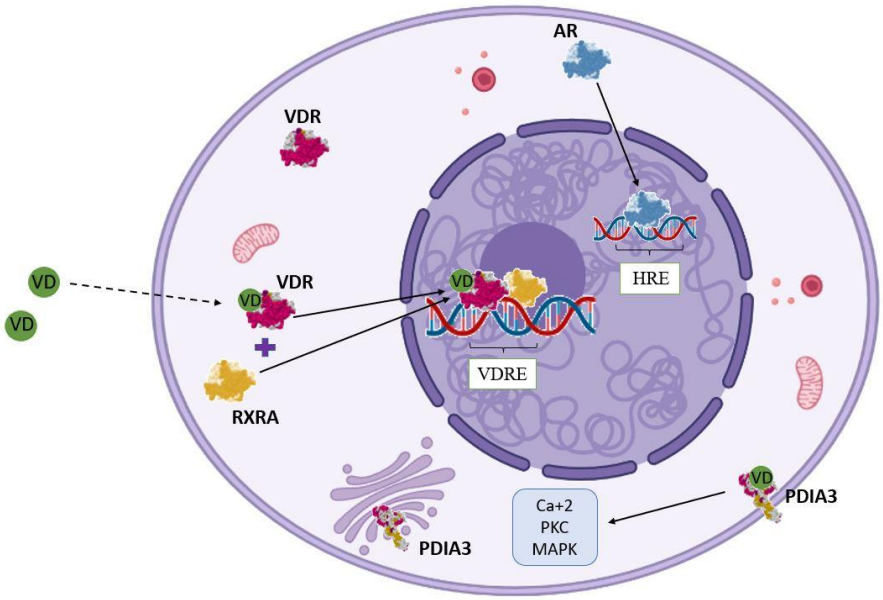

Figure 2. SHRs involved in the activation and mediated effects of the vitamin D signaling pathway and in the androgen's response in the cell.

The PDIA3 receptor is a chaperone localized mainly in the endoplasmic reticulum. Sporadically, this receptor can be localized in the plasma membrane where it is responsible for "non-genomic or rapid actions" of vitamin D in the plasma membrane [22]. These actions include the regulation of intracellular, extranuclear pathways, and signaling cascades, such as the activation of the protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and the calcium transport [22]. The role of PDIA3 in cancer regulation remains controversial since some studies suggest that PDIA3 is responsible for the activation of pro-apoptotic pathways [21, 126], and other studies indicate that it is associated with cancer proliferation, inhibition of apoptosis, and poor prognosis [127, 128]. An explanation could be the existence of two isoforms of PDIA3 playing a different role in cancer progression and the activation of different signaling pathways.

VDR was found to have a lower expression in prostate tissue, whereas PDIA3 had higher expression at protein and transcript levels in both standard and

cancer prostate tissue [129]. Hendrickson et al. showed that high expression of the VDR in prostate tumor tissue is associated with a reduced risk of aggressive prostate cancer [130]. Recent data from our laboratory show that the expression of both receptors increased with the treatment with 25(OH)D and with the inhibition of its metabolism in metastatic prostate cancer cells, suggesting an enhanced sensitivity of the treated cells to the compound [88].

Furthermore, studies with restricted conformation analogs of calcitriol have shown that these compounds bind selectively with different receptor populations. The 6-trans restricted analogs of 1,25(OH)D interact essentially with nVDR, and the 6-cis restricted analogs of 1,25(OH)D interact with PDIA3 or VDR associated with the plasma membrane, inducing distinct cellular effects [6]. This implies different responses that depend on the affinity of the receptors to specific vitamin D metabolites and the balance between receptor pools, which can play a role in the effects of vitamin D regulating prostate cell biology.

3. Rationale

The genetic background and lifestyle habits significantly influence an individual's health status and the probability of acquiring cancer. For example, vitamin D supplementation and vitamin D levels in the blood can contribute to the individual's development of health or disease by repressing or activating specific genes through changes in the methylation of DNA.

Vitamin D exerts a positive effect on several forms of cancer, including prostate cancer. This overall picture of prostate cancer and vitamin D, together with lifestyle, aging, and cellular processes is not well understood. Hence this thesis tries to connect these areas with different model systems. The two model systems used are human and an *in vitro* cell-based system. The human study investigated a population of healthy elderly people belonging to an association of active seniors with a good self-estimated value of health. The *in vitro* cell-based model system used commercially available and well-studied cell lines with different stages of prostate cancer.

Investigating healthy elderly people helps in understanding the regulation of vitamin D receptors in a complex system, the human being, where other factors such as age, mental health, genetic background, and lifestyle interplay. The *in vitro* study of prostate cancer increases the understanding of the molecular and functional mechanisms behind the regulation of the vitamin D receptors.

Hopefully, this broader approach will link lifestyle traits and the regulation of vitamin D receptors with prostate cancer on the path to improved health. This is an essential venue of research since vitamin D deficiency and a higher prevalence of diseases, such as prostate cancer, are very common in the elderly population.

The results of this study may suggest potential implications for the individual connected to healthy aging and therefore improve the understanding and motivations for making healthy lifestyle choices. Last, but not least, connecting these findings on the individual and environmental level to the function on a molecular level will provide us with an insight into the function

of vitamin D and its receptors and with some hints for the connection that aging and lifestyle have on health and cancer.

4. Overall aim

This research project aims to increase our knowledge about steroid hormone receptors, primarily the vitamin D receptors, in both health and disease, focusing on genomic, epigenomic, and transcriptomic perspectives in healthy elderly individuals and in prostate cancer cells.

The specific aims of the studies are listed below:

- **Paper I**: To examine the influence of lifestyle and vitamin D on global methylation levels and the methylation levels of vitamin D receptors and associated metabolic enzymes in a healthy elderly cohort.

- **Paper II**: To characterize single nucleotide polymorphisms (SNPs) in the vitamin D receptors and associated metabolic enzymes in individuals in the healthy elderly cohort and explore potential relations between specific alleles with an intake of vitamin D supplements and health status.

- **Paper III**: To investigate crosstalk between steroid hormone receptors by focusing on receptors involved in the etiology and progression of prostate cancer.

- **Paper IV**: To evaluate steroid hormone receptors involved in vitamin D signaling and their isoforms as candidate biomarkers for clinical diagnosis of prostate cancer.

5. Material and methods

5.1. Study models and population

In this thesis, three sample types or populations were used: an *in vivo* human study, with individuals from a Swedish elderly cohort (Study I and II); an *in silico* study with genomic sequences extracted from a reference database (Study III); and an *in vitro* study with prostate cell lines representing different stages of prostate cancer (Study IV).

5.1.1. Study I and II – The Swedish elderly cohort

The genetic background and lifestyle of adults affect both longevity and health. Genetic makeup plays a significant role in health, but healthy lifestyle choices are vital for older adults. Different lifestyle habits are responsible for the activation or inactivation of the expression of specific genes by methylation [16]. However, few studies report the combined effects of older individuals' lifestyles, genetic backgrounds, and perceived well-being.

The present study is part of a larger cohort study of healthy elderly individuals from Southwest Sweden, previously described by Gillsjö et al. [35]. The cohort consisted of 530 community-dwelling individuals between 70- and 96-years-old living from Southwest Sweden. The individuals participated in collaboration with a program called Active Seniors, a nationwide politically independent program in Sweden for active older adults [35]. This cohort is considered a healthy elderly cohort since the members belong to an association that promotes a healthy lifestyle and most of the individuals estimated their health status from satisfactory to very good. This more extensive study focused on investigating the genetic and the lifestyle factors responsible for successful aging, and how lifestyle factors affect global methylation patterns and epigenetic age.

The participants answered a questionnaire that included background data, self-reported health status (SRHS), medical data, lifestyle habits, family relationships, and meaning in life. Background data and self-reported medical

data among the individuals participating in the questionnaire are presented in Table 1.

Table 1. Background data, SHRS, and medical data in the elderly cohort, Southwest Sweden (N = 530).

General characteristics			
Gender	Female	Male	Total
	(n = 337)	(n = 182)	(n = 519, NA = 11)
Age (mean±SD)	74.9 ± 6.9	75.6 ± 7.3	75.2 ± 7.1
Medical data			
Self – reported health status (SRHS)			
Very good	149	80	229
Satisfactory	147	87	234
Unsatisfactory	7	3	10
Medicine – Lipids			
Yes	201	120	321
No	66	32	98
Medicine – Blood pressure			
Yes	166	98	264
No	125	67	192
Medicine – Heart			
Yes	207	118	325
No	66	31	97

* n: number of samples; NA: no answer.

The cohort was stratified into profile groups based on vitamin supplementation, smoking habits, drinking habits, exercise, sun exposure, and eating habits (Table 2a and Table 2b).

Table 2a: Lifestyle habits in the elderly cohort, Southwest Sweden (N = 530).

Lifestyle habits	Question	Answer	Female (n = 337)	Male (n = 182)	Total (n = 519) (NA = 11)
Supplementation with vitamins	All kinds of vitamins	*Intake*	144	81	225
		No intake	155	93	248
	Specifically vitamin D	*Intake*	49	22	71
		Not intake	265	156	421
Smoking habits	Smoking	*High*	7	4	11
	before retirement	*Intermediate*	21	6	27
		Low	101	70	171
		None	170	94	264
	Smoking habits	*Decreased*	6	2	9
	after retirement	*No change*	281	168	449
		Increased	1	0	1
Drinking habits	Alcohol	*High*	13	3	16
	(SD/week)	*Intermediate*	153	105	258
		Low	136	66	202
	Alcohol	*Low*	246	143	389
	(frequency/week)	*High*	54	31	85
	Alcohol	*Decreased*	70	43	113
	(after retirement)	*No change*	180	89	269
		Increased	25	24	49
Exercise	Physical activity	*High*	163	89	252
	during summer	*Intermediate*	90	53	143
		Low	53	35	88
	Physical activity	*High*	198	87	285
	during winter	*Intermediate*	79	47	126
		Low	40	38	78
	Physical activity	*High*	181	75	256
	during summer	*Intermediate*	100	63	163
	and winter	*Low*	54	42	96
	Physical activity	*High*	107	72	179
	after retirement	*Intermediate*	55	29	84
		Low	139	74	213
Sun exposure	Sun exposure	*High*	147	80	227
		Intermediate	102	64	166
		Low	54	30	84
	Use of sunscreen	*Always*	74	43	117
		Sometimes	167	88	255
		Never	62	45	107
	Sun exposure	*Decreased*	31	21	52
	after retirement	*No change*	171	99	270
		Increased	108	57	165

* n: number of samples; NA: no answer.

Table 2b. Lifestyle habits in the elderly cohort, Southwest Sweden (N = 530).

Lifestyle habits	Question	Answer	Female (n = 337)	Male (n = 182)	Total (n = 519) (NA = 11)
Eating habits	Vegetables and	*High*	92	50	152
	fruits (frequency)	*Intermediate*	154	104	279
		Low	54	22	79
	Fruits (frequency)	*High*	120	75	205
		Intermediate	143	77	237
		Low	39	24	70
	Fish and seafood (frequency)	*High*	30	21	53
		Intermediate	193	115	328
		Low	78	40	130
	Processed food (frequency)	*High*	72	46	124
		Intermediate	111	68	195
		Low	119	62	193
	Fasting >16 h (frequency)	*High*	19	8	33
		Intermediate	30	18	53
		Not	246	141	414
	Calory intake	*High*	46	24	75
		Intermediate	219	128	371
		Low	33	17	56
	Calory intake	*Decreased*	84	24	142
	after retirement	*No changes*	196	128	326
		Increased	19	17	39
	Healthy food habits	*Decreased*	11	5	16
	after retirement	*No changes*	178	100	304
		Increased	111	72	192

* n: number of samples; NA: no answer.

5.1.2. Study III – Human reference genome

The human genome sequence is the largest genome to be extensively sequenced, and it is 25 times as large as any previously sequenced genome. The Human Genome Project started in the early 1990s because of the need to accelerate biomedical research by allowing researchers to solve problems such as explaining the development of previously known genetic diseases [131]. So far, the results of this project have shown that the complete genomic sequence provided relevant information about genes, regulatory regions, and chromosome structure and showed a tremendous amount of DNA variants, much more than previously thought [131, 132].

In our investigation, we used the human reference genome, GRCh38, and GRCh37 as a way to gain knowledge about important regulatory regions through sequence conservation (Study III) and as a tool to map and annotate genes, transcripts, as well as genetic polymorphisms (Study I, II, III, and IV).

5.1.3. Study IV – Cancer cell lines: A model for studying prostate cancer progression

Cancer cell lines, immortalized cells that can be grown for long periods *in vitro,* have been widely used as a model to study molecular mechanisms and test hypotheses related to the improvement of cancer treatments as these cells exhibit all the cancer hallmarks [133, 134]. However, cell lines as a disease and drug treatment model are the subject of debate. One question is if these cells exhibit the same genomic features as tumors. Several studies have evaluated this and confirmed that most cell lines model tumor genomic features, and most cancer subtypes and patterns of tumor heterogeneity are represented in them [133, 134]. One of the drawbacks of using cell lines is that they might have characteristic emerging mutations that tumor cells do not have.

Furthermore, the aggregated clonal cells in cell culture do not resemble the tumor's cell heterogeneity. The cell culture flask conditions are not the same as in the tumor's environment in the human body. Another drawback is finding a suitable control since the normal cell lines retain some of the tumor characteristics, such as immortality. The last drawback is that the *in vitro* cancer cells should be kept sterile to avoid cross-contamination and at a low passage number to ensure a low rate of mutations and to avoid a change of the cell's phenotype [135].

Some of the experiments conducted in Study IV used cell lines for modeling progression of the prostate cancer disease, representing different cancer and hormone dependency. We have selected this *in vitro* model for its reliability, inexpensiveness, and as an alternative to animal studies. The cells were kept at a low number of passages (p2–p12) to prevent genomic instability. The cell lines used in this study were PNT2, P4E6, LNCaP, PC3, and DU145 (Table 3). These cell lines were ordered from Sigma Aldrich and the European Collection of Authenticated Cell Cultures (EACC).

Table 3. Prostate cell lines cultivated for the different experiments in the study.

Name	*Source*	*Stage*	*AR protein*	*Androgen dependency*
PNT2	Prostate epithelial	Normal	Positive-low	Yes
P4E6	Prostate epithelial	Early cancer	Negative	Not reported
LNCaP	Adenocarcinoma-lymph node	Metastatic	Positive-high	Yes
DU145	Primary carcinoma-Brain	Metastatic (II)	Negative	No
PC3	Adenocarcinoma-Bone (vertebral)	Metastatic (IV)	Negative	No

PNT2 was chosen to represent healthy prostate tissue because it is a non-tumorigenic well-differentiated epithelial cell line. P4E6, a human prostate cell line derived from a biopsy of well-differentiated early-stage prostate cancer, was chosen to represent the early stage of the disease (AR-negative). LNCaP, a prostate cancer cell line from a lymph node metastasis, was used in the experiments to represent the metastatic stage (AR-positive). DU145 and PC3, metastatic brain stage II and metastatic bone stage IV prostate cell lines, were used to study the metastatic stage of the disease (AR-negative).

5.2. Overview of the study material and methods

Figure 3 shows a schematic overview of the study models and population and the methodology used in the four studies composing this thesis.

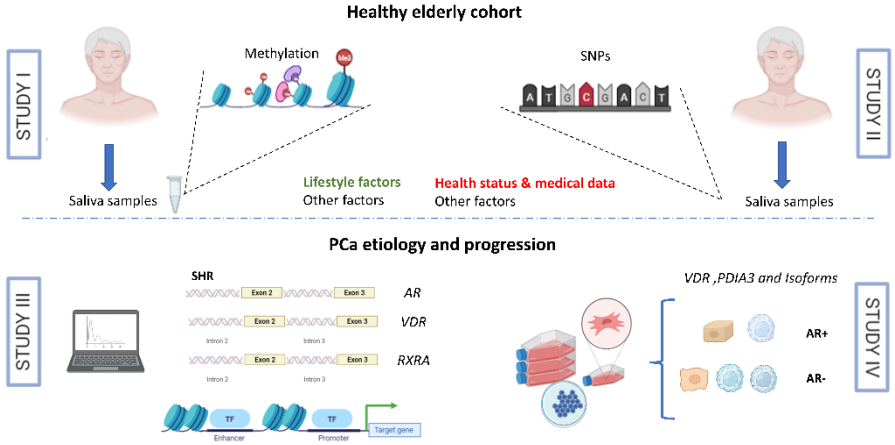

Figure 3. Methodology overview of the thesis.

Study I and II were performed using data from the healthy elderly cohort. These studies are a part of the previously described study with the Swedish elderly cohort, which in this case are focused on the effects of vitamin D supplementation and vitamin D receptors. Saliva samples for subsequent DNA extraction and analyses were collected from 520 of the participants (Figure 3).

In Study I, 277 of the 520 samples were randomly selected and analyzed to explore correlations of methylation levels with lifestyle factors (Table 2a and 2b). Methylation measurements were done using methylation arrays. Global DNA methylation and the methylation of vitamin D-related sites were analyzed with R and the bioinformatic packages to integrate epigenomics data. Functional annotation of the epigenetic markers and associated regulated pathways were analyzed with publicly available tools.

In Study II, all 520 samples were analyzed for potential correlations of SNPs with SRHS, medicine intake, and supplementation of vitamins. Genomic DNA was genotyped using an SNP array. SNPs corresponding to vitamin D-related genes were further analyzed with bioinformatic and statistical packages to integrate genomics data in R.

The study of etiology and progression of prostate cancer, **Study III and IV**, consisted of one *in silico* study with genomic sequences extracted from a reference database (Study III) and one *in vitro* study performed in cell lines representing different stages and androgen dependency of prostate cancer (Figure 3).

Study III analyzed conserved intron and exon sequences among receptors involved in prostate cancer etiology and progression (AR, VDR, and RXRA). We studied their relevance as potential transcription regulatory and binding sequences for other SHRs.

Study IV evaluated the mRNA expression of VDR, RXRA, and PDIA3 in AR-positive (AR+, PNT2, and LNCaP) and androgen negative (AR-, DU145, and PC3) cell lines by Next Generation Sequencing (NGS) and Droplet digital TM PCR (ddPCR). Structural and functional analyses of the predicted protein sequence of these receptors was performed with different bioinformatics tools and software.

5.3. DNA analysis

5.3.1. DNA methylation

DNA methylation is the addition of a methyl group to the fifth carbon of cytosine (C), forming 5-methylcytosine (5mC) by a DNA methyltransferase. These modifications in the DNA play an essential role in regulating gene expression, resulting in a wide range of biological processes and diseases [136]. Methylation microarrays enable the quantitative measurement of specific methylation sites down to a single nucleotide resolution [136]. A total of 277 DNA samples from the elderly cohort were used for epigenome-wide experiments and sent to Swegene Centre for Integrative Biology at Lund University (SCIBLU) in Sweden for analysis. Methylation measurements were done using the Illumina Infinium DNA Methylation EPIC BeadChip array covering approximately 850,000 methylation sites. We performed annotation, pre-processing, and quality control (QC) of the methylation data. Potential variation or batch effects on the pre-processed methylation data were assessed using principal component analysis (PCA). This analysis resulted in 667,259 methylation sites in 274 samples; these samples were analyzed.

Differential methylation analyses allow us to identify positions in the genome that have the largest difference in methylation levels between two groups [137]. This analysis was performed on the 274 samples, which were stratified into different groups according to background data and self-reported medical data (Table 1) and lifestyle (Table 2a and 2b) together with the combination of different lifestyle factors with vitamin D supplementation. Methylated positions and regions with a difference in beta value (β) between groups ≥ 0.2 and ≤ −0.2 and a false discovery rate (FDR) <0.05 were considered differentially methylated and therefore candidate for epigenetic markers. Differentially methylated probes/positions (DMPs) and differentially methylated regions (DMRs) were considered hypomethylated if $\beta \leq -0.2$, and hypermethylated if $\beta \geq 0.2$.

Functional annotation and associated networks

Functional annotation and associated network analysis provided an overview of the pathways associated with the candidate epigenetic markers. Genes overlapping with the DMPs were used as input for these analyses. Functional annotation analysis was performed using the gene ontology (GO) enrichment analysis. Functional regulated pathways were analyzed with different web-based tools. The output pathways were considered down-regulated if the genes were hypermethylated or upregulated if the associated genes were hypomethylated. Associated network analysis was performed to detect related genes, potential interactions, and related networks to the input genes from datasets with available genomics and proteomics data.

Methylation status of vitamin D-related genes

A total of 80 vitamin D-related genes corresponding to a total of 2113 CpG sites were selected for further analyses to assess for correlation between methylation levels and the different groups according to background data and self-reported medical data (Table 1) and lifestyle (Table 2a and 2b). The selected genes included vitamin D receptors (VDR and PDIA3), metabolic enzymes (CYP27A1, CYP27B1, CYP2R1, and CYP24A1), secondary activated receptors (RXRA and TRPV6), vitamin D binding protein (DBP), and primary activated targets of vitamin D.

5.3.2. Single nucleotide polymorphisms (SNPs)

Genotyping is the process of determining variations in an individual's genetic background by examining the DNA sequence. SNPs are single base-pair mutations at a specific locus, usually consisting of two alleles. SNPs are the most frequent type of variation in the genome and are linked to phenotypic changes that might be involved in the etiology of many human diseases and pathologies [9]. SNP microarrays are the most common method to identify genetic variations since they can analyze many SNPs per sample [10]. A total of 520 samples were analyzed in the genome-wide experiments performed at SCIBLU. Genomic DNA was genotyped using the Illumina HumanOmniExpress 24 v.1.1 BeadChip array covering approximately 710,000 known SNPs. We performed pre-processing and PCA in the SNP data, which rendered a total of 565,367 SNPs and 502 samples for further analysis.

Selection of SNP variants and association testing

A total of nine vitamin D pathway candidate genes were selected for further analyses: the main vitamin D receptors VDR and PDIA3; the metabolic enzymes CYP27A1, CYP27B1, CYP2R1, and CYP24A1; the secondary activated receptors RXRA and TRPV6; and the vitamin D binding protein DBP [138-140]. In total, 158 SNPs variants located within or in the proximity of these genes were selected by chromosome and position. The correlation between the genotype of the selected SNPs and the different study variables (SRHS, medicines, and supplements of vitamins; Table 1 and Table 2a) were assessed with a generalized linear model where age and gender were included as model predictors.

Frequency comparison

The minor allele frequency (MAF) was extracted from the genotyping results for each of the selected vitamin D SNPs. Differences in MAFs in these SNPs between our elderly cohort and a European and a Northern Sweden subpopulation were assessed to identify potential risk alleles related to different latitudes.

5.3.3. Steroid hormone receptors (SHRs)

Analysis of conserved sequences

The most studied SHRs family is the NRS, which consists of genes for receptors involved in steroid hormone signaling. Therefore, intron and exon sequences from 49 NRS genes, which were translated from pre-mRNA transcripts to the corresponding DNA sequence, were retrieved from the Ensembl genome database [26] for analysis. Since regulatory elements can be identified through sequence conservation, we selected sequences that belonged to orthologous transcripts from mammalian species with a close phylogenetic relationship, which implies a high probability of sequence conservation.

Conserved sequences between different species were extracted with a basic conserved sequence detection method (BCSDm). BCSDm is based on the combination of three methods: alignment of the different sequences; extraction of the alignment profile and its position score matrix (PSSM); and obtainment of the conserved nucleotide patterns and their position in the alignment. The conserved patterns were extracted by considering a 100% conservation and a sequence length of ≥ 15 consecutive nucleotides.

Analysis of cis-regulatory elements (CREs)

Each conserved intronic and exonic pattern was scanned for CREs. The CREs, located in intron and exon DNA sequences, are often transcription factor binding sites (TFBS) or splicing sites (SS) [83, 141]. Therefore, the intron and exon conserved sequences were scanned for TFBS and SS in the CIS-BP [142] and the Human Splicing Finder (HSF) databases, respectively [32]. The conserved intronic patterns were classified as TFBS or SS depending on their composition in regulatory elements. TFBS and SS found in non-conserved sequences from the same gene intronic regions as the conserved sequences were used as controls. The non-conserved sequences were extracted and scanned for TFBS and SS with the same parameters as for the conserved sequences analysis.

After identifying the TFBS in the conserved sequences, the transcription factor binding domain (TFBD) in these TFBS were analyzed. A small

proportion of all analyzed TFBDs were NR binding domains. NR binding sites were further investigated, and comparisons were done between introns and exons.

5.4. mRNA expression and protein sequence analyses

5.4.1. In vitro cell culture

PNT2 and LNCaP cells were cultured in RPMI-1640. P4E6 cells were maintained in Stemline Keratinocyte Medium II with Stemline Keratinocyte Growth Supplement with 2 mM Glutamine, DU145 cells were cultured in EMEM, and PC3 cells were cultured in DMEM containing 5% of pyruvate sodium. All the media were supplemented with FBS (2%–10%) and PEST (1%).

5.4.2. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from approximately 1×10^6 cells from each cell line, and the genomic DNA was digested with DNase. High-quality RNA (A260/280 = 1.9–2.0, A260/230 = 1.5–1.8, RNA integrity number ≥ 8) from the respective cell line was sent for NGS analysis to The National Genomics Infrastructure (NGI) in The Science Life Lab in Stockholm, Sweden (SciLifeLab). Total RNA was reversed transcribed to cDNA and analyzed using the digital droplet PCR technique.

5.4.3. Next generation sequencing (NGS)

NGS uses massively parallel sequencing that produces millions of short read sequences in short time with high throughput [17]. In contrast to Sanger sequencing, NGS allows the entire genome to be sequenced at once in an automated process. NGS also has more probability of detecting novel or rare variants with deep sequencing [18]. RNA from each cell line were sequenced with NGS Illumina RNAseq HiSeq 2500. Reads were mapped to the human genome assembly, build GRCh37 (hg19). Quantification of normalized expression of VDR, RXRA, PDIA3, and their different transcripts isoforms

were obtained as fragments per kilobase of transcript per million fragments mapped (FPKM).

5.4.4. Droplet digital ™ PCR (ddPCR)

Droplet digital polymerase chain reaction (ddPCR) is a modification of the real-time PCR, which uses a water–oil emulsion droplet system. Each droplet separates template DNA molecules into to 20,000 individual PCR reactions. After PCR amplification, the positive and negative droplets are individually counted on the droplet reader with the aid of a fluorescence detector. The concentration of the amplified target can be determined from the number of positive and negative droplets after amplification using probability analysis [143]. A total of 225 cDNA samples were analyzed to quantify the amount of PDIA3 and PDIA3N. The amount of cDNA for each reaction was 10,000 copies and each sample was partitioned into 10,000–18,000 droplets for successive PCR amplification. After amplification, droplets were read on a QX200 droplet reader and analyzed with QuantaSoft software V1.7.4. To define the number of positive droplets, the amplitude limit was set manually to 2000 for PDIA3 and 4000 for PDIA3N. The mRNA expression or template concentration (copies/µl) and the ratio of mRNA expression between the isoforms (PDIA3N/PDIA3) were calculated using QuantaSoft™ Analysis Pro Software.

5.4.5. Functional analysis

The protein sequences of PDIA3 and PDIA3N were analyzed to create the protein structure model. A prediction of damage or pathogenicity of the 56 variations found in PDIA3N when compared to PDIA3 together with a prediction of the subcellular localization were carried out with different software.

An automated docking of ligand to the macromolecular receptor was performed to assess differences in the binding of 1,25(OH)D to PDIA3 and PDIA3N. Putative binding sites in PDIA3 and PDIA3N were selected by searching for similar free binding energies to the obtained sites in previous experiments with VDR and 1,25(OH)D [144, 145].

5.5. Statistical analyses

5.5.1. DNA methylation

Methylated positions and regions with a difference in beta value (β) between groups ≥ 0.2 and ≤ −0.2 and FDR <0.05 were considered differentially methylated. Functionally enriched pathways and results from the associated network analysis were considered significant if FDR was < 0.05.

Analysis of the association of the methylation levels of vitamin D genes to non-binary factors was performed with Spearman's correlation test. Differences in methylation levels for binary factors were assessed with Welch's two samples t-test considering unequal variances. P values were adjusted for multiple testing with Benjamini-Hochberg correction.

5.5.2. Single nucleotide polymorphisms (SNPs)

SNP variants were considered significantly associated with the variable if the P value was < 0.0005. A two proportions z-test assessed differences on minor allele frequencies (MAFs) with a Yates continuity correction (P-value < 0.05). Statistics for linkage disequilibrium (LD) were calculated between alleles at two loci by the squared Pearson coefficient of correlation (r2).

5.5.3. Steroid hormone receptors (SHRs)

The Mann-Whitney U test was used to assess the distribution of conserved sequences among different introns. The frequency distribution of the four groups (TFBS, TFBS-SS, SS, and not identified) among introns was analyzed using the Chi-square test. The number of TFBS and SS in conserved and non-conserved sequences was analyzed using the Wilcoxon paired non-parametric test. The chi-square test assessed the number of TFBS for each TFBD family between conserved and non-conserved patterns. Statistically significant differences were set to Not significant (ns), *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

5.5.4. *mRNA expression*

Differences between ratios of mRNA expression (PDIA3N/PDIA3) among prostate cancer cell lines were assessed by non-parametric Kruskal-Wallis multiple comparison test. Pairwise comparisons of the mRNA expression of PDIA3 and PDIA3N between prostate cell lines were evaluated using Mann-Whitney U-test. Pairwise comparisons of mRNA expression of PDIA3 and PDIA3N in the same prostate cell line were assessed with Wilcoxon signed-rank test. Levels of significance were set to $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

5.6. Ethical considerations

Ethical considerations in this study were grounded on the ethical principles for medical research involving human subjects in the Declaration of Helsinki [146]. Following the law of the ethical review of research [147], approval for the first and second study (Study I and II), which involved human samples, was obtained by the Regional Ethical Review Board in Gothenburg (Dnr 989-13).

In Study I and II, analysis of genetic, epigenetic, and questionnaire data were performed from a cohort of community-dwelling individuals who were previously recruited in collaboration with the association Active Seniors, a nationwide independent political association in Sweden for older adults. The study invited 800 individuals from the southwest of Sweden, between 70- and 96-years-old [39]. Data collection consisted of two parts: a questionnaire regarding self-evaluation of health, medical data, lifestyle habits, family relationships, and meaning in life and a collection of saliva samples for analyses of DNA. Data collection was part of the routine for ordinary activities and did not cause any discomfort or harm to the participants.

Participants in the study were informed in a presentation about the project. This information was given both in writing and verbally. The participants were informed that they could withdraw their participation at any time without any explanation or any consequence. After the presentation, a total of 530 participants provided informed consent. Both the questionnaire and the saliva samples were coded to protect the confidentiality and integrity of the

participants. All the data were collected and analyzed following data privacy policies, so the individuals could not be identified. Sensitive information, such as the genetic data, was only accessible to the researchers responsible for the investigation. The data were handled by Jönköping University's guidelines for storing sensitive research data.

In Study III and IV, genomic sequences were extracted from a reference database and prostate cell lines representing different stages of prostate cancer, respectively. The genomic sequences were human DNA sequences publicly available in a common repository. As the prostate cancer cell lines are human commercial cell lines developed for research purposes, no ethical approval was needed. Therefore, we only followed the general guidelines and regulations for the culture of cell lines in biomedical research [148].

This research project was done within the framework for a doctoral degree, and the results will be presented in scientific journals and in this dissertation. Research results will also be presented at national and international scientific conferences and disseminated within Jönköping Municipality and other municipalities in the country. All the results will be presented at a group level, and any information that could potentially identify the individuals involved will not be disclosed.

6. Results and discussion

6.1. Methylation patterns associated with lifestyle factors and vitamin D supplementation

Environmental and lifestyle factors such as regular exercise and intake of vitamin D can improve overall health and well-being in the elderly. Lifestyle factors can influence epigenetic mechanisms such as DNA methylation, which is responsible for the activation or repression of the expression of specific genes [3]. Thus, Paper I aimed to evaluate the influence of lifestyle and vitamin D on global DNA methylation patterns in 277 participants from the Swedish elderly cohort.

Vitamin D can be either ingested in the diet and supplements or synthesized in the skin under sunlight exposure. Vitamin D acts like a hormone. Some studies report the influence of vitamin D supplementation and status and sunlight exposure on DNA global methylation and suggest some epigenetic markers [4, 5], although other studies did not find any associations [22, 54, 149, 150].

In the present study, vitamin D intake and sunlight exposure did not influence the methylation levels in the elderly. Questionnaire participants answered whether they were taking vitamin D supplementation (Yes = 33, No = 236) and specified if the vitamin D was part of a multivitamin complex (n = 8). An increase in methylation could be observed when comparing groups with individuals taking vitamin supplements (multivitamin complex), including vitamin D, with individuals not taking vitamin supplements. This increase in methylation was observed in the promoter region of the solute carrier family 25 member 24 gene (*SLC25A24*).

The SLC25A24 protein is a calcium-dependent mitochondrial carrier that mediates the exchange of Mg-ATP or Mg-ADP against phosphate ions [129]. SLC25A24 is inactive at low cytosolic calcium levels and activated by an increase in cytosolic calcium levels, one of the known effects elicited by vitamin D [151]. The promoter region of *SLC25A24* has already been reported as a primary target of vitamin D in human monocytes. However, it is unclear

why in our study this effect in methylation was only observed when the vitamin D was supplemented in a multivitamin complex or if this effect could be from any other supplements in the complex or from the combination of all of them.

We have also investigated the influence of other lifestyle habits on methylation levels, such as general vitamin intake, eating habits, and physical activity. In general, the global methylation patterns did not change according to the lifestyle factors evaluated in this study.

Physical activity affects DNA methylation regulation and is involved in maintaining telomere length (an anti-aging mechanism) [152, 153]. The participants in the questionnaire also answered questions regarding physical activity during the winter or summer. Questions were ranked from 1 to 6 (1 = almost no physical activity and 6 = hard training regularly and several times a week). The physical activity was considered high when the sum of the ranks for the summer and winter exercise was ≥ 9 and ≤ 12, intermediate when it was = 8, and low when it was < 8 (summer + winter). No differences could be observed when comparing high, moderate, and low physical activity. Two DMPs in intergenic regions were found when comparing individuals who exert moderate-high physical activity (n = 264) with those who exert very low or almost no physical activity (n = 8). Several studies have shown that both acute and lifelong physical activity promotes changes of DNA methylation in the skeletal muscle, blood, and saliva [152, 154-156]. However, the epigenetic modifications found in these previous studies were reported as a result of an exercise intervention, and our study is cross-sectional, where the intensity of the physical activity was self-assessed, which might be difficult for the participants to estimate.

Combining vitamin D and physical activity has reported synergistic effects in elderly with muscle loss or atrophy (sarcopenia), such as decreased inflammation and increased fat-free mass, strength, and functionality [157]. The isolated effects of vitamin D supplementation and physical activity on methylation have been explored previously but not their combined effects. When comparing individuals taking vitamin D supplements and exerting high levels of exercise (n = 7) with individuals not taking vitamin D supplements and exerting low levels of exercise (n = 118), we found a total of 357 DMPs

in 221 genes and 108 intergenic regions. Of these DMPs, 21% are within promoter regions implying a role in transcriptional regulation. Functional analyses of these genes confirmed that the main biological process activated was the transcription by the RNA polymerase (Figure 4).

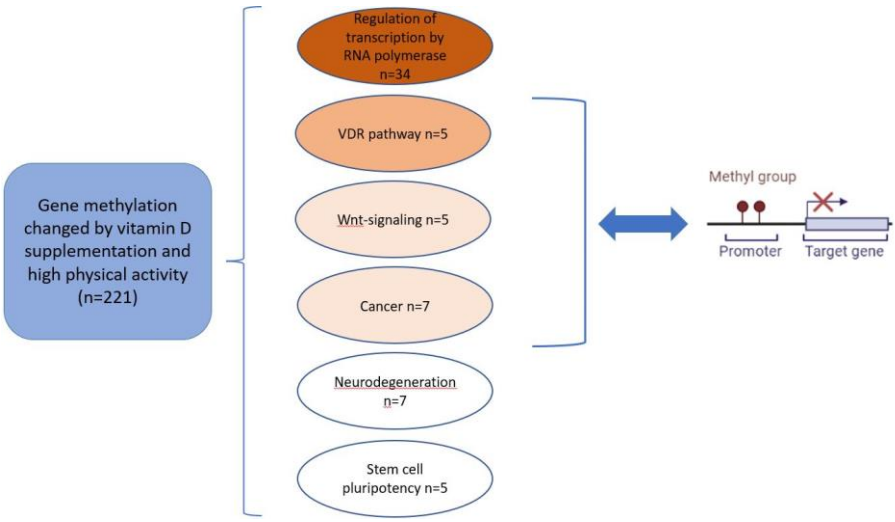

Figure 4. Main functional pathways associated with methylated genes induced by high physical activity and vitamin D supplementation, n: number of samples.

Interestingly, some genes corresponding with the differentially hypermethylated positions were associated with the vitamin D receptor (VDR) pathway (Figure 5). An increase in physical activity has been associated with an increase in circulating 25(OH)D (bioavailability) in men, women, adolescents, and the elderly [158, 159]. Thus, we may speculate that high physical activity increases vitamin D bioavailability and therefore regulates the transcription of genes related to the VDR pathway. Other relevant downregulated pathways were the Wnt-signaling and pathways implicated in cancer development (Figure 4). These results coincide with previous studies, where 1,25(OH)D and physical activity inhibited activation of the Wnt/β-catenin signaling, a pathway frequently hyperactive in cancer [160, 161].

We also analyzed the methylation status of 80 vitamin D-related genes, including well-known receptors, metabolic enzymes, and primary activated targets of vitamin D, previously identified in the literature. The methylation levels of the vitamin D-related genes examined here did not change in individuals with a vitamin D-containing diet, vitamin D supplementation, or sun exposure. Previous studies evaluating methylation of vitamin D receptors and associated metabolic enzymes in response to vitamin D supplementation are inconsistent and contradictory [54, 56, 57]. Zhou et al. reported a negative association of the methylation status of *CYP2R1* and *CYP24A1* with 25(OH)D baseline plasma levels. However, these associations were weak and disappeared when the prediction was corrected for vitamin D intake [56]. Our findings align with this previous study and suggest that the methylation status of vitamin D metabolic enzymes does not change according to vitamin D intake and sunlight exposure.

Chronological age has been shown to have extreme effects on DNA methylation levels, and several epigenetic markers have been suggested to be accurate estimators of biological age [162]. We found an inverse correlation between the methylation status of the analyzed vitamin D-related gene RXRA and age. A previous study using computational networks identified *RXRA* methylation as one of the most critical nodes related to chronological age across multiple tissue types [163]. Our findings and the previous study suggest the methylation of the *RXRA* as an estimator of epigenetic age for future investigations.

6.2. SNPs in vitamin D receptors

Genetic variants of vitamin D pathway-related genes have been associated with vitamin D levels, different phenotypes, or pathological conditions [151-155]. The association between SNPs in the VDR gene and the risk for developing cancer have been investigated extensively [48, 164-167]. Some of these studies examined the association between polymorphisms in the VDR gene and levels of 25(OH)D in the blood [166, 167]. High protein expression of PDIA3 has been observed in glioma, ovarian cancer, and high-risk prostate cancer [126, 168, 169]. However, there are no reports on known PDIA3 SNPs associated with vitamin D levels or a high risk of certain diseases. Thus, in

Paper II, we focused on characterizing SNPs in the vitamin D receptors, VDR and PDIA3, and the pathway-related genes in 520 participants from our Swedish elderly cohort. We also investigated potential correlations between these SNPs and the intake of vitamin D supplements, SRHS, and heart and blood pressure medicines.

Vitamin D deficiency is common in Southern Europe and Eastern Mediterranean populations, despite high levels of sunlight, and it is unclear why this discrepancy exists [170]. An explanation might be that people in Southern Europe generally avoid the sun, cover up more and use more sunscreen protection, but genetic determinants might be the clue. Another reason could be that genetic variants in vitamin D pathway-related genes affect vitamin D levels. Thus, we analyzed minor allele frequencies (MAFs) for each genetic polymorphism and compared the results of our elderly healthy cohort with a general European and Northern Sweden populations.

When comparing the elderly cohort with the European population and the Northern Sweden population, a MAF difference higher than 5% was found in 21 and 18 SNPs variants, respectively (Paper II, Figure 1). More *RXRA* variants were found in the elderly cohort than in the European population and more *VDR* variants than in the Northern Sweden subpopulation. SNP variant rs11103473 in *RXRA* had the most remarkable difference of 8.5% in MAF compared to the European population, and rs34312136 in *RXRA* had a difference of 9.4% compared to the Northern Sweden population (9.4%). Minor alleles are more likely risk alleles for complex diseases [171]. This indicates that the frequency of risk alleles in *VDR* and *RXRA* differ between our cohort versus the Northern Sweden cohort and the European cohort. It is unknown whether genetic variations in *RXRA* influence circulating levels of vitamin D metabolites and through what mechanisms such polymorphism would act, but genetic variations in *RXRA* influence 1,25(OH)D homeostasis by altering its binding with the VDR [172].

Genotype association testing was performed for the 158 selected SNPs and the study variables such as intake of vitamin D supplements, SRHS, and heart medicines. No significant associations were found for most of the variables except for vitamin D supplementation, where we found two SNP variants at the PDIA3 locus, rs2788 and rs12441861. Minor allele frequencies for both

variants were approximately 8% higher in the group that reported having vitamin D supplementation (n = 71) compared to the group that reported not having any vitamin D supplementation (n = 421).

Rs2788 and rs12441861 are located very close to each other on chromosome 15 and are in complete linkage disequilibrium; that is, they have been inherited together and belong to the same haplotype [173]. The rs2788 SNP is in the three prime untranslated region (3'UTR). These regions are well-known for regulating mRNA-based processes, such as mRNA localization, mRNA stability, translation, and mediating protein-protein interaction [174]. Therefore, a mutation in a 3´UTR could greatly affect the modulation of transcriptional responses. PDIA3 has been shown to co-localize with VDR in response to the binding of 1,25(OH)D, evoking rapid cellular responses affecting gene expressions, proliferation, and apoptosis [22]. However, Gauchi et al. suggested potential 1,25(OH)D binding sites in the catalytic active domain of PDIA3, implicating that PDIA3 and 1,25(OH)D may interact directly [145]. A mutation in this region could also affect the dependent rapid responses to 1,25(OH)D.

There are only a few investigations on whether common genetic variants influence the response to vitamin D supplementation [175, 176]. Zhiyong Hu et al. showed that an SNP in *CYP27B1* was associated with the response to long-term vitamin D3 supplementation but not with the baseline levels of 25(OH)D [176]. In our study the *PDIA3* variants with the minor/risk alleles, rs12441861-T and rs2788-G, were associated with the group having vitamin D supplementation. This correlation may relate to a need for vitamin D supplementation experienced in different individuals. However, it is necessary to do additional measurements of the 25(OH)D serum levels to confirm this hypothesis.

Some studies have reported an association of specific polymorphisms in the *VDR* with vitamin D deficiency [177, 178]. Such as a study in a Mediterranean population in Greece characterized by a high prevalence of 25(OH)D deficiency associated with *VDR* polymorphisms in a cumulative effect of three genotypes–*BsmI*, *TaqI*, and *FokI* [177]. The genotype distribution of these polymorphisms did not differ considerably from the European populations in the previous study in Greece nor in the present study. This confirms that

specific VDR gene polymorphisms may influence vitamin D concentrations and may explain the vitamin D deficiency in sunny regions [177]. The rs2060793-A in the *CYP2R1* was associated with low levels of 25(OH)D in a genome-wide association study of 4501 persons from Europe [179]. The frequency for rs2060793-A was 5.7% higher in our elderly cohort from Southwest Sweden than in the European population, but 5.1% lower than in the Northern Sweden population. These results indicate that the Swedish populations may have a higher probability of vitamin D deficiency than the rest of Europe, especially in higher latitudes.

6.3. Crosstalk between steroid hormone receptors

The most studied and numerous SHRs gene family is the nuclear receptor superfamily (NRS). The NRS comprises 49 ligand-activated transcription factors that regulate gene expression by binding to specific DNA sequences and therefore controlling developmental and physiological processes [83]. Several members of the NRS play important roles in the etiology and progression of cancer, which has increased the interest to target them therapeutically [83, 106].

The crosstalk between steroid receptors is relevant to understanding the initiation and progression of hormone-driven cancers [15, 180, 181]. Crosstalk has been described for steroids by Cerliani et al. [182], for steroid NRs by Truong et al. [181], for growth factor receptors by Migliaccio et al. [183] and between kinases within the mitogen-activated protein kinase (MAPK) superfamily by Migliaccio et al. [184], as well as between downstream signaling components of these kinase pathways by Shupnik et al. [185]. These studies showed crosstalk at the protein level but not at the genomic level. Thus, in Paper III, we evaluated the crosstalk between steroid NRs in conserved intron and exon sequences, focusing on steroid NRs involved in prostate cancer etiology. For that purpose, conserved sequences of NR introns and exons were analyzed for their potential as cis-regulatory sequences binding to other NRs.

Conserved intronic sequences (n = 1044) were grouped according to their position in the transcript (intron 1 to intron 11). Of the 25 NRS genes analyzed,

the first intron had higher sequence conservation (35%) when compared to downstream introns. The sequence conservation generally tended to decrease with the position of the intron (Figure 1, Paper III).

The number of conserved sequences decreased with intron position which is in accordance with previous studies [186-188]. The density of conserved patterns was higher when compared with the study by Park et al. [186]. An explanation for this may be the variance in analyzed species (five and 46 mammalian species, respectively). Furthermore, Park et al. excluded sequences within 300 base pairs of the splicing site [186]. The current study included these sequences since they can potentially contain CREs, such as TFBS and SS.

TFBS and SS, were analyzed in both conserved and non-conserved sequences of NRs. There were more putative TFBS within the first introns than in the rest. However, the proportion of TFBS was preserved among introns, a finding that has not been previously reported. This indicates that the number of TFBS is directly associated to the number of conserved sequences and may explain the low number of TFBS obtained in the last introns.

The number of TFBS and SS in the conserved sequences was compared with the same numbers in the non-conserved sequences: 20% more CREs elements (10% of TFBS and 10% of SS) were found in the conserved sequences compared to the non-conserved sequences (Table 4). These results are in line with previous studies [189-191], which show a higher percentage of TFBS within conserved regions than non-conserved regions.

Table 4: Frequency distribution of the conserved and non-conserved patterns identified as TFBS and SS for the introns 1–9.

Intron	1	2	3	4	5	6	7	8	9	**Total**	**%**
Cons patterns	385	147	173	88	121	84	20	8	18	1,044	100
TFBS	294	121	150	67	96	63	14	3	16	824	**79**
SS	137	41	58	28	42	30	6	4	5	351	**33**
TFBS/Cons patterns (%)	76	82	87	76	79	75	70	38	89		
SS/Cons patterns (%)	36	28	34	32	35	36	30	50	28		
Non-cons patterns	385	147	173	88	121	84	20	8	18	1,044	100
TFBS	278	100	124	60	80	50	12	6	7	717	**69**
SS	88	29	41	13	38	17	7	1	2	236	**23**
TFBS/Non-cons patterns (%)	76	82	87	76	79	75	70	38	89		
SS/Non-cons patterns (%)	36	28	34	32	35	36	30	50	28		

*Cons patterns: conserved patterns; TFBS: Transcription factor binding sites identified in the conserved patterns; SS: Splicing sites identified in the conserved patterns; % TFBS/Cons patterns: percentage of TFBS with respect to the total number of conserved patterns; % SS/Cons patterns: percentage of SS with respect to the total number of conserved patterns; % TFBS/Non-cons patterns: percentage of TFBS with respect to the total number of conserved patterns; % SS/Non-cons patterns: percentage of SS with respect to the total number of conserved patterns.

The distribution of conserved patterns in TFBS, TFBS-SS, and SS is shown in Figure 5. An equal distribution of TFBS and SS was observed among introns (Figure 5). The TFBS group showed a similarity with 45–67% of the patterns in most introns. The sum of TFBS-SS and SS was also conserved among introns between 28 and 36% (Figure 5). Only intron 3 and 8 showed different distributions than expected ($P < 0.05$).

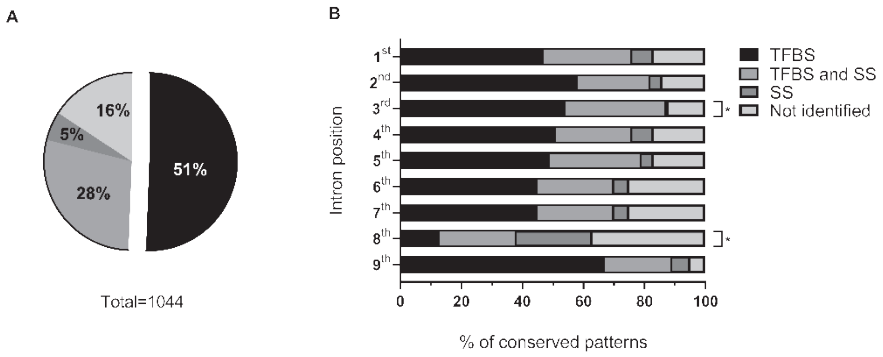

Figure 5. Distribution of conserved pattern groups in total (A) and intron 1–9 for the 25 analyzed genes (B). Conserved patterns were classified into four groups: TFBS, TFBS-SS, SS, and not identified. Frequency distribution of the four groups (TFBS, TFBS-SS, SS, and Not identified) among introns was analyzed with Chi-square test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

A great proportion of SS in conserved sequences is co-localized with TFBS (TFBS-SS group). Some studies have suggested multiple connections between transcription and splicing. However, it is difficult to isolate both processes [192-194]. Therefore, TFBS, which are closely located to SS, may act as transcription factors or splicing binding sequences depending on the conditions. Our results support previous studies that propose a link between the transcriptional and the spliceosome complex.

Mapping of the domains of transcription factors is necessary to understand their molecular function [195]. The analysis of TFBS identified 37 TFBD families (Paper III, Figure 3). One of the TFBD families was the nuclear receptor family (NR) (Paper III, Figure 3). The NR binding domains were 4.8% of the total conserved sequences in introns and 16.3% in the exons.

The crosstalk between steroid receptors is crucial in the initiation and progression of hormone-driven cancers [15, 180, 181]. Analyses of the three genes involved in prostate cancer etiology, *VDR*, *AR*, and *RXRA*, show putative binding sequences for other NRs in introns and exons (e.g., ESR, AR,

PGR, RARA, RORA, RORB, RORC, RXRA, RXRB, and RXRG (Paper III, Table 2 and 3).

NRs are detectable due to similarities of certain protein domains, such as the N-terminal (NTD)-, the DNA binding domain (DBD), and the C-terminal ligand-binding (LBD)-domain [119]. After a ligand binds to the LBD, the NR is conformationally rearranged and translocated to the nucleus. Once there, the NR binds to specific DNA sequences, known as hormone response elements (HREs), through the DBD and assembles with the coregulator proteins. Figure 4 represents NRs binding in the intron and exon conserved sequences of *VDR*, *AR*, and *RXRA*. The HREs correspond to the NR binding sequences in introns 1 and 3 and exons 4, 6, 7, and 9 of *VDR*, *AR*, and *RXRA*. The binding of the NRs to the HREs in *VDR*, *AR*, and *RXRA* occurs in clusters, but the NRs composing the clusters differ between introns and exons (Figure 6).

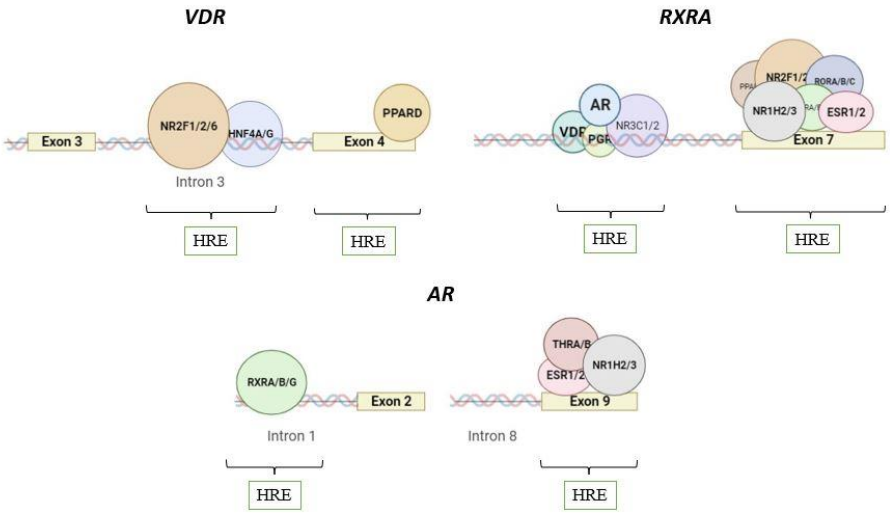

Figure 6. Schematic representation of NR binding in the intron and exon conserved sequences of *VDR*, *AR*, and *RXRA*.

These results indicate a crosstalk between the androgen endocrine system and the VDR, ESR, progesterone receptor (PGR), and the retinoic acid receptors.

There is also an indication of a crosstalk between the retinoic acid endocrine system and ESR and the thyroid hormone receptor (THR), among others (Paper III, Table 2 and 3). These findings follow the same line as previous studies on the crosstalk between steroid receptors of breast and prostate cancer cells [15, 181]. It has been reported that the interaction between VDR and RXR causes antitumoral effects in prostate cancer [196, 197]. Moreover, androgens can either have an antagonistic or a combined effect on the binding of ESR to estradiol, depending on the presence of dihydrotestosterone [198-200]. Thus, the present study and previous studies support the hypothesis that NRs work in crosstalk rather than in isolation, allowing the regulation of transcriptional responses.

Interestingly, some of the TFBDs for the intron conserved sequences of five NR genes were p53 transcription factor domains: the nuclear receptor subfamily 2 group F (NR2F); the estrogen receptor 1 (ESR1); the nuclear receptor subfamily 4 Group A member 3 (NR4A3); the AR; and the nuclear receptor subfamily 1 group D member 1 (NR1D1). A previous investigation that mapped the p53 binding sites in the whole genome, did not detect p53 specific TFBS for NR intronic sequences [201]. In an *in vitro* study, Wei et al. only detected transcriptional regions highly enriched with p53-binding sites [201]. Our study, which was performed *in silico* without transcriptional rates, identified p53 TFBS in five NR genes. Thus, the current finding provides deeper insight into p53 binding locations and indicates that p53 regulates NR-mediated transcription.

6.4. Vitamin D receptors and prostate cancer stage

The vitamin D endocrine system and its receptors–the vitamin D receptor (VDR) and the protein disulfide-isomerase A3 (PDIA3)–are associated with carcinogenesis as well as anti-tumoral effects in several cancer forms, including prostate cancer [128, 130, 202, 203].

Evidence shows that the vitamin D metabolite 1,25(OH)D (calcitriol) interacts with the VDR to decrease proliferation and increase apoptosis of cancer cells [19, 130]. High expression of VDR in prostate tumors has been linked to a reduced risk of lethal cancer [130]. However, in both normal and cancerous

prostate tissues, VDR is lower expressed at the transcription and translation level than PDIA3 [129]. The role of PDIA3 in prostate cancer remains controversial [21, 126-128], and PDIA3 transcript isoforms have not been studied in connection with the progression of prostate cancer. Thus, Paper IV aimed to evaluate mRNA expression of the vitamin D receptors VDR and PDIA3 in cell lines with different stages of prostate cancer. We also aimed to evaluate the vitamin D receptors and their isoforms as potential markers for the clinical diagnosis of prostate cancer.

Five prostate cell lines–PNT2, P4E6, DU145, PC3, and LNCaP–were analyzed using NGS. A novel transcript isoform of PDIA3 (PDIA3N) was identified in these five prostate cell lines. This finding aligns with previous studies where PDIA3N was associated with cancer progression in kidney and colon cells [204, 205]. The novel PDIA3N had higher RNA expression compared to PDIA3 in prostate cells (8.5–14.5 and 44.6–69.3 FPKM, respectively).

The mRNA expression of PDIA3 and PDIA3N was further evaluated by ddPCR in the same cell lines. Among AR-positive cell lines, LNCaP cells showed an exceptionally higher mRNA expression of PDIA3N compared to the expression of PDIA3, whereas PNT2 showed a similar trend (Figure 7). The AR-negative cell lines P4E6, DU145, and PC3 showed a lower or an equally high mRNA expression level of PDIA3N compared to PDIA3 (Figure 7). Expression of PDIA3N in the metastatic cells (LNCaP, DU145, and PC3) was higher than in normal cells (PNT2) and in early cancer (P4E6), but the difference was higher when comparing LNCaP to PNT2 (Figure 7).

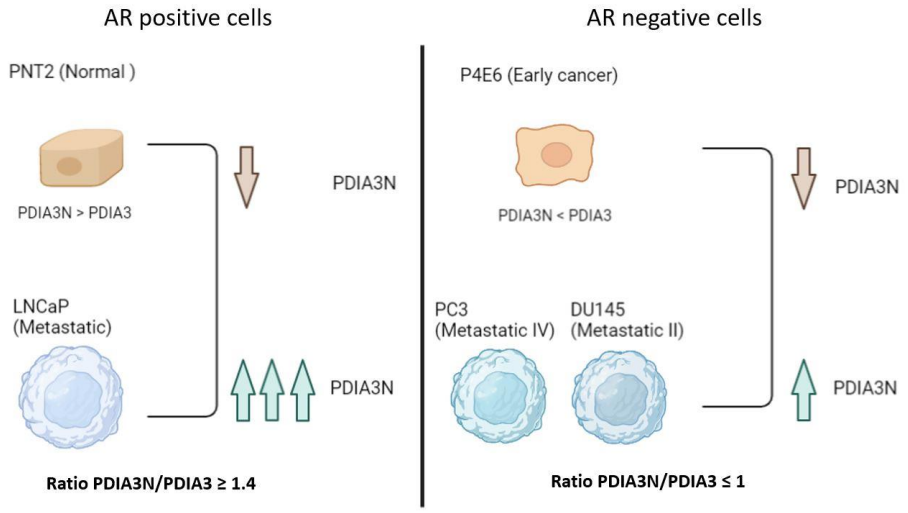

Figure 7. Expression of PDIA3N and PDIA3 in AR-positive prostate cancer cell lines (PNT2 and LNCaP) and AR-negative prostate cancer cell lines (P4E6, DU45, and PC3). The arrows show differences in expression between the LNCaP and PNT2 and between DU145 and PC3 with P4E6.

There was also a correlation between the ratio of PDIA3N/PDIA3 and the cancer stage of prostate cells (Figure 7). AR-positive cell lines showed a higher mRNA expression ratio between PDIA3N and PDIA3, unlike AR-negative cell lines that showed a lower mRNA expression ratio between PDIA3N and PDIA3 (Figure 7).

The analysis of the predicted protein structure of PDIA3 and PDIA3N shows that the first fragment of the N-terminus in PDIA3N differs with 56 amino acids from PDIA3 (Paper IV, Figure 4). Of the 56 altered amino acids in the N-terminus of PDIA3N, 22 were found to alter the structure and function of the protein. At the secondary structure level, PDIA3N missed an α-helix and contains a truncated thioredoxin site in the N-terminus (Paper IV, Figure 4).

The predicted protein for PDIA3N is shorter and shows a different N-terminus sequence compared to PDIA3; this is due to an alternative 5′-proximal translation initiation site (TIS). The ribosomes can recognize several TIS in the sequence at the same time. This mechanism, leaky scanning, is responsible for translating N-truncated proteins containing secretory signals that address

the proteins to different cell compartments [206, 207]. The TIS in PDIA3N leads to an altered protein sequence in the N-terminus, which leads to a change of location to the cytosol for the PDIA3N compared to the typical location for PDIA3, which is in the ER (Figure 8).

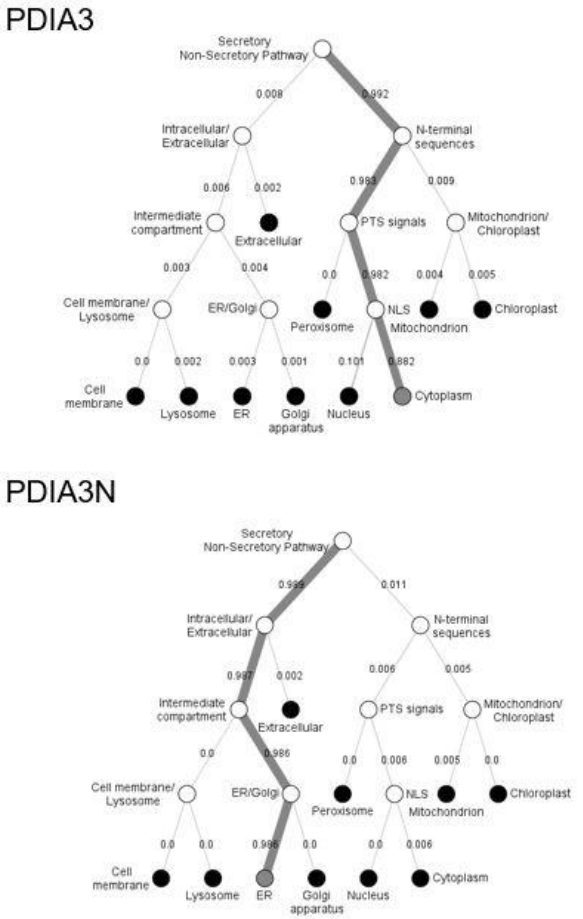

Figure 8. Predicted subcellular localization of PDIA3 and PDIA3 with DeepLoc-1.0. The grey line represents the pathway with the highest probability.

The results of this study also showed that the expression of VDR is low in prostate cells (0–7.4 FPKMs). An imbalance in the ratio between VDR and PDIA3 (including PDIA3 isoforms) might also contribute to the progression of prostate cancer. VDR has a higher affinity to 1,25(OH)D compared to PDIA3 and PDIA3N (Paper IV). The structural change, where one α-helix is lacking in PDIA3N and is truncated in one active thioredoxin site, indicates a defective thioredoxin activity and might be responsible of a lower binding affinity to 1,25(OH)D. Despite this, 1,25(OH)D may preferentially bind to VDR in the normal prostate cells PNT2 (Figure 9A). However, the larger pool of PDIA3N in the metastatic prostate cancer cells LNCaP will bind a considerable part of available 1,25(OH)D (Figure 9B). Therefore, lower levels of VDR and the decreased availability of 1,25(OH)D to VDR may influence the antitumorigenic effects of 1,25(OH)D in prostate cancer cells (Figure 9B).

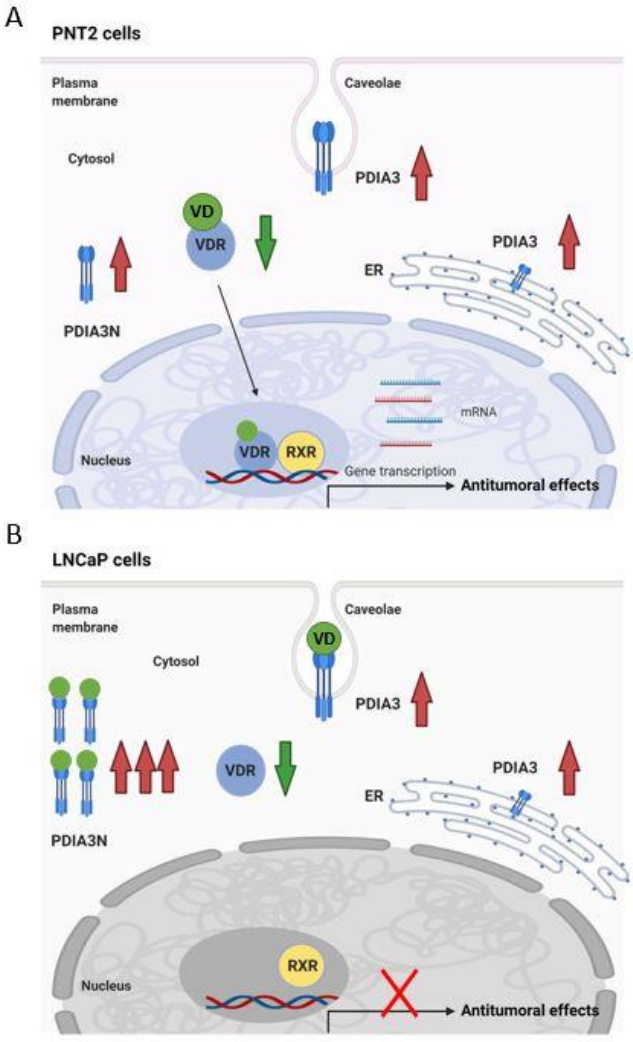

Figure 9. Schematic overview of the vitamin D signaling pathway in prostate cells. A. Signaling pathway in normal prostate cells, PNT2. B. Signaling pathway in metastatic cells, LNCaP. Red arrows represent an increase in expression and green arrows decrease expression. VD: vitamin D (1,25(OH)D).

Several studies associated changes in the expression of PDIA3 with multiple pathologies, including cancer and neurodegenerative disease [208-210]. Aberrant expression of PDIA3 is correlated with poor cancer prognosis and increased cell proliferation mediated by 1,25(OH)D. So far, only one study has evaluated the expression of PDIA3 in prostate cancer tissue. In this study, the tumor samples with a higher Gleason score (GS 8–10) had higher expression of PDIA3 compared to GS 6 tumors [126]. However, a difference in PDIA3 expression between GS 8–10 and benign samples was not observed [126]. This may be explained by the differences in the androgen dependency stage, which also influenced the PDIA3 expression in our experiments. Our result and previous observations point out that PDIA3 is a marker for prostate cancer progression.

The role that PDIA3N could have in tumor samples is still unknown, but it may participate in pathological processes due to its abundant expression, compared to PDIA3, in the metastatic androgen-dependent stage (AR-positive) and as a result of its predicted aberrant structure and function.

Hence, this study demonstrates that PDIA3 isoforms play contrary roles in prostate cancer progression. The shift in expression between these isoforms to a higher abundance of PDIA3N might be associated with a more severe stage of the disease.

7. Conclusions

The overarching contribution of this thesis is that the study of the steroid hormone receptors, mainly vitamin D receptors, needs to be understood to comprehend healthy aging and the etiology and progression of prostate cancer.

The specific conclusions from each study are listed below:

Paper I: Vitamin D supplementation, dietary intake, or sunlight exposure in the healthy elderly did not affect the global DNA methylation or the methylation of the vitamin D receptors and metabolic enzymes. The combination of high levels of physical activity and vitamin D supplementation in the healthy elderly showed a synergistic effect on methylation of the transcription, VDR, Wnt-signaling, and cancer pathways.

Paper II: SNPs of the vitamin D receptors and metabolic enzymes displayed different allele frequencies when comparing our healthy elderly cohort with two other populations at different latitudes (Northern Sweden and Southern Europe). We also identified two SNPs in the PDIA3 locus, rs2788 and rs12441861, that correlated with vitamin D supplementation.

Paper III: The receptors VDR, AR, and RXRA involved in prostate cancer etiology and progression have several HREs in the intron sequences for other SHRs, which is evidence for a crosstalk between SHRs and contributes to novel targets for the regulation of steroid hormones.

Paper IV: PDIA3N, a novel transcript isoform of PDIA3, was detected in prostate cells. PDIA3N has higher transcription expression levels than PDIA3 in prostate cells and exceptionally high levels in the metastatic prostate cancer cells. The ratio between PDIA3N and PDIA3 was related to the androgen dependency and the stage of

prostate cancer. The different structure of PDIA3N suggests an altered function of the protein: a different cell location, thioredoxin activity, and affinity for calcitriol binding.

This thesis has contributed with suggestions of new epigenetic markers associated with a healthy lifestyle combined with high physical activity and vitamin D intake in healthy elderly adults. These epigenetic markers are involved in regulation of several cancer pathways. The isolated effects of vitamin D supplementation and physical activity on methylation patterns have been evaluated previously, but this is the first report on their combined effects. Thus, these biomarkers deserve to be further investigated to elucidate the synergistic effects of regular physical activity and vitamin D supplementation on DNA methylation and regulating cancer pathways.

Crosstalk above the genomic level has been found between steroid hormones and their steroid hormone receptor proteins unlike in the intron sequences where it seems to take place between the steroid hormone receptors in order to regulate their transcription. Hopefully, these results will help clarify the modulation of transcriptional responses in SHRs and provide novel target sequences to investigate the development of steroid hormone-dependent cancer.

Finally, we revealed that genetic and transcriptional markers are associated with the putative vitamin D receptor PDIA3. The genetic markers were specific SNPs alleles detected in the healthy elderly population under vitamin D supplementation. These alleles could be risk alleles indicating that PDIA3 polymorphisms might be involved in impaired vitamin D signaling. These results indicate that SNPs may be used to identify risk individuals who may require higher doses of vitamin D supplementation based on the genetic setup.

The transcriptional markers PDIA3 and PDIA3N were associated with a particular cancer stage in the prostate cancer cells and are potential candidates for the clinical diagnosis of prostate cancer. Overall, these results suggest PDIA3 as a molecular link between health, vitamin D, and prostate cancer, a finding that will be of great interest for future studies.

8. Future perspectives

In the first study, a retrospective observational cohort, we evaluated the influence of lifestyle and vitamin D intake on global DNA methylation patterns in an elderly cohort from Southwest Sweden. The results from this study are in line with previous studies, where vitamin D supplementation, dietary intake, or sunlight exposure did not affect DNA methylation. Future studies should be interventional prospective–i.e., the dose of vitamin D supplements and serum levels of 25(OH) vitamin D3 can be controlled and monitored. Another thing to bear in mind is the number of participants in each group, especially for the group containing individuals taking vitamin D supplements and exerting high levels of physical activity, which was relatively small, compared to those not taking vitamin D supplements and exerting low levels of physical activity. The sample collection for DNA extraction was done in saliva, which reflects the global methylation patterns. A local collection (e.g., skeletal muscle biopsy) would have given another dimension to the study and the possibility to evaluate local DNA methylation elicited by exercise and affected by vitamin D levels. In a future study, further investigation can be done regarding different age groups, an approach that would provide a more thorough investigation of the association between aging and the methylation of *RXRA*.

In the second study, a retrospective observational cohort, we characterized known genetic polymorphisms of vitamin D receptors and related genes. In conclusion, two SNPs in the PDIA3 locus, rs2788 and rs12441861, were correlated with vitamin D supplementation. As in the first study, we could not control the participants' dose of vitamin D supplements. To clarify the role of the *PDIA3* polymorphisms on healthy aging, it would be necessary to compare the serum levels of 25(OH)D3 in the group with the rs2788 (A/G) and rs12441861 (C/T) genotypes and taking vitamin D supplements with the group not taking vitamin D supplements and with the normal genotypes (A/A and C/C respectively). Another track to take could be to investigate vitamin D receptor and metabolic enzymes SNPs in available databases of prostate cancer and compare this with the ones in our healthy cohort. This approach would provide some knowledge of the association of specific SNPs involved in the vitamin D pathway with prostate cancer disease. In addition, this

analysis would provide more results in line with the overall aim of evaluating the vitamin D receptors in both health and disease.

In the third study, an *in silico* study, we found that *VDR*, *AR*, and *RXRA* have several HREs in the exon and the intron sequences for other SHRs. These results provide more evidence for crosstalk between steroid hormone receptors and contributes with novel targets for steroid hormone regulation that might be relevant to understanding the modulation of transcriptional responses and explaining the development of certain cancers. There are several *in vitro* and *in vivo* experimental approaches for future studies in this area. One future strategy to assess that the HREs discovered here truly are transcription factor binding sequences for SHRs could be chromatin immunoprecipitation (ChIP) assay, where an antibody specific for our SHRs is used to immunoprecipitate the DNA-protein complex. This could be done in both normal and prostate cancer samples to compare differences in the crosstalk of the SHRs.

In the fourth study, an *in vitro* study, we assessed the expression of the vitamin D receptors, VDR and PDIA3, in cell lines representing different stages of prostate cancer. We also identified the novel PDIA3N transcript isoform associated with the androgen and cancer stage of prostate cancer cells. To further confirm our previous hypotheses regarding the role of PDIA3N in vitamin D effects and signaling, we would have evaluated the expression of PDIA3 and PDIA3N and antitumoral elicited effects (cell proliferation, invasion, differentiation) at basal levels and in response to different doses of calcitriol. These experiments could be performed with different prostate cancer cells but the same prostate cancer stages to validate the association of PDIA3 and PDIA3N with the prostate cancer progression.

To verify these markers for diagnosis and prognosis of prostate cancer the expression of PDIA3 and PDIA3N needs to be assessed in a second stage in patient samples with different Gleason scores and androgen dependency stage of prostate cancer. This investigation and more extensively verified studies are crucial to determine whether PDIA3N is a suitable biomarker for advanced prostate cancer as well as the role of PDIA3, PDIA3N, and the vitamin D endocrine system in regulating prostate cell biology.

9. Sammanfattning

9.1. Bakgrund

En individs hälsotillstånd påverkas av individens genetisk bakgrunden tillsammans med miljöfaktorer och livsstil. Den genetiska bakgrunden är nedärvd och irreversibel om inte nya mutationer eller andra genetiska förändringar inträffar. Till exempel kan genetiska polymorfier påverka åldrandeprocessen och är även kopplade till uppkomst av kroniska sjukdomar. Livsstilsvanor, till exempel kost, tillskott av vitaminer, beteende, stress, fysisk aktivitet, rökning och alkoholkonsumtion, är alla variabler som också bidrar till individens utveckling av hälsa eller sjukdom genom att inaktivera eller aktivera uttrycket av specifika gener genom förändringar i metyleringen av DNA. Det finns dessutom studier som visar att D-vitamintillskott och D-vitaminnivåer i blodet påverkar den globala DNA-metyleringen.

Vitamin-D tillhör en grupp av fettlösliga steroider som fungerar som hormoner i kroppen. Dessa steroider kan tas upp via kosten, kosttillskott eller genom syntes i huden från 7-dehydrokolesterol genom att huden exponeras för solljus (UV-B strålar). Historiskt sett är D-vitamin känt för att bota rakitis eller bendeformiteter eftersom det kan öka kalcium-reabsorptionen och därmed benmineraliseringen. D-vitaminbrist kan uppstå på grund av en minskning av en eller flera av följande faktorer; kostintag, syntes i huden, absorption av D-vitamin eller exponering för solljus. D-vitaminbrist har blivit ett globalt folkhälsoproblem då omkring en miljard människor världen över har D-vitaminbrist och 50 % av befolkningen har D-vitamininsufficiens. Förekomst av D-vitaminbrist är vanlig hos högpigmenterade individer, överviktiga och patienter men är även utbrett hos äldre. I Europa är D-vitaminbrist så hög som 80% hos institutionaliserade äldre men endast 2–30% hos vuxna individer. D-vitaminbrist hos äldre kan leda till flera hälsoproblem, såsom kardiovaskulära och autoimmuna sjukdomar, osteoporos, depression och olika cancersjukdomar som bröst-, bukspottkörtel- och prostatacancer.

Prostatacancer kännetecknas av okontrollerad tillväxt av celler i prostatakörteln, vilken är lokaliserad under urinblåsan i det manliga reproduktionssystemet. Prostatacancer är den högst frekvent diagnostiserade

sjukdomen och den näst vanligaste dödsorsaken bland män i USA och Europa. Riskdeterminanter är miljöfaktorer där kosten har en stor betydelse. Dessutom finns nya bevis för att utveckling av prostatacancer också beror på genetiska faktorer. Humana prostatakarcinom är känsliga för androgener, och hormonbehandling har visats ge en tillfällig remission, följt av ett återfall till en androgenokänslig fas. Detta indikerar att steroidhormoner, särskilt androgener, spelar en betydande roll i human prostatakarcinogenes. Vitamin D har flera antitumöreffekter, såsom minskad celldelning hos prostatacancerceller både *in vitro* och *in vivo*, oberoende av androgenstadiet. Effekterna av vitamin D som steroidhormon på molekylär nivå är dock inte helt fastställda och inte heller vilken av steroidhormonreceptorerna som förmedlar dess effekt.

Steroidhormonreceptorer (SHR) är intracellulära (cytoplasmatiska eller nukleära) och initierar cellsignaleringskaskader för steroidhormoner, som slutligen leder till förändringar i genuttryck. Androgenreceptorn (AR) är ansvarig för att upprätthålla normala förhållanden i epitelet hos prostatan. Ett överuttryck av AR stimulerar dock cancercellsdelningen hos prostatacancerceller hos patienter med prostatacancer, även om androgener inte är närvarande. I likhet med alla steroidhormonreceptorer kan AR translokera in i cellkärnan för att reglera transkriptionen av specifika gener. Vitamin Ds nukleära receptor (VDR) är den huvudsakliga receptorn som binder D-vitamin. När D-vitamin kommer in i cellen, binder den till VDR i cytoplasman och sekundärt även till retinoid X receptor-alfa (RXRA). Komplexet translokeras in i cellkärnan, där det binder specifika DNA-sekvenser och aktiverar de genomiska effekterna av D-vitamin, vilket föranleder antitumör-effekterna. En annan SHR är disulfid-isomeras familj A medlem 3 (PDIA3). PDIA3 är den förmodade receptorn för D-vitamin i det endoplasmatiska retiklet som sporadisk även kan binda D-vitamin i plasmamembranet. Bindningen aktiverar snabba svar via proteinkinasvägar och kalciumfrisättning i cellen.

Rollen för AR i frisk prostatavävnad samt vid uppkomst och progression av prostatacancer är tydlig, däremot inte rollen för vitamin D-receptorerna VDR och PDIA3. Ökad kunskap om effekterna av dessa receptorer kan gynna den äldre befolkningen, som vanligtvis är i riskzonen för D-vitaminbrist och till detta kopplade kroniska sjukdomar som prostatacancer.

9.2. Syfte

Det övergripande syftet med detta forskningsprojekt var att öka kunskapen om steroidhormonreceptorer, i första hand D-vitaminreceptorer, inom både hälsa och sjukdom, med fokus på genomiska, epigenomiska och transkriptomiska perspektiv hos friska äldre individer och i prostatacancerceller.

De specifika syftena med studierna var:

- Att undersöka påverkan av livsstil och D-vitamin-intag på globala metyleringsnivåer samt metyleringsnivåer av vitamin D-receptorer och vitamin D-associerade metabola enzymer hos individer i en frisk äldre kohort (Studie I).

- Att karakterisera single nucleotide polymorphisms, SNPs (enbaspolymorfier) i vitamin D-receptorerna och associerade metabola enzymer hos individer i en frisk äldre kohort och utforska potentiella samband mellan specifika alleler med intag av vitamin D-tillskott och hälsotillstånd (Studie II).

- Att undersöka samverkan mellan steroidhormonreceptorer, med fokus på receptorer involverade i etiologi och progression av prostatacancer (Studie III).

- Att utvärdera steroidhormonreceptorer involverade i vitamin D-signalering och deras isoformer som kandidater till biomarkörer för klinisk diagnos av prostatacancer (Studie IV).

9.3. Material och metod

Studie I och II ingår i en större tvärsnittsstudie som tidigare beskrivits av Gillsjö et al. Kohorten bestod av 530 individer i Sydvästra Sverige med en ålder från 70 till 95 år. Alla deltagare svarade på en undersökning relaterad till allmänna attribut så som; intag av hjärtmediciner, fysisk och mental hälsa,

tillskott av vitaminer, livsstilsvanor och social status. Salivprover samlades in från 520 deltagare i kohorten och DNA extraherades från saliven för SNP- och metyleringsanalyser.

I den första studien (Studie I) analyserades 277 prover för att undersöka korrelationer av metyleringsnivåer med livsstilsfaktorer såsom tillskott av vitaminer och en rad andra faktorer i undersökningen. Metyleringsmätningar gjordes med hjälp av metylerings-arrayer. Totalt valdes 2113 metyleringsplatser ut i 80 vitamin D-relaterade gener. Globalt metyleringsmönster och metylering av vitamin D-relaterade gener analyserades med bioinformatiska programmeringsverktyg i R, där funktionella annoteringar och associerade reglerade cellulära vägar analyserades.

I Studie II analyserades 520 prover för potentiella korrelationer mellan SNPs med hälsotillstånd, intag av hjärtmediciner samt tillskott av vitaminer. Genomiskt DNA genotypades genom användning av en SNP-arrayer. Totalt 158 SNPs i 9 vitamin D-relaterade gener analyserades vidare med bioinformatiska och statistiska metoder för integrering av genomisk data i R.

Studien av uppkomst och progression av prostatacancer (Studie III och IV) bestod av en *in silico* studie med genomiska sekvenser extraherade från en referensdatabas (Studie III) och en *in vitro* studie utförd i cellinjer som representerade olika stadier av cancer och androgenberoende hos prostatacancer celler (Studie IV).

Studie III utvärderade konserverade intron- och exon-sekvenser bland receptorer involverade i uppkomst och progression av prostatacancer (AR, VDR och RXRA). Vi studerade deras relevans som potentiella transkriptionsreglerande och bindande sekvenser för andra SHR. De konserverade sekvenserna extraherades med en nyutvecklad metod och regulatoriska sekvenser analyserades med olika allmänt tillgängliga program och bioinformatiska verktyg.

Studie IV utvärderade mRNA-uttrycket av VDR och PDIA3 i PNT2-, P4E6-, LNCaP-, DU145- och PC3-cellinjer med hjälp av Next Generation Sekvensering (NGS) och som verifierades med Droplet digital TM PCR

(ddPCR). Dessutom utfördes strukturella och funktionella analyser av dessa receptorers predikterade proteinsekvens med olika bioinformatiska verktyg.

9.4. Resultat och Diskussion

Denna avhandling består av fyra vetenskapliga artiklar, där två är publicerades i vetenskapliga tidskrifter (Studie III och IV), en är inskickad till en tidskrift, och en är för närvarande i manuskriptform (Studie I och II).

Resultaten i den första studien (Studie I) visar på nya potentiella epigenetiska markörer kopplade till en hälsosam livsstil där hög fysisk aktivitet kombineras med intag av D-vitamin hos vuxna friska äldre. Dessa markörer är involverade i reglering av flera cellulära signalvägar för cancer. De enskilda effekterna av D-vitamintillskott och fysisk aktivitet avseende metyleringsmönster har utvärderats i tidigare studier, och detta är den första studien som visar deras kombinerade effekter. Således skulle dessa biomarkörer behöva undersökas ytterligare för att verifiera och förklara de synergistiska effekterna som regelbunden fysisk aktivitet och D-vitamintillskott kan ha på DNA-metylering och reglering av cancer-relaterade signalvägar.

I den andra studien (Studie II) kunde vi identifiera olika SNPs i D-vitamin receptorerna och i D-vitamin associerade metabola enzymer som visar olika allel-frekvenser vid jämförelse av vår friska äldre kohort med två andra populationer på olika breddgrader (Norra Sverige och Sydeuropa). Att studera SNPs i D-vitamin receptorer och enzymer i populationer som lever på olika breddgrader kan ge oss möjlighet till bättre förståelse av de olika D-vitaminnivåerna på olika breddgrader. Dessutom identifierade vi två SNPs i PDIA3, rs2788 och rs12441861, som korrelerade med D-vitamintillskott. Detta är den första studien som antyder att specifika SNP-alleler i PDIA3-receptorn är kopplade med D-vitamintillskott. Dessa alleler kan vara risk-alleler som tyder på att PDIA3-polymorfier kan vara involverade i en försämrad D-vitaminsignalering. Framtida studier skulle kunna utvärdera om dessa risk-alleler har potentiella hälsoeffekter och därmed stödja en rekommendation om D-vitamintillskott till dessa individer, särskilt till äldre med en hög förekomst av D-vitaminbrist.

Genanalyser av de tre receptorerna som är involverade i prostatacancer, dvs *VDR*, *AR* och *RXRA*, visade förmodade bindningssekvenser för andra SHR i introner och exoner (t.ex. ESR, AR, PGR, RXRA, RXRB och RXRG (Studie III). Interaktion ovan den genomiska nivån har hittats mellan steroidhormoner och deras steroidhormon-receptor-proteiner, men inte intron-sekvenserna, där det verkar ske mellan steroidhormonreceptorer för att styra deras transkription. Förhoppningsvis kommer dessa resultat att bidra till att klargöra moduleringen av transkriptionssvar i SHR. Dessutom finns möjlighet att detta kan bidra till nya målsekvenser för att undersöka utvecklingen av cancer som är steroidhormon-beroende.

I Studie IV undersöktes uttrycket av en ny isoform av PDIA3 (PDIA3N) som kopplas till androgen- och cancerstadium i prostatacancercellinjer. Proteinstrukturen för PDIA3N skiljer sig från PDIA3, vilket tyder på en förändrad funktion hos proteinet. Detta motsvarar en annan lokalisering i cellen, tioredoxin-aktivitet och förändrat affinitet för bindning till kalcitriol. Dessa resultat pekar på att PDIA3, och särskilt PDIA3N, kan vara nya kandidatmarkörer för klinisk diagnos av prostatacancer. Framtida studier bör utvärdera uttrycket av PDIA3 och PDIA3N i kliniska prover, korrelerat till Gleason-skalan och det androgen-beroende stadiet i prostatacancer.

9.5. Slutsats

Sammantaget stödjer dessa fynd relevansen av att studera D-vitamin och steroidhormon-receptorer, särskilt PDIA3-receptorn, för att förstå en del av faktorerna relaterade till hälsosamt åldrande och uppkomst samt utvecklingen av prostatacancer.

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Exploring vitamin D and steroid hormone receptors

– from healthy elderly to prostate cancer cells

The genetic background and lifestyle habits significantly influence an individual's health status and the probability of acquiring cancer. For instance, vitamin D supplementation and vitamin D levels in the blood can contribute to the individual's development of health or disease by repressing or activating specific genes through changes in the methylation of DNA.

Vitamin D is a steroid hormone that positively affects several forms of cancer, including prostate cancer. However, the molecular effect of vitamin D as a steroid hormone and which steroid hormone receptor (SHR) mediates this effect are not fully understood.

The overall aim of this research project was to increase our knowledge about SHRs, primarily the vitamin D receptors, in both health and disease, focusing on genomic, epigenomic, and transcriptomic perspectives in healthy elderly individuals and prostate cancer cells.

The results presented in this research project could help us understand the importance of a healthy lifestyle that includes vitamin D for health. In this sense, we found specific methylation markers involved in the downregulation of cancer pathways that are associated with high physical activity and vitamin D supplementation. We have further clarified the modulation of transcriptional responses in SHRs related to prostate cancer progression and etiology. Moreover, we suggested genetic and transcriptional markers associated with the putative vitamin D receptor PDIA3, which are interesting for future studies assessing vitamin D levels and prostate cancer progression.

Altogether, these findings may ultimately be helpful to suggest potential implications for the individual connected to healthy aging and thus improve the understanding and motivations for making healthy lifestyle choices. Last, but not least, connecting these findings on the individual and environmental level to the function of vitamin D and its receptors on a molecular level.

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