Vitamin D, PTH, and Calcium in relation to Prostate Cancer

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DOCTORAL DISSERTATION

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To be defended at lecture theatre Lilla Aulan at Jan Waldenströms gata 5, Skåne University Hospital, Malmö on Friday 6th of May 2016 at 09.00 am

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| Abstract  Prostate cancer is one of the most common malignancies. There are only a few established risk factors and none of them are modifiable. In 1990 it was hypothesised that vitamin D deficiency was correlated with prostate cancer. Since then it has been shown that vitamin D indeed has anti carcinogenic effects but the epidemiologic results have been conflicting. Vitamin D is closely related to PTH and calcium, which both have been implicated in prostate cancer.  The aim of this thesis was to study these metabolites in relation to prostate cancer, more specifically risk, aggressiveness and mortality. Further we sought to investigate the association between SNPs associated with vitamin D, PTH, and calcium in relation to prostate cancer. We conducted a prospective investigation within the Malmö Diet and Cancer Study. Papers I and II and IV used a nested case control setting with 943 cases and 943 controls. In Paper III we only studied the cases.  We found a statistically significant increased risk of prostate cancer with high vitamin D levels (paper I), but no difference according to the aggressiveness of the tumour. There was however an increased risk of intermediate grade tumours with high vitamin D levels (paper II). We also found that sufficient levels of pre-diagnostic vitamin D may improve survival in prostate cancer. Several SNPs previously associated with vitamin D were found to change the risk for prostate cancer and this risk was affected by the level of vitamin D. | | |
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To Jenny, Noel, Nils, and Sixten

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# List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

1. Brandstedt J, Almquist M, Manjer J, Malm J: Vitamin D, PTH, and calcium and the risk of prostate cancer: a prospective nested case-control study. *Cancer Causes Control 2012*: 23 (8): 1377-1385.
2. Brandstedt J, Almquist M, Ulmert D, Manjer J, Malm J: Vitamin D, PTH, and calcium and tumor aggressiveness in prostate cancer: a prospective nested case-control study. *Cancer Causes Control 2016*: 27 (1): 69-80.
3. Brandstedt J, Almquist M, Manjer J, Malm J. Vitamin D, PTH, and calcium in relation to survival following prostate cancer. *Cancer Causes Control 2016*:*00 (0):00-00 (Epub ahead of print)*
4. Brandstedt J, Almquist M, Manjer J, Malm J. SNPs associated with vitamin D, PTH, and calcium in relation to prostate cancer risk: overall association and risk in different strata of vitamin D levels. *Manuscript*

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# Abbreviations

ASAP Atypical Small Acinar Proliferation

BMI Body Mass Index

BRCA1 Breast cancer gene 1

BRCA2 Breast cancer gene 2

BPH Benign Prostatic Hyperplasia

CaSR Calcium Sensing Receptor

CI Confidence Interval

CRPC Castrations Resistant Prostate Cancer

CT Computed Tomography

CV Coefficient of Variation

DRE Digital Rectal Examination

DHT Di Hydroxy Testosterone

GnRH Gonadotropin Releasing Hormone

HPLC High-Pressure Liquid Chromatography

HR Hazard Ratio

HT Hormonal Therapy

LUTS Lower Urinary Symptoms

MDCS Malmö Diet and Cancer Study

MRI Magnetic Resonance Imaging

OR Odds Ratio

PET Positron Emission Tomography

PIA Proliferative Inflammatory Atrophy

PIN Prostatic Intraepithelial Neoplasia

PSA Prostate Specific Antigen

PTH Parathyroid Hormone

PTHrP Parathyroid Hormone related Peptide

RDA Recommended Daily Allowance

RT Radiation Therapy

SNP Single Nucleotide Polymorphism

TNM Tumour Node Metastasis

TRUS Trans Rectal Ultrasonography

VDR Vitamin D Receptor

WHO World Health Organisation

# Introduction

Prostate cancer is the most common cancer in Swedish males with 11000 new cases in 2014 [1]. Increasing age, family history and ethnicity are the only established risk factors, and none of these are modifiable. Three decades ago ecological studies reported lower prostate cancer mortality in southern latitudes in the U.S. It was hypothesised that vitamin D, which is mainly obtained from sunlight exposure, may protect against prostate cancer [2]. Since then it has been shown that vitamin D has a supressing effect on carcinogenesis and development of prostate cancer, as well as other cancers, by reducing cell proliferation, invasiveness, and angiogenesis as well as inducing apoptosis and cell differentiation [3,4]. Hence, there is support for a causal relation between low levels of vitamin D and risk of prostate cancer.

This has however been hard to demonstrate in the numerous epidemiological studies that have been conducted, indicating that the association is not that clear and straight forward. Vitamin D is closely related to calcium and parathyroid hormone (PTH) levels, which both by themselves have been linked to cancer outcomes [5,6]. There is also evidence that single nucleotide polymorphisms (SNPs) may affect the risk for prostate cancer, possibly modified by serum levels of vitamin D.

The aim of the work presented in this thesis was to investigate the relationship between Vitamin D, PTH, and calcium in relation to the subsequent risk of prostate cancer, prostate cancer mortality and associations with prostate cancer aggressiveness. We further sought to investigate the relationship between SNPs related to vitamin D, PTH, and calcium and their association with prostate cancer risk.

# Background

## The prostate gland

The normal human adult prostate is walnut shaped with a weight of about 20g. It is located beneath the bladder and in front of the rectum and surrounds the beginning of the urethra. The main function is to store and secrete an alkaline fluid that nourishes and protects sperm. The prostate is divided into different zones with different histology: central zone, peripheral zone, transition zone and anterior zone (Figure 1). The transition zone is the site of benign prostatic hyperplasia, which can cause a tightening of the urethra and voiding problems in ageing men. Approximately 70% of prostate cancer comes from the peripheral zone [7], which is next to the rectum. This enables the palpation of tumours during a digital rectal examination (DRE).

The male sex hormone, testosterone, is converted to dihydroxytestosterone (DHT), a five times more potent androgen, in the prostate.

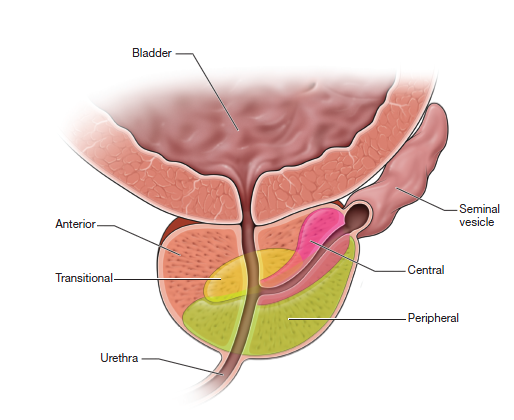


Figure 1. An illustration of different zones of the prostate gland. Reprinted with permission from *The Whole Life Prostate Book.*

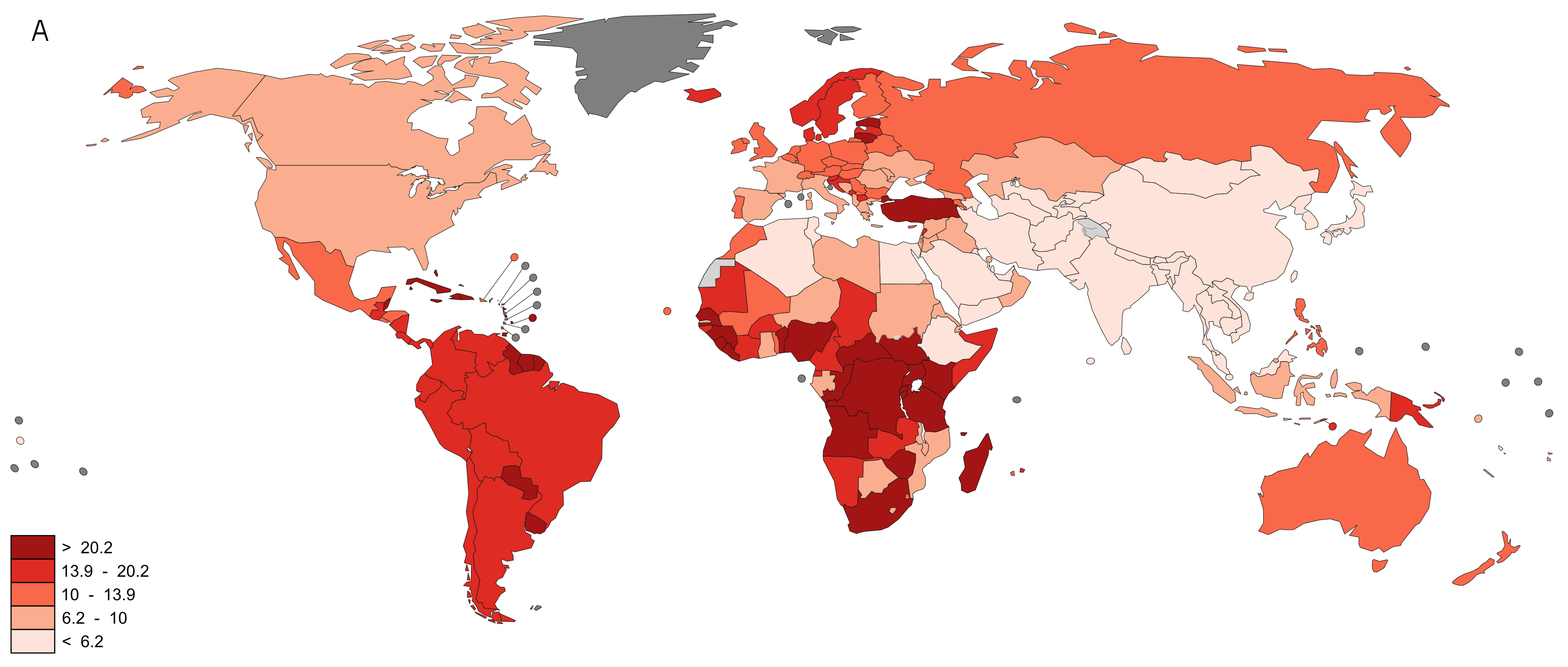
## Prostate cancer

Prostate cancer is the second most common cancer in men worldwide [8] and the most common cancer form in Sweden with 11000 new cases in 2014. The cumulative risk to be diagnosed with prostate cancer is 11.8% by the age of 75 years, but the lifetime risk of dying from prostate cancer is only about 3-5%, which means that most men with prostate cancer will die from other causes [9,10]. The incidence varies greatly between different parts of the world. Afro Americans have the highest incidence followed by white Americans and Scandinavian men, whereas prostate cancer is unusual in Asia. The variation in incidence can in part be explained by the different use of prostate specific antigen (PSA) screening [11], environmental differences but also by genetic susceptibility (Figure 2).

### Risk factors

There are not many established risk factors for prostate cancer besides age, ethnicity and family history. Prostate cancer is rarely seen in men aged below 40, but as age increases, the risk of developing prostate cancer also increases. The modern western lifestyle, with greater consumption of animal fats, excess body weight, and physical inactivity are also understood to be risk factors [12,13]. Smoking increases the risk of aggressive prostate cancer, relapse after curative treatment and death from prostate cancer [14-16]. Regarding alcohol there is limited evidence [17].

A family history of prostate cancer is also a risk factor, and men with a first degree relative diagnosed with prostate cancer have twice the risk of developing prostate cancer compared to men with no prostate cancer in their family [18]. A mutation in BRCA1 or BRCA2 may be associated with an increased risk. For men with BRCA2 mutation there is an increased risk for aggressive cancer [19]. Fruit and vegetables and polyphenols (i.e. green tea and soy-products) may be preventive, but need to be further investigated before any recommendations can be made [13,20]



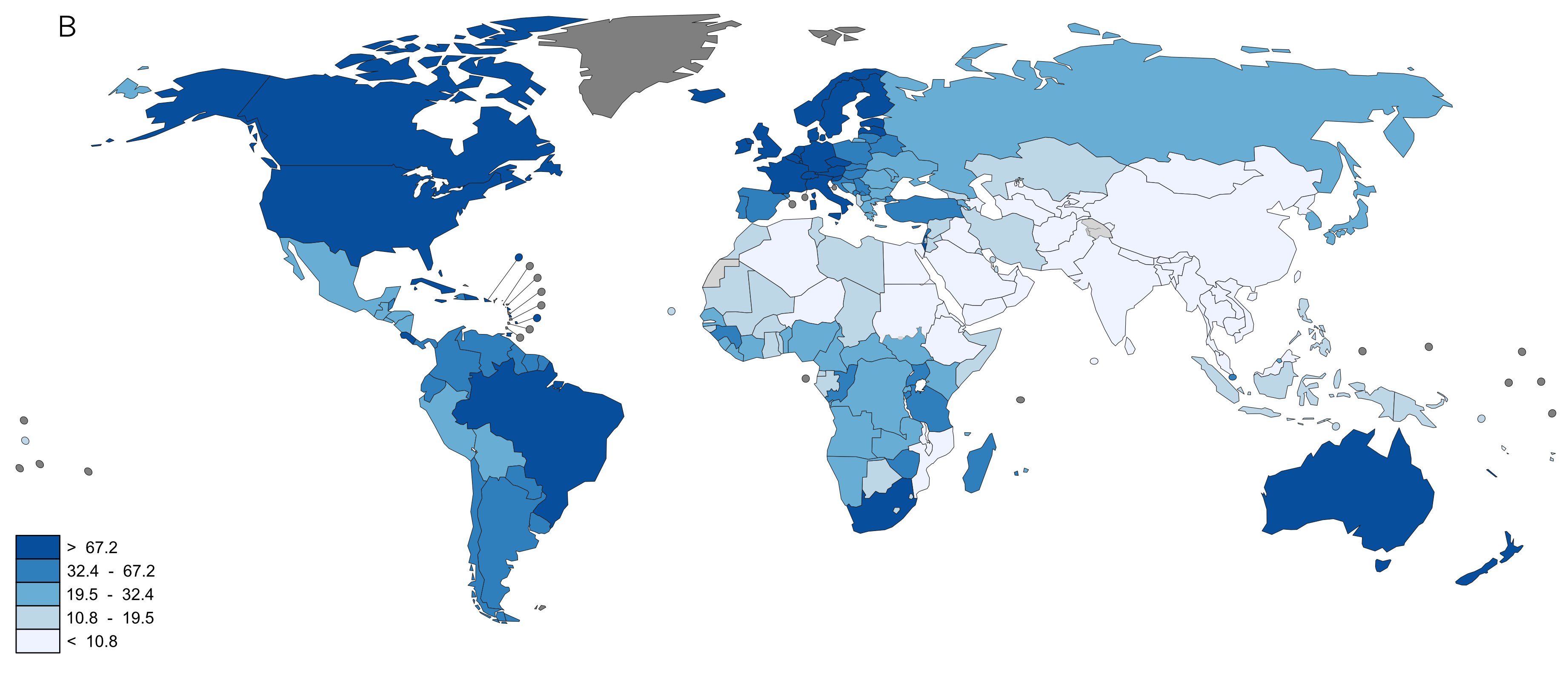


Figure 2. Prostate cancer mortality (A) and incidence (B) worldwide 2012. Age-standardized rates per 100000. Adapted from GLOBOCAN 2012 [21].

### Carcinogenesis

Most of the prostate cancers are adenocarcinomas. The mechanism behind the initiation of prostate cancer is unclear. In 1999 De Marzo et al [22] proposed that chronic inflammation could be a possible mechanism. He described atrophic areas with epithelial cells that failed to differentiate into secretory cells, proliferative inflammatory atrophy (PIA). The prostate cells are affected by oxidants secreted by the inflammatory cells, causing genomic damage and subsequently the transformation from PIA to prostatic intraepithelial neoplasia (PIN) and prostate cancer (Figure3).

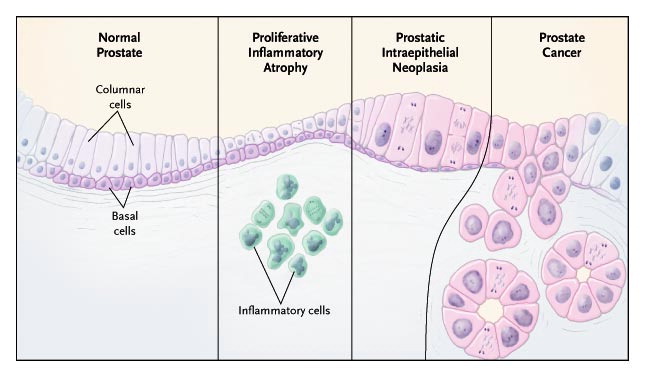


Figure 3. Proliferative inflammatory atrophy as a precursor to prostatic intraepithelial neoplasia and prostate cancer. Reproduced with permission from [23] Copyright Massachusetts Medical Society.

Since testosterone is converted to DHT in the prostate and androgen deprivation therapy shrinks prostate tumours, androgens are also thought to be part of the initiation of prostate cancer. This has however been hard to demonstrate and it is most likely that the pathway to prostate cancer is heterogeneous.

### Diagnosis of prostate cancer

In the early stages, prostate cancer is asymptomatic and most often diagnosed through PSA testing and DRE. PSA is fairly prostate specific, but is not specific for prostate cancer. An enlarged prostate due to BPH or inflammation in the prostate, i.e. prostatitis, can also influence PSA levels. For a patient with lower urinary symptoms (LUTS), PSA and DRE are included in the work up. They are also mandatory in patients with symptoms associated with prostate cancer, i.e. skeletal pain, skeletal metastasis without known primary tumour or B-symptoms (fatigue or loss of appetite). If there is suspicion of prostate cancer, a transrectal ultrasound (TRUS) guided biopsy is performed. The biopsies are examined by a pathologist and classified according to the Gleason system if cancer is found [24]. It has been shown that organised PSA testing in men 50-70 years of age reduces the mortality of prostate cancer, but is also generating an over diagnosis of clinically insignificant cancers [25-27]. Screening for prostate cancer is not recommended according to The Swedish Board of Health [28].

### Classification

In 1966 Donald Gleason created a grading system based on the architectural pattern of the tumour. The grade was defined as the sum of the two most common grade patterns and reported as the Gleason score. In 2005 this was altered to represent the most common grade and the highest grade [29]. Staging of prostate cancer is done according the tumour-node-metastasis (TNM) system [30] (Table 1).

Table 1. TNM classification according UICC 2009.

|  |  |
| --- | --- |
| Tx | Primary tumour cannot be assessed |
| T0 | No primary tumour detected |
| T1 | Tumour not clinically apparent, neither by palpation nor imaging |
| T1a | Incidental finding in ≤ 5% of material from resection |
| T1b | Incidental finding in ≥ 5% of material from resection |
| T1c | Identified by needle biopsy |
| T2 | Tumour confined within prostate |
| T2a | Tumour involves ≤ half of one lobe |
| T2b | Tumour involves ≥ half of one lobe |
| T2c | Tumour involves both lobes |
| T3 | Tumour extends through the prostatic capsule |
| T3a | Extraprostatic extension including microscopic bladderneck involvement |
| T3b | Seminal vesical invasion |
| T4 | Tumour is fixed or invades adjacent structures other than seminal vesicles |
| Nx | Regional lymph nodes have not been assessed |
| N0 | No regional lymph node metastasis |
| N1 | Regional lymph node metastasis |
| M0 | No distant metastasis |
| M1 | Distant metastasis |

T stadium is assessed by DRE and TRUS, and in special cases by magnetic resonance imaging (MRI), i.e. high-risk cancer prior to curative treatment. N stadium is evaluated by different imaging technics (PET/CT with choline or acetate or MRI) or surgical lymph node extraction in high-risk cancer prior to curative treatment. Distant metastasis, M stadium, is most commonly located in the skeleton and the standard method for evaluation is scintigraphy [24].

### Treatment

If the cancer is confined to the prostate, there are several curative treatment options available: radical prostatectomy, which is the surgical removal of the prostate, or radiation therapy (RT), either as brachytherapy or as external beam RT. Based on the risk of recurrence of the disease, patients are categorised into different risk groups based on TNM classification, Gleason score and PSA level: low, intermediate or high risk. This risk stratification was first described by D`Amico et al in 1998 [31], and is still the basis for treatment recommendations [24] (Table 2).

Table 2. Risk groups.

|  |  |
| --- | --- |
| Risk Group | Criteria |
| Low | PSA < 10μg/L and  Gleason score ≤6 and  T1a-T2a |
| Intermediate | PSA 10-19.90μg/L and/or  Gleason score 7 and/or  T2b |
| High | PSA ≥ 20 μg/L and/or  Gleason score 8-10 and/or  T2c - T3 |

In low risk cancer the risk of dying from prostate cancer within 10-15 years is less than 10% without treatment and 20% in the intermediate risk group. For high risk cancer the risk is 29% after 10 years and 36% after 15 years [32]. Therefore it is important to consider age and other comorbidities when discussing the treatment options. For patients with low risk localised prostate cancer, active surveillance may also be an option. These patients are monitored closely for disease progression, and if so definite treatment can be discussed. In case of distant metastatic disease there is no curative treatment and hormonal treatment (HT) is used to delay the progression of the disease and prolong survival [33]. The options include surgical excision of the testis or medical castration by either antagonists or analogues of gonadotropin releasing hormone (GnRH). A third option is inhibiting the androgen receptor with anti-androgens. The median overall survival with HT is 6 years if it only is lymph node engagement, in case of skeletal metastasis at diagnosis it is 3 years [34].

After a period of HT, the cancer becomes insensitive to androgen treatment, and fatal castrations-resistant prostate cancer (CRPC) develops. The standard treatment is then chemotherapy, but in recent years there have been new HTs, arbiterone acetate and enzalutamide, available [35,36]. The sequence of these new drugs and chemotherapy is not clear, and there are on-going studies examining this.

## Vitamin D

Vitamin D is not a true vitamin, but refers to a group of fat-soluble steroids. The most important compounds in this group are Vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). They are responsible for enhancing the intestinal absorption of calcium, phosphate and zinc. The major source of vitamin D is endogenous synthesis in the skin. When the skin is exposed to UV-B radiation, 7-dehydrocholesterol is converted to vitamin D3. The amount of vitamin D produced depends on several factors such as exposed skin surface, season, latitude, skin pigmentation, and age. Dermal production is reduced by pigmentation of the skin and with increasing age. Experience demonstrates that under the living conditions and latitude of the Nordic countries (55°N-72°N), vitamin deficiency can occur if the diet is devoid of the vitamin.

However, vitamin D can also be obtained through the diet, either as vitamin D2 (from plants) or as vitamin D3 (from animals). The dietary sources of vitamin D include food and dietary supplements. Food sources with naturally occurring vitamin D are fatty fish, egg yolk and fish liver oil. Some foods, such as cereals, milk, milk products and fruit juices are, however, fortified with vitamin D. The recommended daily allowance (RDA) for vitamin D in Sweden is 400 IU up till 75 years of age, thereafter 800 IU [37].

Vitamin D deficiency has traditionally been defined as 25 OHD levels < 25 nmol/L, because levels below this cause the skeletal disease rickets. In the last decades however vitamin D has gained attention in other health outcomes, and the definition has changed, although still no consensus has been met. In 2010 the Institute of Medicine in the U.S. stated that 50 nmol/L is the serum level that covers the needs of 97.5% of the population [38]. In 2011 the Endocrine Society however stated that the desirable serum concentration is above 75 nmol/L to maximise the effect on calcium, bone and muscle metabolism. Under 50 nmol/L is classified as deficiency, 50-75 nmol/L is insufficient and considered inadequate for bone and overall health [39].

Vitamin D has been associated with multiple acute and chronic disorders over the past decade including cancer, multiple sclerosis, depression and respiratory tract infections. A recent review stated however that many of the studies investigating this suffer from being small and of poor quality. The evidence supports vitamin D supplementation to help prevent fractures, possibly to prevent falls and slightly reduce mortality [40]. Vitamin D deficiency has also been found to contribute to diabetes and cardiovascular disease, such as hypertension, coronary artery disease, stroke, and atherosclerosis, but again the studies supporting this are not clear and further investigation is necessary [41].

### Vitamin D metabolism

Vitamin D from the diet or dermal synthesis from sunlight is biologically inactive and requires enzymatic conversation. Vitamin D2 and D3 are first hydroxylated to 25OHD in the liver and subsequently to the active form, 1,25-dihydroxyvitamin D (1,25(OH)2D), in the kidneys. The biologically active vitamin D has a short serum half-life and is under strict homeostatic control [42]. Vitamin D is involved in both calcium and parathyroid hormone (PTH) regulation. A low level of calcium causes a rise in PTH that activates 25OHD. 1,25(OH)2D then increases calcium concentration through enhanced intestinal absorption, reabsorption in the kidneys and bone resorption (Figure 4).

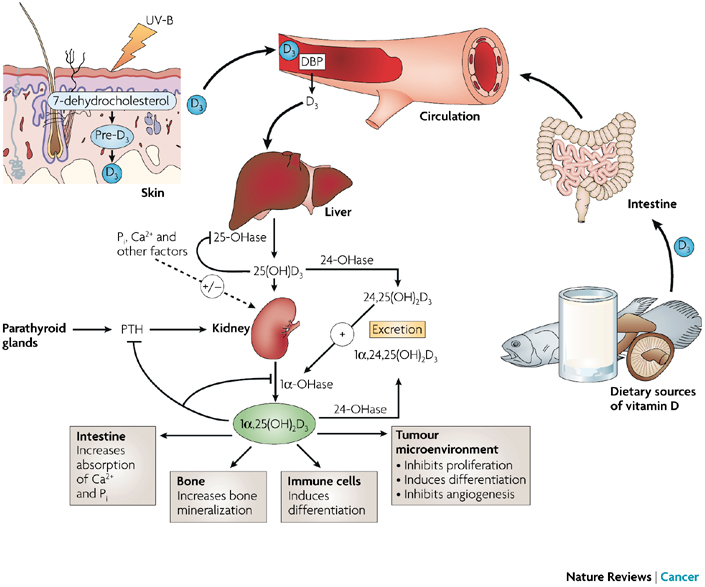


Figure 4. Vitamin D metabolism. Reprinted with permission from Nature Reviews [43].

The production of active vitamin D, i.e. the addition of a second hydroxyl group, was formerly presumed to take place exclusively in the kidneys and the active vitamin D thought to control mineral metabolism (calcium) only. In recent years it has become clear that local synthesis of active vitamin D occurs in an autocrine or paracrine fashion in many non-renal tissues, including keratinocytes, colon, and prostate cells. In these tissues active vitamin D (i.e. 1,25-dihydroxyvitamin D) controls key processes involving cell differentiation and proliferation [44].

Vitamin D is transported to different target organs by the vitamin D-binding protein (DBP), which also mediates the effect of vitamin D. It has been hypothesized that levels of DPB may affect the delivery of 25OHD and 1,25(OH)2D [45].

### Vitamin D binding receptor

Active vitamin D, 1,25(OH)2D, exerts its effects by binding to vitamin D receptors, VDRs. These are found in the cell nucleus, where the ligand-receptor complex interacts with genomic DNA and selectively induces transcription and expressions of certain genes [46]. VDRs are also found in the cell membrane, and are responsible for the more immediate actions of 1,25(OH)2D, such as the enhancement of intestinal calcium absorption [46]. VDR has been shown to be widely expressed in human prostate cells, especially in the peripheral zone of the prostate, the site of origin for most prostate cancers [47].

## Parathyroid hormone (PTH)

PTH is secreted by the parathyroid glands, which are located close to the thyroid, and act to increase the concentration of calcium. There is a complex feedback system regulating the PTH levels. The parathyroid gland senses the calcium level by means of the calcium sensing receptor (CaSR) [48]. Extracellular calcium is the main parathyroid regulator, and low levels of calcium stimulate the secretion of PTH within a few minutes, while elevated levels inhibit the release of the hormone. The receptors for PTH (PTHR1) are found mainly in the kidneys and skeleton [49].

1,25(OH)2D, the active form of vitamin D, is also an important regulator of PTH. It exerts a direct effect by inhibiting the mRNA synthesis through the VDR receptor [50]. PTH has a half-life of only three to eight minutes and is rapidly degraded in circulation.

### PTH-related peptide

PTH-related peptide (PTHrP) is a hormone, originally discovered as a tumour product causing hypercalcemia. Since then it has been shown to promote tumour cell growth, aggressiveness and metastasis [51]. PTH and PTHrP bind to the same receptor, which is expressed in prostate cancer cells [52,53].

## Calcium

Over 99% of total body calcium is stored in bones and teeth, where it provides hard tissue with its strength. Extracellular calcium is critical for mediating vascular contraction and vasodilatation, muscle function, nerve transmission, intra cellular signalling, and hormonal secretion. The skeleton serves as a reservoir of calcium, which can be used as a source of calcium for these metabolic needs by bone remodelling. Of the small fraction of circulating calcium only 47% is free (ionised), 46% is bound to different proteins, mainly albumin, and the rest is bound to small ions. It is the ionised form that is the active form.

Total calcium concentration in serum is tightly regulated to remain between 2.12-2.62 mmol/L. If this level deviates slightly, PTH is secreted and stimulates the kidneys to produce 1,25(OH)2D, as well as to activate bone resorption, which will increase extracellular calcium. 1,25(OH)2D acts in an endocrine manner on the kidneys, bones and the intestines to raise serum levels. As the serum level rises, the PTH secretion drops due to negative feedback. If there is an overshoot in serum calcium levels, the parafollicular cells of the thyroid gland can secret calcitonin, which can block bone calcium resorption and help keep calcium levels in the normal range.

Calcium is classically associated with dairy products such as milk, yoghurt, and cheese. Fruit juices, soft spreads, wheat flour are often fortified with calcium. Calcium is also available as non-prescription multivitamin supplements, and is prescribed as prophylaxis and treatment for several conditions, most notably osteoporosis [54].

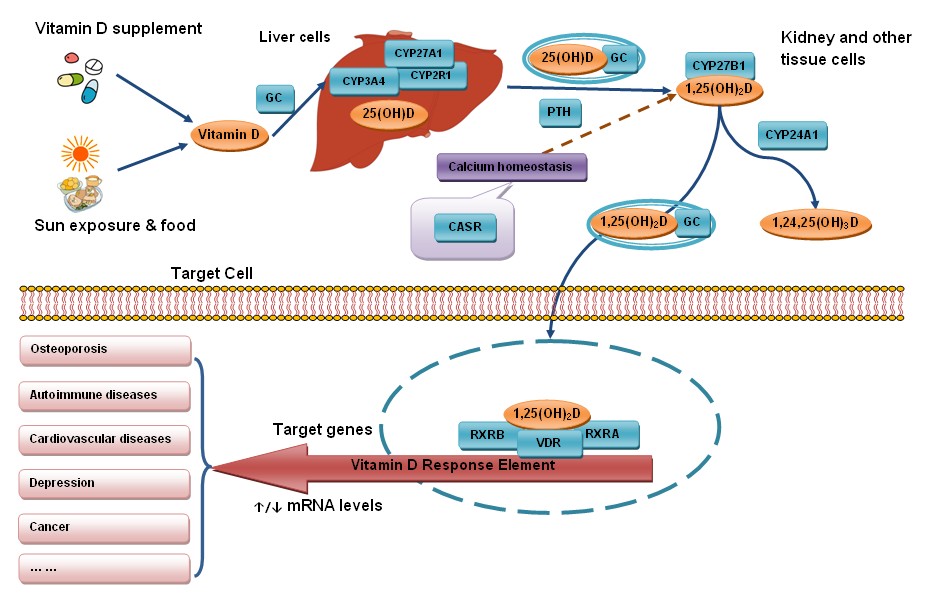
### Calcium sensing receptor

The CaSR is the main regulator of calcium homeostasis by regulating PTH secretion and urinary calcium excretion. It also controls numerous other processes, such as blood pressure, regulates insulin secretion and synthesis of enteroendocrine hormones. On a cellular level CaSR is a key regulator for gene expression, inflammation, proliferation, differentiation and cell death [55]. Abnormal CaSR function has been associated with several cancer forms, and depending on the site of the cancer it acts either as an oncogene or a tumour suppressor gene. In prostate cancer it acts as an oncogene by promoting proliferation and inhibiting apoptosis, although the mechanisms are not yet completely understood [56].

## 

## The vitamin D pathway

The transformation of vitamin D from synthesis in the skin to the active form is often referred to as the vitamin D pathway (Figure 5). It involves proteins coded by several genes. For instance CYP27B1, responsible for the enzyme 1-α-hydroxylase that converts 25OHD to its active form; CYP24A1 which codes for an enzyme inactivating 25OHD; GC, the vitamin D binding protein that transports 1,25(OH)2D to the target tissue and VDR, where the effect is executed [3].



**Figure 5.** Genes involved in vitamin D metabolism. Reprinted with permission from Tulane University, copyright 2011. All rights reserved [57].

## Single nucleotide polymorphism

A single nucleotide polymorphism (SNP) is a variation in a single nucleotide at a specific position in the genome. They occur often in a population and can have modest but real biological effects. They can lead to different cellular effects due to enhanced or reduced transcription of a protein. Genome wide associations studies (GWAS) have identified SNPs that are associated with vitamin D status and the vitamin D pathway. In the VDR gene there are several known SNPs. Since everyone carries two copies of the gene there can be three different alleles, e.g. FF, Ff and ff, for every SNP. One of these alleles could code for a receptor less sensitive to 1,25(OH)2D levels, which could explain the conflicting results of vitamin D and prostate cancer risk, i.e. levels of vitamin D sufficient to prevent cancer in one individual may be insufficient in someone with the less sensitive receptor.

There are also multiple GWAS studies that have identified SNPs associated with an increased risk of prostate cancer [58], and several that thereafter have been validated in well-conducted case-control studies [59]. One of the problems with these SNPs is the understanding of how they contribute to disease, which is the target gene and what is the functional consequence [60].

## The vitamin D hypothesis

In 1990 Schwartz and Hulka presented the vitamin D hypothesis [2] based on the observation that the risk factors for prostate cancer, older age, ethnicity (African American), residence at northern latitude all are associated with a decreased vitamin D synthesis: Age – with increasing age the prevalence of vitamin D deficiency increases; ethnicity – in African Americans the melanin inhibits the synthesis of vitamin D and their vitamin D levels are often only half or less of those in Caucasians; Asians often have a diet high in vitamin D (fish oil) and the protection wanes as migrants adopt a western diet.

They hypothesised that vitamin D maintained the normal phenotype of prostatic cells and that vitamin D deficiency permitted the development of clinical prostate cancer from its preclinical precursors. They could shortly after show that mortality rates for prostate cancer in the USA were inversely correlated with the availability of UV radiation, the major source of vitamin D [61].

The effect of the active form of vitamin D, 1,25(OH)2D, on normal and cancerous prostate cells were investigated and several anticancer effects have been reported, such as inhibition of cell proliferation [62], cell migration [63] and angiogenesis [64]. In 1998 it was demonstrated that prostate cells possess the activating enzyme 1α-OHase, and thereby synthesise 1,25(OH)2D from 25OHD[44], and also that the expression of 1α-OHase is significantly reduced in prostate cancer cells [65].

### Sunlight and prostate cancer

Several epidemiologic studies have examined the relationship between exposure to sunlight and prostate cancer. A large U.S. study by Schwartz et al [66] showed that there was a significant inverse correlation between UV radiation and prostate cancer mortality, and that the observations were more pronounced at locations north of 40° latitude. The same inverse correlation has been shown in other countries [67,68]. In the United Kingdom, different studies have shown that a high sunbathing score was significantly protective for prostate cancer, and similar results was seen for multiple sunburns during childhood. Furthermore, among prostate cancer cases it was demonstrated that men with low sunlight exposure were diagnosed at significantly younger ages then men with high exposure [69]. As concluded in the review by Schwartz et al [5], epidemiologic studies on sunlight exposure on prostate cancer risk generally showed a protective effect.

## Vitamin D and prostate cancer

### Prostate cancer incidence

Serum levels of vitamin D and prostate cancer incidence have been assessed in several prospective studies with conflicting results over the last 20 years. Of these, only two have reported a clear and statistically inverse relationship for overall risk of prostate cancer [70,71]. Five studies have reported an increased risk for prostate cancer with higher concentrations [72-76] and the remaining studies have reported null results. However, an updated meta-analysis of 21 studies published in 2014, reported a statistically significant association that higher vitamin D concentrations were related to a higher risk of developing prostate cancer [77]. In summary, the evidence that there is a protective effect of higher vitamin D concentrations regarding the risk of developing prostate cancer is weak. In fact, there is stronger evidence of the opposite, i.e. higher vitamin D levels may be associated with an increased risk of prostate cancer [78].

### Prostate cancer aggressiveness

Due to the conflicting results regarding vitamin D and prostate cancer incidence, attempts have been made to distinguish between low-risk and advanced prostate cancer. The reason for this may be the heterogeneity of prostate cancer, and the high prevalence of clinically insignificant tumours, which may dilute the net effect when observing all cancers as one group [79]. The results are however not consistent, with some reporting an inverse association between vitamin D and more advanced cases [80-82], some reporting no association [83-85] and others an increased risk for aggressive prostate cancer with higher levels [75,74,86].

### 

### Prostate cancer mortality

The literature regarding vitamin D status and prostate cancer specific mortality is limited and has yielded mixed results. There have been reports of a lower mortality with higher vitamin D levels [81,87-89] as well as studies with no associations [75,85,90,91]. All but two of these studies were conducted with prospectively collected serum; Holt et al used serum collected approximately 10 months after diagnosis and demonstrated no association [91]; Tretli et al used serum collected within a month after treatment and could show that vitamin D levels at medium or high levels were significantly related to better prognosis [88]. In summary, there is no convincing evidence that vitamin D will prevent prostate cancer, some evidence that it may prevent progression of early stage disease, and inconsistent findings for mortality [78].

## Calcium and prostate cancer

The idea of an association between calcium and prostate cancer came from a concept similar to that of vitamin D and prostate cancer, an ecological study showing a correlation between prostate cancer mortality rates and dairy consumption [92]. Most of the studies on calcium and prostate cancer concerned dietary calcium and only few investigated the association with serum calcium. Two relatively small studies reported an association between high calcium levels and increased prostate cancer mortality [93,94], whereas two other found no association [95,96]. Several publications demonstrated an association between high dietary intake and both increased risk of prostate cancer [97-99] and increased prostate cancer mortality [100-102]. Laboratory analysis showed that calcium promotes the growth of prostate cancer cells [103] and the CaSR, which plays a pivotal role in calcium metabolism, is believed to promote metastasis and act as an oncogene in prostate cancer if affected [56].

## PTH and prostate cancer

PTH is believed to be a mitogen in prostate cancer by promoting cell growth both in vitro and in vivo and by increasing the proliferation of human prostate cancer cells in tissue culture [6,103]. PTH and parathyroid related protein (PTHrP) share the same receptor, and it has been demonstrated that PTHrP may play a significant role in the growth of prostate cancer [104].

The association between serum levels of PTH and prostate cancer risk is sparse. To the best of my knowledge our publication (paper I) was the first. Since then there has been one more publication, also reporting no associations [105]. There have been reports of a positive correlation between PTH and PSA, this was however seen in both men with prostate cancer and in controls [106].

# The present investigation

## Aims of the thesis

The general aim of this thesis was to study vitamin D, PTH, and calcium in relation to prostate cancer.

The specific aims of each paper are listed below:

* To examine the relationship between pre-diagnostic serum levels of vitamin D, PTH, and calcium and risk of prostate cancer;
* To examine the relationship between pre-diagnostic serum levels of vitamin D, PTH, and calcium and risk of prostate cancer according to tumour aggressiveness;
* To examine the association of pre-diagnostic serum levels of vitamin D, PTH, and calcium with prostate cancer specific survival; and
* To investigate the association between selected vitamin D, PTH, and calcium SNPs and subsequent prostate cancer risk.

# Material and methods

## Malmö Diet and Cancer Study (MDCS)

This prospective cohort was initiated in 1991 with the primary objective to study the association between dietary factors and cancer incidence [107]. All residents of Malmö born between 1923 and 1950 were invited to participate. Participation included a first visit with measurements of weight, height, blood pressure, and body composition. Blood was also drawn and stored at -80°C. The subjects filled out a questionnaire, which assessed socioeconomic factors and life style factors, medications, and previous disease. It also contained questions on subjective well-being, weight changes and physical activity. Dietary assessment consisted of a menu book, a diet history questionnaire and a dietary interview. All baseline examinations were performed between 1991 and 1996. The total number of participants included 28098 individuals, 38% of eligible subjects, of whom 11063 were men and 17035 were women [108].

## Study population and follow-up

We identified 943 incident prostate cancer cases by linkage to the Swedish Cancer Registry until the 31st of December 2005 and with the Southern Swedish Regional Cancer Registry up to the year 2006 (time discrepancy due to a one year delay in reporting to the latter Registry). The Swedish Cause of Death Registry was used to collect vital status. The cases were matched to controls according to age (± 2 years) and calendar time at inclusion (± 15 days). In all, 940 case-control pairs were exactly matched and age at inclusion were relaxed to ±3 years in three pairs. This case-control set was used in papers I, II and IV. For paper III we only used the cases and updated the vital status until 31st December 2013.

The cause of death was retrieved from the Cause of death form. Men with prostate cancer as the cause of death were used for the analysis regarding prostate specific mortality. For overall mortality we combined the men with prostate cancer as an underlying factor to death and the men who died from causes unrelated to prostate cancer.

## Laboratory analyses

Serum from cases and controls was retrieved from the MDCS biobank. Blood samples had been drawn from non-fasting subjects, and serum had been separated within one hour of collection [109] and thereafter stored at -80˚C. The samples were used for the first time, i.e. had not previously been thawed. Serum was analysed for 25OHD₂, 25OHD₃, PTH, and calcium creatinine and albumin. Case-control pairs were analysed in random order with regard to case control status and time of baseline examination. Cases and controls in the same pairs were examined in the same batch.

Analysis of 25OHD₂ and 25OHD₃ was done by high-pressure liquid chromatography and PTH was determined using an Immulite® 2000 Intact PTH immunoassay (Diagnostic Products Corporation, Los Angeles, CA). Total calcium was analysed using a neutral carrier ion-selective electrode [110] on a Synchron LX System (Beckman Coulter Inc., Brea, CA).

Coefficient of variation (CV) values were as follows: for 25OHD₂, 8.0% at 65 nmol/L and 6.8% at 190 nmol/L; for 25OHD₃, 8.5% at 70 nmol/L and 7.1% at 210 nmol/L; for PTH, 4% at both 5.9 and 40.3 pmol/L; for calcium, 2% at both 2.00 and 3.10 mmol/L. The laboratory of the Department of Clinical Chemistry at Skåne University Hospital is accredited by SWEDAC (the Swedish Board for Accreditation and Conformity Assessment) and participates in the external quality assurance program of INSTAND e.V. (Düsseldorf, Germany).

## Classification of tumour aggressiveness

Data on tumour characteristics was obtained from medical records and pathology reports, which were obtained for over 90% of the cases. Data included TNM stage according to the 2002 classification [111], tumour differentiation and serum PSA at diagnosis. In patients at risk of having extra prostatic disease, the lymph nodes were evaluated by histologic examination of obturator lymph nodes obtained at surgery. Bone scan was used to evaluate distant metastasis. Clinically low risk patients, i.e. PSA < 20 and T1-T2, who did not undergo lymph node surgery and/or bone scan were classified as N0 or M0.

Tumour differentiation was based on the histopathological or cytological examination of the core biopsy/aspirate that led to the diagnosis of prostate cancer. In 78% of the cases, the Gleason score was available, but in some of the earliest cases pathology reports were based on the World Health Organization (WHO) grading system [112]. We merged the two classification systems such that WHO G1 and Gleason score ≤6 were classified as low-grade tumours, WHO G2 and Gleason score 7 as intermediate, and WHO G3 and Gleason score ≥8 as high grade. ASAP (atypical small acinar proliferation) lesions and PIN (high grade prostatic intraepithelial neoplasia), four and two cases respectively, were regarded as low-grade cancer. For our main analysis in paper II we defined a non-aggressive tumour as T1-T2, N0 and M0, and an aggressive tumour as T3-T4 or N1 or M1 or Gleason score ≥8. This definition was used to be able to compare our results with other studies [75].

## Statistical methods

All statistical analyses were performed using SPSS version 16.0 through 23.0 (SPSS institute, Chicago, IL, USA). In papers I-III albumin adjusted calcium was used according to the formula: adjusted calcium (mmol/L) = measured total calcium (mmol/L) + 0.02(40- serum albumin (g/L)) [113]. In all papers we assessed the risk of reverse causality by repeating all the analyses excluding men diagnosed with prostate cancer within two years (n=72) from baseline. In paper IV we expanded this to five years (n=229) as well.

### Papers I and II

The cohort was divided into quartiles and deciles (paper I) based on serum levels of 25OHD, PTH, and calcium in controls. Missing values were coded as separate categories for all these factors and for other categorical covariates. Crude and multivariate conditional logistic regression analyses were used to calculate the odds ratio (OR) with 95% confidence interval (CI) for prostate cancer in different quartiles and deciles of 25OHD, PTH and calcium. The trend over quartiles was calculated by including the quartile variable as a continuous factor. In paper I both matched and unmatched analyses were performed.

To be able to perform analyses stratified for BMI, 25OHD, PTH, and calcium, it was necessary to conduct unmatched analyses using unconditional logistic regression adjusted for matching factors (age at baseline as continuous variable, screening month and year). Both matched and unmatched analyses were further adjusted for potential risk factors, such as educational level, alcohol consumption, and smoking status.

In paper I we performed stratified analyses in different groups defined by age (<55 years, 55–65 years, >65 years), BMI (<25 vs. >25), and low vs. high levels of 25OHD, PTH, and calcium. The cut-off for low vs. high 25OHD was set at 50 nmol/L, which is the minimum level considered to be sufficient by several authors and first proposed in the original hypothesis by Schwartz & Hulka [114,2]. Cut-offs for low vs. high PTH and calcium were set at the median in order to split the cohort into groups that were as equal in size as possible.

In paper II we performed stratified analyses according to different aggressiveness of the tumours in different quartiles of 25OHD, PTH and calcium and further investigated the relationship by stratifying for low vs. high levels of the metabolites. Since 25OHD is affected by season, i.e. UV radiation, we adjusted for screening month in the multivariate analysis. We also created season specific quartiles of vitamin D by taking into account the month of blood draw. These analyses were not adjusted for month of screening. To exclude the impact of renal disease and/or primary hyperparathyroidism, we also repeated analyses after excluding individuals with creatinine above 105 µmol/L, or with PTH > 6.8 pmol/L and ca >2.5 pmol/L.

### Paper III

Levels of 25OHD, PTH and calcium were divided into quartiles (Q). Missing values were coded as separate categories for these factors. Cox proportional hazards regression models were used to estimate HR and their 95 % CIs to assess the relation of the metabolites with total and prostate cancer-specific mortality. The lowest quartile (Q1) was used as a reference. Trend over quartiles was calculated by including the quartile variable as a continuous factor. The proportional hazard assumption was tested visually using log-log plots and found to hold.

Because the metabolites, especially 25OHD, vary largely with season and age, we adjusted for month and year of inclusion (HR2). In the HR2 model we also adjusted for age at inclusion, continuously, and BMI (<25, 25-30, >30 Kg/m). In HR3 we further adjusted the model for age at diagnosis and factors known to affect survival, i.e. tumour stage according to the TNM classification (T1-T2/T3-T4, N0/N1, M0/M1), Gleason score (<7/≥7). To further address the seasonal properties of 25OHD, we created season specific quartiles, both taking into account the month of blood drawn and the season. These analyses were not adjusted for month of screening.

We investigated the relationship between the metabolites and prostate cancer specific mortality further by stratifying for low versus high levels of vitamin D, PTH, and calcium. The cut-off level for low versus high vitamin D was first set at 50 nmol/L, but due to the number of cases being too small it had to be increased to 75 nmol/L. Cut-offs for low versus high PTH and calcium were set at the median in order to split the cohort into equally sized groups.

Since there have been reports of better prostate cancer survival if diagnosed during summer [115], we created two groups: one diagnosed during November through May (winter), and one group diagnosed June through October (summer). We performed Cox proportional hazards regression analysis using the models previously described to stratify if survival from prostate cancer was affected by season of diagnosis.

### Paper IV

All SNPs were tested for consistency with Hardy-Weinberg equilibrium. Unconditional binary logistic regression was used to investigate the association between the SNPs and risk of prostate cancer, yielding ORs with 95% CIs. For each SNP we classified the major homozygote as the referent group, and combined the minor homozygote and the heterozygote as the exposure group. Since the metabolites, especially 25OHD, vary largely with season and age, we adjusted for age at baseline and season of inclusion, i.e. year and month. We further adjusted the model for alcohol consumption and smoking status. In order to see if the level of vitamin D affected the results we stratified the analyses on low vs. high levels of 25OHD, using a cut-off at 50nmol/L.

### Ethical approval

The Research Ethics Review Board of Lund University approved all four of the investigations: LU 51-90 and Dnr 268/2007.

# Results

## Paper I

We found a weak trend toward increasing prostate cancer risk with rising vitamin D levels (p-trend 0.048). Dividing the cohort into deciles showed a nonlinear association. Compared to decile one, the prostate cancer risk was highest in deciles seven and eight, which corresponded to vitamin D levels of 91-97 nmol/L OR 1.68 (1.06-2.68), and 98-106 nmol/L OR 1.80 (1.13-2.85). Calcium was positively associated with an increased risk for prostate cancer among men aged 55-65 and with a BMI <25, OR in Q3 2.07 (1.08-3.97). No association was observed between PTH and subsequent prostate cancer incidence.

## Paper II

We found no significant association when comparing aggressive to non-aggressive disease regarding vitamin D, PTH or calcium. There was a trend toward an increased risk in low grade tumours, i.e. Gleason score ≤ 6, and a significant association regarding Gleason score 7 tumours with OR 1.70 (1.09-2.65) in the highest quartile of vitamin D. Stratifying the analysis yielded several significant findings demonstrating a non-specific interaction between the metabolites. In men with PTH above the median the risk of aggressive prostate cancer was double in the highest vitamin D quartile, OR 2.01 (1.24-3.25) and for non-aggressive cancer 1.82 (1.25-2.66). There was an inverse effect on risk of prostate cancer in men with PTH above median and vitamin D ≤ 50 nmol/L, OR 0.25 (0.09-0.71) and calcium ≤ 2.37 mmol/L, OR 0.53 (0.34-0.82) for aggressive cancer.

## Paper III

We observed a trend towards a lower prostate specific mortality with sufficient vitamin D in the unadjusted analysis. This became statistically significantly in the third quartile of 25OHD (85-102nmol/L) compared to the first, HR 0.54 (0.34-0.85) when adjusting for age, time of inclusion and BMI. The association was further strengthened when adjusted for age at diagnosis, Gleason score and TNM classification with a HR in Q3 0.36 (0.22-0.60). P for trend was 0.03. Regarding calcium, there was a significantly lower HR for the second quartile (2.35-2.39 mmol/L) compared to the first with a HR of 0.54 (0.32-0.86) in the unadjusted analysis. However, this association disappeared when adjusting for tumour characteristics. There were no associations between levels of PTH and prostate cancer mortality.

## Paper IV

Two VDR SNPs, ApaI and BglI, were associated with a lower risk of prostate cancer with ORs 0.77 (0.62-0.97) and 0.77 (0.61-0.97) in the adjusted analyses respectively. These associations were strengthened in men with high vitamin D levels, ORs 0.72 (0.57-0.92) and 0.72 (0.56-0.92). There were no corresponding statistically significant associations in men with low levels of vitamin D. One VDR SNP, BsmI showed a decreased risk of prostate cancer in the analyses among men with low levels of vitamin D, OR 0.25 (0.09-0.69). Among men with high levels of vitamin D, there was a borderline significant finding in one of the calcium SNPs, rs 4306808, with OR 1.31 (1.00-1.73). We observed no significant finding in the PTH SNPs.

# Discussion

## Methodological issues

### Misclassification

We have used prospectively collected serum in all the papers. The serum was collected at inclusion of the study; hence there is only a single determination for the metabolites. If this sample is representative several years later could be questioned. This has however been investigated, and there is a high correlation between levels of 25OHD measured on two separate occasions several years apart [116-118] and seasonal variation seems to be larger than the actual longitudinal change [119]. Vitamin D levels in serum are affected by season and decrease with increasing age and with high BMI [120]. We have adjusted our analysis for these factors. To further address the seasonality of vitamin D we created season specific quartiles, which were used in paper II and III. The mean 25OHD levels in our cohort were somewhat higher when compared to other Nordic studies, and only 5% of the cases had a 25OHD level below 50 nmol/L, and only 30% below 75 nmol/L. This is however comparable to another study from the same region [121].

25OHD is not the active form of vitamin D, but is considered the best indicator of vitamin D status. The active form, 1,25(OH)2D, is tightly regulated and in healthy individuals not correlated with serum levels of 25OHD [122]. As described previously, prostate cells themselves can convert 25OHD to 1,25(OH)2D, but how the balance of intraprostatic cellular vitamin D is in relation to circulating levels is unknown. It is therefore possible that the true effects of vitamin D cannot be evaluated by studying 25OHD.

Regarding calcium there is also little short-term [123] and long-term [124] intra-individual variation. Free (ionised) calcium is considered the best marker of calcium status because it is biologically active and tightly regulated. However, in healthy individuals, where the albumin level is expected to be normal, total calcium has been considered a good measure of calcium homeostasis. This was the case in our cohort with only 65 men (3.4%) having an albumin level outside the reference range. The samples were collected in a standardised manner, which minimised the difference in albumin levels due to diurnal variation or fasting status [125]. We also conducted all the analyses with albumin-adjusted calcium as described in statistical methods. Hence calcium measurement can be considered reliable. Another concern is that undetected prostate cancer could influence the serum level towards a lower level, due to diversion of calcium into bone metastasis [93], which sometimes can be seen years before metastatic disease [126]. We have addressed this by also conducting the analysis by excluding patients diagnosed within 2 years of inclusion.

PTH has been reported to have intra-individual variations of about 25% over short periods (up to 6 weeks) [127,128]. There is also a relatively large circadian fluctuation in PTH, with a reported twofold difference in nadir to peak concentrations [127]. We did not have information about the time of day for blood collection, and as the intra individual fluctuations can be quite high, it could lead to a non-differential misclassification of the true levels. This may, hence, lead to an attenuation of true risk in the statistical analysis.

Incomplete follow-up and poor quality of end point data may affect the results. However, the Swedish Cancer Registry has been validated and found to have a completeness of about 97% [129], and the Swedish Cause of Death Registry has been shown to be correct in 90% of cases where malignant tumour is the cause of death [130]. Therefore, it is expected that the data concerning cause of death (paper III) is complete and correct to a great extent. Furthermore, if the cause of death is misreported, i.e. non-prostate cancer deaths are reported as prostate cancer deaths, it would lead to an attenuation of true risk.

### Selection bias

The representativity of our cohort may be questioned. Only 38% of those invited participated in the study, and as we have no information about the other men and their exposure and risk factors, the results may not be applicable to the general population. However, as there was a wide distribution of vitamin D, PTH and, calcium levels it was possible to make internal comparison between subjects with high and low values. Therefore, relative risks were probably not affected by selection bias to any large extent.

### Detection bias

It has been shown that men from higher socio-economic background are more health conscious and may be more likely to undergo PSA screening [131]. In our cohort there was a larger per cent with higher education among the cases, 24.7 vs. 20.2, which may have affected the results in such that we see higher vitamin D levels and more prostate cancers.

### Confounding

A confounding factor correlates with both exposure and outcome variables without being caused by either of them. We had information in all papers on most established risk factors: age, BMI, education, smoking and alcohol consumption. The results were similar when adjusting for them in the multivariate analysis; hence the results are probably not confounded by these factors. A limitation of the present investigation is that we had no information about family history of prostate cancer and ethnicity.

### Chance findings and statistical power

A common issue in epidemiology is multiple hypothesis testing. The more tests that are performed, the higher the risk that some of the observed associations are coincidental. We have used a 95%CI, which means there is a 5% risk of observing false positive results due to chance. For instance in paper IV, where several SNPs were investigated, it is possible that some of the findings were due to chance. By using SNPs previously associated with vitamin D, PTH, and calcium and prostate cancer, the likelihood of this diminishes, i.e. we had an a priori hypothesis. We decided against using Bonferroni´s correction since it could increase the false negative results (type II error).

Overall, the statistical power can be considered high, since the number of cases was almost 1000. However, in some of our stratified analysis there was only a small number and real associations might not be observed, i.e. a type II error. An indicator of this is a wide CI. For instance the significant association in young men with high BMI in paper I should be regarded with caution. Another example is the analysis in paper IV stratified for low levels of vitamin D there were only a small number of cases, and hence a fairly wide CI.

### Reverse causality

It is possible that prostate cancer itself can cause alteration of the studied metabolites, for example due to bone metastasis where calcium is trapped in the skeleton. This could camouflage the true association between levels of the metabolites and prostate cancer. We have addressed this by excluding cases with prevalent prostate cancer and furthermore conducted sensitivity analysis, where cases diagnosed within 2 years of inclusion were excluded. In paper III we extended the sensitivity analysis to five years without any major difference, making reverse causality unlikely.

### Previous studies and possible explanations

The finding in paper I, a statistically significant positive association of a 1.7 fold increased risk of prostate cancer with vitamin D levels 91-107nmol/L, was contradictory to the original hypothesis. Similar results have however been reported in five other studies [75,73,72,74,76] and a recent meta-analysis suggested a positive association between high level of 25OHD and increased risk of prostate cancer [77]. The authors of the meta-analysis speculated that the association is U shaped, as reported by Tuohimaa et al [73] and in the Prostate Cancer Prevention Trail (PCPT) [82], in a similar manner as folate and colon cancer [132]. We did not observe this U shaped association, but one possible explanation to this could be the small number of cases with actual vitamin D deficiency in our cohort, which could camouflage the association with low vitamin D levels. In fact, many of the studies did not test the hypothesis of vitamin D deficiency, but rather addressed whether different degrees of vitamin D sufficiency were associated with an altered risk of prostate cancer [5]. There is no known biological explanation for the increased risk, and all the laboratory evidence pointed in the other direction: inhibition of cell proliferation [62], cell migration [63] and angiogenesis [64].

We suggested that our finding could, at least partly, be contributed to detection bias. Men from higher socio-economic groups tend to be more health conscious and may have higher vitamin D levels [133] and also be more likely to undergo PSA screening [131]. Jacobs et al [134] proposed that vitamin D is a biomarker for a healthier lifestyle, and showed that an individual with low BMI and high physical activity has higher vitamin D levels. However, since both a smaller body size and greater physical activity are related to lower cancer risk, it is difficult to separate the effects of these characteristics from those attributed to vitamin D levels. We have in our analysis adjusted for BMI, but not physical activity.

We also speculated that the reason for the previous conflicting results has been that many studies did not distinguish between the aggressiveness of the disease and since prostate cancer is a very heterogeneous disease the net effect may be concealed when studying prostate cancer as an entity. We addressed this in paper II but could not show any difference according to the aggressiveness of the cancer, defined as a T3/T4 or N1 or M1 or Gleason score ≥8, in relation to vitamin D levels. We did however find a significant increased risk when dividing the cohort according to the Gleason score. This can however also be explained by detection bias, similar to paper I.

Based on our first two studies, Jacobs et al could be right. Vitamin D could be just a marker for a healthier lifestyle, and the findings represent medical aware individuals with screening detected tumours. Unfortunately we do not have information about the screening rate in our cohort, and could not take this into account accordingly. However, most of the cases were diagnosed 1998-2004, and 2004 the screening detected cancers in our cohorts region was 29% [135].

There have been questions as to where in the prostate carcinogenesis vitamin D exerts its effects. According to Schwartz [5], the inhibitory role is more likely to be in the stages of cancer progression rather than in tumour initiation. If this is correct, then the effects of vitamin D should be greater on the risk of advanced or fatal cancer. In paper III we explored this and found that high levels of vitamin D may improve survival in prostate cancer, a finding coherent with the original hypothesis. The literature regarding prostate cancer specific mortality and vitamin D is limited and has been inconsistent. This could partly be attributed to different settings; one study was not aimed at investigating mortality [75] and one study used serum taken after diagnosis, which makes the results harder to interpret due to reverse causality [91].

In paper III we also investigated the reported association of a survival benefit if diagnosed with cancer during the summer, which has been shown for prostate cancer [115], as well as for other cancers [136-138]. We could however only show a marginally lower risk. More interestingly there was a 75% risk reduction for prostate specific mortality in the highest vitamin D quartile (≥ 103nmol/L) if the diagnosis was made in the winter. Hence, a high level of vitamin D seems to be more important in the winter. There are also reports of an improved survival if treatment for the cancer starts during summer [139], but as we have no information about this we could not adjust the analysis accordingly.

The effects of Vitamin D are mediated through the VDR receptor and GWAS studies have implicated genetic polymorphisms as a possible explanation [140]. In paper IV we investigated the association between SNPs in the vitamin D pathway, PTH and calcium in different strata of vitamin D and subsequent risk of prostate cancer. We could show that there indeed was an association, and in two SNPs there was a protective effect that was further strengthened with high vitamin D levels. In one SNP we observed the opposite, i.e. a decreased risk of prostate cancer only manifesting with low levels of vitamin D. In a meta-analysis by Raimondi et al similar results was reported [141] but an updated report showed no association [142]. Another study demonstrated an increased risk of prostate cancer in rs4588 and rs7041, which we could not replicate [143]. The reason for the inconsistencies in results could be the ethnicity of the studied cohort, as it is well known that polymorphic alleles differ by ethnicity [144]. We could not find any association between prostate cancer risk and SNPs associated with PTH, which to the best of my knowledge never has been investigated.

It is clear that the association between vitamin D and prostate cancer is complex, and in an attempt to clarify this we therefore also investigated the associations regarding PTH and calcium in all our papers.

When only observing calcium or PTH in relation to prostate cancer risk or tumour aggressiveness there was no associations (paper I and II). In paper II we conducted stratified analyses with low and high levels of the metabolites, which revealed several significant associations demonstrating that there most probably was an interaction between prostate cancer and these metabolites. The results implied, that when the natural feedback system works, the risk of prostate cancer is lower both for the aggressive cancers and the non-aggressive ones. When stratifying for high PTH levels there was twofold risk of prostate cancer in relation to the vitamin D quartiles. This association seemed linear for the aggressive cases, whereas for the non-aggressive cases the risk was about the same in all quartiles, indicating that the elevated PTH rather then the vitamin D level caused the risk. This was however a subgroup analysis and the results must be interpreted cautiously.

In the overall analysis regarding PTH in relation to prostate specific mortality (paper III) there were no significant associations, however, the risk reduction demonstrated for vitamin D was strengthened stratifying for low PTH levels.

## Strengths and limitations

|  |  |  |
| --- | --- | --- |
| Paper | Strengths | Limitations |
| I | Prospective study. Large number of patients. Including metabolites related to vitamin D metabolism. Adjusted for several known risk factors. Reliable follow up. | Only one serum sample. Non fasting PTH samples. |
| II | Prospective study. Large number of patients. Data on tumour characteristics. Adjusted for several known risk factors. Reliable follow up. | Only one serum sample. Non fasting PTH samples. Few cases in some of the stratified analyses. No re-evaluation of PAD. |
| III | Prospective study. Large number of prostate cancer specific deaths. Adjusted for several known risk factors. Reliable follow up. | Only one serum sample. Non fasting PTH samples. No information about treatment. |
| IV | Prospective study. Including Vitamin D levels. Adjusted for several known risk factors. Reliable follow up. | Small number of cases in the stratified analyses. Multiple testing. |

## Conclusions

* Prospectively measured vitamin D levels are positively associated with an increased risk of prostate cancer (paper I).
* There is no difference between aggressive and non-aggressive prostate cancer in relation to vitamin D, PTH and calcium. A possible relationship was observed between Gleason score 7 tumours and vitamin D (paper II).
* Sufficient levels of pre-diagnostic vitamin D may improve survival in men with prostate cancer (paper III).
* SNPs in the VDR gene (ApaI and Bg1I) are associated with a decreased risk for prostate cancer and high vitamin D levels strengthen these association. One VDR SNP (BsmI) showed a decreased risk of prostate cancer in men with low levels of vitamin D.

## Implications and future perspectives

We could, based on the population at risk in MDCS, show that high vitamin D levels are associated with a non-linear increased risk of prostate cancer. We could not find any difference in aggressiveness of the cancer in relation to the investigated metabolites, although there was a significant finding with Gleason score 7 tumours and high vitamin D levels. Currently vitamin D supplementation is common, but perhaps caution should be taken before recommending vitamin D for prostate cancer prevention.

Our finding in paper III, that sufficient vitamin D levels may affect survival of prostate cancer could have important public health implications. Perhaps a higher dose of vitamin D supplementation should be considered for men diagnosed with prostate cancer. It has been demonstrated that patients under active surveillance may benefit from vitamin D supplementation [145] and there are studies investigating the effect of vitamin D in addition to currently available drugs to potentiate their anticancer effect [146].

The finding, that vitamin D levels above 100nmol/L provided a 75% lower prostate cancer specific mortality if diagnosed during the winter is remarkable. This may be a group that could benefit from vitamin D supplementation. Considering our results in paper IV, where vitamin D levels modified the risk of prostate cancer, it would be interesting to analyse the SNPs in relation to prostate cancer mortality.

# Populärvetenskaplig sammanfattning

Prostata är en körtel som ligger under urinblåsan och omsluter urinrörets övre del. Den producerar ett sekret som är viktigt för spermierna. Prostatans funktion och tillväxt styrs av testosteron, det manliga könshormonet. Med åldern växer prostata ofta och kan orsaka vattenkastningsbesvär.

Prostatacancer är den vanligaste manliga cancerformen med över 11000 nya fall 2014. Fram till för ett par år sedan var det också den cancerform som ökade mest, vilket berodde på en ökad diagnostisk aktivitet med PSA testning, men också pga. en ökning av antalet äldre män i befolkningen. Sjukdomen är ovanlig före 50 års ålder. De senaste 15 åren har det skett en minskning av män med prostatacancer som dödsorsak i åldern 60-80 år med upp till 35 %, men med tanke på den åldrande befolkningen har den totala dödligheten i prostatacancer varit oförändrad. Förutom ålder, ärftlighet och etnicitet finns det inte så många kända riskfaktorer.

I början på 1980-talet upptäckte två forskare i USA att mortaliteten i prostatacancer var omvänt korrelerade med breddgraden, d.v.s. UV exponeringen. De kopplade ihop detta med vitamin D, som man till största del får från solljuset. Sedan dess har man kunnat visa experimentellt att vitamin D har en hämmande effekt på prostatacancer och prostatacancerns tillväxt.

Många har försökt visa detta i befolkningsstudier, men resultaten har varierat: några studier har visat en skyddande effekt, många kan inte påvisa något samband och andra hittar ett U format samband. Forskare har också studerat genetiska variationer (SNPar), och kunnat visa att det finns vissa variationer som påverkar risken för prostatacancer.

Syftet med denna avhandling har varit att undersöka förhållandet mellan vitamin D, PTH och kalcium och 1) risk för prostatacancer, 2) risk för olika grader av aggressiv cancer, 3) död i prostatacancer samt 4) samband mellan olika SNPar associerade med Vitamin D, PTH och kalcium i förhållande till prostatacancer.

Till samtliga delarbeten har vi använt oss av material från Malmö Kost Cancer Studien, som är en stor databas och biobank i Malmö. I början på 1990-talet tillfrågades alla män i Malmö födda mellan 1923 och 1950 om de ville vara med i ett projekt där man skulle studera sambandet mellan kost och cancer. Totalt deltog 11063 män. I projektet ingick ett frågeformulär om livsstil och socioekonomiska faktorer, fysisk undersökning samt blodprovstagning. Fram till och med december 2005 hade 943 av dessa män utvecklat prostatacancer och det är de män som studerats.

Sammantaget har vi kunnat visa att det finns en ökad risk för prostatacancer vid relativt höga nivåer av vitamin D. Det finns ingen skillnad i hur aggressiv prostatacancern är beroende på nivåerna av de studerade metaboliterna. Det finns en minskad risk för död i prostatacancer vid höga nivåer av vitamin D. Inga övertygande samband observerades med PTH och kalcium. I det sista arbetet kunde vi visa att vissa av de studerade SNParnas riskeffekt på prostatacancer påverkas av vitamin D nivåerna.

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