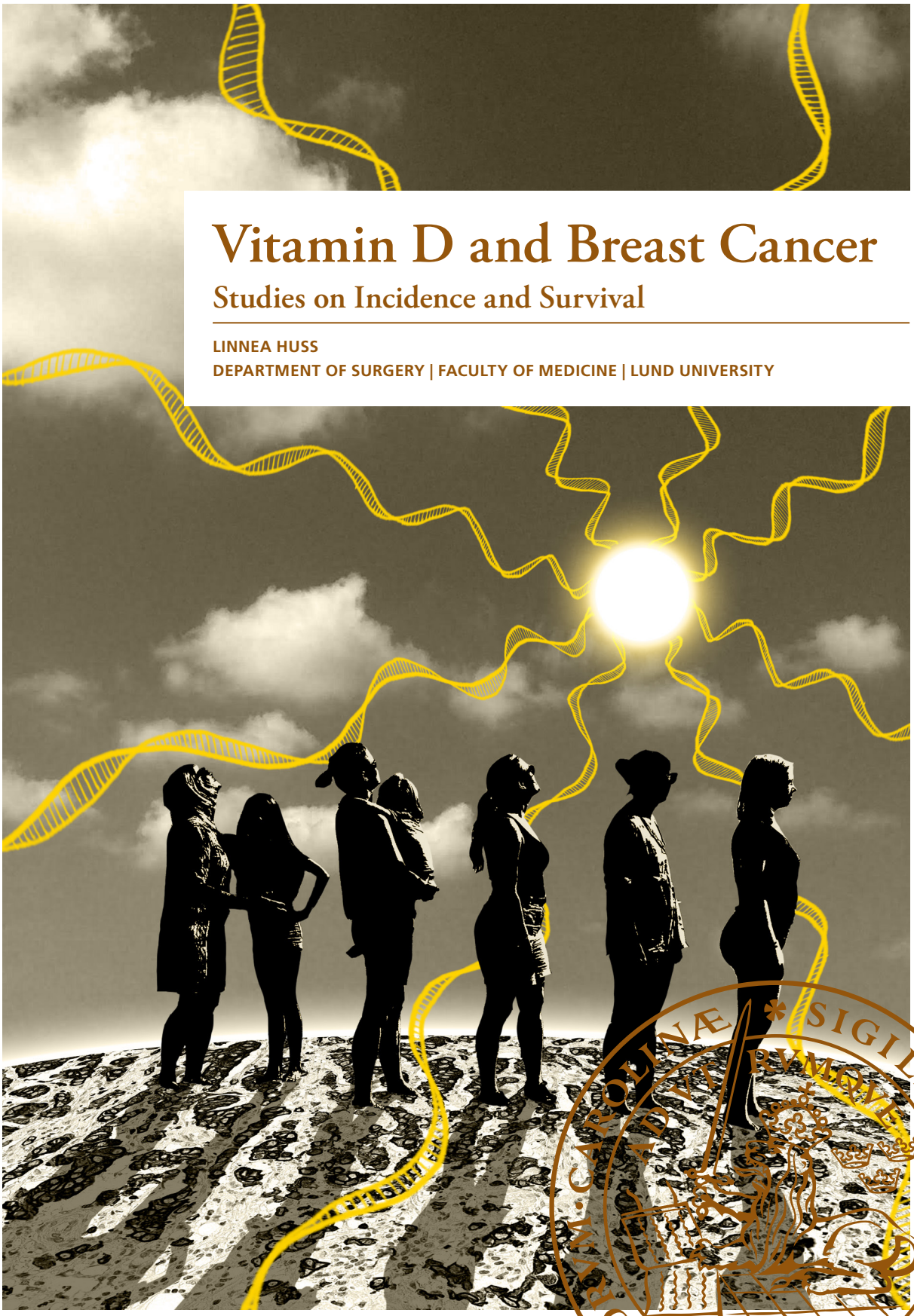
Vitamin D and Breast Cancer. Studies on Incidence and Survival.

Huss, Linnea

2019

Document Version:
Publisher's PDF, also known as Version of record



Vitamin D and Breast Cancer

Studies on Incidence and Survival

LINNEA HUSS
DEPARTMENT OF SURGERY | FACULTY OF MEDICINE | LUND UNIVERSITY

Vitamin D and Breast Cancer

Studies on Incidence and Survival

Linnea Huss

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Lilla Aulan, Jan Waldenströms gata 5, plan 1,
Skånes Universitetssjukhus, Malmö.

Friday May 10th 2019 at 1.00 p.m.

Faculty opponent

Professor Peer Christiansen, Aarhus University, Denmark

Organization: LUND UNIVERSITY	Document name: Doctoral Dissertation
Department of Clinical Sciences	Date of issue: 2019-05-10
Author: Linnea Huss	Sponsoring organization

Title and subtitle: **Vitamin D and Breast Cancer – Studies on Incidence and Survival**

Abstract

Previous research has suggested beneficial effects of vitamin D on both breast cancer risk and prognosis. The overall aim of this research project was to investigate associations between vitamin D and breast cancer. The population-based prospective cohort, the Malmö Diet and Cancer Study, recruited 17,034 women in the first half of the 1990s. Studies in in the current thesis are based on blood samples collected at baseline, analyzed for levels of vitamin D, parathyroid hormone (PTH), calcium and later also used for genetic sequencing. Breast tumors that developed in women within the cohort were included in a tissue microarray and analyzed for expression of the vitamin D receptor (VDR).

Specific aims were to investigate:

I. Serum levels of vitamin D, PTH and calcium in relation to breast cancer survival, i.e. mortality among women diagnosed with breast cancer.
II. Vitamin D-related single nucleotide polymorphisms (SNPs) and breast cancer risk.
III. Expression of VDR in association with breast cancer mortality.
IV. Levels of vitamin D in relation to expression of VDR in subsequent breast tumors.

Results and conclusions:

I. Compared to intermediate levels of vitamin D, low levels and high levels were associated with a poor survival, i.e. high risk of death related to breast cancer. No association was found between PTH and breast cancer mortality. Relatively high serum calcium levels were associated with relatively low breast cancer mortality.
II. SNPs associated with levels of vitamin D did not affect breast cancer risk. One SNP, related to the vitamin D binding protein, was associated with breast cancer risk.
III. VDR expression was associated with a favorable breast cancer prognosis.
IV. There were indications that vitamin D levels were associated with VDR expression in a subsequent breast tumor.

The association between low vitamin D levels and high breast cancer mortality may be mediated through development of a VDR-negative tumor. There was no evidence to suggest an additional beneficiary effect of vitamin D levels higher than intermediate levels.

Key words: Breast cancer, risk, prognosis, vitamin D, PTH, calcium, SNP, VDR.

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title 1652-8220		ISBN 978-91-7619-772-1
Recipient's notes	Number of pages 74	Price
	Security classification	

Vitamin D and Breast Cancer

Studies on Incidence and Survival

Linnea Huss

LUND
UNIVERSITY

Cover photo by Per Huss, Linnea Huss

Faculty of Medicine
Department of Surgery

ISSN 1652-8220
ISBN 978-91-7619-772-1

Lund University, Faculty of Medicine Doctoral Dissertation Series 2019:43

Printed in Sweden by Media-Tryck, Lund University
Lund 2019

Media-Tryck is an environmentally certified and ISO 14001:2015 certified provider of printed material.
Read more about our environmental work at www.mediatryck.lu.se

MADE IN SWEDEN

To Anja, Uno, Egon and Ina,

and all others to whom the future belongs.

Contents

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Huss L**, Butt S, Borgquist S, Almquist M, Malm J, Manjer J: **Serum levels of vitamin D, parathyroid hormone and calcium in relation to survival following breast cancer**. *Cancer Causes Control.* **2014** Sep;25(9):1131-40.

II. **Huss L,** Butt S.T, Almgren P, Borgquist S, Brandt J, Försti A, Melander O, Manjer J: **SNPs related to vitamin D and breast cancer risk: a case control study.** *Breast Cancer Res.* **2018** Jan 2;20(1):1.

III. **Huss L**, Butt S.T, Borgquist S, Elebro K, Sandsveden M, Rosendahl A, Manjer J: **Vitamin D receptor expression in invasive breast tumors and breast cancer survival**. *Submitted, February 2019.*

IV. **Huss L**, Butt S.T, Borgquist S, Elebro K, Sandsveden M, Manjer J, Rosendahl A: **Levels of vitamin D and expression of the vitamin D receptor in relation to breast cancer risk and survival.** *Manuscript.*

Abbreviations and Definitions

Abbreviations

BMI	Body Mass Index	LD	Linkage Disequilibrium
BRCA	BReast CAncer gene	MDCS	Malmö Diet and Cancer Study
CaSR	Calcium Sensing Receptor	MI	Multiple Imputation
CI	Confidence Interval	MR	Mendelian Randomization
Cis	Cancer in situ	OC	Oral Contraceptives
EGF	Epidermal Growth Factor	OEE	HumanOmniExpressExome BeadChip
ER	Estrogen Receptor		
DNA	DeoxyriboNucleic Acid	OR	Odds Ratio
FGF	Fibroblast Growth Factor	PgR	Progesterone Receptor
GWAS	Genome Wide Association Study	PTH	Parathyroid Hormone
		PTHrP	Parathyroid Hormone-related Protein
HER2	Human Epidermal Growth Factor 2	RCC	Regional Cancer Centers
HR	Hazard Ratio	SNP	Single Nucleotide Polymorphism
HRT	Hormone Replacement Therapy		
IGF	Insulin-like Growth Factor	TMA	Tissue MicroArray
ISH	In Situ Hybridization	TNM	Tumor, Node, Metastases
Ki67	Proliferation Index Factor of Kiel	VDB	Vitamin D Binding protein
		VDR	Vitamin D Receptor

Definitions

Vitamin D_3	Cholecalciferol
$25(OH)_2D_3$	Calcifediol (or calcidiol)
$1,25(OH)_2D_3$	Calcitriol
Breast cancer mortality	Breast cancer as underlying or contributing cause of death.

Thesis at a glance

Paper	Research Questions	Material and Methods	Results and Conclusions
I	Are there any associations between levels of vitamin D, parathyroid hormone, calcium and breast cancer survival?	Pre-diagnostic levels on the analytes available on 672 women with invasive breast cancer were investigated in a Cox regression analysis to find associations with breast cancer mortality.	Low and high levels of vitamin D were associated with relatively high breast cancer mortality, compared to intermediate levels. No association between levels of PTH and breast cancer mortality was found. A high level of calcium was associated with relatively low breast cancer mortality.
II	Are there any associations between vitamin D-related SNPs and risk of breast cancer? Are the associations between SNP variants and breast cancer risk, modified by levels of vitamin D?	SNPs previously associated with vitamin D available in 865 breast cancer cases and 3193 controls were analyzed in a binary logistic regression analysis to find associations with risk of breast cancer. The analyses were repeated, stratified by high/low level of vitamin D.	One out of ten investigated SNP-variants was associated with a relatively low risk of breast cancer. For three out of ten investigated SNP variants the risk of breast cancer was relatively low or high, depending on high/low level of vitamin D.
III	Are there any associations between expression of VDR in an invasive breast tumor, and breast cancer survival?	718 tumors in a TMA were evaluated for VDR expression. The results of the evaluation were analyzed in a Cox analysis to find associations with breast cancer mortality.	A breast cancer expressing VDR was associated with favorable prognostic factors and better survival.
IV	Are there any associations between levels of vitamin D and VDR expression in a subsequent breast tumor? Are the associations between VDR expression and breast cancer death, modified by levels of vitamin D?	Levels of vitamin D in 1482 women were used in binary logistic analyses to find associations with VDR-negative/positive breast cancers. Cox analyses stratified for tertiles of vitamin D were used to find differing associations between VDR expression and breast cancer mortality, depending on the tertile of vitamin D.	Pre-diagnostic levels of vitamin D may have influenced the tendency of a subsequent breast tumor to express VDR. Pre-diagnostic levels of vitamin D did not modify the association between VDR expression and breast cancer mortality.

Abbreviations: PTH – parathyroid hormone, SNP – single nucleotide polymorphism, VDR – vitamin D receptor, TMA – tissue microarray.

Populärvetenskaplig sammanfattning på svenska

D-vitamin är ett hormonliknande ämne som bildas i huden under inverkan av solljus. D-vitamin finns även i vissa födoämnen som till exempel fet fisk, ägg, kött och vild svamp. Mjölkprodukter, och växtbaserade drycker av tex havre och soja berikas med D-vitamin.

När D-vitamin först upptäcktes, var det för sin unika egenskap att göra skelett hårt genom att styra kalkinlagring i skelettet. Om det inte fanns tillgång till D-vitamin drabbades barn av engelska sjukan, som bland annat gav upphov till skelettmissbildningar.

I slutet av 1900-talet upptäckte man att det mottagarprotein som aktivt D-vitamin binder till i kroppen (D-vitaminreceptorn, VDR), finns på en mängd andra platser i kroppen, och sedan dess har D-vitamin tillskrivits en rad positiva egenskaper. En del mer underbyggda än andra. Bland annat noterades man att kvinnor som bor längre från ekvatorn oftare drabbas av bröstcancer, än de som har bättre tillgång till solljus. Man tyckte sig också se att kvinnor som drabbas av bröstcancer under sensommaren och hösten, då D-vitaminnivåer i blodet är som högst, överlever sin sjukdom i högre grad än de som drabbas under våren.

I det här forskningsprojektet har vi undersökt om det finns samband mellan D-vitamin och risk att drabbas av bröstcancer, samt om D-vitamin påverkar möjligheten att överleva sin sjukdom om man drabbas av bröstcancer.

Samtliga delarbeten som ingår i projektet bygger på information och prover som man samlat in från 17,034 kvinnor som under första halvan av 90-talet valde att delta i en stor studie som heter Malmö Kost Cancer. Av alla dessa kvinnor har en liten andel senare drabbats av bröstcancer, och information om deras tumörer samt prover från tumörer har också använts i projektet.

Det första delarbetet undersökte om det fanns samband mellan nivåer av D-vitamin, i blodprover tagna då kvinnorna gick med i kost-cancer-studien, och risken att dö av sin bröstcancer. Utöver D-vitamin undersöktes även nivåer av parathormon och kalcium, som samverkar med D-vitamin i kroppen. Resultaten från de statistiska analyserna visade att kvinnor med medelhöga nivåer av D-vitamin i högre utsträckning överlever sin sjukdom, jämfört med de som har lägre eller högre nivåer. Vad gällde parathormon kunde vi inte finna något samband, men höga kalciumnivåer verkade vara förenat med en bättre överlevnad.

I delarbete nummer två identifierades små genetiska variationer, single nucleotide polymorphisms (SNPar), som tidigare kopplats samman med D-vitamin på något sätt. Dessa variationer användes sedan för att räkna på om de hade ett samband med risken att insjukna i bröstcancer. Utifrån dessa beräkningar kunde vi komma fram

till att SNP-varianter som verkade påverka nivåer av D-vitamin i kroppen, antagligen inte påverkar risken att insjukna i bröstcancer i någon större utsträckning. Däremot kunde vi finna att en SNP-variant som tidigare kopplats samman med förmågan att binda D-vitamin i blodet, verkade påverka risken att drabbas av bröstcancer. Dessutom såg vi att för några av de SNP-varianter som vi undersökte, verkade sambandet med risken att drabbas av bröstcancer styras av vilken nivå av D-vitamin man hade, innan man fick sjukdomen.

Inför delarbete tre och fyra genomfördes en mikroskopisk bedömning av förekomsten av D-vitaminreceptorn i bröstcancertumörer från kvinnor i Malmö Kost Cancer Studien. I arbete tre undersöktes sedan samband mellan förekomst av D-vitaminreceptorn i bröstcancercellernas olika delar och risken att dö av bröstcancer. Då fann vi att om det förekom D-vitaminreceptorer i tumörcellernas kärnor och/eller cytoplasma (cellens kropp) hade man en betydligt ökad chans att överleva sin sjukdom.

I delarbete fyra länkades information om D-vitaminnivå i blodet samman med informationen om D-vitaminreceptorförekomst i bröstcancer. Utifrån statistiska analyser kunde vi konstatera att det möjligen är så att D-vitaminnivåer i blodet påverkar om det bildas D-vitaminreceptorer i en bröstcancertumör. Vi kunde dock inte se att den nivå man har av D-vitamin i blodet interagerar med förekomsten av D-vitaminreceptorer med hänsyn till överlevnad i bröstcancersjukdom.

Sammanfattningsvis konstaterar vi att nivåer av D-vitamin sannolikt inte påverkar den generella risken att drabbas av bröstcancer, men för en individ med ett visst genetiskt uttryck skulle nivån av D-vitamin kunna ha betydelse. Lagom nivåer av D-vitamin i blodet verkar vara bäst om det gäller att överleva sin bröstcancersjukdom. D-vitaminreceptorer i en bröstcancer tycks vara förenat med en bättre överlevnad.

Introduction

Since the turn of the millennium, vitamin D has gained a reputation for being some sort of miracle drug. According to providers of supplements, nine out of ten people around the world are vitamin D-deficient, and vitamin D reduces a person's risk of flu, diabetes, muscle aches, cardiovascular disease, depression, autoimmune disease, osteoporosis and cancer [1, 2]. No wonder sales have soared [3].

The science backing up these statements is not at all conclusive, although there has been an increasing scientific interest in studying associations between vitamin D and different aspects of health [4-6].

This thesis is based on four papers analyzing epidemiologic associations between vitamin D and different aspects of breast cancer, in an attempt to provide more scientific results to help determine whether or not an association between vitamin D and breast cancer can be established.

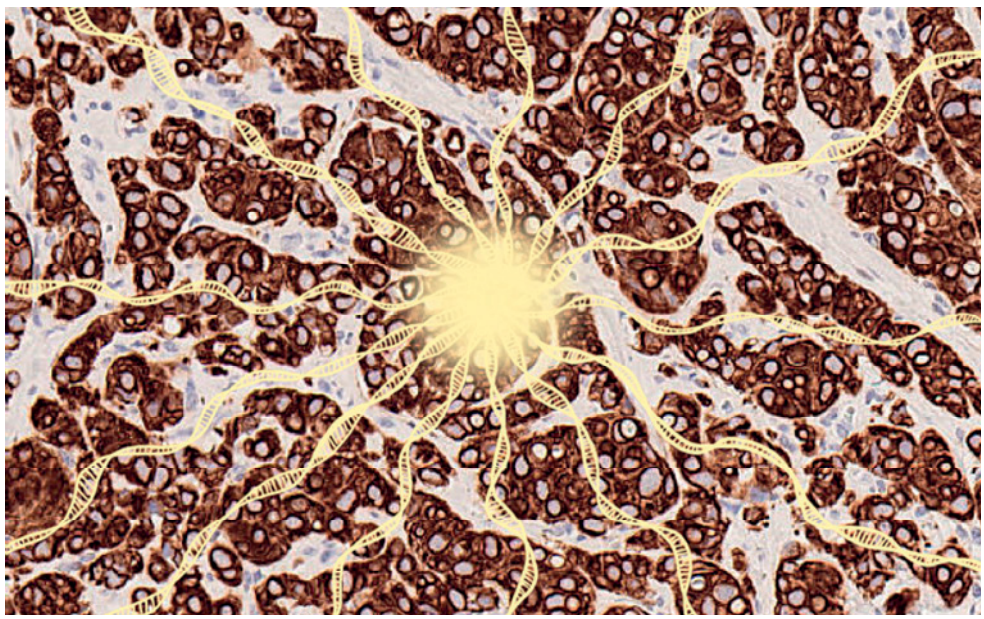


Figure 1: Breast cancer with vitamin D receptor expression. Influenced by vitamin D?

Breast cancer

In 2018, over two million women worldwide were diagnosed with breast cancer, and although modern treatment can cure the disease for many, over 625,000 women died from it [7]. This means that breast cancer is the leading cause of death from cancer for women globally, and cancer is the leading cause of premature death (deaths before the age of 70) in most European countries, according to the World Health Organization [7].

In Sweden, 7,558 women were diagnosed with invasive breast cancer during 2016, and the disease caused 1,319 female deaths that same year [8]. At the same time there were 108,579 women living with the disease and the relative 10-year survival after a breast cancer diagnosis is as high as 86.1% [8].

Clinical breast cancer

For 60% of women affected by breast cancer in Sweden, the diagnosis is discovered as a result of mammographic screening, and may therefore not have given any symptoms prior to diagnosis [8]. Most other breast cancers are first recognized after a woman notices a lump in her breast [9]. Other symptoms that may indicate a cancer are secretion from the nipple, a retracted nipple, a change in size or shape of the breast, a discolored skin or changed skin structure. A cancer that has metastasized may present a range of other symptoms related to the location of metastases [10].

Triple diagnostics is the norm, when any woman (or man) presents symptoms that could be associated with breast cancer. This includes; clinical examination, with palpation of breasts and lymph-nodes in axilla and along claviculae; imaging, usually with both mammography and ultra-sound; and histopathological evaluation of a tissue sample [9].

In Sweden, every breast cancer diagnosis is discussed in a multidisciplinary conference, attended by surgeons, oncologists, pathologists, radiologists and contact nurses. All aspects of the patient, such as history of other disease, size and location of the tumor, suspicion of lymph-node involvement, histology of the tumor, are taken into consideration when the conference suggests a treatment. Usually surgery is the primary treatment of choice, but for large tumors, neoadjuvant oncologic treatment is sometimes suggested [9, 11].

Surgery may be performed as a mastectomy when the whole breast is removed, or as a partial mastectomy, when the tumor and surrounding tissue is excised. Usually there is also some sort of axillary surgery at the same time, often a sentinel node biopsy, when a marker injected in the breast is traced to the lymph node/-s primarily

draining lymph from the tumor area. The sentinel node is sent for pathological evaluation and if metastatic spread is discovered in this examination, a subsequent axillary dissection may be performed [9].

After surgery the patient is again discussed in a multidisciplinary conference which suggests if and what adjuvant treatment should be recommended to the patient. Depending on the histopathology of the tumor and on other patient factors, a range of adjuvant oncological treatments are available, including radiation, endocrine therapy, chemotherapy, and immunological therapy, to reduce the risk of recurrent disease. To aid in the recommendation of treatment there is a national care program for breast cancer in Sweden, with adaptations by regional cancer centers (RCC) [9, 11].

Development of a breast tumor

The breast is an epithelial organ composed of various proportions of glandular and adipose tissue [10]. Its normal growth and function (lactation) is influenced by sex hormones (estrogen and progesterone), growth factors (epidermal growth factor (EGF), fibroblast growth factors (FGF) and insulin-like growth factors (IGF)) and other agents which up- and down-regulate different genetic pathways in order to make cells proliferate or regress. When a tumor evolves the initial tumor cells have to overcome a series of normal defenses over a long period of time. Starting with a genetic predisposition, new defects in the transcriptional process lead to a cascade which in turn leads to a deregulated cell cycle, when tumor cells acquire an ability to avoid regulating feedback loops and sustain a chronic state of proliferation without inducing apoptosis [12].

A breast cancer in situ has acquired the ability to divide endlessly, but does not have the ability to traverse the basement membrane [12]. In order to do so the tumor must produce specific gene products, such as hyaluronidase. When the tumor is invasive it can invade surrounding tissue, and disseminate either via the lymphatic drainage system or by intravasation through blood vessels to secondary sites, usually the skeleton or the liver [8, 9].

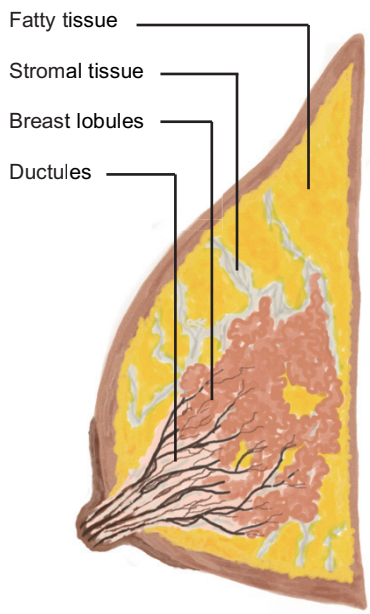


Figure 2: Illustration of a female breast.

Most cancers of the breast are derived from the epithelial tissues, the lobules and ductules that the glandular tissue consists of [10]. Only a very small proportion of cancers emanate from stromal tissue, and such phyllodes tumors exhibit different characteristics compared to the more common ductal carcinomas (90% of breast cancers) and lobular carcinomas (8% of breast tumors) [10].

Epidemiology

Over the past 50 years the incidence of breast cancer has increased but during the same time mortality has decreased (Figure 3) [13]. Mammography screening was gradually introduced in Sweden during the 1980s through the 1990s and is thought to have had an effect on both breast cancer incidence and mortality [9]. Some tumors that would never otherwise have been diagnosed are discovered during the screening program, but as tumors are discovered early in the course of the disease, treatment is more successful.

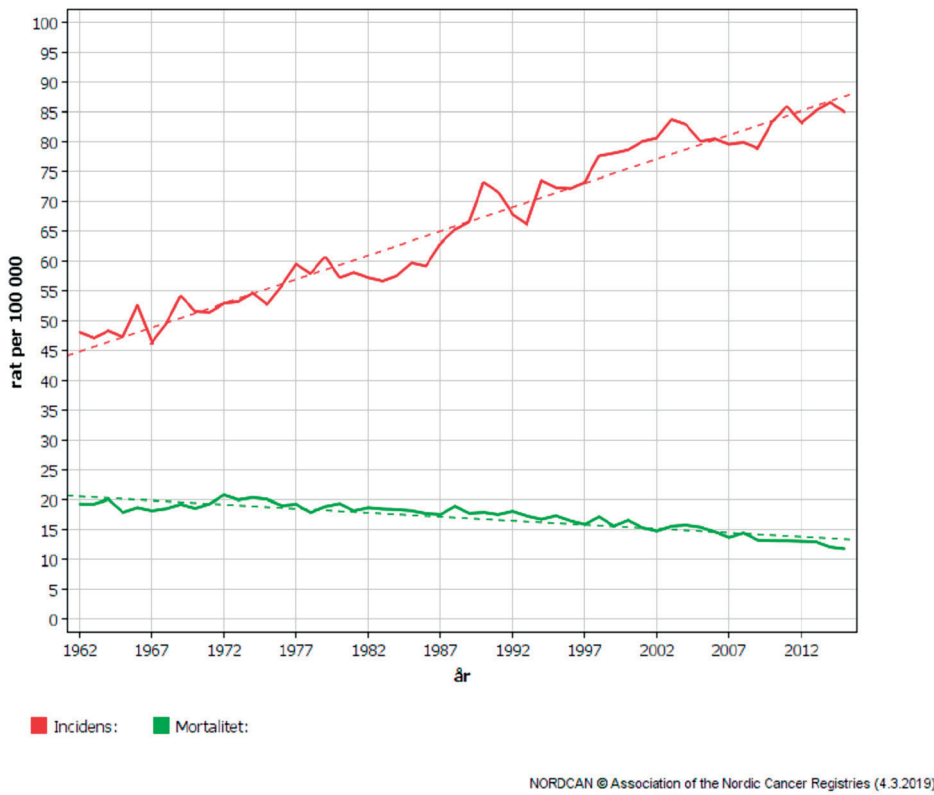


Figure 3: Breast cancer incidence and mortality in Sweden.

Since the increase in breast cancer incidence was seen prior to the introduction of the screening program, other contributing factors, such as changes in lifestyle, in child-bearing patterns and in socioeconomic factors are believed to cause the high breast cancer incidence of today [9].

Improved adjuvant cancer therapy contributes to the decreased mortality rates of breast cancer [9].

Risk factors

The main risk factors of breast cancer are summarized in Table 1 [10]. Breast cancer is typically a woman´s disease, and one out of ten Swedish women is diagnosed with breast cancer before the age of 75 [8]. The second most important risk factor is age, and there is a greater risk of breast cancer with increasing age, but the highest incidence is seen in those aged 50-69 [14].

A family history of breast cancer increases the risk of the disease. When two close relatives have had the disease, and they have been diagnosed at a young age, the risk of being diagnosed with breast cancer is four times higher compared to the risk of breast cancer with no heredity of the disease [10]. Several genes responsible for increased risk have been identified. The high-penetrance genes BRCA1 and BRCA2 are associated with a very high individual risk of breast cancer. If you are a carrier of BRCA1 your lifetime risk of getting breast cancer is 72% [15], but fortunately these genes are fairly uncommon and account for only approximately 4% of all breast cancers [10]. The remainder of familial breast cancers originate from moderate penetrance genes (for example, CHEK2 and ATM) and low penetrance genes, such as SNPs, which each only give a slightly increased breast cancer risk for an individual, but since they are quite common in the population they may lead to a considerable number of tumors [16].

Estrogen also plays an important role in breast cancer development [8, 9]. It has been shown that both exogenous supply from oral contraceptives and replacement therapy [17-20], and a comparably high endogenous production during a lifetime, due to early menarche and late menopause, increase the risk of breast cancer [21, 22]. Also related to the hormonal influence of the breasts are the risk factors of nulliparity, high age at first childbirth, and absence of breast feeding [23, 24].

Also, high BMI after menopause may cause an increased risk of breast cancer mediated through higher levels of estrogen in the breast due to storage of hormone in adipose tissue [10]. Another risk factor related to body constitution is that taller women have a comparably high risk of breast cancer. Women that have a greater proportion of glandular tissue in their breast also have a higher mammographic density, which has been associated with an increased risk of breast cancer.

A few risk factors for breast cancer are modifiable. For example, it has been shown that not drinking alcohol is associated with a low breast cancer risk and exercise also reduces the risk [10, 25].

Table 1: Risk factors for breast cancer and associations with prognosis.

Risk factor	High risk	Poor prognosis
Gender	Female	Male
Age	Increasing age	Age < 40 or > 80
Genetic	High-penetrance genes (BRCA1, BRCA2) Moderate penetrance genes (ex. CHEK2) Low penetrance genes (SNPs)	BRCA1, BRCA2
Body constitution	High BMI (post menopause) Tall height High mammographic density	High BMI
Medical history	Previous breast disease	
Socio-demographic factors	High socioeconomic status High educational level Life in developed countries Urban life	Low socioeconomic status
Radiation	High dose to the chest	
Hormonal factors	Early menarche (<12 years) Late menopause (>55 years) Oral contraceptives (OC) Hormone replacement therapy (HRT)	OC and/or HRT => good prognosis
Parity	No children Late first pregnancy (>30 years) No breastfeeding	
Lifestyle factors	Alcohol Low level of physical activity	Physical inactivity

Prognostic factors

Many well-known risk factors for breast cancer are also associated with breast cancer prognosis, sometimes related to a worse prognosis, but sometimes, in contrast, related to a favorable prognosis (Table 1) [10, 26].

Other prognostic factors for breast cancer are mainly found in the tumor itself at the time of diagnosis. The tumor – lymph node – distant metastases (TNM) classification provides a measure of how advanced the tumor is [9]. This can be

translated into a grading scale of stages, which ranges from stage 0, pre-invasive disease, to stage IV which is metastatic.

The strongest factor for a favorable prognosis is a disease-free axilla, i.e. no cancer in regional lymph nodes (N in the TNM-classification). The size of the tumor is also a strong prognostic factors (T in the TNM-classification) [9].

Information that a histopathological examination contributes, also predicts outcome to a great extent [27]. A modern Swedish pathological report includes information on histological type (cellular origin), histological grade (aggressiveness), hormonal receptor status (estrogen receptor (ER) and progesterone receptor (PgR)), human epidermal growth factor 2 (HER2) and the proliferation index factor of Kiel (Ki67). All factors are weighed together in order to predict an outcome, and the prediction is used to suggest adjuvant therapy [9].

Vitamin D

During the early 1920s it was discovered that both sunlight and fish liver oil could be used to treat children with rickets, and shortly thereafter it was discovered that the steroid named vitamin D was present in some foods but could also be synthesized in human skin under the influence of UV light [28]. Since then it has been suggested that vitamin D plays a role in several other aspects of health, and associations between vitamin D and skeletal, malignant, cardiovascular, auto-immune, infectious and metabolic diseases, among others have been studied, but no evidence of a clear association with any outcome has been found [29].

Since vitamin D can be synthesized in the skin when it is exposed to sun, there is a seasonal variation of vitamin D levels in countries far away from the equator, such as Sweden. During the winter months, Swedes have to rely on food sources such as fortified dairy products, fatty fish, meat, eggs and wild mushrooms to maintain adequate levels of vitamin D [30]. It is debated what adequate levels are, and to date

Figure 4: Dietary sources of vitamin D.

there is no clear consensus, although older guidelines of The Committee of the Institute of Medicine state that 40-50 nmol/liter is the lower acceptable level and levels above 125 nmol/liter should be regarded as too high [31].

Vitamin D, parathyroid hormone and calcium

The first discovered function of vitamin D was its association with calcification of the skeleton. Most calcium, 99%, is deposited in the skeleton but it takes part in a myriad of molecular functions in the body [32, 33]. Calcium is needed to make muscles contract, to transmit neurologic signals, to regulate the secretion of enzymes and hormones, and also to regulate the cell cycle and it is involved in the expression of genes. The blood level of calcium is tightly regulated by vitamin D in interaction with parathyroid hormone (PTH) (Figure 5), and calcium has a narrow reference interval in plasma (2.20-2.60 mmol/liter) [32, 33].

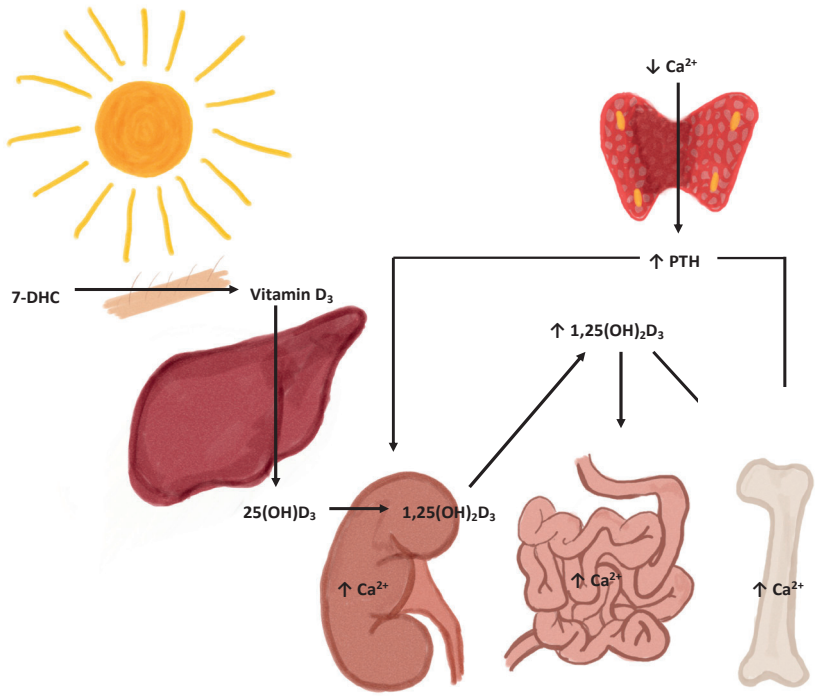


Figure 5: Regulation of calcium-levels: Parathyroid glands respond to lowered levels of calcium and produce PTH. PTH induce the kidney to activate vitamin D ($1,25(OH)_2D_3$), and reabsorb calcium from the urine. PTH in conjunction with $1,25(OH)_2D_3$ stimulates the skeleton to release calcium. $1,25(OH)_2D_3$ increases calcium absorption in the small intestine.

7-dehydrocholesterol (7-DHC) is converted into vitamin D_3 by ultraviolet light from the sun.

Parathyroid hormone was also discovered in the 1920s and is produced in the very small, parathyroid glands located adjacent to the thyroid gland. It is secreted when the level of calcium in the blood is low, and has a half-life of about four minutes. Aside from being part of calcium regulation PTH is also involved in the regulation of magnesium and phosphor levels.

Vitamin D and breast cancer

Prior to the first study included in this thesis, there was some research to suggest a possible association between vitamin D and breast cancer risk and survival [34-39]. Animal and in vitro studies had shown that vitamin D had anti-proliferative effects on breast tumor cells [34]. Others suggested an association between sunlight exposure and risk of breast cancer and cancer survival [36-40]. Studies on levels of vitamin D and risk of breast cancer had shown conflicting results [34, 35, 41, 42], but more recent studies suggest that there is no linear association between higher levels of vitamin D and a reduced risk of breast cancer [43-45].

Studies on vitamin D levels and breast cancer mortality are more conclusive. Low levels measured either at the time of diagnosis or pre-diagnostically have been associated with a relatively high breast cancer mortality, compared to sufficient vitamin D levels [46, 47]. Results concluding a beneficial effect of sufficient vitamin D levels on breast cancer survival has since then been replicated [48].

Parathyroid hormone and breast cancer

Regarding PTH, epidemiological studies prior to ours suggested an association between primary hyperparathyroidism, i.e. an overproduction of PTH, and a high risk of breast cancer [49-52]. Previous experimental studies showed PTH or the PTH-related protein (PTHrP) or their common receptor to have carcinogenic and tumor-promoting effects [53-56]

More recent studies have shown that a high expression of PTHrP in breast cancer nuclei is associated with a better breast cancer prognosis [57], and that PTH may increase skeletal tumor growth in a breast cancer in vivo model [58]. Intermittent PTH administration on the other

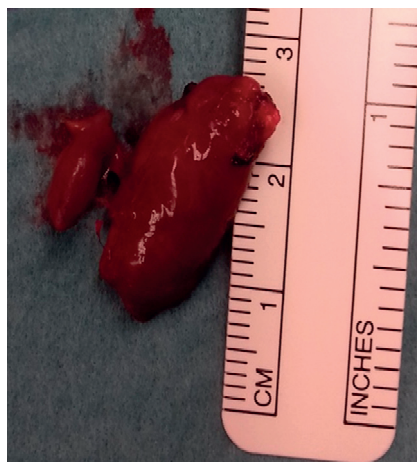


Figure 6: Adenomas of the parathyroid. A possible cause of overproduction of PTH.
Photo by: Erik Nordenström, Daniel Ansari.

hand, seem to decrease skeletal metastases and improve survival in mice models with disseminated breast cancer [59]. Hence, there are reasons to believe that levels of PTH might influence breast cancer prognosis.

Calcium and breast cancer

It is known that high serum levels of calcium may follow a malignant diagnosis [33]. It has also been shown that increasing levels of calcium, reduce proliferation and increase differentiation of breast cancer cells in vitro [60].

The calcium sensing receptor (CaSR) was first recognized by its ability to sense changes in extracellular free calcium levels and thereby regulate secretion of PTH [61]. CaSR is also expressed in normal [62] and breast cancer cells [63]. Results from previous studies on the direct effect of CaSR signaling on cancer development and progression have been inconsistent [64], but it appears that PTHrP production in breast cancer cells is regulated by CaSR, and therefore CaSR might have an indirect effect on the ability of a tumor to cause bone metastases [65].

Single nucleotide polymorphisms

The human genome is typically to more than 99% identical between two individuals [66]. The differences may consist of variants of complete genes that are built from a long sequence of nucleotides. Some differences are due to very small variants when only a single nucleotide is exchanged for another. These small variations are

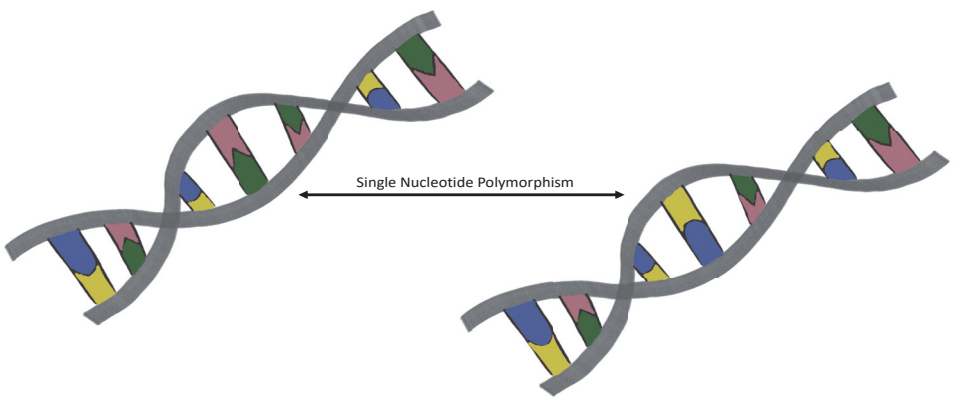


Figure 7: A SNP is a variation of only one nucleotide in a DNA sequence.

called single nucleotide polymorphisms (SNP) (Figure 7). Although small, it has been shown that such small variations may affect the susceptibility to a wide range of diseases, and breast cancer is one of them [67]. SNPs may also be associated with other traits, (for example levels of vitamin D), [68-72] and in genome wide association studies (GWAS) researchers have scanned whole genomes to identify SNPs associated with different traits [67-72].

In a Mendelian Randomization study a pattern of SNPs associated with a specific trait is studied in association with a disease [73]. This way an unbiased estimate of an effect of a modifiable factor on an outcome can be obtained, since genotypes are assigned randomly at conception (Figure 8).

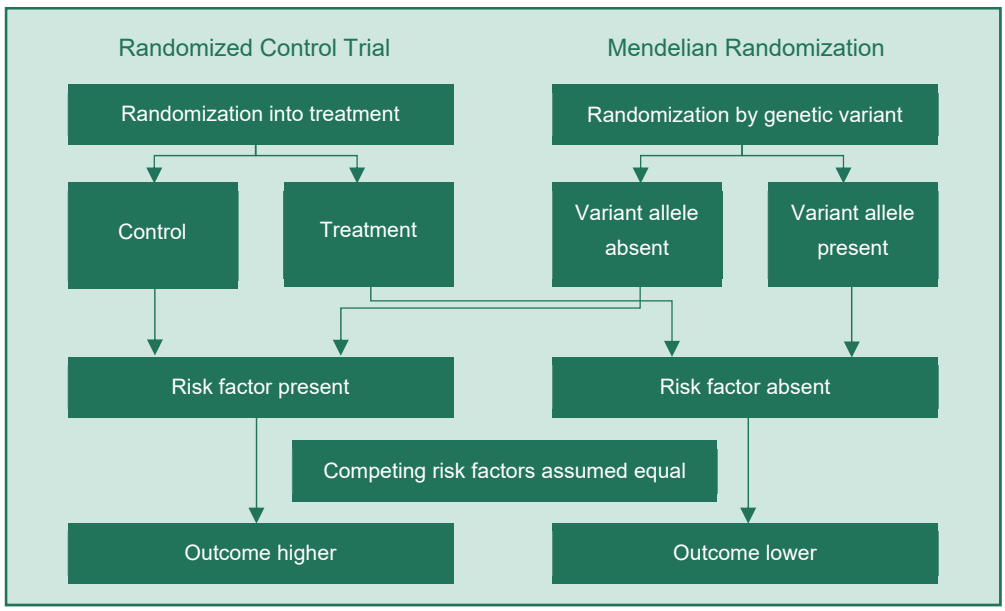


Figure 8: The theory of Mendelian Randomization.

Vitamin D receptor

Active vitamin D (1,25-(OH)2 vitamin D3) exerts its effects through binding to the VDR [74, 75]. VDR is regarded a nuclear receptor with regulatory effects on gene expression. VDR was first identified in breast cells using radioactive 1,25-(OH)2 vitamin D3 [76], and has also been found in breast tumor cells [77-80].

Previous studies on expression of VDR receptor in association with breast cancer prognosis have shown somewhat conflicting results [81-86]. Early studies were

fairly small and could not establish any associations with other prognostic factors or breast cancer survival [84, 85]. Neither could a study from 2002, in which 228 breast cancers were evaluated [86], but in a study of 82 patients (published in 2012) associations were found with tumor size, lymph node involvement, progression-free survival and overall survival which indicated that VDR expression in a breast tumor was associated with a favorable prognosis [83]. The largest study to date involved 1,114 female patients and showed an association between VDR expression in breast tumors and less aggressive tumor characteristics, but not with better survival [81].

Study aims

The overall aim was to investigate associations between vitamin D and breast cancer. Papers included in the thesis more specifically had the following aims:

- *Paper I*
  - To study whether pre-diagnostic levels of vitamin D and the closely related parathyroid hormone and calcium were associated with breast cancer mortality.

- *Paper II*
  - To study whether or not SNPs associated with vitamin D could also be related to breast cancer risk.
  - To study if any association between vitamin D SNPs and breast cancer risk was modified by levels of vitamin D.

- *Paper III*
  - To study whether the expression of VDR in invasive breast tumors was associated with breast cancer mortality.

- *Paper IV*
  - To study whether pre-diagnostic levels of vitamin D were associated with the expression of VDR in a subsequent breast tumor.
  - To study whether an association between VDR expression and breast cancer mortality was modified by pre-diagnostic levels of vitamin D.

Materials and methods

The Malmö Diet and Cancer Study

Malmö is the third largest city of Sweden. At the beginning of the nineties, all women living in Malmö, born between 1923-1950, were invited to take part in a population-based cohort study, the Malmö Diet and Cancer Study (MDCS). A total of 17.035 women (43% of eligible subjects) completed baseline examinations during 1991-1996 [87, 88]. Blood samples were drawn, anthropometric measurements performed by a registered nurse, and all participants completed a questionnaire including questions on previous diseases, medications, diet and lifestyle factors. All participants signed a written informed consent [87].

Study populations

Women included in the MDCS were and are followed by record-linkage to the Swedish Cancer Registry and the Swedish Cause of Death Registry. All studies included in this thesis are based on the MDCS. As 576 women had been diagnosed with breast cancer prior to the baseline examinations, the cohort available for studies on incident breast cancer included 16.459 women. Figure 9 is a flowchart overviewing study populations in papers I-IV.

Paper I

This study included women diagnosed with breast cancer before December 31st 2006, n=766. Two of these women had not donated blood at baseline examinations, hence 764 women were included. Since this was a mortality study and in situ cancers are associated with very low (if any) breast cancer mortality, all tumors that were only ductal cancer in situ were excluded, n=77. Due to difficulties in pairing pathological information bilateral tumors, n=15, were also excluded. The final study population included 672 women. The end of follow up was December 31st 2010.

Paper II

In 2012-2013 genotyping was performed on women diagnosed with breast cancer until December 31st 2009 (n=946) [41], on women included as controls in a previous case-control study on breast cancer risk (n=704) and on women included in a

randomized subsample of the MDCS, the cardiovascular cohort (n=2,658) [89]. Some of these women, 45 of the cases and 27 of the controls, had no samples available for genotyping. Quality control of the genotyped material, led to exclusion 14 of the cases and 37 of the controls. Further exclusions were made (22 cases, 105 controls) since some women in the cohort were related. The final study population included 865 cases and 3,193 controls with available SNP data, and 700 cases and 643 controls with data on both SNPs and pre-diagnostic levels of vitamin D.

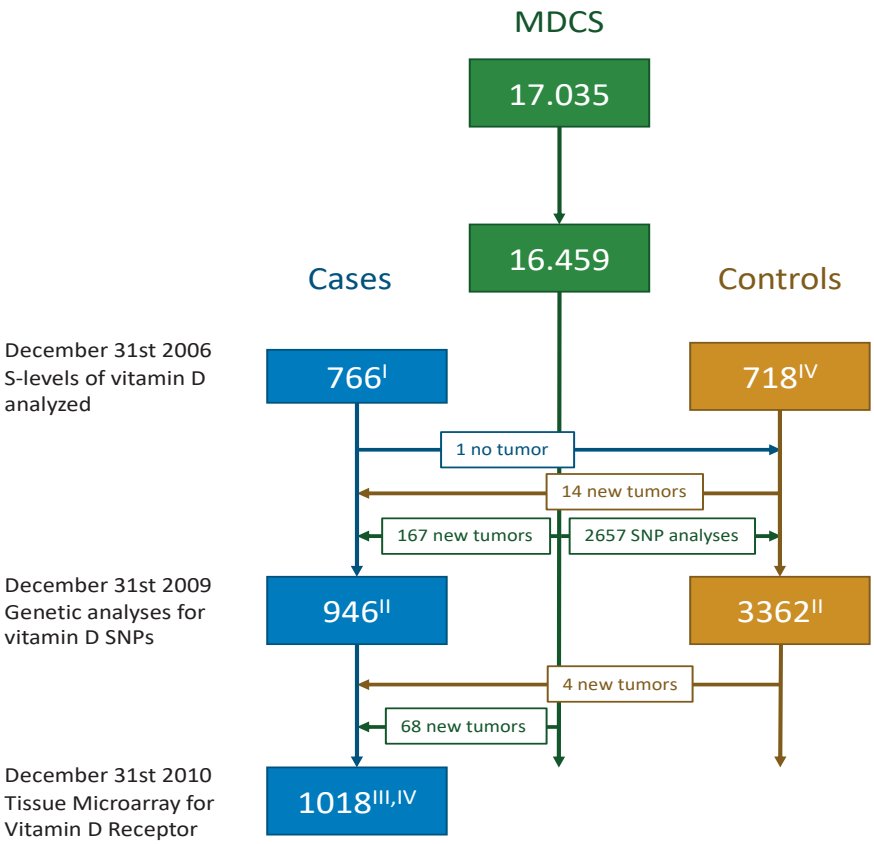


Figure 9: Flowchart, overview of women included in study populations in papers I-IV.

Paper III

Until December 31st 2010, 1,018 women within the MDCS were diagnosed with breast cancer. Invasive tumors from this group were included in a TMA. Excluded from the study were women diagnosed with bilateral breast cancer (n=17), women who had distant metastasis at the time of diagnosis (n=14), women who had received

oncologic treatment prior to surgery (n=4), one woman who died before surgery was performed, one who refused treatment for four years, and another one who was diagnosed post mortem. This meant that a total number of 912 possible tumors were included in the TMA, but there was no available tissue to use for 194 tumors. It was not possible to score VDR expression regarding nuclear fraction in 40 tumors, or cytoplasmic fraction and intensity in 39 tumors. The final study population consisted of 678/679 women who were included in the mortality analysis.

Paper IV

VDR scores of nuclear fraction from paper III, and S-levels of vitamin D used in papers I and II, were combined for analyses in paper IV. Out of the 678 cases with available nuclear VDR expression, the original vitamin D analysis had failed in 13 cases, and on 168 of the cases (diagnosed after December 31st 2006) no vitamin D analysis was ever performed. Out of the 718 women with vitamin D measurements included as controls in a previous case-control study, 18 developed a breast cancer until December 31st 2010. There was one woman who was later concluded misdiagnosed, and she was instead included in the control-group. Hence the control-group contained 701 women, although the vitamin D analysis had failed with 10 of them. The final study population included 497 cases and 691 controls with valid information on vitamin D levels and VDR expression, but after multiple imputation (MI, see below) all 912 cases and 701 controls were included.

Ethical approval

All studies included in this thesis were approved by the ethical committee in Lund. The registration numbers given were for the MDCS (LU51-1990), for papers I, III and IV (LU652-2005 and LU23-2007) and for paper II (LU153-2004 and LU682-2009).

Clinical information

Baseline examinations in the MDCS provided information on weight and height of all included women [87]. The questionnaire that was completed at baseline, included questions on previous disease and treatments, and on lifestyle, diet and reproductive factors, and provided data on such information. Medical records and clinical notes were consulted to retrieve information on type of surgery, axillary surgery, lymph node status and planned adjuvant treatments on all women diagnosed with breast cancer.

Histopathological analyses

Papers I, III and IV included information from histopathological analyses of invasive tumors. The first primary breast cancer within the MDCS was diagnosed in December 1991, and the last included in papers III and IV was diagnosed in December 2010. During these 19 years, histopathology had evolved. Therefore, one senior pathologist reevaluated all tumors diagnosed before December 31st 2004 regarding size, histological type, grade, hormone receptor status, Ki67 and HER2 status [90, 91]. From 2005 until December 31st 2007 a tissue microarray (TMA) was used to obtain information on HER2 and Ki67 [92], but information on size, grade, histological type and hormone receptor status was retrieved from histopathological reports. From 2008 and onwards, all histopathological information used in the studies was retrieved from histopathological reports.

Hormone receptor status; ER and PgR were regarded as positive when the proportion of stained malignant cells exceeded 10%. HER2 status was defined using in situ hybridization (ISH) when available. When immunohistochemistry was used, a HER2 score of 3+ was regarded as positive, 0 or 1+ was negative and 2+ was categorized as missing if ISH was not used to confirm a result [93]. When the expression of Ki67 was evaluated, the distribution differed between different periods of diagnosis (1991-2004, 2005-2007, 2008-2010). Within these time periods, the expression of Ki67 was divided into tertiles, and set as low, medium or high, based on the tertile.

The first paper did not use HER2 and Ki67, as the TMA evaluation contributing this information for the time period 2005-2007 was not complete at the time of the study. Papers III and IV used data on HER2 and Ki67, in adjusted statistical analyses. The molecular subtypes used in these papers adheres to a local modification of intrinsic subtypes used by the South Swedish Health Care Region for prognostic evaluation of tumors diagnosed within this region [14]. The categories are defined as follows:

- Luminal A like: ER-positive and Low proliferative:
    - Histological grade 1
    - Histological grade 2 and low Ki67
    - Histological grade 2, intermediate Ki67 and PgR-positive
- Luminal B like: ER-positive and High proliferative:
    - Histological grade 3
    - Histological grade 2 and high Ki67
    - Histological grade 2, intermediate Ki67 and PgR-negative
- HER2-positive: all HER2-positive as defined above.
- Triple negative: ER-negative, PgR-negative and HER2-negative

Levels of vitamin D, parathyroid hormone and calcium

At baseline examinations of MDCS, blood was collected and within one hour, serum was extracted and the samples were thereafter stored in a biological bank at -80°C [94]. Serum from women diagnosed with breast cancer before December 31st 2006 and from controls matched on time of sample (±15 days), menopausal status and age at inclusion (± 2 years) were retrieved from the biobank and analyzed for $25OHD_3$, PTH and calcium. The blood samples had not been thawed previously and the samples were examined randomly regarding case-control order and time of baseline examinations [41]. $25OHD_3$ was analyzed using high pressure liquid chromatography (HPLC), PTH with an Immulite® 2000 Intact PTH immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA), and total calcium by using a neutral carrier ion-selective electrode [95].

Genetic analyses

For paper II, there were blood samples available from baseline examinations of MDCS for 901 cases and a total of 3,335 controls, which were used to extract DNA. The population of controls were a combination of controls used in a previous case-control study [41] and women with no breast cancer diagnosis from a randomly selected subsample of the MDCS, the cardiovascular cohort [89]. Genotyping was performed using the HumanOmniExpressExome BeadChip (OEE) version 1.0 or 1.1 CH37 and iScan System (Illumina, San Diego, CA, USA). The Broad institute of MIT and Harvard University (Cambridge, MA, USA) performed genotyping on 804 cases and 3,244 controls, and the remaining 97 cases and 91 controls were genotyped at the Clinical Research Center, Skåne University Hospital, Malmö, Sweden [96]. All genotyping was performed during 2012-2013.

Quality control

Quality control of genotyped data was performed using PLINK version 1.07 software [97].

- SNPs were excluded if:
    - There was a lack of variation in a European population (monomorphic).
    - There was a deviation from Hardy Weinberg equilibrium in the controls ($p<10^{-6}$).
    - The variant call rate was <95% in all samples.

- Subjects were excluded if:
    - There was an individual call rate of <95%.
    - There was an individual excess of heterozygosity.
    - There was a first- or second-degree relationship between individuals in the cohort. (The person with the highest call rate was kept in the population.)

SNP selection

The GWAS catalog was used to identify SNPs previously associated with vitamin D [98]. The search string "vitamin D" rendered 20 SNPs, and out of those, nine were genotyped on the OEE that was used in the study. For eight additional SNPs it was possible to identify proxies on the basis of linkage disequilibrium (LD), and physical distance to the selected SNP, using the web-based tool SNAP proxy [99]. Sometimes more than one proxy was identified for a selected SNP, which rendered a total of 20 SNPs representing 10 different genetic loci that were tested in the study.

Tissue Microarray

A tissue microarray (TMA) was constructed for previous studies [93], and included in this microarray were invasive breast tumors diagnosed in the MDCS cohort before December 31st 2010. Two 1 mm cores were extracted from a paraffinized tumor and inserted into a recipient paraffin block (Beecher, WI, USA). Sections of 4 µm were cut and placed on glass slides, and incubated in a heat chamber at 60°C for 60 minutes. A PT Link system (Agilent/Dako A/S) was used for deparaffination and antigen retrieval.

Figure 10: Tissue microarray.
Reprinted with permission from Karin Jirström.

Antibodies for VDR and CaSR were selected after literature research. Vitamin D receptor antibodies had been previously evaluated and validated and the mouse monoclonal D-6 antibody (sc-13133, Santa Cruz Biotechnology) was selected for analyses of VDR since this antibody was described as superior to alternatives regarding specificity and sensitivity [100, 101]. For CaSR, an antibody previously used by others in a similar setting was selected, a rabbit polyclonal antibody (HPA039686, Atlas antibodies) [102].

Microscopy assessment

A light microscope was used to obtain an introductory overview of the immunohistochemistry stained tissue samples. It was then noted that staining regarding CaSR was sparse: only 3.7% of tumors expressed any CaSR, and for 2.8% there were only single cells expressing CaSR. In 3.8% of samples there was expression of CaSR in non-tumorous cells. It was therefore concluded that the TMA regarding CaSR could not be used for any statistical analysis.

VDR, on the other hand, was expressed in multiple subcellular compartments in a majority of tumors, and after an initial overview in a light microscope, the digital pathology platform PathXL (http://www.pathxl.com, PathXL Ltd., UK) was used for microscopic assessment. The evaluation of VDR was based on a semi-quantitative scale of the proportion (0=0%, 1=1-10%, 2=11-50%, 3=51-75%, 4=76-100%) of staining in nuclei, nuclear membranes, cytoplasm and cytomembranes. There were also evaluations of intensity in nuclei, nuclear membranes and cytoplasm. Evaluation was carried out twice, by LH in order to achieve scores that were as valid as possible. A description of how discordance between evaluations was handled is included in paper III. Scores regarding nuclear fraction, cytoplasmic fraction and cytoplasmic intensity were used for statistical analyses in paper III, and nuclear fraction scores were used in paper IV.

Endpoint retrieval

As mentioned previously, the MDCS is regularly crosslinked to the Swedish Cancer Registry and the Swedish Cause of Death Registry [103, 104]. An invasive breast cancer registered in the Swedish Cancer Registry and included in any of the papers in this thesis was confirmed in medical records and/or clinical notes, as information on histopathology and treatment was retrieved.

Data on date of death and cause of death relies entirely on information retrieved from the Swedish Cause of Death Registry. Papers I, III, and IV, studied breast cancer mortality and in all papers death from breast cancer was defined as breast cancer registered as the underlying or contributing cause of death. The end of follow-up in these papers was, date of death, date of emigration, or date of last follow-up (paper I – December 31st 2010, papers III and IV – December 31st 2016).

Statistical analyses

All statistical analyses were performed using the statistical software program SPSS versions 19 to 25 (IBM).

Table 2: Overview of the statistical methods used in different papers

	Paper I	*Paper II*	*Paper III*	*Paper IV*
Descriptive tables	X	X	X	X
Cox proportional hazards	X		X	X
Kaplan-Meier			X	X
Binary logistic regression		X		X
Multiple imputation			X	X

Descriptive statistics

Descriptive tables are used in all papers to describe the distribution of factors that might influence an outcome. This is done in order to present the study population and to aid in the decision on whether or not to include such a factor as a confounder in adjusted analyses. Descriptive tables normally show distributions in column percentages. In paper III, chi2-tests were used to show the statistical significance of differences in distributions between categorical variables, and a Kruskal-Wallis test was used to evaluate differences in distribution between continuous variables.

Cox proportional hazards analyses

To investigate breast cancer survival in papers I, III and IV, Cox proportional hazards analyses yielding Hazard Ratios (HR) and 95% confidence intervals (CI) were used.

In paper I, pre-diagnostic levels of vitamin D ($25OHD_3$), PTH and calcium were divided into tertiles, and different tertiles were compared regarding breast cancer mortality. Adjustments were included in two multivariable analyses, the first including factors known to influence levels of vitamin D, PTH and calcium such as season of blood draw, age at baseline and also for storage time of the blood sample, (i.e. year of baseline examination). The second multivariable analyses also included factors known to be associated with breast cancer survival (age at diagnosis, tumor size, lymph node status, presence of distant metastases, Nottingham grade, histological type, ER-status and PgR status). Missing values on adjustment factors

were coded in a separate category and included in the analyses. The assumption of proportional hazards was confirmed as tested by log - minus log plots.

Paper III investigated the expression of VDR in different subcellular compartments in association with breast cancer mortality. These Cox analyses were also performed crude and adjusted in two different multivariable analyses, the first one for factors known to be associated with vitamin D levels (season and age at diagnosis), and the second one for factors known to influence breast cancer prognosis (tumor size, lymph node status, histological type and molecular subtypes). In this paper MI (see below) was used to handle missing data on confounders included in adjusted analyses.

The Cox analyses performed in paper IV were similar to the Cox analyses of papers I and III. As a first analysis, tertiles of vitamin D were compared in relation to breast cancer mortality, (similar to paper I). In this paper, tertiles of vitamin D were calculated dependent on month of inclusion, and therefore no adjustment was made for season of inclusion in MDCS in the multivariable model. The multivariable model differed regarding adjustment factors compared to adjusted models in paper I, and was adjusted for age at and season of diagnosis, size of tumor, lymph node status, and molecular subtypes. The second Cox analysis within paper IV, concerned nuclear VDR expression in relation to breast cancer mortality, and was very similar to the Cox analysis described above for paper III. The only difference between these statistical models was that imputed values also for main exposure (VDR expression) was included. The third Cox analyses in paper IV tested associations of VDR expression and breast cancer mortality, with the addition that these analyses were stratified by tertiles of vitamin D, in order to test if pre-diagnostic levels of vitamin D would modify the association between VDR expression and breast cancer mortality.

Kaplan-Meier

In papers III and IV, Kaplan-Meier estimates were plotted to visualize unadjusted survival associations and to confirm the assumption of proportional hazards.

Binary logistic regression

To investigate whether SNP variants influenced the risk of low vitamin D levels and the risk of breast cancer (paper II), an unconditional binary logistic regression analyses, yielding odds ratios (OR) and 95% CIs, was used. Major allele homozygotes were used as reference groups in all analyses and compared to heterozygotes and minor homozygotes. After crude analyses, adjustment factors (year and age of inclusion in MDCS, level of education, type of occupation, age at menarche, age at first childbirth, exposure to oral contraceptives and hormone replacement therapy, height, BMI, alcohol consumption and smoking) were added to the analyses. The risk of breast cancer in association with SNP variants was also

investigated in groups stratified on low versus high vitamin D levels and a test of interaction was performed, to investigate if levels of vitamin D might modify an OR of breast cancer risk.

Binary logistic regression was also used in paper IV, when pre-diagnostic levels of vitamin D (in tertiles) were investigated in relation to risk of breast cancer, risk of VDR-negative breast cancer and VDR-positive breast cancer. These analyses were performed crude and with adjustment for possible confounders (year and age of inclusion in MDCS, type of occupation, age at first childbirth, exposure to oral contraceptives and hormone replacement therapy and alcohol consumption). They were also performed both including only cases and controls with complete data on all variables (complete case analyses), and after MI (see below) when all cases and controls were included.

Multiple imputation

MI is a method to handle missing data on some variables in a cohort [105, 106]. Before deciding to use this method, one should consider which mechanism lies behind missingness. If data is missing completely at random, it means that all individuals within the study population have the same probability for a missing value of a variable [106]. Then there is no need to impute missing data, as the missing data does not change the result of a statistical analysis; it only gives less power to it since the numbers are smaller. On the other end of the range is when data is missing but not at random, that is, when data is missing systematically, and there are no other variables that can predict why a value is missing or what the value might be if it was not missing. MI can be used, when missing values are missing at random, i.e. when the missing data is systematically missing, but within groups of defined data the data is missing completely at random. This means, that there are values on other variables in the cohort that can help predict a missing value [105, 106].

A MI process is when statistical computer software (SPSS version 25) uses known values on other variables and/or other individuals, to calculate and impute missing values in several new imputed datasets. These imputed datasets can be used in statistical analyses which calculates results from individual imputed datasets and thereafter give a pooled result, that considers the uncertainty that imputed values by default inflicts on a cohort with missing values (Figure 11).

The method was used to impute missing values on several variables in paper III and paper IV. On all occasions a MI model was fitted in order to work with the statistical analysis intended to study an association. It therefore included all variables meant to be included in the adjusted analysis, as well as variables that predicted missing values and/or, predicted missingness.

In paper III we chose only to use imputed values on confounding factors in the final analyses, but in paper IV vitamin D levels and nuclear expression of VDR were also imputed and used in the analyses (main exposures and outcome).

The imputation models are described in further detail in papers where they were used.

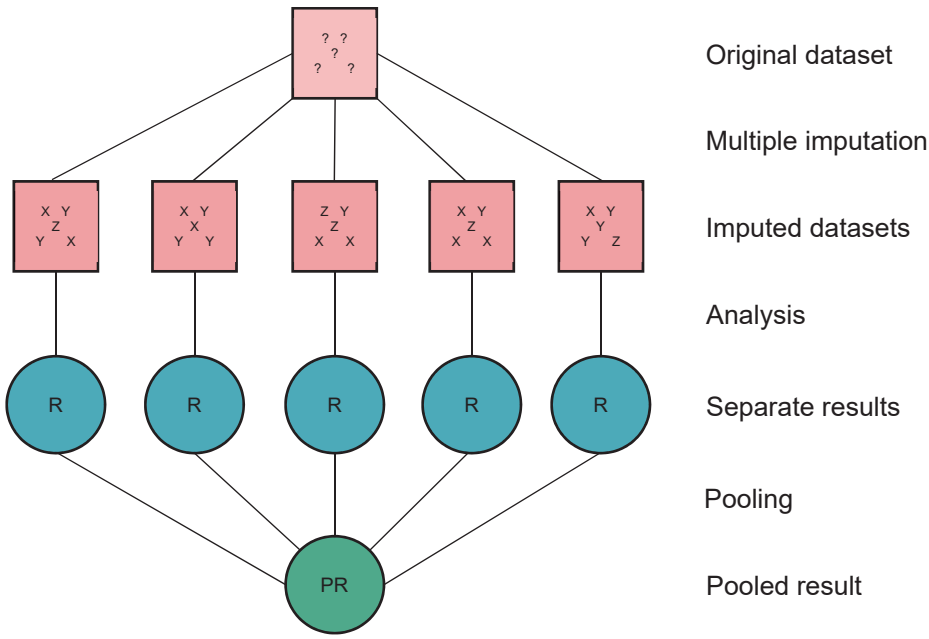


Figure 11: Illustration of the process of multiple imputation.

Results and discussion

Levels of vitamin D and breast cancer survival

In the study presented in paper I, we found a U-shaped relationship between levels of vitamin D and breast cancer mortality, with the best prognosis for women with intermediate levels of pre-diagnostic vitamin D (Table 3). A similar pattern, but one that was neither as strong, nor statistically significant, was seen when the analysis was repeated for paper IV (Table 3). In paper IV we had less power since women who had received neoadjuvant treatment or had distant metastases at diagnosis were excluded from the analyses. Also, when the MI technique was used to handle missing data (paper IV), all women with a missing value on any of the covariates included in the statistical model were withdrawn from the complete case analysis, and when the missing indicator method was used (paper I), all women were included.

Table 3: Tertiles of vitamin D in relation to breast cancer mortality

Tertile/level vitamin D	HR[1] (CI 95%)	HR[2] (CI 95%)
1st Low	2.46 (1.38-4.37)	1.51 (0.96-2.38)
2nd Medium	1.00 (ref)	1.00 (ref)
3rd High	1.99 (1.14-3.49)	1.41 (0.88-2.26)

[1]Results from paper I: Adjusted for season and year of blood sample, age at baseline, age at diagnosis, size of tumor, Elston-Ellis grade, histological type, ER-status, PgR-status, lymph node status and distant metastasis at diagnosis.
[2]Results from paper IV: Adjusted for age at and season of diagnosis, size of tumor, lymph node status, and molecular subtypes.

Nevertheless, we still find it plausible that there is a non-linear association between pre-diagnostic vitamin D levels and breast cancer mortality. A growing body of research supports the finding that low levels of vitamin D, (deficiency or insufficiency) are associated with unfavorable prognostic factors and prognosis [48, 107-113], but our finding that higher pre-diagnostic levels also lead to a relatively high breast cancer mortality has not been reproduced. The reason for that may be that most other studies have used blood samples taken at the time of diagnosis, which might not reflect an individual´s habitual vitamin D level, and/or that they have not used the same cut-offs. Most other studies distinguished only between high

vs. low levels, but one other study (with blood-samples from the time of diagnosis) compared overall mortality for women in tertiles, similarly as we did. They used the highest tertile as a reference and found statistically significant, relatively high overall mortality in the lowest tertile, but it can also be noted in that study that the intermediate tertile seemed to have the lowest risk of mortality, although it was not statistically significant [114].

Levels of parathyroid hormone and breast cancer survival

There was no association found between different tertiles of pre-diagnostic PTH and breast cancer mortality. That does not necessarily mean that PTH has nothing to do with breast cancer progression. Looking at recent studies of PTHrP and the common PTH and PTHrP receptor there seems to be evidence that PTH actually does have an effect, but it might depend on whether this level is constantly high or if PTH is released intermittently from the parathyroid glands [59, 61].

The reason we found no association is probably because one single sample of PTH is not an adequate measurement, due to intra-individual variation of PTH levels [115, 116]. It might be better to use the Mendelian Randomization approach, to conclude or disregard an association. It is also possible that an association between PTH and breast cancer progression cannot be found at all when studying levels of PTH, but that PTH is still part of an important pathway, and studies should instead be concentrated on expression of PTH/PTHrP receptors.

Levels of calcium and breast cancer survival

There was an association between a relatively low risk of breast cancer mortality and a high pre-diagnostic level of calcium (HR:0.53 95% CI:0.30-0.92), in the adjusted analysis. This is congruent with the previous findings that extracellular free calcium inhibits cell proliferation and invasion [117]. Others have also found the calcium sensing receptor (CaSR) to have tumor suppressive effects [118-120].

The previous case-control study on breast cancer risk by Almquist et al., showed that higher levels of serum calcium were associated with high risk of breast cancer for overweight and/or post-menopausal women [41]. Together with our findings on associations with breast cancer mortality, this might imply that serum calcium has similar associations as estrogen regarding breast cancer, i.e. high levels increase the risk of breast cancer but a cancer that develops under such circumstances is associated with a better prognosis.

Vitamin D SNPs and breast cancer risk

This study (paper II) was not a Mendelian Randomization. It merely investigated whether SNPs previously related to vitamin D in any way, were associated with vitamin D levels and/or breast cancer risk.

Results from statistical analyses are summarized in Table 4. Previously observed associations between SNPs and vitamin D levels [68-72, 121] were confirmed with most SNPs for which such an association had been found. The only exceptions were for a few SNPs which a previous study, conducted on a cohort of Australian children, had found to be associated with vitamin D levels [69]. Considering all factors that differ between the Australian population and the one in our study, there are several plausible explanations for the conflicting results.

None of SNPs previously associated with levels of vitamin D showed any significant association with breast cancer risk (Table 4). Hence, if the study would have had a Mendelian Randomization approach the conclusion would have been that there was no association between vitamin D levels and breast cancer risk. Since this study was published, there have been several large studies whose results also strengthens this hypothesis [44, 122-124].

SNPs from only one of the tested genetic loci in our study, were statistically significantly associated with breast cancer risk (rs12239582 and rs2209458, see Table 4). The gene where SNPs were positioned is called *ST6GALNAC3* and encodes a sialyltransferase that is expressed excessively in fat-tissue, in the thyroid and in the kidney [20]. Regarding the association with vitamin D, prior to our study, one study had found an association with circulating vitamin D-binding protein (VDB) [121]. To our knowledge, these SNPs, or expression of *ST6GALNAC3* have not been studied in association with breast cancer risk, and therefore this result should be replicated.

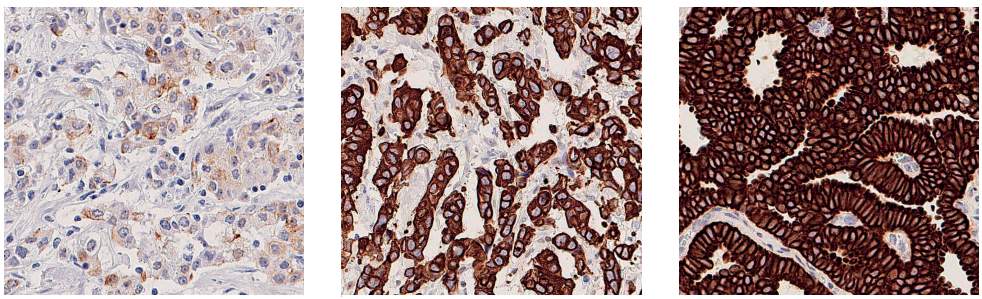
In the study of vitamin D SNPs, we also conducted an exploratory analysis in which we stratified women based on low vs high vitamin D levels and thereafter investigated breast cancer risk dependent on selected SNPs. Intriguingly, we found that the SNPs of three tested loci (rs198300, rs10485165 and rs2060793) showed different ORs of breast cancer based on whether the women were in the group with high or low levels of vitamin D. This might indicate that depending on what genomic traits you have, the level of vitamin D may interact with your risk of breast cancer.

Table 4: Vitamin D SNPs in relation to risk of low vitamin D and breast cancer			
SNPs[1]	Allele	OR[2] (95% CI) low vitamin D	OR[2] (95% CI) breast cancer
rs12239582 *(rs2209458)*	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	0.87 (0.67-1.12)	0.82 (0.68-0.99)
	Minor homozygote	0.72 (0.52-1.00)	0.93 (0.73-1.17)
rs7041 *(rs4588, rs705117, rs228267)*	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	1.39 (1.09-1.77)	0.88 (0.74-1.05)
	Minor homozygote	2.12 (1.51-2.99)	0.89 (0.70-1.13)
rs10485165	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	1.03 (0.79-1.34)	0.99 (0.82-1.20)
	Minor homozygote	1.04 (0.49-2.21)	1.00 (0.60-1.74)
rs198300 *(rs13245518)*	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	1.08 (0.84-1.38)	1.10 (0.91-1.30)
	Minor homozygote	1.09 (0.79-1.52)	0.96 (0.75-1.22)
rs4751058	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	0.88 (0.68-1.13)	1.05 (0.88-1.27)
	Minor homozygote	0.77 (0.36-1.63)	0.66 (0.38-1.17)
rs12295888 *(rs10832275)*	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	1.22 (0.96-1.54)	1.05 (0.88-1.24)
	Minor homozygote	1.80 (1.24-2.63)	0.93 (0.71-1.23)
rs1007392 *(rs10832299)*	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	1.31 (1.03-1.67)	0.99 (0.83-1.18)
	Minor homozygote	1.78 (1.26-2.52)	0.91 (0.71-1.17)
rs2060793 *(rs1993116)*	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	0.78 (0.61-1.00)	1.10 (0.92-1.33)
	Minor homozygote	0.58 (0.42-0.81)	1.04 (0.82-1.32)
rs7944926 *(rs12791871, rs3829251)*	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	1.30 (1.03-1.64)	0.91 (0.77-1.08)
	Minor homozygote	2.39 (1.59-3.59)	0.77 (0.57-1.02)
rs2302190	Major homozygote	1.00 (ref)	1.00 (ref)
	Heterozygote	1.24 (0.97-1.58)	1.09 (0.91-1.30)
	Minor homozygote	1.45 (0.69-3.02)	0.99 (0.62-1.58)

[1]Results for analysis of SNP in **bold** are presented. SNPs in *italics* are from the same genetic locus and showed similar results.
[2]Adjusted for age at baseline, year of inclusion, level of education, type of occupation, age at menarche, age at first childbirth, exposure to oral contraceptives, exposure to hormonal replacement therapy, height, BMI, alcohol consumption and smoking.
Arrows indicating statistically significant results.

Vitamin D receptor expression and breast cancer survival

Nuclear and cytoplasmic expression of VDR was associated with other histopathological factors considered prognostically positive. For example, tumors with a nuclear VDR expression up to ten percent (negative), were related to larger tumors ($p=0.002$), with a higher Nottingham grade ($p<0.001$), ER negativity ($p<0.001$), PgR negativity ($p<0.001$) and high Ki67 ($p<0.001$). We also found a statistically significant association between VDR positivity and a relatively low breast cancer mortality (Figure 12), indicating that VDR positivity means a better breast cancer survival.



Nuclear VDR 0-10%
1.00 (ref)*

Nuclear VDR 11-50%
0.54 (0.32-0.89)*

Nuclear VDR 51-100%
0.66 (0.34-1.28)*

Figure 12: Example of nuclear VDR expression.
*HR (95% CI) for breast cancer mortality, adjusted for age at and season of diagnosis, size of tumor, lymph node status, histological type and molecular subtypes.

Only one study had previously made any associations between VDR expression and survival, and this study showed results similar to ours [83]. Other studies that have investigated similar associations have not shown any statistically significant associations, but several showed associations with positive prognostic factors [81, 82], and a longer disease-free interval for women with VDR-positive tumors was seen in one study [82]. There are no studies showing opposite associations. With one exception, our study included a larger number of women with breast cancer and with no exception our study had a longer follow-up, compared to previous studies.

In accordance with our findings there is research on cell-lines and mice models which have found that activated VDR regulates genes that induce autophagy [125]. In addition, treatment with vitamin D and androgen receptor (AR) agonists on triple negative breast cancer cell-lines positive for VDR and AR reduces the viability of cancer cells [126].

Vitamin D and expression of the vitamin D receptor

Results from statistical analyses indicated a possible association between low levels of pre-diagnostic vitamin D and a subsequent breast tumor with negative VDR expression. Women with vitamin D levels in the second and third tertiles of vitamin D had a comparably low risk of VDR-negative tumors: HR (95% CI) adjusted for age at baseline, year of baseline, type of occupation, age at first child birth, exposure to oral contraceptives, exposure to hormone replacement therapy and alcohol consumption was 0.73 (0.47-1.12) for the second tertile and 0.66 (0.38-1.15) for the third tertile of vitamin D. Also, only 14% of tumors developed in women within the third tertile were VDR negative, compared to 21% of tumors developed in women within the first and second tertiles. No results were statistically significant, possibly due to a relatively low frequency of VDR-negative tumors.

To our knowledge this association has not been studied previously, and our study should be replicated, in order to confirm or refute our results.

Hypothesizing that the levels of vitamin D while a tumor is developing affect the tendency of a tumor to express VDR, this could indicate that this is the pathway by which low vitamin D levels are associated with a poorer breast cancer outcome. Several studies have shown that activated VDR has tumor suppressive effects [125, 126], which support this hypothesis.

In this hypothesis, high vitamin D levels would mean a tendency of a tumor to express more VDR, which should be favorable. But there has been at least one study that shows unliganded VDR to have adverse, tumor proliferative effects [79]. If a tumor was stimulated with high levels of vitamin D while it was developed, it could mean that it expresses more VDR which is not activated if levels of vitamin D after the tumor has developed are not high enough to fill its needs. This would imply, that vitamin D supplementation should be given to women with tumors that are VDR-positive.

Interaction of vitamin D levels and expression of vitamin D receptor on breast cancer mortality

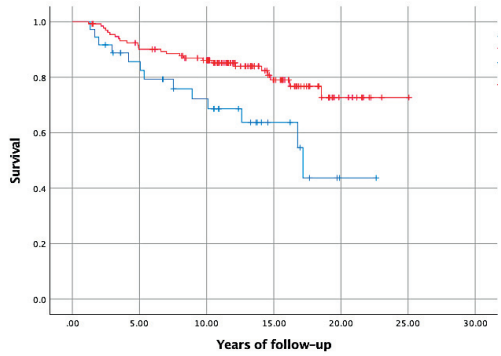
In paper IV we wanted to investigate whether levels of vitamin D modify the positive association between VDR expression and breast cancer survival found in paper III. Results from the mortality analyses stratified by pre-diagnostic vitamin D levels differed between the tertiles (see Figure 13), but not to an extent that allowed us to conclude that an interaction existed.

Studies on vitamin D levels combined with expression of VDR in relation to breast cancer prognosis have not been published previously, to our knowledge. Therefore, it is not possible to compare our results with others, and in order to establish if vitamin D in any way can affect the outcome of a VDR-negative or VDR-positive breast tumor, future studies are of great importance

Since vitamin D levels might be different at the time of a breast cancer diagnosis, and may possibly change due to tumor development, it would probably be more accurate to use blood samples from the time of diagnosis for such analyses.

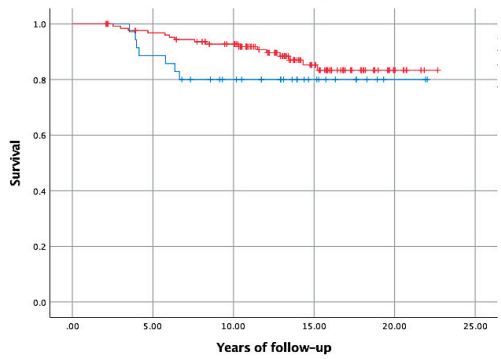
1st tertile of vitamin D.

1.68 (0.76-3.71)*



2nd tertile of vitamin D.

1.44 (0.57-3.64)*



3rd tertile of vitamin D.

1.97 (0.88-4.41)*

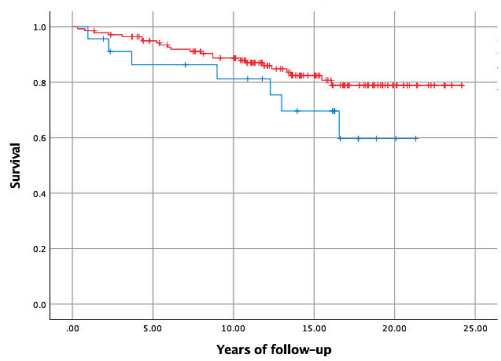


Figure 13: Kaplan Meier curves comparing survival for positive vs negative VDR expression.
*HR (95% CI) for breast cancer mortality of VDR-negative tumors compared to VDR-positive. Adjusted for age at and season of diagnosis, size of tumor, lymph node status and molecular subtypes.

Methodological considerations

Serum levels of vitamin D, parathyroid hormones and calcium

Levels of vitamin D, PTH and calcium were analyzed from blood samples collected at baseline examinations of the MDCS. Therefore, it is not likely that a subsequent breast tumor has influenced the measured levels. On the other hand, when investigating mortality from a subsequent breast cancer in relation to serum levels, it is not certain that the environment in which the breast cancer has developed is reflected.

Levels of vitamin D, measured as $25OHD_3$, are known to vary over the year, as vitamin D is produced in the skin when it is exposed to sunlight [30, 127]. Therefore, all analyses including vitamin D levels were in some way adjusted for the season of sampling. In paper I, the season was included as an adjustment factor in Cox analyses, but in papers II and IV we choose to dichotomize/divide into tertiles of vitamin D based on the month of baseline examinations, before any statistical analysis was performed.

In previous studies, the intra-individual variation of vitamin D levels has been found to be low [128, 129], but over the past decade there has been an increase in oral vitamin D supplementation [130] and after baseline examinations (1991-1996) there is no information on usage of supplements. Therefore, it must be considered that a measured level of vitamin D may not reflect the habitual level for a woman, nor the environment in which a tumor develops if this tumor was diagnosed a very long time from baseline examinations.

Compared to $25OHD_3$, PTH is unstable and has a half-life in the body of only four minutes. It has also been shown to have an intra-individual variation of about 25%, and fluctuates considerably over the day [115, 116]. Misclassification regarding PTH levels may therefore be considered, which might have obscured a possible true effect of PTH levels on breast cancer mortality that could not be observed in paper I.

Serum levels of total calcium are stable under normal physiological conditions and the intra-individual variation is low [131, 132]. After menopause, serum levels of

calcium are known to rise [133, 134], but it seems that the ranking of calcium levels between women remains the same [135]. The risk of misclassification of calcium levels when included in an analysis comparing tertiles of calcium level can therefore be regarded as low.

SNPs

As previously described, SNPs with a known effect on a trait, for instance vitamin D level, can be used as a stable approximation of that trait, not affected by temporary fluctuation [73]. At the time of the study described in paper II, there was no information which could be used to determine a pattern of SNP variants associated with vitamin D levels. Therefore, SNPs that had been associated with vitamin D in any way in a GWAS study were used and studied individually in relation to breast cancer risk.

All SNPs investigated in relation to breast cancer risk in paper IV, were investigated with the assumption that if there was an association with vitamin D there would possibly also be one with risk of breast cancer. Therefore, we decided not to adjust our definition of statistical significance with a higher CI, although several SNPs were tested.

Change in risk due to a SNP variation is expected to be very small, and large groups are needed in order to have enough power to find statistically significant associations. A recent Mendelian Randomization study investigating levels of vitamin D on breast cancer risk used previous GWASs to determine vitamin D associations with breast cancer risk. The population used to identify associations between SNPs and vitamin D included 73,699 individuals, and the researchers had access to information on SNPs on 122,977 breast cancer cases [44]. In comparison, our population included 1,343 women with information on vitamin D levels, and "only" 865 breast cancer cases.

Tissue microarray

A tissue microarray enables the evaluation of markers with limited use of tissue and is less time-consuming than evaluating a whole section of the tumor. Of course, it must be considered that 1 mm cores might not reflect the complete pattern of expression of a marker, and a heterogenous expression in a tumor might lead to misclassification. The TMA used for evaluation of VDR (and CaSR) was designed to include two cores from each tumor. Usage of duplicate cores has been shown to

reduce the impact of heterogeneity [136, 137], and an adequately constructed TMA is considered a good representation of a cohort [136].

When TMA sections were cut for immunohistochemical staining for this project, the research group decided to stain for both CaSR and VDR as well as other markers included in other projects. After a literature search, antibodies were selected for staining. The CaSR antibody used in this project had been previously used in similar tissue [102], but the VDR antibody was selected since it was considered well-validated and the best one available for immunohistochemistry on paraffinized tissue [100, 101]. The VDR antibody selected had not, to our knowledge been used on breast tumor tissue before.

Scoring of CaSR and VDR was performed by LH, who had no previous experience of immunohistochemical evaluation. Therefore, early evaluations were considered not as valid as later ones. All cores were scored twice regarding VDR expression, and when there were small differences in scores between scoring rounds, the second score was regarded as correct. Details on how scores were handled are included in an appendix of paper III.

Vitamin D receptor expression in invasive breast tumors

The vitamin D receptor was expressed in several subcellular compartments in the majority of tumors (Figure 14). Previous studies evaluating VDR expression in breast tumors mainly identified only nuclear expression [77, 80-82], although expression in the cytoplasm was reported in one study [83]. Early studies used

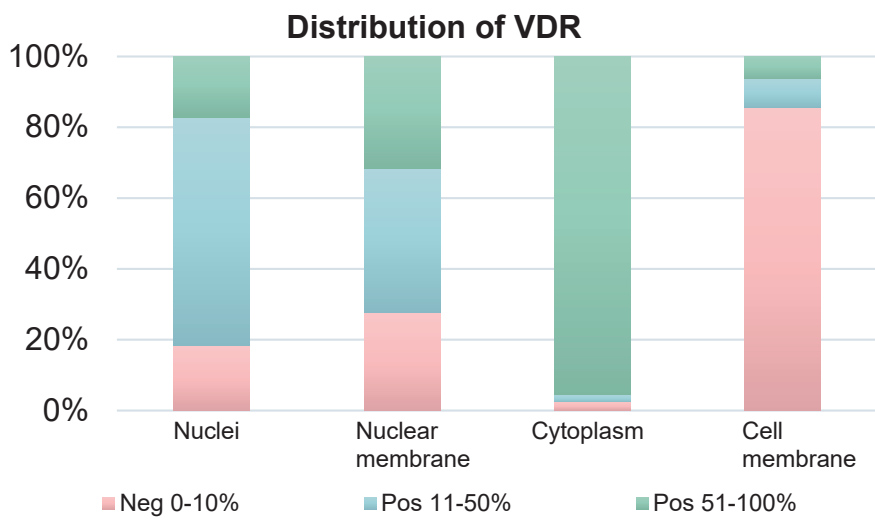


Figure 14: Chart showing expression of VDR in different subcellular compartments.

radioactive calcitriol ($1,25(OH)_2D_3$) to identify VDR in benign and malignant breast tissue [84, 85]; hence, they only identified the ligand-activated protein. Others who used the rat monoclonal (9A7 gamma) antibody also recorded only nuclear expression [77, 80-82], whereas a different antibody was used by Ditch et al. who recognized cytoplasmic expression as well [83].

We find it likely that studies that saw only nuclear expression, identified only ligand-activated VDR, whereas we also visualized unliganded VDR. The Santa Cruz D-6 antibody is directed toward a section of the VDR protein containing one of three binding sites of calcitriol ($1,25(OH)_2D_3$) [138, 139]. Therefore, it is likely that the antibody shows unliganded expression, but it is probable that it also binds to liganded VDR.

Calcium receptor expression in invasive breast tumors

We found almost no expression of CaSR in our material on 718 invasive tumors. This was unexpected since a previous study on a Chinese population distinguished between high and low CaSR expression in breast tumors and observed a high expression in 89 of 148 investigated tumors [102]. It is also known that the CaSR is present in normal breast tissue as it has functions needed for the lactation process [140], and the expression in normal breast tissue was almost as weak as in malignant tissue. Therefore, we considered the immunohistochemistry process of CaSR staining as failed, although the reason was not clear.

Confounders

When studying associations, confounders are added to an analysis to adjust the analysis for a factor that is linked to both the exposure and the outcome, in order to see that not all the impact an exposure has on an outcome is due to the exposure's covariation with another risk factor. When deciding to add a confounder to an analysis it should also be considered that the confounder is not on the causal pathway between an exposure and an outcome, i.e. the confounder is not explained by the exposure and the association that the confounder has on an outcome is actually due to the exposure [141].

There are known relations between overweight and vitamin D levels, and there are known associations between overweight and risk of breast cancer, and breast cancer prognosis [142-148]. Therefore, we considered including BMI in adjusted analyses when investigating associations between vitamin D and breast cancer risk and survival. As we believed low vitamin D levels could in part be due to overweight,

we concluded that vitamin D may actually be on the causal pathway between overweight and breast cancer outcomes. Therefore, we did not primarily include BMI as a confounder in our mortality analyses.

Physical activity is another known risk/prognostic factor for breast cancer, that has similar associations with vitamin D as BMI. The same argument could be used not to include values for physical activity in our adjusted analyses. At the same time, the information we had on physical activity on individuals of the MDCS has a questionable validity [149], so there was yet another reason not to include values for physical activity in the adjusted analyses.

In Mendelian Randomization (MR) the hypothesis is that genotypes assigned randomly at meiosis, act in a similar manner to randomization in a trial comparing for example different treatment regimes [73]. Therefore, MR studies usually do not include confounding factors. Since there is a chance of covariation between selected SNPs and other risk factors of breast cancer, we decided to perform adjusted analyses along with unadjusted (crude) analyses.

One weakness with the study population used in all four papers is that we had no information on family history of breast cancer and no information on previous benign breast disease, both of which are known risk factors for breast cancer [150-155]. Hence, no adjustments were made in any analysis for these rather important risk factors of breast cancer. On the other hand, there is nothing to suggest that family history of breast cancer is associated with vitamin D levels. It is probable that adjustments for family history and/or benign breast disease would have minor effects on outcome estimates.

Conclusions

The overall conclusion of this thesis is that there was no linear association between levels of vitamin D and breast cancer survival, but both low and high levels were associated with an increased risk of breast cancer death. The association between low levels and high breast cancer mortality may be mediated through development of a VDR-negative tumor. There was no evidence to suggest an additional beneficiary effect of vitamin D levels higher than intermediate, regarding breast cancer risk or prognosis.

Paper I

- Low and high levels of pre-diagnostic vitamin D were associated with relatively high breast cancer mortality, compared to intermediate levels.
- There was no association between pre-diagnostic levels of PTH and breast cancer mortality.
- A high pre-diagnostic level of calcium was associated with a relatively low breast cancer mortality.

Paper II

- SNPs previously associated with levels of vitamin D, were associated with vitamin D also in this study, but were not associated with breast cancer risk.
- One SNP previously associated with vitamin D-binding protein was associated with breast cancer risk.
- Levels of vitamin D may modify the breast cancer risk associated with certain SNPs.

Paper III

- A breast cancer expressing VDR was associated with favorable prognostic factors and better survival.

Paper IV

- Pre-diagnostic levels of vitamin D may have influenced the expression of VDR in a subsequent breast tumor.
- Pre-diagnostic levels of vitamin D did not modify the association between VDR expression and breast cancer mortality.

Clinical implications and future perspectives

The differing results between different analytes and breast cancer mortality presented in paper I suggest different implications for each result. Regarding the U-shaped association between vitamin D levels and breast cancer mortality, this suggests that vitamin D supplementations should only be considered for individuals with low vitamin D levels. The finding that measured levels of PTH have no impact on breast cancer mortality, indicates that further studies using this method may have little importance. Instead future studies should consider using more stable measurements of PTH, for example SNPs associated with hyperparathyroidism to investigate associations with breast cancer risk and prognosis. The Mendelian Randomization approach could also be used to confirm the result that high levels of calcium seemed to be associated with a better breast cancer prognosis.

It seems that there is no strong association between vitamin D levels and breast cancer risk. Therefore, there is no use putting vitamin D levels or SNPs with associations to vitamin D levels, into a general risk prediction model. However, the exploratory part of paper II indicated that levels of vitamin D may modify a breast cancer risk related to genetic predisposition. One hypothesis is therefore that if future research confirms those results and identifies genomic traits that benefit from high levels of vitamin D, vitamin D supplementation could be used to reduce breast cancer risk for women with that genetic set.

Perhaps VDR could be included as a prognostic factor at breast cancer diagnosis, but randomized clinical trials are warranted in order to investigate whether vitamin D could be used as a part of adjuvant cancer treatment based on VDR expression of the tumor, or serum vitamin D levels.

One might argue that vitamin D supplementation is rather cheap and has few side effects, and therefore should be prescribed to every woman with a breast cancer diagnosis, but we do not know if some tumors actually prosper with an abundance of vitamin D, as indicated by the U-shaped relationship between pre-diagnostic serum levels of vitamin D and breast cancer mortality shown in paper I.

Future studies should include serum vitamin D levels analyzed at the time of diagnosis, studied in association with expression of VDR in the breast cancer at diagnosis. Another suggested study would include randomized prescription of

vitamin D supplements at the time of diagnosis and would compare patterns of VDR expression in the tumor, and/or surrounding breast tissue before and after vitamin D distribution. This might be troublesome to implement, since there is no way to control for sun exposure, dietary habits and voluntary vitamin D supplementation. Measurements of vitamin D level at the time of diagnosis and at the time of surgery might be an option.

Vitamin D is not a miracle drug, and should not be prescribed to each and every person. No positive effects can be seen with excessively high vitamin D levels. On the other hand, it is possible that vitamin D has a positive effect on some breast cancers.

Acknowledgements

Mitt personliga tack!

Det finns så många så många som har bidragit till att den här avhandlingen har författats och förfärdigats: De som har format mig som person, de som har stöttat mig i min vardag, de som deltagit i studier, framför allt Malmö Kost Cancer, de som genomfört tidigare studier och de som bidragit ekonomiskt till att studier kunnat genomföras. Jag vill tacka er alla, men det är svårt att nämna er alla. I vilket fall som helst så finns det några som är värda ett extra tack!

Tack! Jonas Manjer, min handledare. Det smartaste jag gjort i hela doktorandprojektet, var att välja just dig till handledare. Alltid precis lagom med stöd, utmaningar och återkoppling. Du har gjort detta till ett rent nöje.

Tack! Salma Butt, min bihandledare, min SPSS-guru, mitt bollplank och mitt stöd. Det enda felet med dig är att du är för trevlig, så våra möten blir alltid lite längre än tänkt.

Tack! Alla mina medförfattare: Signe Borgquist, förebilden som bidragit till projektets utbredning och lärt mig känna igen tumörceller; Martin Almquist och Karin Elebro, föregångare som generöst delat med sig av datafiler och ovärderlig information; Asta Försti, Peter Almgren och Jasmine Brandt Bratlie, som hjälpt mig förstå det här med SNPar; Ann Rosendahl som förklarat hur antikroppar och immunohistokemi fungerar, så att till och med jag förstår; Olle Melander och Malte Sandsveden vars arbete bidragit till att det blev så här bra.

Tack! Till er som gjort så mycket ”bakom kulisserna”; Anna Hwasser, Björn Nodin, Carina Forsare och Kristina Lövgren.

Tack! Anne-Marie Rorstock, som fixat så mycket inför disputationen.

Tack! Alla ni andra goa forskningskollegor som gör forskandet roligt: Helena Jernström, Sophia Zackrisson, Hanna Sartor, Kristina Lång, Shabaz Majid, Looket Dihge, Lisa Rydén, och Per-Ola Bendahl för att bara nämna några.

Tack! Alla fina kollegor på Kirurgen i Malmö och Lund. Ni gör det kul att gå till jobbet. Ett särskilt tack till bästa kliniska handledaren Peter Mangell.

Tack! Till mina vänner; Erica vars fel det nog är att jag hamnade i den här branschen (det var du som skulle bli läkare); gamla kursarna, Anna, Anna, Cecilia, Stina, Åsa och Caroline som gjorde utbildningen uthärdlig, och som jag kan dela farhågor och våndor med än idag; AK-syjuntan för att ni ser till att jag bibehåller någon slags normal social kompetens; Metropolis BigBand som fungerar som min ventil (även om det är saxofon jag spelar) och alla ni andra härliga människor som jag får kalla vänner.

Tack! Mamma Brita och pappa Åke, för att ni lärt mig att jag kan bara jag vill, och att det inte är farligt att göra bort sig. Ni ställer alltid upp för mig och min familj, ovärderligt.

Tack! Till bästa syskonen Matilda, Linus, Tobias och Tove som lurat mig, retat mig och format mig till den jag är och som jag vet finns där, om utifall att… Så glad att ni är de ni är, och att vi har så roligt ihop. Era makar och barn får också ett särskilt tack!

Tack! Till min fina svärfamilj, Monika, Gunilla, Anders och Jenny. Ni ställer upp och rycker ut i akuta lägen.

Tack! Anja, Uno, Egon och Ina. Mina fina, kloka barn som visar mig vad som verkligen är viktigt i livet.

Tack! Per, för att du vet när jag behöver lyftas, när jag behöver pushas, när jag behöver backa, när jag behöver vila, när jag behöver hjälp och när jag behöver kärlek. Jag behöver dig.

Funding

No research is possible without financial support. I am especially thankful towards ALF, due to which it was possible for me to combine research and clinical work. Other funders of projects within this thesis are: The Swedish Research Council (K2007-69X-20487-01-3), The Gunnar Nilsson Cancer Foundation, The Ernhold Lundström Foundation, The Einar and Inga Nilsson Foundation, The Maggie Stephen Foundation, The Malmö University Hospital Cancer Research Fund, The Malmö University Hospital Funds and Donations, The Crafoord Foundation, The Region Skåne and The Mossfelt Foundation.

References

1. AlgaeCal: **Top vitamin D benefits.** Available at: https://www.algaecal.com/algaecal-ingredients/vitamin-d/benefits/ Access date: 20-03-2019.
2. Brunet: **The many benefits of vitamin D.** Available at: https://www.brunet.ca/en/advices/benefits-vitamin-d.html Access date: 20-03-2019.
3. Markets and Markets: **Vitamin D market worth $2.5 billion by 2020.** Available at: https://www.marketsandmarkets.com/PressReleases/vitamin-d.asp Access date: 20-03-2019.
4. Hossein-nezhad A, Holick MF: **Vitamin D for health: a global perspective**. *Mayo Clinic proceedings* 2013, **88**(7):720-755.
5. Prentice RL, Pettinger MB, Jackson RD, Wactawski-Wende J, Lacroix AZ, Anderson GL, Chlebowski RT, Manson JE, Van Horn L, Vitolins MZ *et al*: **Health risks and benefits from calcium and vitamin D supplementation: Women's Health Initiative clinical trial and cohort study**. *Osteoporosis international* 2013, **24**(2):567-580.
6. Rejnmark L, Bislev LS, Cashman KD, Eiriksdottir G, Gaksch M, Grubler M, Grimnes G, Gudnason V, Lips P, Pilz S *et al*: **Non-skeletal health effects of vitamin D supplementation: A systematic review on findings from meta-analyses summarizing trial data**. *PloS one* 2017, **12**(7):e0180512.
7. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: **Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries**. *CA: A Cancer Journal for Clinicians* 2018, **68**(6):394-424.
8. Socialstyrelsen & Cancerfonden: **Cancer in numbers 2018. [In Swedish: Cancer i siffror 2018].** Available at: https://www.socialstyrelsen.se/Lists/Artikelkatalog/Attachments/20976/2018-6-10.pdf Access date: 28-02-2019.
9. Regionala Cancercentrum i Samverkan: **Valid care program breast cancer. [In Swedish: Gällande vårdprogram bröstcancer].** Available at:https://www.cancercentrum.se/samverkan/cancerdiagnoser/brost/vardprogram/gallande-vardprogram/ Access date: 04-03-2019.
10. Vaidya JS, Joseph D: **Fast Factes - Breast Cancer**, 5 edn; 2014.

11. Sydsvenska Bröstcancergruppen: **Guidelines for surgical and oncologic treatment of breast cancer - regional adaption of the national treatment guidelines, Region West and South [In Swedish: Lathund för kirurgisk och onkologisk behandling av bröstcancer – Regional anpassning av nationellt vårdprogram, Region Väst och Syd].** Available at: https://www.cancercentrum.se/globalassets/cancerdiagnoser/brost/syd/regional-anpassning-vast-och-syd_ssbcg_180601-190531.pdf?v=9acd7c93591740c2ab7c4d7796ee0f47 Access date: 01-03-2019.
12. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.
13. Engholm G FJ, Christensen N, Hansen HL, Hertzum-Larsen R, Johannesen TB, Kejs AMT, Khan S, Ólafsdóttir E, Petersen T, Schmidt LKH, Virtanen A, Storm HH. : **NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 8.1 (28.06.2018).** Available at: http://www.ancr.nu Access date: 04-03-2019.
14. Sydsvenska Bröstcancergruppen.: **Guidelines for surgical and oncologic treatment of breast cancer - regional adaption of the national treatment guidelines, Region West and South [In Swedish: Lathund för kirurgisk och onkologisk behandling av bröstcancer – Regional anpassning av nationellt vårdprogram, Region Väst och Syd].** Available at: https://www.cancercentrum.se/globalassets/cancerdiagnoser/brost/syd/sydsvenska-brostcancergruppens-lathund-170401-180331.pdf. Access date: 09-05-2018.
15. Kuchenbaecker KB, Hopper JL, Barnes DR, Phillips KA, Mooij TM, Roos-Blom MJ, Jervis S, van Leeuwen FE, Milne RL, Andrieu N *et al*: **Risks of Breast, Ovarian, and Contralateral Breast Cancer for BRCA1 and BRCA2 Mutation Carriers**. *Jama* 2017, **317**(23):2402-2416.
16. Eccles SA, Aboagye EO, Ali S, Anderson AS, Armes J, Berditchevski F, Blaydes JP, Brennan K, Brown NJ, Bryant HE *et al*: **Critical research gaps and translational priorities for the successful prevention and treatment of breast cancer**. *Breast cancer research* 2013, **15**(5):R92.
17. **Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies**. *Lancet* 1996, **347**(9017):1713-1727.
18. Chlebowski RT, Anderson GL, Gass M, Lane DS, Aragaki AK, Kuller LH, Manson JE, Stefanick ML, Ockene J, Sarto GE *et al*: **Estrogen plus progestin and breast cancer incidence and mortality in postmenopausal women**. *Jama* 2010, **304**(15):1684-1692.
19. Key TJ: **Endogenous oestrogens and breast cancer risk in premenopausal and postmenopausal women**. *Steroids* 2011, **76**(8):812-815.

20. Narod SA: **Hormone replacement therapy and the risk of breast cancer**. *Nature reviews Clinical oncology* 2011, **8**(11):669-676.
21. McPherson K, Steel CM, Dixon JM: **ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics**. *BMJ (Clinical research ed)* 2000, **321**(7261):624-628.
22. Singletary SE: **Rating the risk factors for breast cancer**. *Annals of surgery* 2003, **237**(4):474-482.
23. **Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease**. *Lancet* 2002, **360**(9328):187-195.
24. Hinkula M, Pukkala E, Kyyronen P, Kauppila A: **Grand multiparity and the risk of breast cancer: population-based study in Finland**. *Cancer Causes & Control* 2001, **12**(6):491-500.
25. American Institute for Cancer Research World Cancer Research F: **How diet, nutrition and physical activity affect breast cancer risk.** Available at: https://www.wcrf.org/dietandcancer/breast-cancer Access date: 20-03-2019.
26. American Institute for Cancer Research World Cancer Research F: **How diet, nutrition and physical activity affect breast cancer survival.** Available at: https://www.wcrf.org/dietandcancer/breast-cancer-survivors Access date: 20-03-2019.
27. Fitzgibbons PL, Page DL, Weaver D, Thor AD, Allred DC, Clark GM, Ruby SG, O'Malley F, Simpson JF, Connolly JL *et al*: **Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999**. *Archives of pathology & laboratory medicine* 2000, **124**(7):966-978.
28. Ajanki T: **Discovery of the Vitamins [In Swedish: Vitaminernas upptäckt].** *Populär Historia* 2018(4).
29. Theodoratou E, Tzoulaki I, Zgaga L, Ioannidis JP: **Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials**. *BMJ (Clinical research ed)* 2014, **348**:g2035.
30. Livsmedelsverket: **Vitamin D [In Swedish: D vitamin].** Available at: https://www.livsmedelsverket.se/livsmedel-och-innehall/naringsamne/vitaminer-och-antioxidanter/d-vitamin Access date: 27-02-2019.
31. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G *et al*: **The 2011 Report on Dietary Reference Intakes for Calcium and Vitamin D from the Institute of Medicine: What Clinicians Need to Know**. *The Journal of Clinical Endocrinology & Metabolism* 2011, **96**(1):53-58.
32. Törring O: **Hypocalcemia. [In Swedish: Hypokalcemi].** Available at: https://www.internetmedicin.se/page.aspx?id=2659 Access date: 20-03-2019.

33. Ganrot PG, A; Stenflo, J: **Clinical Chemistry in Practical Medicine. [In Swedish: Klinisk kemi i praktisk medicin]**, 7 edn: Studentlitteratur; 1997.
34. Colston KW: **Vitamin D and breast cancer risk**. *Best practice & research Clinical endocrinology & metabolism* 2008, **22**(4):587-599.
35. Gorham ED, Mohr SB, Garland FC, Garland CF: **Vitamin D for Cancer Prevention and Survival**. *Clinical Reviews in Bone and Mineral Metabolism* 2009, **7**(2):159-175.
36. Grant WB: **An ecological study of cancer incidence and mortality rates in France with respect to latitude, an index for vitamin D production**. *Dermato-endocrinology* 2010, **2**(2):62-67.
37. Porojnicu A, Robsahm TE, Berg JP, Moan J: **Season of diagnosis is a predictor of cancer survival. Sun-induced vitamin D may be involved: a possible role of sun-induced Vitamin D**. *The Journal of steroid biochemistry and molecular biology* 2007, **103**(3-5):675-678.
38. Robsahm TE, Tretli S, Dahlback A, Moan J: **Vitamin D3 from sunlight may improve the prognosis of breast-, colon- and prostate cancer (Norway)**. *Cancer Causes & Control* 2004, **15**(2):149-158.
39. Rohan T: **Epidemiological Studies of Vitamin D and Breast Cancer**. *Nutrition reviews* 2007, **65**(Supplement 1):80-80.
40. Chen W, Armstrong BK, Rahman B, Zheng R, Zhang S, Clements M: **Relationship between cancer survival and ambient ultraviolet B irradiance in China**. *Cancer Causes & Control* 2013, **24**(7):1323-1330.
41. Almquist M, Bondeson AG, Bondeson L, Malm J, Manjer J: **Serum levels of vitamin D, PTH and calcium and breast cancer risk-a prospective nested case-control study**. *International Journal of Cancer* 2010, **127**(9):2159-2168.
42. Shao T, Klein P, Grossbard ML: **Vitamin D and breast cancer**. *Oncologist* 2012, **17**(1):36-45.
43. Atoum M, Alzoughool F: **Vitamin D and Breast Cancer: Latest Evidence and Future Steps**. *Breast cancer : basic and clinical research* 2017, **11**:1-8.
44. Jiang X, Dimou NL, Al-Dabhani K, Lewis SJ, Martin RM, Haycock PC, Gunter MJ, Key TJ, Eeles RA, Muir K *et al*: **Circulating vitamin D concentrations and risk of breast and prostate cancer: a Mendelian randomization study**. *International journal of epidemiology* 2018.
45. Vojdeman FJ, Madsen CM, Frederiksen K, Durup D, Olsen A, Hansen L, Heegaard AM, Lind B, Tjonneland A, Jorgensen HL *et al*: **Vitamin D levels and cancer incidence in 217,244 individuals from primary health care in Denmark**. *International Journal of Cancer* 2019.
46. Freedman DM, Looker AC, Chang SC, Graubard BI: **Prospective study of serum vitamin D and cancer mortality in the United States**. *Journal of the National Cancer Institute* 2007, **99**(21):1594-1602.

47. Rose AA, Elser C, Ennis M, Goodwin PJ: **Blood levels of vitamin D and early stage breast cancer prognosis: a systematic review and meta-analysis**. *Breast cancer research and treatment* 2013, **141**(3):331-339.
48. Jacobs ET, Kohler LN, Kunihiro AG, Jurutka PW: **Vitamin D and Colorectal, Breast, and Prostate Cancers: A Review of the Epidemiological Evidence**. *Journal of Cancer* 2016, **7**(3):232-240.
49. Michels KB, Xue F, Brandt L, Ekbom A: **Hyperparathyroidism and subsequent incidence of breast cancer**. *International Journal of Cancer* 2004, **110**(3):449-451.
50. Nilsson IL, Zedenius J, Yin L, Ekbom A: **The association between primary hyperparathyroidism and malignancy: nationwide cohort analysis on cancer incidence after parathyroidectomy**. *Endocrine-related cancer* 2007, **14**(1):135-140.
51. Palmer M, Adami HO, Krusemo UB, Ljunghall S: **Increased risk of malignant diseases after surgery for primary hyperparathyroidism. A nationwide cohort study**. *American journal of epidemiology* 1988, **127**(5):1031-1040.
52. Pickard AL, Gridley G, Mellemkjae L, Johansen C, Kofoed-Enevoldsen A, Cantor KP, Brinton LA: **Hyperparathyroidism and subsequent cancer risk in Denmark**. *Cancer* 2002, **95**(8):1611-1617.
53. Birch MA, Carron JA, Scott M, Fraser WD, Gallagher JA: **Parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor expression and mitogenic responses in human breast cancer cell lines**. *British journal of cancer* 1995, **72**(1):90-95.
54. Cataisson C, Lieberherr M, Cros M, Gauville C, Graulet AM, Cotton J, Calvo F, de Vernejoul MC, Foley J, Bouizar Z: **Parathyroid hormone-related peptide stimulates proliferation of highly tumorigenic human SV40-immortalized breast epithelial cells**. *Journal of bone and mineral research* 2000, **15**(11):2129-2139.
55. Hoey RP, Sanderson C, Iddon J, Brady G, Bundred NJ, Anderson NG: **The parathyroid hormone-related protein receptor is expressed in breast cancer bone metastases and promotes autocrine proliferation in breast carcinoma cells**. *British journal of cancer* 2003, **88**(4):567-573.
56. Linforth R, Anderson N, Hoey R, Nolan T, Downey S, Brady G, Ashcroft L, Bundred N: **Coexpression of parathyroid hormone related protein and its receptor in early breast cancer predicts poor patient survival**. *Clinical Cancer Research* 2002, **8**(10):3172-3177.
57. Tran TH, Utama FE, Sato T, Peck AR, Langenheim JF, Udhane SS, Sun Y, Liu C, Girondo MA, Kovatich AJ *et al*: **Loss of Nuclear Localized Parathyroid Hormone-Related Protein in Primary Breast Cancer Predicts Poor Clinical Outcome and Correlates with Suppressed Stat5 Signaling**. *Clinical Cancer Research* 2018, **24**(24):6355-6366.
58. Brown HK, Allocca G, Ottewell PD, Wang N, Brown NJ, Croucher PI, Eaton CL, Holen I: **Parathyroid Hormone (PTH) Increases Skeletal**

Tumour Growth and Alters Tumour Distribution in an In Vivo Model of Breast Cancer. *Int J Mol Sci* 2018, **19**(10).
59. Swami S, Johnson J, Bettinson LA, Kimura T, Zhu H, Albertelli MA, Johnson RW, Wu JY: **Prevention of breast cancer skeletal metastases with parathyroid hormone**. *JCI insight* 2017, **2**(17).
60. Cui Y, Rohan TE: **Vitamin D, calcium, and breast cancer risk: a review**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2006, **15**(8):1427-1437.
61. Brown EM, MacLeod RJ: **Extracellular calcium sensing and extracellular calcium signaling**. *Physiological reviews* 2001, **81**(1):239-297.
62. Cheng I, Klingensmith ME, Chattopadhyay N, Kifor O, Butters RR, Soybel DI, Brown EM: **Identification and localization of the extracellular calcium-sensing receptor in human breast**. *The Journal of clinical endocrinology and metabolism* 1998, **83**(2):703-707.
63. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Butters RR, Brown EM: **Extracellular calcium-sensing receptor expression and its potential role in regulating parathyroid hormone-related peptide secretion in human breast cancer cell lines**. *Endocrinology* 2000, **141**(12):4357-4364.
64. Tennakoon S, Aggarwal A, Kallay E: **The calcium-sensing receptor and the hallmarks of cancer**. *Biochimica et biophysica acta* 2016, **1863**(6 Pt B):1398-1407.
65. Kim W, Wysolmerski JJ: **Calcium-Sensing Receptor in Breast Physiology and Cancer**. *Frontiers in physiology* 2016, **7**:440.
66. National Institute of Health;: **Biological Sciences Curriculum Study. NIH Curriculum Supplement Series.Understanding Human Genetic Variation.** Available at: https://www.ncbi.nlm.nih.gov/books/NBK20363/ Access date: 18-03-2019.
67. Sud A, Kinnersley B, Houlston RS: **Genome-wide association studies of cancer: current insights and future perspectives**. *Nature Reviews Cancer* 2017, **17**:692.
68. Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCullough ML, Gallicchio L, Jacobs EJ, Ascherio A, Helzlsouer K, Jacobs KB *et al*: **Genome-wide association study of circulating vitamin D levels**. *Human molecular genetics* 2010, **19**(13):2739-2745.
69. Anderson D, Holt BJ, Pennell CE, Holt PG, Hart PH, Blackwell JM: **Genome-wide association study of vitamin D levels in children: replication in the Western Australian Pregnancy Cohort (Raine) study**. *Genes Immun* 2014, **15**(8):578-583.

70. Benjamin EJ, Dupuis J, Larson MG, Lunetta KL, Booth SL, Govindaraju DR, Kathiresan S, Keaney JF, Jr., Keyes MJ, Lin JP *et al*: **Genome-wide association with select biomarker traits in the Framingham Heart Study**. *BMC Med Genet* 2007, **8 Suppl 1**:S11.
71. Jorde R, Schirmer H, Wilsgaard T, Joakimsen RM, Mathiesen EB, Njolstad I, Lochen ML, Figenschau Y, Berg JP, Svartberg J *et al*: **Polymorphisms related to the serum 25-hydroxyvitamin D level and risk of myocardial infarction, diabetes, cancer and mortality. The Tromso Study**. *PloS one* 2012, **7**(5):e37295.
72. Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL *et al*: **Common genetic determinants of vitamin D insufficiency: a genome-wide association study**. *Lancet* 2010, **376**(9736):180-188.
73. Emdin CA, Khera AV, Kathiresan S: **Mendelian Randomization**. *Jama* 2017, **318**(19):1925-1926.
74. Margolis RN, Christakos S: **The nuclear receptor superfamily of steroid hormones and vitamin D gene regulation. An update**. *Annals of the New York Academy of Sciences* 2010, **1192**:208-214.
75. Wang Y, Zhu J, DeLuca HF: **Where is the vitamin D receptor?** *Archives of biochemistry and biophysics* 2012, **523**(1):123-133.
76. Narbaitz R, Sar M, Stumpf WE, Huang S, DeLuca HF: **1,25-Dihydroxyvitamin D3 target cells in rat mammary gland**. *Hormone research* 1981, **15**(4):263-269.
77. Berger U, Wilson P, McClelland RA, Colston K, Haussler MR, Pike JW, Coombes RC: **Immunocytochemical detection of 1,25-dihydroxyvitamin D3 receptor in breast cancer**. *Cancer Res* 1987, **47**(24 Pt 1):6793-6799.
78. Cui X, Pertile R, Eyles DW: **The vitamin D receptor (VDR) binds to the nuclear matrix via its hinge domain: A potential mechanism for the reduction in VDR mediated transcription in mitotic cells**. *Molecular and cellular endocrinology* 2018, **472**:18-25.
79. Trivedi T, Zheng Y, Fournier PGJ, Murthy S, John S, Schillo S, Dunstan CR, Mohammad KS, Zhou H, Seibel MJ *et al*: **The vitamin D receptor is involved in the regulation of human breast cancer cell growth via a ligand-independent function in cytoplasm**. *Oncotarget* 2017, **8**(16):26687-26701.
80. Friedrich M, Axt-Fliedner R, Villena-Heinsen C, Tilgen W, Schmidt W, Reichrath J: **Analysis of vitamin D-receptor (VDR) and retinoid X-receptor alpha in breast cancer**. *The Histochemical journal* 2002, **34**(1-2):35-40.
81. Al-Azhri J, Zhang Y, Bshara W, Zirpoli GR, McCann SE, Khoury T, Morrison CD, Edge SB, Ambrosone CB, Yao S: **Tumor Expression of Vitamin D Receptor and Breast Cancer Histopathological Characteristics and Prognosis**. *Clinical Cancer Research* 2016, **23**(1):97-103.

82. Berger U, McClelland RA, Wilson P, Greene GL, Haussler MR, Pike JW, Colston K, Easton D, Coombes RC: **Immunocytochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis**. *Cancer Res* 1991, **51**(1):239-244.
83. Ditsch N, Toth B, Mayr D, Lenhard M, Gallwas J, Weissenbacher T, Dannecker C, Friese K, Jeschke U: **The association between vitamin D receptor expression and prolonged overall survival in breast cancer**. *The journal of histochemestry and cytochemestry* 2012, **60**(2):121-129.
84. Eisman JA, Suva LJ, Martin TJ: **Significance of 1,25-dihydroxyvitamin D3 receptor in primary breast cancers**. *Cancer Res* 1986, **46**(10):5406-5408.
85. Freake HC, Abeyasekera G, Iwasaki J, Marcocci C, MacIntyre I, McClelland RA, Skilton RA, Easton DF, Coombes RC: **Measurement of 1,25-dihydroxyvitamin D3 receptors in breast cancer and their relationship to biochemical and clinical indices**. *Cancer Res* 1984, **44**(4):1677-1681.
86. Friedrich M, Villena-Heinsen C, Tilgen W, Schmidt W, Reichrat J, Axt-Fliedner R: **Vitamin D receptor (VDR) expression is not a prognostic factor in breast cancer**. *Anticancer research* 2002, **22**(3):1919-1924.
87. Manjer J, Elmstahl S, Janzon L, Berglund G: **Invitation to a population-based cohort study: differences between subjects recruited using various strategies**. *Scandinavian Journal of Public Health* 2002, **30**(2):103-103.
88. Sandsveden M, Manjer J: **Selenium and breast cancer risk: A prospective nested case-control study on serum selenium levels, smoking habits and overweight**. *International Journal of Cancer* 2017, **141**(9):1741-1750.
89. Fernandez C, Sandin M, Sampaio JL, Almgren P, Narkiewicz K, Hoffmann M, Hedner T, Wahlstrand B, Simons K, Shevchenko A *et al*: **Plasma lipid composition and risk of developing cardiovascular disease**. *PloS one* 2013, **8**(8):e71846.
90. Borgquist S, Anagnostaki L, Jirstrom K, Landberg G, Manjer J: **Breast tumours following combined hormone replacement therapy express favourable prognostic factors**. *International Journal of Cancer* 2007, **120**(10):2202-2207.
91. Butt S, Borgquist S, Anagnostaki L, Landberg Gr, Manjer J: **Parity and age at first childbirth in relation to the risk of different breast cancer subgroups**. *International journal of cancer* 2009, **125**(8):1926-1934.
92. Elebro K, Butt S, Dorkhan M, Jernstrom H, Borgquist S: **Age at first childbirth and oral contraceptive use are associated with risk of androgen receptor-negative breast cancer: the Malmo Diet and Cancer Cohort**. *Cancer Causes & Control* 2014, **25**(8):945-957.

93. Elebro K, Bendahl PO, Jernstrom H, Borgquist S: **Androgen receptor expression and breast cancer mortality in a population-based prospective cohort**. *Breast cancer research and treatment* 2017, **165**(3):645-657.
94. Wallström P: **Diet, lifestyle, antioxidants, and biomarkers of cancer risk: an epidemiological report from the Malmö Diet and Cancer cohort**. *Dissertation/Thesis.* Malmö: Univ.-sjukhuset MAS; 2002.
95. Anker P, Wieland E, Ammann D, Dohner RE, Asper R, Simon W: **Neutral carrier based ion-selective electrode for the determination of total calcium in blood serum**. *Analytical chemistry* 1981, **53**(13):1970-1974.
96. Soderholm M, Almgren P, Jood K, Stanne TM, Olsson M, Ilinca A, Lorentzen E, Norrving B, Engstrom G, Melander O *et al*: **Exome array analysis of ischaemic stroke: results from a southern Swedish study**. *European journal of neurology* 2016, **23**(12):1722-1728.
97. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira M A R, Bender D, Maller J, Sklar P, de Bakker P I W, Daly M J *et al*: **PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses**. *Am J Hum Genet* 2007, **81**(3):559-575.
98. Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, Klemm A, Flicek P, Manolio T, Hindorff L *et al*: **The NHGRI GWAS Catalog, a curated resource of SNP-trait associations**. *Nucleic Acids Res* 2014, **42**(Database issue):D1001-1006.
99. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI: **SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap**. *Bioinformatics* 2008, **24**(24):2938-2939.
100. Costa PL, Franca MM, Ferraz-de-Souza B: **Nonspecific binding of a frequently used vitamin D receptor (VDR) antibody: important implications for vitamin D research in human health**. *Endocrine* 2016, **54**(2):556-559.
101. Wang Y, Becklund BR, DeLuca HF: **Identification of a highly specific and versatile vitamin D receptor antibody**. *Archives of biochemistry and biophysics* 2010, **494**(2):166-177.
102. Li X, Li L, Moran MS, Jiang L, Kong X, Zhang H, Zhang X, Haffty BG, Yang Q: **Prognostic significance of calcium-sensing receptor in breast cancer**. *Tumour biology* 2014, **35**(6):5709-5715.
103. Brooke HL, Talback M, Hornblad J, Johansson LA, Ludvigsson JF, Druid H, Feychting M, Ljung R: **The Swedish cause of death register**. *European journal of epidemiology* 2017, **32**(9):765-773.
104. Pukkala E, Engholm G, Hojsgaard Schmidt LK, Storm H, Khan S, Lambe M, Pettersson D, Olafsdottir E, Tryggvadottir L, Hakanen T *et al*: **Nordic Cancer Registries - an overview of their procedures and data comparability**. *Acta oncologica (Stockholm, Sweden)* 2018, **57**(4):440-455.

105. Donders AR, van der Heijden GJ, Stijnen T, Moons KG: **Review: a gentle introduction to imputation of missing values**. *Journal of clinical epidemiology* 2006, **59**(10):1087-1091.
106. Newgard CD, Haukoos JS: **Advanced statistics: missing data in clinical research--part 2: multiple imputation**. *Academic emergency medicine* 2007, **14**(7):669-678.
107. Shirazi L, Almquist M, Borgquist S, Malm J, Manjer J: **Serum vitamin D (25OHD3) levels and the risk of different subtypes of breast cancer: A nested case-control study**. *Breast* 2016, **28**:184-190.
108. de Sousa Almeida-Filho B, De Luca Vespoli H, Pessoa EC, Machado M, Nahas-Neto J, Nahas EAP: **Vitamin D deficiency is associated with poor breast cancer prognostic features in postmenopausal women**. *The Journal of steroid biochemistry and molecular biology* 2017, **174**:284-289.
109. Ismail A, El-Awady R, Mohamed G, Hussein M, Ramadan SS: **Prognostic Significance of Serum Vitamin D Levels in Egyptian Females with Breast Cancer**. *Asian Pacific journal of cancer prevention* 2018, **19**(2):571-576.
110. Karthikayan A, Sureshkumar S, Kadambari D, Vijayakumar C: **Low serum 25-hydroxy vitamin D levels are associated with aggressive breast cancer variants and poor prognostic factors in patients with breast carcinoma**. *Archives of endocrinology and metabolism* 2018, **62**(4):452-459.
111. Wu Y, Sarkissyan M, Clayton S, Chlebowski R, Vadgama JV: **Association of Vitamin D3 Level with Breast Cancer Risk and Prognosis in African-American and Hispanic Women**. *Cancers* 2017, **9**(10).
112. Yao S, Kwan ML, Ergas IJ, Roh JM, Cheng TD, Hong CC, McCann SE, Tang L, Davis W, Liu S *et al*: **Association of Serum Level of Vitamin D at Diagnosis With Breast Cancer Survival: A Case-Cohort Analysis in the Pathways Study**. *JAMA oncology* 2017, **3**(3):351-357.
113. Yao S, Sucheston LE, Millen AE, Johnson CS, Trump DL, Nesline MK, Davis W, Hong CC, McCann SE, Hwang H *et al*: **Pretreatment serum concentrations of 25-hydroxyvitamin D and breast cancer prognostic characteristics: a case-control and a case-series study**. *PloS one* 2011, **6**(2):e17251.
114. Vrieling A, Hein R, Abbas S, Schneeweiss A, Flesch-Janys D, Chang-Claude J: **Serum 25-hydroxyvitamin D and postmenopausal breast cancer survival: a prospective patient cohort study**. *Breast cancer research* 2011, **13**(4):R74.
115. Viljoen A, Singh DK, Twomey PJ, Farrington K: **Analytical quality goals for parathyroid hormone based on biological variation**. *Clinical chemistry and laboratory medicine : CCLM / FESCC* 2008, **46**(10):1438-1442.

116. Ankrah-Tetteh T, Wijeratne S, Swaminathan R: **Intraindividual variation in serum thyroid hormones, parathyroid hormone and insulin-like growth factor-1**. *Annals of clinical biochemistry* 2008, **45**(Pt 2):167-169.
117. Liu G, Hu X, Chakrabarty S: **Calcium sensing receptor down-regulates malignant cell behavior and promotes chemosensitivity in human breast cancer cells**. *Cell calcium* 2009, **45**(3):216-225.
118. El Hiani Y, Ahidouch A, Lehen'kyi V, Hague F, Gouilleux F, Mentaverri R, Kamel S, Lassoued K, Brule G, Ouadid-Ahidouch H: **Extracellular signal-regulated kinases 1 and 2 and TRPC1 channels are required for calcium-sensing receptor-stimulated MCF-7 breast cancer cell proliferation**. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2009, **23**(4-6):335-346.
119. El Hiani Y, Lehen'kyi V, Ouadid-Ahidouch H, Ahidouch A: **Activation of the calcium-sensing receptor by high calcium induced breast cancer cell proliferation and TRPC1 cation channel over-expression potentially through EGFR pathways**. *Archives of biochemistry and biophysics* 2009, **486**(1):58-63.
120. Manning AT, O'Brien N, Kerin MJ: **Roles for the calcium sensing receptor in primary and metastatic cancer**. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* 2006, **32**(7):693-697.
121. Moy KA, Mondul AM, Zhang H, Weinstein SJ, Wheeler W, Chung CC, Mannisto S, Yu K, Chanock SJ, Albanes D: **Genome-wide association study of circulating vitamin D-binding protein**. *The American journal of clinical nutrition* 2014, **99**(6):1424-1431.
122. Dimitrakopoulou VI, Tsilidis KK, Haycock PC, Dimou NL, Al-Dabhani K, Martin RM, Lewis SJ, Gunter MJ, Mondul A, Shui IM *et al*: **Circulating vitamin D concentration and risk of seven cancers: Mendelian randomisation study**. *BMJ (Clinical research ed)* 2017, **359**:j4761.
123. Chandler PD, Tobias DK, Wang L, Smith-Warner SA, Chasman DI, Rose L, Giovannucci EL, Buring JE, Ridker PM, Cook NR *et al*: **Association between Vitamin D Genetic Risk Score and Cancer Risk in a Large Cohort of U.S. Women**. *Nutrients* 2018, **10**(1).
124. Ong JS, Gharahkhani P, An J, Law MH, Whiteman DC, Neale RE, MacGregor S: **Vitamin D and overall cancer risk and cancer mortality: a Mendelian randomization study**. *Human molecular genetics* 2018, **27**(24):4315-4322.

125. Tavera-Mendoza LE, Westerling T, Libby E, Marusyk A, Cato L, Cassani R, Cameron LA, Ficarro SB, Marto JA, Klawitter J *et al*: **Vitamin D receptor regulates autophagy in the normal mammary gland and in luminal breast cancer cells**. *Proceedings of the National Academy of Sciences of the United States of America* 2017, **114**(11):E2186-e2194.
126. Thakkar A, Wang B, Picon-Ruiz M, Buchwald P, Ince TA: **Vitamin D and androgen receptor-targeted therapy for triple-negative breast cancer**. *Breast cancer research and treatment* 2016, **157**(1):77-90.
127. Hansen L, Tjonneland A, Koster B, Brot C, Andersen R, Cohen AS, Frederiksen K, Olsen A: **Vitamin D Status and Seasonal Variation among Danish Children and Adults: A Descriptive Study**. *Nutrients* 2018, **10**(11).
128. Meng JE, Hovey KM, Wactawski-Wende J, Andrews CA, Lamonte MJ, Horst RL, Genco RJ, Millen AE: **Intraindividual variation in plasma 25-hydroxyvitamin D measures 5 years apart among postmenopausal women**. *Cancer epidemiology, biomarkers & prevention* 2012, **21**(6):916-924.
129. Platz EA, Leitzmann MF, Hollis BW, Willett WC, Giovannucci E: **Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and subsequent risk of prostate cancer**. *Cancer Causes & Control* 2004, **15**(3):255-265.
130. O'Brien KM, Sandler DP, House M, Taylor JA, Weinberg CR: **The influence of a breast cancer diagnosis on serum 25-hydroxyvitamin D**. *American journal of epidemiology* 2019.
131. Gallagher SK, Johnson LK, Milne DB: **Short-term and long-term variability of indices related to nutritional status. I: Ca, Cu, Fe, Mg, and Zn**. *Clinical chemistry* 1989, **35**(3):369-373.
132. Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M: **Current databases on biological variation: pros, cons and progress**. *Scandinavian journal of clinical and laboratory investigation* 1999, **59**(7):491-500.
133. Young MM, Nordin BE: **Calcium metabolism and the menopause**. *Proceedings of the Royal Society of Medicine* 1967, **60**(11 Part 1):1137-1138.
134. Prince RL, Dick I, Devine A, Price RI, Gutteridge DH, Kerr D, Criddle A, Garcia-Webb P, St John A: **The effects of menopause and age on calcitropic hormones: a cross-sectional study of 655 healthy women aged 35 to 90**. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 1995, **10**(6):835-842.
135. Nordin BE, JM WI, Clifton PM, McArthur R, Scopacasa F, Need AG, Morris HA, O'Loughlin PD, Horowitz M: **A longitudinal study of bone-related biochemical changes at the menopause**. *Clinical endocrinology* 2004, **61**(1):123-130.

136. Camp RL, Neumeister V, Rimm DL: **A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers**. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2008, **26**(34):5630-5637.
137. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Kochli OR, Mross F, Dieterich H, Moch H, Mihatsch M *et al*: **Tissue microarrays for rapid linking of molecular changes to clinical endpoints**. *Am J Pathol* 2001, **159**(6):2249-2256.
138. SantaCruz Antibodies: **VDR Antibody (D-6): sc-13133.** Available at: https://www.scbt.com/scbt/sv/product/vdr-antibody-d-6?requestFrom=search Access date: 21-03-2019.
139. UniProt: **UniProtKB - P11473 (VDR_HUMAN)** Available at: https://www.uniprot.org/uniprot/P11473 Access date: 21-03-2019.
140. Vanhouten JN, Wysolmerski JJ: **The calcium-sensing receptor in the breast**. *Best practice & research Clinical endocrinology & metabolism* 2013, **27**(3):403-414.
141. Katz MH: **Multivariable Analysis, A Practical Guide for Clinicians**, 2 edn: Cambridge; 2008.
142. Biganzoli E, Desmedt C, Fornili M, de Azambuja E, Cornez N, Ries F, Closon-Dejardin MT, Kerger J, Focan C, Di Leo A *et al*: **Recurrence dynamics of breast cancer according to baseline body mass index**. *European journal of cancer* 2017, **87**:10-20.
143. Gershuni V, Li YR, Williams AD, So A, Steel L, Carrigan E, Tchou J: **Breast cancer subtype distribution is different in normal weight, overweight, and obese women**. *Breast cancer research and treatment* 2017, **163**(2):375-381.
144. Majeed W, Aslam B, Javed I, Khaliq T, Muhammad F, Ali A, Raza A: **Breast cancer: major risk factors and recent developments in treatment**. *Asian Pacific journal of cancer prevention : APJCP* 2014, **15**(8):3353-3358.
145. Neuhouser ML, Aragaki AK, Prentice RL, Manson JE, Chlebowski R, Carty CL, Ochs-Balcom HM, Thomson CA, Caan BJ, Tinker LF *et al*: **Overweight, Obesity, and Postmenopausal Invasive Breast Cancer Risk: A Secondary Analysis of the Women's Health Initiative Randomized Clinical Trials**. *JAMA oncology* 2015, **1**(5):611-621.
146. Picon-Ruiz M, Morata-Tarifa C, Valle-Goffin JJ, Friedman ER, Slingerland JM: **Obesity and adverse breast cancer risk and outcome: Mechanistic insights and strategies for intervention**. *CA Cancer J Clin* 2017, **67**(5):378-397.
147. Savastano S, Barrea L, Savanelli MC, Nappi F, Di Somma C, Orio F, Colao A: **Low vitamin D status and obesity: Role of nutritionist**. *Reviews in endocrine & metabolic disorders* 2017, **18**(2):215-225.
148. Walsh JS, Bowles S, Evans AL: **Vitamin D in obesity**. *Current opinion in endocrinology, diabetes, and obesity* 2017, **24**(6):389-394.

149. Li C, Aronsson CA, Hedblad B, Gullberg B, Wirfalt E, Berglund G: **Ability of physical activity measurements to assess health-related risks**. *European journal of clinical nutrition* 2009, **63**(12):1448-1451.
150. Adraskela K, Veisaki E, Koutsilieris M, Philippou A: **Physical Exercise Positively Influences Breast Cancer Evolution**. *Clinical breast cancer* 2017, **17**(6):408-417.
151. Brewer HR, Jones ME, Schoemaker MJ, Ashworth A, Swerdlow AJ: **Family history and risk of breast cancer: an analysis accounting for family structure**. *Breast cancer research and treatment* 2017, **165**(1):193-200.
152. Dethlefsen C, Pedersen KS, Hojman P: **Every exercise bout matters: linking systemic exercise responses to breast cancer control**. *Breast cancer research and treatment* 2017, **162**(3):399-408.
153. Hirschey R, Docherty SL, Pan W, Lipkus I: **Exploration of Exercise Outcome Expectations Among Breast Cancer Survivors**. *Cancer nursing* 2017, **40**(2):E39-e46.
154. Kraschnewski JL, Schmitz KH: **Exercise in the Prevention and Treatment of Breast Cancer: What Clinicians Need to Tell Their Patients**. *Current sports medicine reports* 2017, **16**(4):263-267.
155. Shiyanbola OO, Arao RF, Miglioretti DL, Sprague BL, Hampton JM, Stout NK, Kerlikowske K, Braithwaite D, Buist DSM, Egan KM *et al*: **Emerging Trends in Family History of Breast Cancer and Associated Risk**. *Cancer epidemiology, biomarkers & prevention* 2017, **26**(12):1753-1760.

Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Surgery
Lund University, Faculty of Medicine
Doctoral Dissertation Series 2019:43
ISBN 978-91-7619-772-1
ISSN 1652-8220