Selenium and Breast Cancer. Is It Protective? Epidemiologic Evidence Regarding Risk and Prognosis

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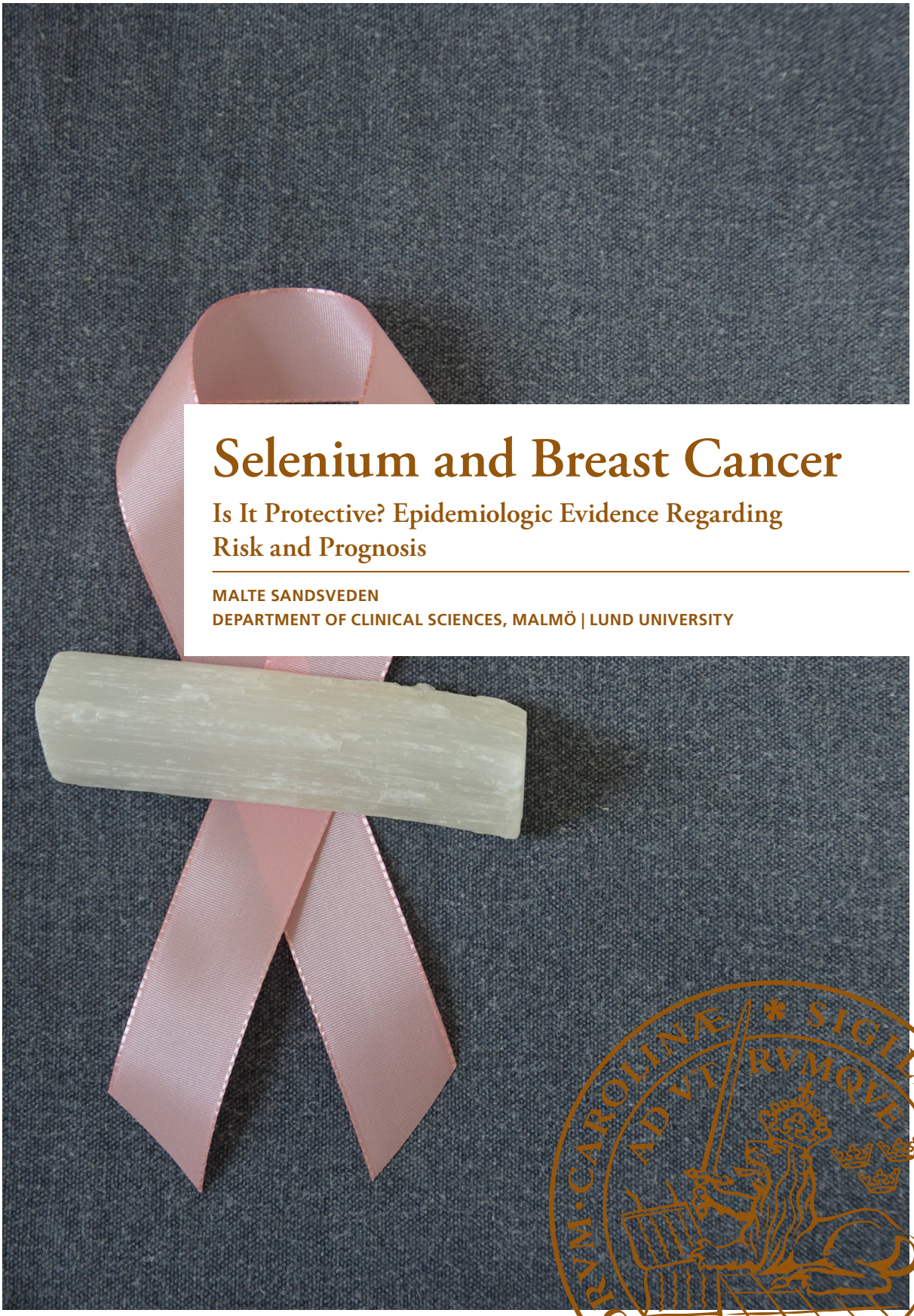

Selenium and Breast Cancer

Is It Protective? Epidemiologic Evidence Regarding Risk and Prognosis

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Is It Protective? Epidemiologic Evidence Regarding Risk and Prognosis

Malte Sandsveden

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Title and subtitle: **Selenium and Breast Cancer – Is It Protective? Epidemiologic Evidence Regarding Risk and Prognosis.**

Background

Previous research has suggested that the essential nutrient selenium might be protective regarding both risk of, and mortality in, breast cancer. However, the evidence has not been convincing. Selenium-dependent proteins are mainly involved in antioxidation and thyroid hormone function. The overall aim of this thesis was to investigate if selenium or selenium-related genes and proteins, including the thyroid hormone receptor alpha 2 (THRα-2), have any role in breast cancer development or prognosis.

Method

This thesis is based on the results of four epidemiological research studies. We used data from the Malmö Diet and Cancer Study, a population-based cohort that included 17,035 women during 1991-1996. Selenium levels were measured from stored serum samples, from dietary assessment and by a genetic score. In one study we evaluated the intra-tumor expression of THRα-2 in stored tumor samples by using immunohistology. Information regarding breast cancer diagnosis and death was collected from the Swedish Cancer Registry and the Swedish Cause of Death Registry. Tumor data was collected both by histologic evaluation of the tumors and from clinical records.

Results

No difference in overall risk of breast cancer was seen for women with high or low levels of selenium in serum or in dietary intake or by genetic score. BMI or smoking did not affect the association; however, for women with genetic variation in the selenium-dependent protein glutathione peroxidase 1 (GPx-1), a protective effect was seen among women with intermediate and high intake of selenium. Women with a genetic variation in GPx-1 also had an overall lower risk of breast cancer compared to those with the standard alleles. Women with the highest levels of selenium in serum had a lower mortality in breast cancer compared to women with the lowest levels. However, no difference was seen regarding favorable or unfavorable tumor characteristics. A low THRα-2 expression in breast tumors was associated with prognostically unfavorable tumor characteristics and also a higher mortality in breast cancer.

Conclusion

The results of this doctoral thesis do not support the claim that there is any overall protective effect of selenium regarding breast cancer risk. However, they do suggest that selenium might be protective from death in breast cancer, since pre-diagnostic serum levels are inversely correlated with mortality. Furthermore, the selenium-dependent protein GPx-1 might be involved in breast cancer development, since carriers of alternative alleles of the GPx-1 gene have a lower risk of breast cancer and show a protective effect from selenium intake. Additionally, the expression of THRα-2 in breast tumors is likely to be prognostically favorable. Future studies should focus on mechanistic evidence and whether the associations found are causally related to breast cancer risk and prognosis.

Key words Breast Cancer, Selenium, THRα-2, Risk, Prognosis, Epidemiology, SNP, The Malmö Diet and Cancer Study

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Cover photo by Malte Sandsveden and Julia Larsson. A selenite crystal on top of a pink ribbon. The pink ribbon is an international symbol for the fight against breast cancer. The selenite crystal is supposed to symbolize the element selenium. Despite its name, selenite does not contain any significant amount of selenium. However, it is also named after the Greek word for moon, just like the element selenium studied in this thesis.

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MADE IN SWEDEN

Den här avhandlingen tillägnar jag min farmor

Table of Contents

List of papers

The thesis is based on the following papers.

I. **Sandsveden M**, Manjer J. Selenium and breast cancer risk: A prospective nested case-control study on serum selenium levels, smoking habits and overweight. *Int J Cancer*. 2017;141(9):1741-50.

II. **Sandsveden M**, Nilsson E, Borgquist S, Rosendahl AH, Manjer J. Prediagnostic serum selenium levels in relation to breast cancer survival and tumor characteristics. *Int J Cancer*. 2020; 147:2424-2436.

III. **Sandsveden M**, Borgquist S, Rosendahl AH, Manjer J. Low thyroid hormone receptor alpha-2 (THRα-2) tumor expression is associated with unfavorable tumor characteristics and high breast cancer mortality. Submitted manuscript.

IV. **Sandsveden M**, Bengtsson Y, Melander O, Rosendahl AH, Manjer J. Genetic variation interacts with selenium exposure regarding breast cancer risk: Assessing dietary intake, serum levels and genetically elevated selenium levels. Submitted manuscript.

List of abbreviations

BMI = Body mass index, kg/m^2

ER = Estrogen receptor

GPx-1 = Glutathione peroxidase 1

GPx-3 = Glutathione peroxidase 3

GPx-4 = Glutathione peroxidase 4

GWAS = Genome-wide association studies

HER2 = Human epidermal growth factor receptor 2

HR = Hazard ratio

ICP-SFMS = Inductively coupled plasma sector field mass spectrometry

MDCS = The Malmö Diet and Cancer Study

MKC = Malmö Kost Cancer

MR = Mendelian randomization

PgR = Progesterone receptor

SNP = Single nucleotide polymorphism

SOD-2 = Superoxide dismutase 2 (also known as manganese-dependent superoxide dismutase)

THRα-1 = Thyroid hormone receptor alpha 1

THRα-2 = Thyroid hormone receptor alpha 2

THRß = Thyroid hormone receptor beta

TMA = Tissue microarray

Populärvetenskaplig sammanfattning (In Swedish)

Bakgrund

Den här avhandlingen handlar om sambandet mellan selen och bröstcancer. Kan selen vara skyddande? Tidigare forskning har sett samband mellan låga selennivåer och ökad risk för att insjukna och dö i bröstcancer. Men många av de studierna har haft begränsningar, samtidigt som resultaten inte varit entydiga. Därför har det varit svårt att fastställa om det finns ett orsakssamband.

Selen är ett grundämne som människor får i sig via kosten. Det finns i flertalet livsmedel, men mängden selen i grönsaker och växter avgörs av hur mycket selen som det finns i jorden där de växer. I Sverige är halterna i jorden låga och därför är intaget av selen lågt i befolkningen. De främsta källorna till selen i Sverige är animaliska, så som kött, fisk, mjölk och ägg, men även nötter, frön och importerade grönsaker kan innehålla rikligt med selen.

Selen behövs hos människor som byggsten i så kallade selenoproteiner. Det finns 25 sådana proteiner som har olika funktioner och de är viktiga för bland annat kroppens antioxidation och sköldkörtelfunktion. Med låga selennivåer sjunker aktiviteten i dessa proteiner. Antioxidation är ett viktigt försvar mot cellskada, som kan leda till cancerutveckling. Detta lyfts ofta som en möjlig mekanism där selen kan verka skyddande. Men även sköldkörtelfunktion har kopplats till bröstcancer och selen kan vara en möjlig länk.

Bröstcancer är en cancerform som ökar och sedan 2020 är det den vanligaste cancerdiagnosen globalt sett. I Sverige drabbas var tionde kvinna av bröstcancer innan 75-års ålder. Av de som diagnosticerats med bröstcancer i Sverige är prognosen idag bättre än den tidigare varit, nästan 9 av 10 överlever, räknat 10 år efter diagnos, men det motsvarar trots det att 1353 kvinnor dog i bröstcancer i Sverige 2019. Uppskattningsvis orsakas minst var femte bröstcancer av påverkbara riskfaktorer. Hög ålder, kvinnligt kön och ärftlighet är starka riskfaktorer för bröstcancer som inte går att påverka. Hormonersättning med kvinnligt könshormon, hög alkoholkonsumtion, låg fysisk aktivitet och övervikt är exempel på riskfaktorer som är påverkbara. Selen skulle möjligtvis kunna vara en påverkbar riskfaktor.

Metod

Denna avhandling baseras på resultatet av fyra olika studier som separat undersöker selen och selenrelaterade gener och proteiners roll gällande risk och prognos för bröstcancer. Studierna utgår ifrån 17035 kvinnor som medverkade i befolkningsstudien Malmö Kost Cancer (MKC) 1991-1996. Alla deltagare intervjuades, fyllde i frågeformulär om kost och livsstil samt lämnade blodprover.

I den första studien undersöktes om risken att insjukna i bröstcancer påverkas av mängden selen i blodet. Genom Cancerregistret identifierade vi de kvinnor som fått diagnosen bröstcancer i MKC till och med 2013 (1186 kvinnor) och analyserade mängden selen i deras sparade blodprover samt hos lika många som inte fått bröstcancer. Vi undersökte också om övervikt eller rökning påverkade sambandet.

I den andra studien undersöktes risken att få brösttumörer med egenskaper som kopplas till dålig prognos, så som större tumör eller avsaknad av hormonreceptorer. Vi jämförde även överlevnaden i bröstcancer hos de med låga och höga selennivåer i blodet. Samma individer som i den första studien användes och selennivåerna i blodet fanns därmed tillgängligt. Information om tumörernas egenskaper samlades in på flera olika vis, bland annat hade en del av tumörerna undersökts tidigare i MKC, men även information från patientjournaler samlades in. Dödsorsak och dödsdatum hämtades från dödsorsaksregistret.

I den tredje studien undersöktes om en specifik receptor för sköldkörtelhormon (THRα-2) var associerad till egenskaper i brösttumören som påverkar prognosen, så som storlek och hormonreceptorer. Även överlevnaden bland de kvinnor med lågt respektive högt uttryck av THRα-2 jämfördes. För att mäta uttrycket av receptorn färgades den i tunna snitt av sparad tumörvävnad och undersöktes sedan i mikroskop för att avgöra vilka som hade högt eller lågt uttryck av receptorn.

I den sista studien i avhandlingen undersöktes om genetisk variation i selenrelaterade proteiner kunde påverka sambandet mellan selen och bröstcancerrisk, så att selen är skyddande för vissa kvinnor men inte för andra. Vi undersökte även om dessa genetiska variationer enskilt hade någon effekt på bröstcancerrisken. Arvsmassan hos alla deltagare i MKC analyserades från celler i sparade blodprover. Selen mättes på tre vis; 1) kostintag av selen, 2) selennivåer i blodet, 3) genetiska faktorer som ger högre selennivåer i blodet. Selens effekt på bröstcancerrisk undersöktes för alla och separat för de olika genetiska variationerna.

Resultat

Vi fann ingen generell skillnad i bröstcancerrisk bland de kvinnor som exponeras för lite eller mycket selen, oavsett om det mäts i kosten, i blodet eller genetiskt. Övervikt och rökning påverkade inte sambandet. Däremot sågs en skyddande effekt mot bröstcancer av ett medelhögt eller högt intag av selen hos de kvinnor med en genetisk variation i ett viktigt selenoprotein. Vi fann inga bevis för att selen påverkar risken för att diagnosticeras med brösttumörer med dålig prognos. Däremot hade de kvinnor med högst selenvärden i blodet också den lägsta dödligheten. Vidare så var ett lågt uttryck av THRα-2 i brösttumörer korrelerat till dåliga prognostiska markörer och ökad dödlighet i bröstcancer.

Slutsats

Resultaten i den här avhandlingen talar emot att det finns en generell skyddseffekt av selen gällande bröstcancerrisk. Däremot är det sannolikt att både högre selennivåer i blodet och THRα-2 uttryck i tumörer är associerat med överlevnad i bröstcancer. Det finns också indikationer att genetiska variationer i selenoproteinet GPx-1 kan minska bröstcancerrisken och att kvinnor med denna variant har en skyddande effekt av högre selenintag. Framtida forskning bör fokusera ytterligare på hur selenrelaterade proteiner och gener påverkar utvecklingen och prognosen i bröstcancer. Genom att påverka de processerna kan förhoppningsvis ytterligare alternativ till att förebygga och behandla bröstcancer utvecklas.

The thesis papers in one minute

Paper	Research question	Material and methods	Results and conclusion
I	Are there any associations between selenium levels and breast cancer risk? Can smoking and BMI affect that association?	Pre-diagnostic selenium levels in serum were compared between 1186 women with breast cancer and an equal number of controls using logistic regression, adjusted for established breast cancer risk factors. Analyses were stratified for smoking and BMI.	We found no overall association and no interaction by smoking or BMI. If an association exists, it is dependent on other factors than those investigated.
II	Can selenium affect survival in breast cancer or the risk of more aggressive tumors?	Survival among 1066 breast cancer patients was compared over serum selenium tertiles with Cox's regression. Risk of specific breast tumor characteristics, such as size and hormone receptor status were compared between 1003 women with breast cancer and 1186 controls.	We found an inverse association between mortality and selenium, but no association with specific tumor characteristics. The findings suggest that selenium might be an independent prognostic factor for survival in breast cancer.
III	Is thyroid hormone receptor alpha 2 expression in breast cancer tumors relevant for prognosis?	The expression of the thyroid hormone receptor alpha-2 (THRα-2) was evaluated in breast cancer tumors from 654 women using immunohistochemistry. Survival and association with prognostic factors were compared between those with a low and a high expression of the receptor using Cox's regression and logistic regression.	THRα-2 was associated with prognostically unfavorable features such as larger tumor size, and a lack of estrogen receptors. High THRα-2 expression was also associated with higher mortality. The results suggest that thyroid hormone receptors are involved in breast cancer progression.
IV	Does genetic variation affect the association between selenium and breast cancer risk? Can those genetic variants affect breast cancer risk on their own? How are different exposure measurements of selenium affected by these genetic variations and how do they affect the overall risk?	Breast cancer risk from three different selenium exposure measurements (A genetic score n= 16,429, dietary intake n=15,891 and serum levels n=2,037) were evaluated using Cox regression analysis and analyses were stratified by single nucleotide polymorphisms (SNPs) in selenium-related genes. The same SNPs individual effects on breast cancer risk were also evaluated.	Women with alternative alleles in a SNP in the gene for glutathione peroxidase 1 both had a lower breast cancer risk overall and also showed a protective effect from selenium intake that was not present among women with the standard alleles. The results suggest that selenium exposure might be protective against breast cancer for some women, but not others, depending on genetic variation. Genetic markers of increased selenium exposure were not associated with breast cancer risk.

Introduction

In this doctoral thesis the possible protective effect of selenium regarding breast cancer risk and prognosis is discussed.

Selenium is a trace element that is naturally occurring in the environment and necessary in small amounts for vital functions in the human body, including antioxidation and thyroid function. The main source of exposure to selenium is through diet. However, intake can vary significantly between individuals, both depending on type of diet and where in the world one's food is produced.

Evidence from preclinical studies indicates that selenium and selenium-dependent proteins could have a protective effect on the development and progression of several cancers, including breast cancer. However, in observational studies the evidence has not been convincing that any association between selenium exposure and breast cancer risk exists. But there are limiting factors in the current evidence and individual studies have reported conflicting results. Some have found no effect from selenium while others have found that women with breast cancer have lower levels of selenium in the blood and that a low intake of selenium is associated with a higher mortality in breast cancer.

When I started this project, my colleagues and I concluded that although there are exciting preclinical indications regarding selenium and breast cancer, there was weak evidence of any effect on actual risk or prognosis. Further evidence was needed to draw conclusions. One problem could be that the previous studies had too low statistical power to detect an effect. But we also wanted to explore if lifestyle factors, such as obesity and smoking, could act as bias or influence the effect of selenium. There were also indications that genetics were important, and should also be considered. And although antioxidation was most frequently mentioned as the probable causative pathway, selenium also plays a central role in thyroid hormone function. Could that be another pathway by which it influences breast cancer?

With all of those questions, I started on a six-year-long research journey that has been both challenging and rewarding and that now culminates in this doctoral thesis. For me, the results and conclusions have brought clarity to some questions and have also led me to new ones. Whether you only read the introduction to this doctoral thesis or study every part of it in detail, I hope you find it interesting!

Background

Breast cancer

Background

Breast cancer is defined as a cancer formed in the breast, but most commonly refers to epithelial tumors in the glandular elements of the breast.[2] With over 2.2 million new breast cancer cases in 2020, it surpassed lung cancer as the most commonly diagnosed cancer worldwide, accounting for 11.7% of new cancer cases globally.[3] It also causes more deaths than any other cancer among women, with the total estimated at over 680,000 deaths globally in 2020.[3] In Sweden, there were 7620 women who were diagnosed with a first time breast cancer in 2019, and the number is increasing every year. The risk for a Swedish woman of being diagnosed with breast cancer before 75 years of age is now 10.2%.[4] However, although the incidence is increasing, the survival rate is also increasing. The overall 10-year survival has gone from 60% 40 years ago to over 86% today.[5, 6]

Figure 1. Incidence and mortality of breast cancer during 1970 to 2019 in Sweden, statistics from the Swedish Cancer Registry.[6]

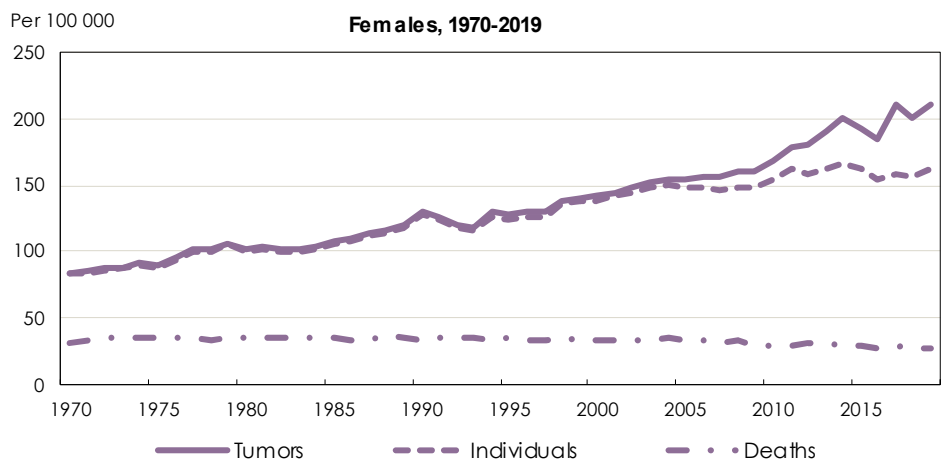
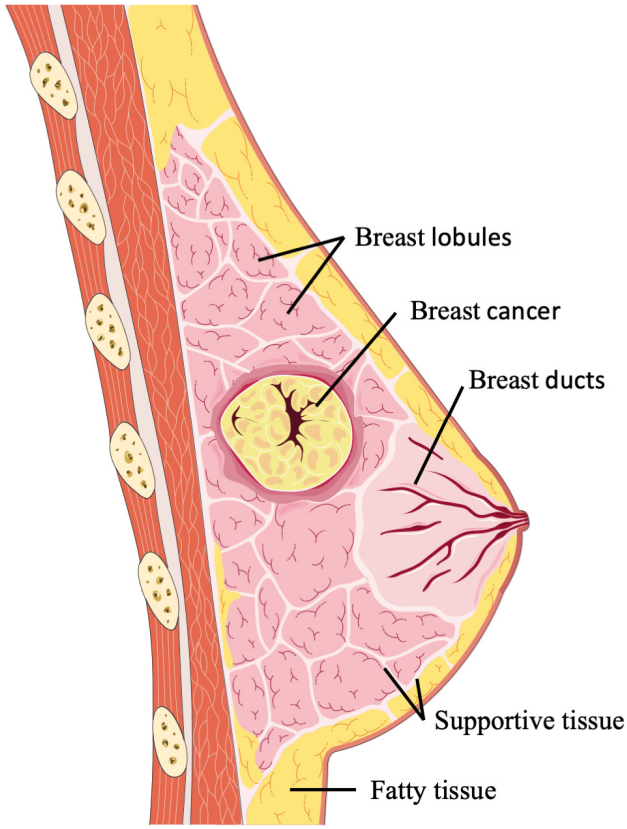


Figure 2. Schematic picture of a breast cancer.



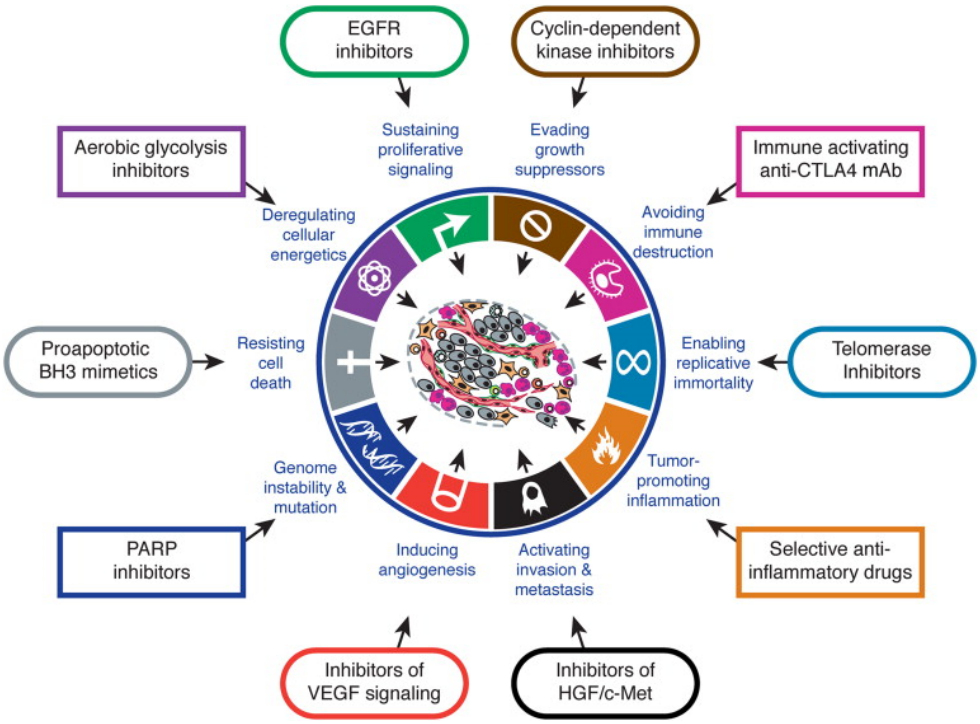
Most breast cancers develop from cells in the terminal ducts or in the lobules, and can present as a lump in the breast. The figure is an adaption of an illustration from Servier Medical Art, reprinted under CC By-3.0 license.[7]

Breast carcinogenesis

The female breast consists of lobules where milk can be produced and of ducts that can lead the milk out of the nipple. Both the lobules and the ducts consist of epithelial cells. In addition to those structures there is also supportive tissue and fat. The main morphological development of the female breast is initiated by female sex hormones during puberty but the full differentiation of the breast, mainly regarding the lobular structure, is not complete until the end of the first pregnancy.[8] Breast cancer most commonly develops from cells in the so-called terminal duct lobular units.[9] It changes from a normal breast epithelial cell to a breast cancer cell due to stepwise genetic changes that give the cells features such as increased proliferation signaling, activation of invasion and metastasis, increased genome instability and avoidance of cell death, described as the hallmarks of cancer.[10] The reasons these

changes develop can vary and are multifactorial. One reason is damage to the cell's DNA, e.g. from radiation for individuals that have been treated with radiotherapy for childhood cancers.[11] However, the more common reason is that mutations in important genes are inherited or spontaneously developed.

Figure 3. The hallmarks of cancer.



The hallmarks of cancer and possible therapeutic options as proposed by Hanahan and Weinberg (2011).[10] Reprinted with permission from Elsevier Inc.

Risk factors

Many risk factors for breast cancer are related to exposure to female sex hormones, mainly estrogen but also progesterone. They are key proliferative signals in breast tissue and most breast cancers are dependent on this signaling.[12] Female sex and high age are two of the strongest risk factors for breast cancer. Women have approximately 100 times the risk of males and the risk of breast cancer increases in women after menopause, with 60 to 69 years being the most common age of diagnosis in Sweden. [4, 13] Other risk factors include high age at menarche, low age at menopause, high age at first childbirth, a low number of pregnancies, short time breast feeding and use of hormone replacement after menopause.[14-18] Hormonal

contraceptives with combined estrogen and progesterone slightly increase the risk of being diagnosed with breast cancer during use, while there is no excess risk ten years after discontinuing the use.[19]

Lifestyle also seems to affect the risk of breast cancer, and might be a reason for the increasing incidence.[20-22] Some factors increase risk on their own and some may be markers of increased risk. Examples include an increased risk due to high alcohol intake and a decreased risk from high physical activity that both have a probable causal effect on risk while a high socioeconomic status and a long education are markers of increased risk that at least in part is attributable to reproductive factors.[21, 23, 24] Another well documented risk factor for breast cancer is body fatness, mostly documented as BMI or waist-hip ratio. A high body fatness seems to be protective against pre-menopausal breast cancer, while increasing weight through adulthood and high BMI are risk factors for post-menopausal breast cancer.[21] This can be explained by higher levels of circulating estrogen. After menopause, the estrogen produced by the ovaries dramatically decreases and the main source of estrogen instead becomes the conversion of other steroid hormones to estrogen by the aromatase enzyme that is present in adipose tissue.[21] However, there is some evidence that specific dietary factors also might decrease breast cancer risk, including non-starchy vegetables, food containing carotenoids and diets high in calcium.[21] In the Nurses' Health Study including 121,700 women, of which 8,421 were diagnosed with breast cancer, it was estimated that 30% of all post-menopausal breast cancers were caused by modifiable risk factors, mainly high alcohol intake, weight increase, post-menopausal hormone use and low physical activity. [25]

Genetics are also important. Women with a mother, sister or daughter with breast cancer have twice the risk of breast cancer compared to someone without a family history of breast cancer.[26] Most notable are the BRCA-1 and BRCA-2 genes, which are estimated to cause up to 15% of all hereditary breast cancers.[27] The cumulative lifetime risk of breast cancer among BRCA-1 and BRCA-2 mutation carriers is estimated to be 57-72% and 49-69% respectively.[28, 29] Other mutations in individual genes that increase breast cancer risk, such as TP53 and CHEK2, are either less common or have lower penetrance.[27] However, a majority of the genetic factors are yet not characterized or give only a slight increase in risk on their own, but can, together with other factors, contribute a significant risk.[27] Single nucleotide polymorphisms (SNPs), described later in this thesis, are an example of that.[27] Genetic factors can also affect breast cancer risk through other risk factors such as age at menopause, breast density or acquired adult height.[30-33] A high breast density means that the breast has a lower percentage of fatty tissue, which both increases the risk of breast cancer as well as decreases the sensitivity of mammography.[32, 34] The complexity of the genetic risk factors are yet to be fully understood, but it is known that women diagnosed with breast cancer also have an increased risk of a second cancer, which might in part be due to shared genetic risk factors for different cancers.[35]

Diagnosis

Breast cancer has few symptoms and can go undetected for a long time. The typical presentation is a lump in the breast, but there can also be secretion from the nipple or changes in the skin on the breast. Due to the lack of symptoms, screening for breast cancer is established in some countries around the world to detect early disease. In Sweden, all women between the ages of 40 and 74 are offered mammography at least every two years, and around 50% of all breast cancers are diagnosed within the screening program.[22] When a breast cancer is suspected, a clinical examination and a needle biopsy or cell aspiration is part of the diagnostic routine as well as some sort of imaging, most commonly a mammography or ultrasound.[22]

Prognostic and treatment predictive factors

Breast cancer is a heterogeneous disease and several factors affect prognosis and treatment choices. Early breast cancer, that has not spread outside of the breast, has a five-year survival rate of >99%, regionally spread breast cancer to lymph nodes has a survival rate of between 80-90% while metastatic disease has a survival rate of only 28%.[36] Breast cancer can be staged according to its anatomical features according to the TNM system by the American Joint Committee on Cancer. Tumor size/growth (T), lymph node invasion (N) and distant metastases (M) are considered, and based on those parameters the cancer can be given a stage between I to IV.[37] Although the staging system provides prognostic information, it is no longer used as extensively as a clinical tool. The individual factors are more important for clinical decisions and are prognostically important on their own, with the presence of lymph node invasion and distant metastasis being prognostically unfavorable, as is a larger tumor size.[38, 39] Ductal carcinoma in situ (DCIS) is a variant of breast cancer that is not locally invasive.[2] Yet the breast cancer mortality following a DCIS diagnosis is around 4-5% after 15 years, but a majority of those who die have a recurrence of invasive breast cancer prior to death.[40]

Biological features of the tumor are also clinically important both for predicting treatment success and for prognosis.[41] There are distinctly different subtypes of breast cancers. They are either genetically closer to luminal breast epithelium (luminal-like), and often express estrogen receptors (ER) and progesterone receptors (PgR), or closer to basal/myoepithelium (basal-like) and do not express these receptors.[42] The luminal type can be divided into two subgroups (A and B) that have different intrinsic properties that are highly correlated with outcome.[43] In both luminal-like and basal-like breast cancer, some tumors also overexpress the human epidermal growth factor receptor 2 (HER2), and those tumors are distinctly different in their biology and clinical presentation, and can be treated with specific antibodies that target HER2.[42, 43] These intrinsic subtypes (luminal A, luminal B, HER2+ (luminal or non-luminal) and basal-like) are defined by their genetic

expression by analyses of tumor DNA, but surrogate intrinsic subtypes can also be derived from their immunohistological expression.[41] In Sweden, the surrogate intrinsic subtypes define five corresponding subtypes that are derived from the expression of estrogen receptors, progesterone receptors, HER2-receptors, the Nottingham histological grade and the expression of the proliferation marker Ki67.[22, 41, 44] The specific subtypes are presented in Table 1. It has been known for a long time that the expression of ER and PgR is prognostically favorable, although perhaps their most important role is that tumors expressing these receptors respond better to anti-hormonal treatment.[45] Indeed, several of these factors are not only of prognostic value but are also predictive of treatment success for different therapeutic regimes. The difference between luminal A from B is that luminal B tumors are more aggressive and proliferative, histologically defined by a higher histological grade and a higher expression of Ki67. Tumors defined as luminal A-like have the best prognosis and the basal-like tumors that do not express any of the hormonal receptors or HER2 are called triple-negative breast cancers and have the worst prognosis and the fewest treatment options.[41]

Table 1. A simplified table of prognostic and treatment predictive factors and their relation to tumor staging and subtype.[22, 37]

Anatomical staging I-IV (TNM)	Tumor size		Lymph node invasion	Distant metastasis	
I	≤20mm		No	No	
II	>20-50mm		No	No	
	≤20mm		Yes	No	
III	>50mm		Any	No	
	>20-50mm		Yes	No	
IV	Any		Any	Yes	
Surrogate intrinsic subtype	**ER**	**HER2**	**Histological grade***	**Ki67**	**PgR**
Luminal A-like	+	-	1	Any	Any
	+	-	2	Low	Any
	+	-	2	Intermediate	+
Luminal B-like, HER2-	+	-	3	Any	Any
	+	-	2	High	Any
	+	-	2	Intermediate	-
Luminal B-like, HER2+	+	+	Any	Any	Any
Non-luminal HER2+	-	+	Any	Any	Any
Triple-negative breast cancer	-	-	Any	Any	-

The staging and subtype is based on the combination of values on a single row.

Treatment

In Sweden, all breast cancer diagnoses will be discussed by a multidisciplinary team and an individually tailored treatment regime will be recommended. Surgical removal of the tumor is standard for most breast cancers and will alone or in combination with local radiotherapy be the standard invasive treatment.[22] If radical excision with a good cosmetic result is possible, a partial mastectomy will be performed. Otherwise, complete mastectomy is an alternative. Axillar surgery, most often being a sentinel node biopsy, is also included in standard treatment and part of the disease staging. Historically, surgical treatment of breast cancer did not extensively consider the cosmetic result and was often mutilating and led to suffering for the operated women.[46] Now, breast conserving surgery or reconstructive surgery is offered as standard to all breast cancer patients in Sweden.[22]

The tumor grade and intrinsic subtype of the tumor will guide additional therapy. Treatment choices usually include anti-hormonal therapy with either an estrogen receptor antagonist like tamoxifen or an aromatase inhibitor, reducing the overall estrogen levels. Local radiotherapy, specific antibodies like Trastuzumab that blocks HER2, and chemotherapy are other standard treatment options.[22, 41]

Selenium

Historic background

Selenium is an essential mineral for humans and the 34th element of the periodic table. It was discovered by the Swedish chemist Jöns Jacob Berzelius in 1817 as a residue after the production of sulfuric acid. The name relates to the Greek word for moon, *Selene*. The reason is supposed to be that before Berzelius could prove it was a new element, he disputed with a colleague who believed the reddish dye they had examined consisted of the element tellium, named after the earth, *Tellus*.[47] The residue was foul smelling and caused skin blisters, and selenium was therefore first described as a toxin.[47] And indeed selenium is toxic to animals when ingested in large amounts, which mainly happens to plant-eating animals who feed on seleniferous soils.[48] Anecdotally, selenium is believed to have caused the military defeat of General Custer at the battle of Little Big Horn due to acute selenium poisoning of the horses in his cavalry by eating plants growing in seleniferous soil.[49]

However, the importance of selenium in human health was gradually understood during the latter half of the 20th century. In 1957 Schwarz and Foltz were the first to suggest that selenium could be an essential trace element after observing that only small levels of selenium could prevent liver necrosis in selenium deficient rats.[50] Keshan's disease, causing necrotic heart muscle death, was at the time endemic to

areas in China where the soil content of selenium was very low, and supplementing children with selenium convincingly lowered the incidence.[51] The results regarding Keshan's disease further indicated that selenium intake is essential for human health.

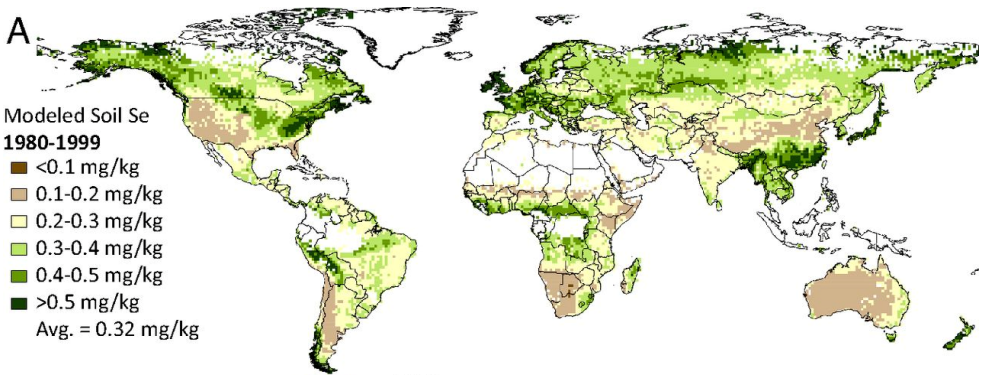
Selenium in health

Humans are mainly exposed to selenium via diet, but also from drinking water and inhalation of fumes, e.g. tobacco smoke. The recommended daily intake of selenium is 50 μg/day for women and 60 μg/day for men.[52] Crops grown in Sweden have low levels of selenium due to the low selenium content of the soil, and consequently the main sources of selenium in a Swedish diet are fish, meat and other animal products.[52] However, this can differ between and within countries. In Spain, cereals and grains are the main source of selenium.[53] In China, the selenium content of the soil can vary between 0.005 mg/kg in areas with selenium deficiency up to 79 mg/kg in seleniferous areas, resulting in a thousand-fold difference of the selenium concentration in rice and other food items grown in different areas.[54]

After ingestion and uptake, selenium is mainly incorporated in proteins via the amino acid selenocysteine.[47] It can also be non-specifically inserted as selenomethionine instead of methionine.[55] Only 25 known proteins include selenocysteine, and they are called selenoproteins.[56] These proteins are considered to be where selenium mainly exerts its biological functions. A majority of the selenoproteins have antioxidant roles, but other important functions include production and activation/deactivation of thyroid hormones.[57, 58] Glutathione peroxidase 1 (GPx-1) was the first characterized selenoprotein.[59] There are five selenium-dependent GPx enzymes and all have antioxidant functions.[60] GPx-1 is the most abundantly expressed, present in all human cells.[61] Other notable selenoproteins are Selenoprotein P and GPx-3, which make up most of the selenium in the blood, and GPx-4, a membrane bound enzyme important for lipid peroxidation.[57, 60]

In addition to Keshan's disease, severe selenium deficiency can cause bone and joint deformity (Kashin-Beck disease) and is believed to cause thyroid dysfunction and cretinism if combined with iodine deficiency.[62, 63] A high intake on the other hand can cause loss of hair, fatigue and acute GI symptoms such as vomiting and nausea, but has also been linked with increased risk of diabetes mellitus type-2, although severe toxicity is rare among humans.[48] It does seem that other factors than just selenium intake affect the levels of selenium in the body, e.g. a high BMI and smoking seems to lower the selenium levels in the blood.[64-66]

Figure 4. A modeled map of how soil selenium content differs around the world.



Published by permission of the National Academy of Science. This figure is previously published by Jones et al. (2017).[67]

Selenium and cancer

The first anti-carcinogenic evidence of selenium was published in 1949 when Clayton and Baumann reported a decreased incidence of spontaneous breast tumors in mice that were fed extra selenium.[68] In the 1970s, more evidence from animal studies and some observational studies were published. In the article *Selenium and cancer: A review* by Schrauzer (1976), the author concluded that evidence suggested a protective effect of selenium against cancer in humans.[69] In 1983 a randomized control trial, the Nutritional Prevention of Cancer trial (NPC-trial), investigated whether 200µg selenium yeast a day could lower the recurrence of non-melanoma skin cancer. The authors found a significantly reduced cancer incidence in the treatment arm for several cancers, including prostate cancer. [70] When the results were summarized in 2002, they sparked new enthusiasm regarding a possible protective effect of selenium. The optimistic results however could not be replicated in the following SELECT trial, which investigated the protective effect of selenium and vitamin E on prostate cancer incidence among 35,533 American men, as the authors reported no effect on incidence from selenium supplementation.[71] In addition to that, selenium supplementation among American men diagnosed with non-metastatic prostate cancer has been found to be associated with an increased mortality in prostate cancer.[72] However, in contrast to those findings, an observational study found an overall lower cancer mortality and all-cause mortality among 13,887 Americans with higher serum selenium levels.

But selenium has not only been studied regarding cancer risk and progression. The antioxidant activity of selenium is also important for regulating cell death, and preclinical studies have found that selenium supplementation to breast cancer cell lines inhibits their growth and induces apoptosis.[73-75] And indeed, combining selenium with chemotherapy does seem to increase cancer cell death.[76] Although evidence suggests that selenium could be used in cancer therapy, the effect depends

on both the dose and chemical form of selenium, and there is not yet enough evidence for a standardized treatment.[77]

Selenium in breast cancer

Breast cancer incidence has not specifically been studied in any randomized trial, but some cases have been reported as secondary outcomes without providing any clear evidence.[70, 78] One selenium supplementation trial with breast cancer as the outcome was started in a BRCA-1-positive population, with 1135 women randomized either to 250 μg selenite daily or placebo. However, only a meeting abstract has been published from that trial, reporting 60 incident breast cancers in the supplementation arm and 45 in the placebo arm.[79]

Most published studies regarding the incidence and outcome have instead been from observational studies. The available evidence does not support that an overall association between selenium exposure and breast cancer risk exists, although individual studies have found lower selenium levels among women with breast cancer compared to controls.[80-82] However, there is evidence that women with lower selenium levels in the blood or with a lower selenium intake have a higher mortality in breast cancer compared to women with higher levels or intake.[83, 84]

The central antioxidative role of selenium is often regarded as the most probable mechanism involved in cancer development.[57] GPx-1 is a potent intracellular antioxidant and reduces intracellular hydrogen peroxide, a reactive oxygen species with the potential to cause DNA damage, to water.[61] It contributes to DNA stability by reducing DNA oxidation, DNA adducts and DNA breakage, thus theoretically protecting from carcinogenesis.[85] Loss of heterozygosity in the GPx-1 gene is a common event in breast cancer, as well as in other cancers, indicating that it acts as tumor suppressor gene.[86] Also, preclinical studies have found that overexpression of GPx-1 seems to protect cells from DNA damage.[87] The GPx-1 levels are also among the most sensitive to changes in selenium status.[88] However, other selenoproteins might be important as well. Downregulation of GPx-3 is also common in several cancers, and the levels are sensitive to selenium intake.[89, 90] Selenium-binding protein 1 is less expressed in breast cancer compared to controls, and low expression is associated with increased mortality in breast cancer.[91] Its expression also seems to be regulated by estrogen.[91]

Selenium, breast cancer and thyroid function

The thyroid is a vital human organ producing thyroid hormone, which regulates cell activity through the thyroid hormone receptors.[92] The function of the thyroid is intimately connected with both selenium and breast cancer and there might be a mechanistic link.

Thyroid hormone has an estrogen-like effect on breast cancer cells.[93-95] Higher levels of thyroid hormone both as a single factor and in hyperthyroidism have indeed been associated both with an increased incidence and mortality in breast cancer.[96-98] However, other studies have instead found thyroid hormones to be associated with less aggressive tumors and a lower mortality.[99, 100] A recent meta-analysis found an increased risk of breast cancer among hyperthyroid women, but the authors argued that since information regarding treatment and other possible sources of bias is often lacking the causality is not clear.[101]

The thyroid is the most selenium-rich organ, and selenoproteins are essential both for the production of thyroid hormones as well as to activate and inactivate thyroid hormone. [58] Iodine has long been known to be essential for thyroid function, and deficiency leads to hypothyroidism. However, evidence suggests that selenium deficiency can also cause thyroid dysfunction. Cretinism, a development disorder due to congenital thyroid dysfunction, is more common among individuals with selenium deficiency in addition to iodine deficiency, compared to selenium-replete individuals.[63, 102] Furthermore, our own research also suggests that a combination of both high iodine and high selenium levels might have an overall protective effect against breast cancer, while neither one of the trace elements had that effect alone.[103]

Most of the circulating thyroid hormone is in an inactive form, thyroxine (T4), and the activation and inactivation of thyroid hormones is regulated by three selenoproteins called deiodinases (D1, D2 and D3).[56, 104] The local levels of these also affect the local levels of active thyroid hormone (T3). D2 is the most expressed of the deiodinases and activates T4 to T3. D1 does the same with less affinity while D3 inactivates T3.[104] It has been demonstrated that D1 is more abundantly expressed in breast cancer tissue compared to normal breast tissue and that the D2 gene is upregulated in breast cancer.[105, 106] In a study of healthy elderly, a low selenium status was correlated with a reduced T3/T4 ratio.[107] Although selenium availability seems to affect the function of some selenoproteins, mild or moderate selenium deficiency has not been proven to reduce the deiodinase function according to another study.[108] Thus, exactly how selenium might affect breast cancer through thyroid function is not fully understood.

Thyroid hormone receptor alpha 2 (THRα-2)

Thyroid hormones exert their functions through thyroid hormone receptors. There are two separate genes coding for these receptors called thyroid hormone receptor alpha (THRα) and beta (THRß). There are also several isoforms of those receptors.[109] The THRα-1 binds T3 which leads to the thyroid hormone's proliferative signaling, while THRα-2 acts as an antagonist for that signaling.[109] Isoforms of THRß also mediate T3 signaling, and loss off heterozygosity has been reported in breast cancer for the THRß-gene.[110] Although only a few studies with few included cases have been published, THRα-2 expression has been positively associated with prognostically favorable tumor characteristics and improved

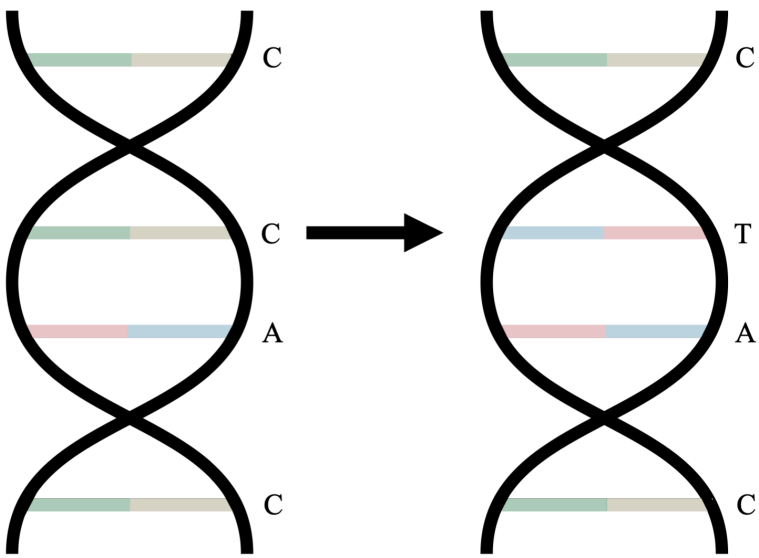
survival in breast cancer. [111, 112] A recent publication further strengthens the theory that there may be an association with low expression of THRα -2 and a worse prognosis in breast cancer.[113]

Single nucleotide polymorphisms

SNPs are normal variations of a single DNA base in the genome that is present in >1% of the population.[114] There are over 1.4 million known SNPs and although many of them are not localized within genes they represent most of the variation in human genetics.[115] By conducting so called genome-wide sequencing studies (GWAS), associations between SNPs and disease or individual traits can be identified. This has been done to identify SNPs that are associated with increased serum selenium levels or with breast cancer risk. [116, 117]

SNPs can be markers of risk, without functional consequences for the protein produced by the gene. But a SNP can also change the amino acid at the location of the change, and thus possibly also changes the protein's function. Moscow et al. (1994) identified one such functional SNP in the GPx-1 gene; a T allele instead of a C allele led to leucine instead of proline at codon 198, and seemed to be more common in lung cancer.[118] In more recent studies, it was found that SNP as well as other SNPs in selenoproteins could be linked to increased breast cancer risk.[119, 120] There is also evidence of interaction between SNPs in selenoproteins and selenium in prostate cancer.[121]

Figure 5. A single nucleotide polymorphism exemplified by a change from DNA base C to T.

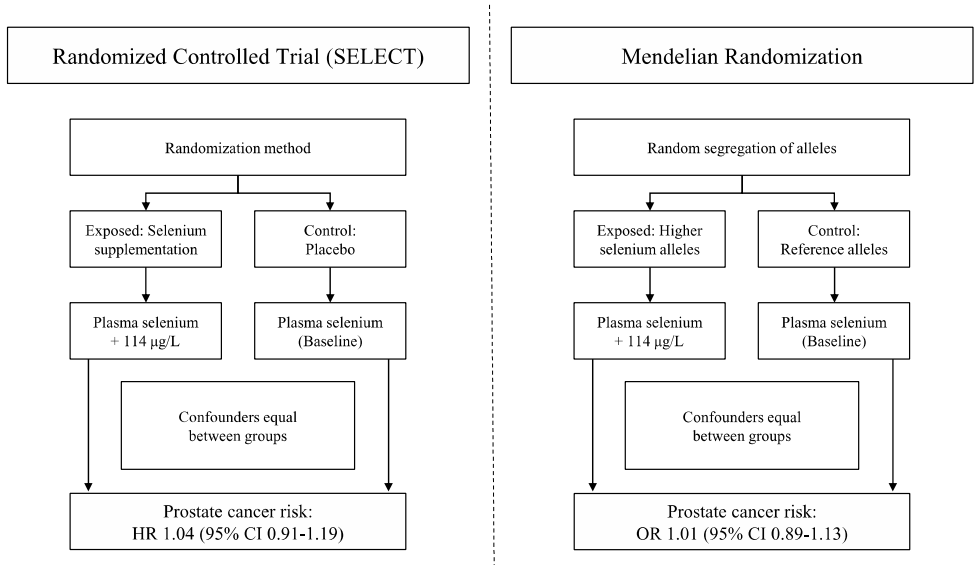


Mendelian randomization

Randomized controlled trials can study causal effects with a low risk of bias.[122] As mentioned above, no such completed trial has specifically studied selenium supplementation and breast cancer risk. However, just as a randomized control trial can investigate the effect of additional selenium exposure, so can a genetic trait (a SNP) that is correlated to selenium levels. Instead of comparing individuals randomized to selenium supplements or placebo, individuals "randomized" to higher selenium levels at birth are compared to those who are not. Following a null result in the SELECT trial, studying selenium supplementation and prostate cancer risk, Yarmolinski et al. (2018) argued that the same research question could be answered with less cost and less risk, by adopting a Mendelian randomization (MR) study design, and indeed they found similar results.[71, 123, 124] The MR method utilizes SNPs strongly associated with the exposure (serum selenium levels), but not associated with the outcome (breast cancer) or confounders, as instrumental variables, reducing the risk of both residual confounding and reverse causation, similar to randomized control trials.[124] Every individual can have 0, 1 or 2 alleles of a specific SNP, and the effect of having an additional allele can be quantified. That effect can then be used in the statistical analyses and the interpretation of results. Furthermore, several SNPs associated with the exposure can be combined into an allele score for a more powerful instrument.[125] This can be done by just adding the number of alleles of one individual and then dividing by the number of SNPs included, giving a continuous value between 0 and 2. However, the allele score can also be weighted, by multiplying every allele with its effect on the exposure.[125]

The MR method, with a weighted allele score, is thus a feasible and powerful alternative to a randomized trial. The method was used by Papadimitriou et al. (2021) regarding selenium and breast cancer risk; however, no effect of genetically elevated selenium could be seen in their study.[126]

Figure 6. Design and results of an MR compared to a randomized controlled trial regarding plasma selenium and prostate cancer risk.



Originally published by Yarmolinski et al. (2018).[123] Reprinted in line with CC BY license.

Rationale for the thesis

Breast cancer is one of the most pressing health issues globally causing considerable morbidity and mortality. Selenium and THRα-2 have both biological and epidemiological indications as protective factors in breast cancer, but high quality evidence has been lacking.

Aims

The overall aim of this thesis was to investigate selenium exposure in breast cancer. Selenium and related factors, including THRα-2 expression, were evaluated regarding risk, prognosis and interaction in the individual studies. Specific aims were to:

- Investigate if the overall risk of breast cancer is affected by selenium exposure. (Paper I and IV)
- Evaluate the effect of selenium exposure regarding treatment predictive factors and prognostically important characteristics in breast cancer. (Paper II)
- Investigate if breast cancer survival is affected by selenium exposure. (Paper II)
- Study if factors such as smoking, BMI or genetics interact with the association between selenium exposure and breast cancer risk. (Paper I and IV)
- Study if genetic variation in selenoprotein genes is associated with increased risk of breast cancer. (Paper IV)
- Evaluate the prognostic importance of THRα-2 expression in breast cancer (Paper III)

Material and methods

Methodology overview

Different epidemiological study designs were used in this thesis. The same population cohort was the base for all studies and Swedish national registries were used for collecting information regarding breast cancer diagnoses and cause of death. One strength that comes with a population cohort design is that the exposure is measured in a healthy state, meaning that the breast cancer later diagnosed is unlikely to have affected the exposure measurement at baseline. The cohort also made it possible to have comprehensive data regarding sources of bias, breast tumor characteristics, and genetics, and to have a large number of participants enabling qualified study designs and conclusions. However, there are also limitations, including residual confounding, which are further discussed under the section methodological considerations.

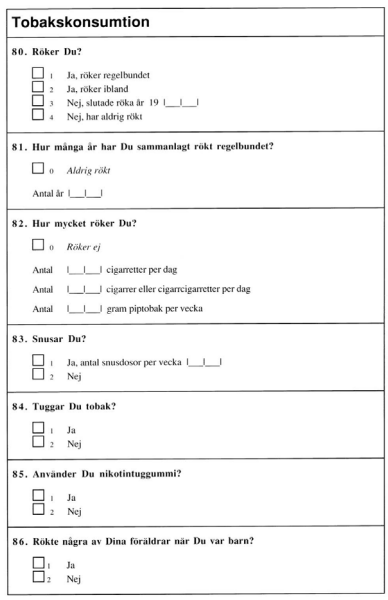
The Malmö Diet and Cancer Study (MDCS)

The MDCS is a prospective population cohort conducted in the Swedish city of Malmö. The primary objective was to investigate possible links between diet and cancer, but the MDCS was also intended as a resource for new hypotheses to be tested, by collecting high quality information and biological samples at baseline.[127] Starting on January 1st 1991, all inhabitants in Malmö born in the period 1926-1945 were initially invited, both through advertisements (e.g. posters, pamphlets and ads in the local newspaper) and through mail sent to randomly selected individuals eligible for inclusion.[128] In 1995 the study was extended to include individuals born in the period 1923-1950 and inclusion stopped at the end of 1996.[128] In total, 17,035 women and 11,063 men were included, corresponding to

Figure 7. A picture of one of the posters used during the MDCS recruitment campaign.

a participation rate of 40.8%.[128] The baseline data collection included an extensive questionnaire regarding lifestyle, health, occupation and other subjects. Measurements of height and weight, collection of blood samples, and collection of dietary data were also performed. Characteristics between participants and non-participants were compared in a study to investigate selection bias. Participants were similar to non-participants regarding socioeconomic status, BMI and smoking, but had a lower incidence of cancer before baseline and a higher mortality during inclusion and follow-up.[129] In 1993, the MDCS was also included as one of 22 cohorts in an international collaboration, the European Prospective Investigation into Cancer and Nutrition.[130] The inclusion of participants to the four papers in this thesis was based on the 17,035 women in the MDCS. Due to differences in the designs, the final study population differed between all four papers. Detailed descriptions can be found in the method section of each paper.

Figure 8. Pictures from the MDCS questionnaire.



Tobakskonsumtion

80. Röker Du?
☐ 1 Ja, röker regelbundet
☐ 2 Ja, röker ibland
☐ 3 Nej, slutade röka år 19 |__|__|
☐ 4 Nej, har aldrig rökt

81. Hur många år har Du sammanlagt rökt regelbundet?
☐ 0 *Aldrig rökt*
Antal år |__|__|

82. Hur mycket röker Du?
☐ 0 *Röker ej*
Antal |__|__| cigarretter per dag
Antal |__|__| cigarrer eller cigarrcigarretter per dag
Antal |__|__| gram piptobak per vecka

83. Snusar Du?
☐ 1 Ja, antal snusdosor per vecka |__|__|
☐ 2 Nej

84. Tuggar Du tobak?
☐ 1 Ja
☐ 2 Nej

85. Använder Du nikotintuggummi?
☐ 1 Ja
☐ 2 Nej

86. Rökte några av Dina föräldrar när Du var barn?
☐ 1 Ja
☐ 2 Nej

22

Serum analyses

At MDCS baseline 45ml of blood was drawn and blood components were separated and then stored in 2ml vials in freezers, and serum was stored in -80°C.[127] Selenium levels in serum was initially analyzed by ALS Scandinavia AB, Sweden, for 1186 women with breast cancer and 1186 controls in study I.[1] For each individual, 0.15ml

serum was thawed and diluted to 10ml with an alkalic medium including 0.1% NH_3 and 0.005% EDTA/Triton-X. The samples were then analyzed by using single element standards traceable to *National Institute of Standards and Technology* with ICP-SFMS using the instrument Element 2™ from Thermo Scientific™. Reference material Seronorm™ *(Trace Elements Serum level 1, lot 0608414*), from Sero AS, Norway, was used. Interbatch variation was low, with a coefficient of variation of 0.03. [1]

Dietary data

When designing MDCS it was concluded that the best known dietary assessment, weighed food records, would not be feasible in such a large population cohort. Therefore, a trial was set up to compare two alternative methods to a reference method, weighed food records for three days every two months over one year. The method with the highest validity was chosen and it included a combination of a 168-food item semi-quantitative questionnaire, a two-week food record, and an interview.[131, 132] During the interview, photographs were used to estimate portion sizes and information regarding e.g. food preparation and fats used in cooking was requested.[133] The interviewer entered the information gathered from the different sources into a computer program that calculated a mean g/day intake of food items and that information was then converted to intake of specific nutrients by utilizing the Swedish Food Database PC KOST2 -93 from the Swedish National Food Administration.[133] Due to an unforeseen reduction of grants, the interview method was adjusted in 1994 to reduce interview time, by reducing the number of portion size pictures from 180 to 75, a study found the effect of this change to be small.[134]

Genetic data collection

Genetic analyses were performed by using stored blood components (buffy coats and granulocytes) from MDCS participants in the chip Illumina GSA v1 genotyping array including ~640,000 SNPs. Individuals were excluded if they had low quality samples, defined as either a difference between reported and genetically inferred sex or that <90% of the SNPs in the chip could not be analyzed adequately. Furthermore, SNPs were excluded if they were not in Hardy-Weinberg equilibrium ($p<1x10^{15}$), suggesting a low quality of that SNP. Individually and collectively missing SNPs were then imputed using a reference panel from the Haplotype Reference Consortium.[135, 136] Out of 17,035 women in MDCS, 38 individuals had a missing lab number and 568 were excluded after lab analyses, resulting in 16,429 women with complete SNP information available after analyses and quality control.

The Hardy-Weinberg equilibrium is an ideal state of Mendelian inheritance where no genetic drift of the population by mutations, fertility differences, natural selection or migration may occur, and it was described both by the mathematician

Hardy and the medical doctor Weinberg in the early 20th century.[137] If a population is in a true Hardy-Weinberg equilibrium, the allele distribution will be the same as in the previous generation. The Hardy-Weinberg equilibrium never occurs in nature, but serves as a reference for the genetic drift of a population. It is usually one of the quality controls in studies including genetics. By being close to the expected ideal value, the quality of the genotyping is supposedly good, while if a certain genetic variant differs greatly, the quality of the genotyping can be questioned.[138]

SNP selection and genetic score

SNPs were selected for two reasons in paper IV. First, we selected SNPs to test for genetic interaction between selenium exposure and breast cancer risk. Those SNPs were selected from the literature due to their potential mechanistic effects in important selenoproteins and in another major antioxidant enzyme, superoxide dismutase 2 (SOD-2), that previously has been shown to interact with selenium and GPx-1. [120, 139, 140]

The second reason for selecting SNPs was to create a genetic score that would serve as an instrumental variable for selenium exposure, as in an MR study design. We used the same SNPs as the only MR study conducted so far evaluating selenium and breast cancer risk by Papadimitriou et al. (2021).[126] Those SNPs were selected from two GWAS, using a p-value threshold of $p=5x10^{-8}$ and excluding SNPs with a minor allele frequency <5% and SNPs in linkage disequilibrium.[116, 141] Linkage disequilibrium is the non-random association between SNPs, usually due to co-inheritance of those loci.[142] Without excluding such SNPs the results could be skewed. In our study design, another GWAS of serum selenium was also explored but did not add any additional SNP when adopting the same methodology and p-value threshold.[143]

Table 2. SNPs selected for interaction analyses in paper IV.

SNP	Gene/Protein	Protein function	SNP effect
rs1050450	GPx-1	One of the major antioxidant enzymes and a key selenoprotein present in all human cells. Has been associated with breast cancer and is susceptible to dietary changes.[144]	Amino acid change from proline to leucin at codon 198. Lower proportion of GPx-1 in the cytoplasm. [145]
rs713041	GPx-4	A membrane bound selenoprotein important for lipid peroxidation.[57]	Basepair change in 3'UTR of mRNA. The alternative allele is more susceptible to selenium deficiency, decreases GPx-4 levels and increases GPx-1 levels.[146]
rs3877899	Selenoprotein P	Contributes the largest proportion of selenium in serum. Has antioxidant capabilities and works as a selenium transporter.[147]	Alanine to threonine amino acid change at codon 234. Affects serum selenium levels.[148]
rs7579	Selenoprotein P	See above.	Basepair change in 3' UTR of mRNA. Affects serum selenium levels.[148]
rs4880	SOD-2	Not a selenoprotein, but a key intracellular antioxidant enzyme, mostly present in mithocondria.[149]	Amino acid change from valine to alanine at codon 16. Gene-gene interaction with rs1050450 and breast cancer risk.[140]

Tissue microarray and immunohistochemistry

To efficiently evaluate the breast tumors in MDCS, for e.g. expression of different receptors or proteins, tissue microarray (TMA) blocks were constructed from stored tumor material. The construction of the TMAs we utilized in paper III has been previously described by Elebro et al. (2017) and included two 1-mm core biopsies from each available breast cancer diagnosed in the MDCS population from 1991 up until 2010.[150] In paper III, 3-4 µm sections were cut from the TMA and subsequently deparaffinized and immunohistochemically stained for the THRα-2 receptor. The expression was then scored regarding intensity and fraction in two separate readings using digital microscopy and classified as low or high expression for statistical analyses by multiplying intensity and fraction as described in paper III.

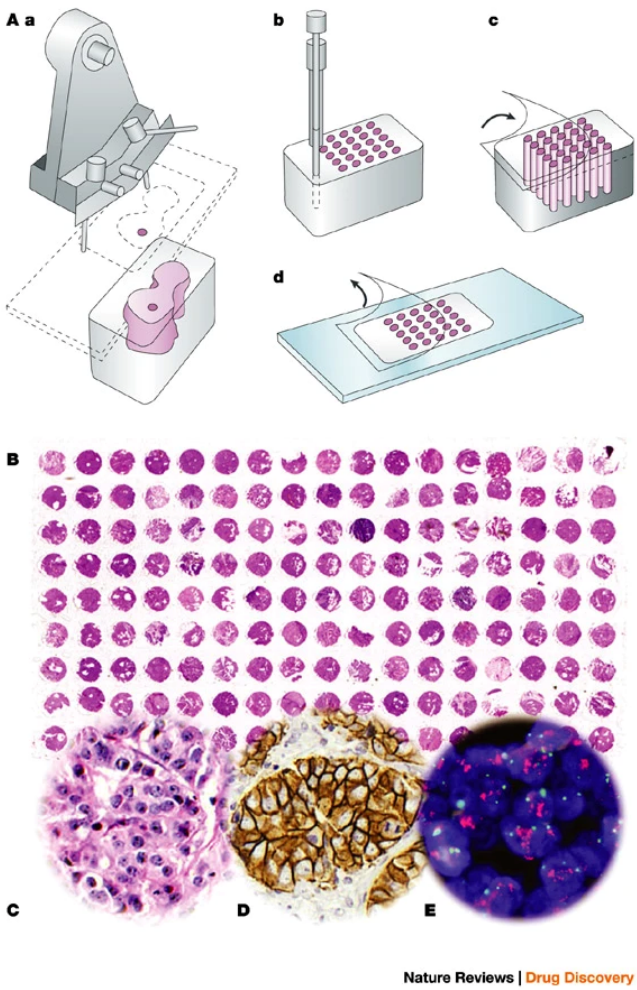


Figure 9.

A) An illustrative explanation of how a tissue microarray is constructed, published by Sauter et al. (2003).[151] Reprinted by permission from Nature Publishing Group.

a) A core biopsy is taken from a stored tumor.

b) The core is inserted into a donor block, along with core biopsies from other tumors, creating a TMA block.

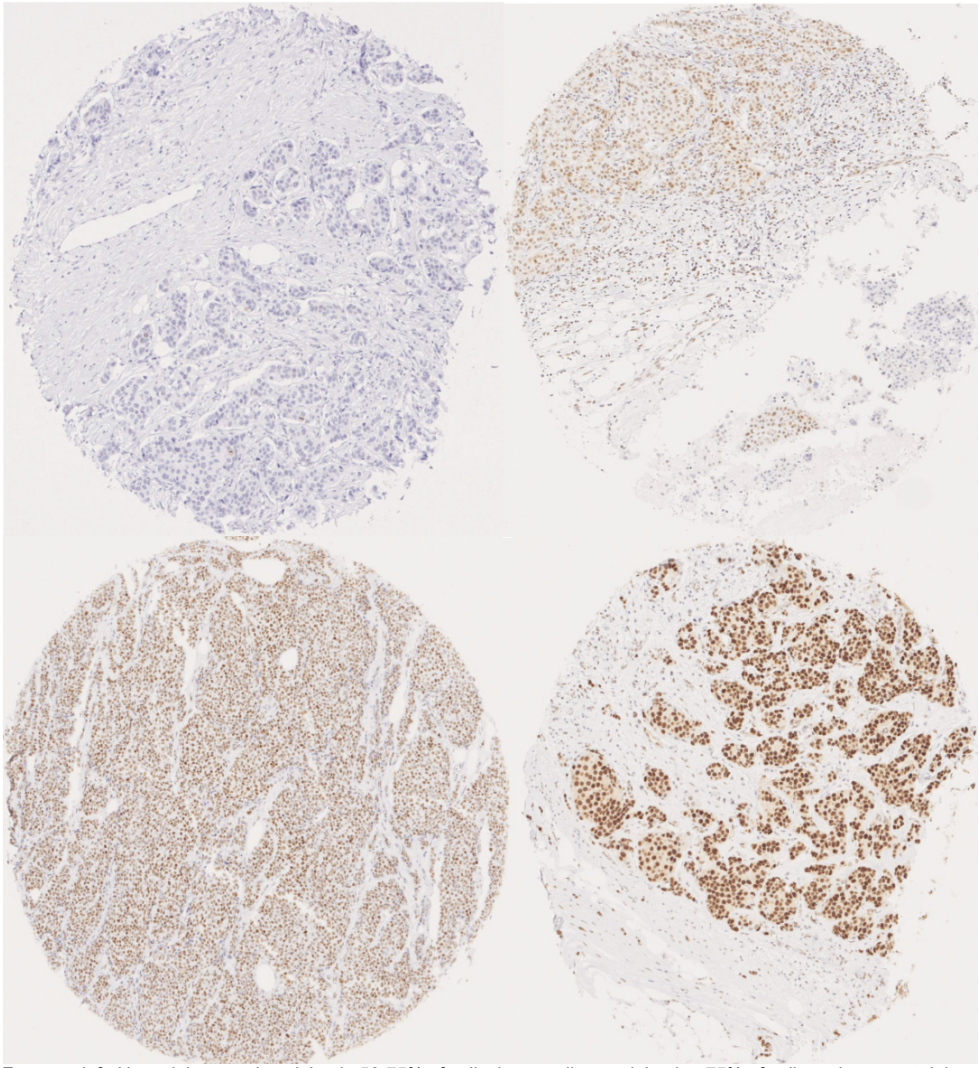
c) A thin slice is cut from the TMA block, including cores from all tumors in the block. This section can be stained for different proteins like THRα-2, ER or HER2.

d) A glass with cores from all tumors in the block is now available for analysis.

B) Shows a complete TMA-glass.

C,D and E) Shows examples of cells stained in different ways.

Figure 10. Example pictures from paper III, the TMA evaluation of intra-tumor THRα-2 expression.



From top left: No staining, weak staining in 50-75% of cells, intermediate staining in >75% of cells and strong staining in >75% of cells for THRα-2. The top two were categorized as low intra-tumor expression of THRα-2 and the bottom two as high.

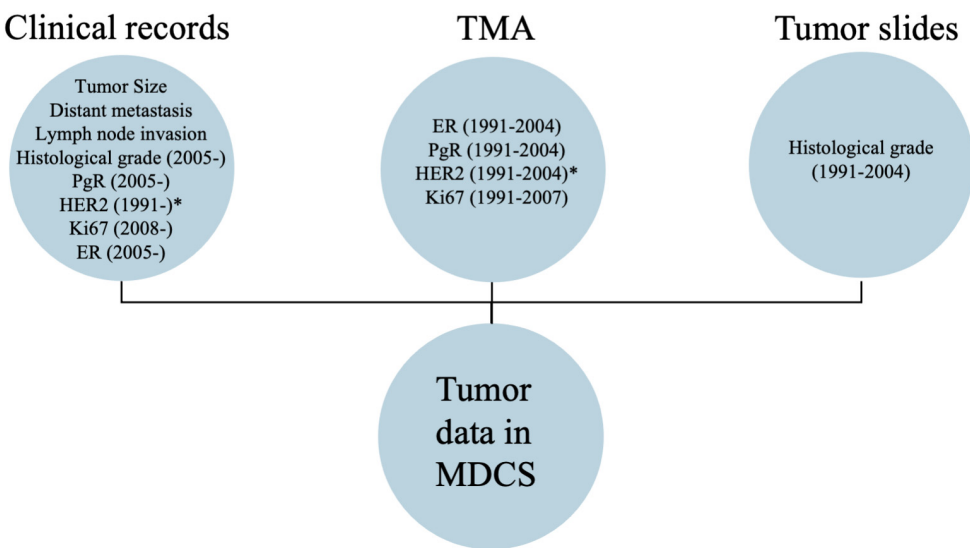
Table 3. Categorization of the THRα-2 expression.

Intensity				Fraction				
0	1	2	3	0	1	2	3	4
No staining	Weak	Moderate	Strong	<1%	1-10%	11-50%	51-75%	76-100%
Total score = Intensity * Fraction								
Low (0-7)				**High (8-12)**				

Tumor data

Characteristics of the breast tumors have been collected from different sources during different time periods of the MDCS follow-up, as visualized in Figure 11. All tumors diagnosed from 1991 to the end of 2004 had their histological grade and receptor status (ER, PgR, HER2 and Ki67) scored in a histological re-examination and by TMA as described by Borgquist (2007) and Butt (2014).[152, 153] Immunohistochemical receptor status was also evaluated by TMA during the period 2005-2007; however, due to questions regarding the quality of the PgR data in that TMA, a new data collection from clinical records was performed for ER and PgR covering that time period. However, all data regarding tumor characteristics except HER2 and Ki67 were collected from clinical records from 2005 and onwards. The HER2 data was collected by data from national registries when available. In the case of missing information, data was obtained from clinical records and if missing there, it was obtained from the TMA.[154] The Ki67 was based on TMA up until 2007. Parameters such as tumor size and axillary lymph node invasion were collected from clinical records throughout the follow-up.

Figure 11. Data collection methods for tumor characteristics in MDCS.



*HER2 data was collected from national registries, and if no conclusive data was found there, clinical records or TMA were used. No TMA data was used after 2005 for the HER2 variable.

Surrogate intrinsic subtypes

The tumor intrinsic subtypes are, as mentioned in the background, important clinical and prognostic information. However that data was not available in the MDCS data set. In paper II a surrogate intrinsic subtype was constructed from the available tumor data based on the local criteria in the south Swedish health care region, adapted for the MDCS data set.[155, 156] Subtypes were assigned as presented in Table 1, with the exception that all women with HER2-positive tumors were grouped together regardless of whether they were ER+ or not, creating four categories: luminal A-like, luminal B-like, HER2+ and triple-negative breast cancer. The reason for this is discussed under the section methodological considerations.

Endpoint data

The Swedish personal identity number was used to link MDCS participants to official registries. The Swedish Cancer Registry was used to identify women with breast cancer and their date of diagnosis.[6] The Swedish Cause of Death Registry was used for the date and cause of death.[157] Breast cancer-specific death was defined as a death where breast cancer was the cause or a contributing factor to the death.

Statistical methods

A range of statistical methods were used in the papers leading to this dissertation. All papers include regression analyses, which has the strength of quantifying and giving a direction to possible associations, either with a time variable or without. Analyses were performed both unadjusted and adjusted for possible confounders. The presentation and identification of possible confounders were handled through descriptive tables. Missing values were handled either through the missing indicator method or multiple imputation. All statistical work has been done in IBM SPSS Statistics, versions 23 to 27.

Table 4. A summary of the statistical methods in each paper.

Paper	Descriptive statistics	Logistic regression	Cox regression	Interaction statistics	Missing indicator	Multiple imputation
I	X	X		X	X	
II	X	X	X		X	
III	X	X	X	X		X
IV	X		X	X		X

Descriptive statistics

Describing and comparing the included cases and controls is a standard method to detect any skewness in the data that might be due to errors and to visualize possible confounders. This was done in all four papers. Indeed, a common approach is to conduct statistical testing in descriptive tables to detect statistical differences that reach $p<0.05$ and then adjust for them in the main analysis. However, the American Statistical Association and other leading experts have strongly argued against such use of "statistical significance" testing.[158, 159] Instead, arguments should be made for weak or strong evidence for or against a possible confounder, based both on reasoning and statistics. Thus, in all four papers my principle has been to present descriptive statistics of established factors affecting risk (or prognosis) in breast cancer as well as possible factors that could bias the relationship between selenium and breast cancer.

Logistic regression

As the output in paper I was dichotomous (breast cancer or no breast cancer), and the study design was a nested case-control study without a time variable, a binary logistic regression was a suitable statistical choice.[160] The exposure, serum levels of selenium, was divided into quartiles, and the lowest quartile was chosen as reference. Analyses were performed both unadjusted and adjusted for breast cancer risk factors as well as for factors affecting the selenium levels (smoking and time of year that blood samples were collected). The resulting odds ratio presented for each quartile are the odds of having breast cancer for women in that quartile, divided by the odds of having breast cancer in the reference quartile. When the odds are small, meaning the outcome is a rare event, the odds ratio can, for practical purposes, be interpreted as a relative risk, which is more intuitive for most people. And even in scientific literature, the 'risk of disease' is a more commonly used and understood choice of words, although the results are presented as an odds ratio. The same model was also used in papers II and III when investigating the risk of having a certain tumor characteristic depending on serum selenium or THRα-2 respectively. There is a rule of thumb that there should be at least 10 events per category in a regression model, which is important regarding how many factors can be adjusted for, although that rule of thumb can be relaxed to at least half.[161] Thus, all possible confounders could be adjusted for without doubting the robustness of the model, except for small subgroup analyses.

Cox regression

Cox regression, or the Cox proportional hazards model, is a regression model that compares time-dependent risks (hazards) and yields a hazard ratio.[162] Each

individual is given a time at risk which, in a survival analysis, is the time from diagnosis to the time of censoring in the model. Censoring can in principle either occur when the studied event happens (the individual dies from breast cancer) or when no more data is available for the individual without the event ever happening (e.g. end of follow-up or lost to follow-up). This is a standard model to use in survival analyses, and more precise than logistic regression when the time from exposure to outcome is available. The hazard ratio can for practical purposes also be interpreted as a relative risk, e.g. a hazard ratio of 2 means that there is twice as great a risk of getting the studied outcome in any given time period compared to the reference group. The model assumes that the hazards compared (e.g. breast cancer death in the lowest and the highest selenium quartiles) are proportional to each other through the follow-up period. This assumption can be tested, either statistically e.g. by a log rank test of the unadjusted survival data or by graphical methods, most commonly screening for non-proportionality in a Kaplan-Meier curve. The Kaplan-Meier curve has a theoretical advantage over the log rank test and is a popular method, although in simulation studies, they are similarly good.[163] In paper III, the visual assessment of the Kaplan-Meier curves indicated an assumption violation since the curves converged at the end of the follow-up. Due to this we conducted sensitivity analyses for the follow-up period 0 to 15 years where there were no indications of assumption violation.

Interaction

Interaction was tested in papers I, III and IV, first by stratifying the data and analyzing the different strata separately. Then, the main analysis was performed with the addition of an interaction term, along with the individual variables, to statistically test possible effect modification.

Statistical methods for the exposure variables

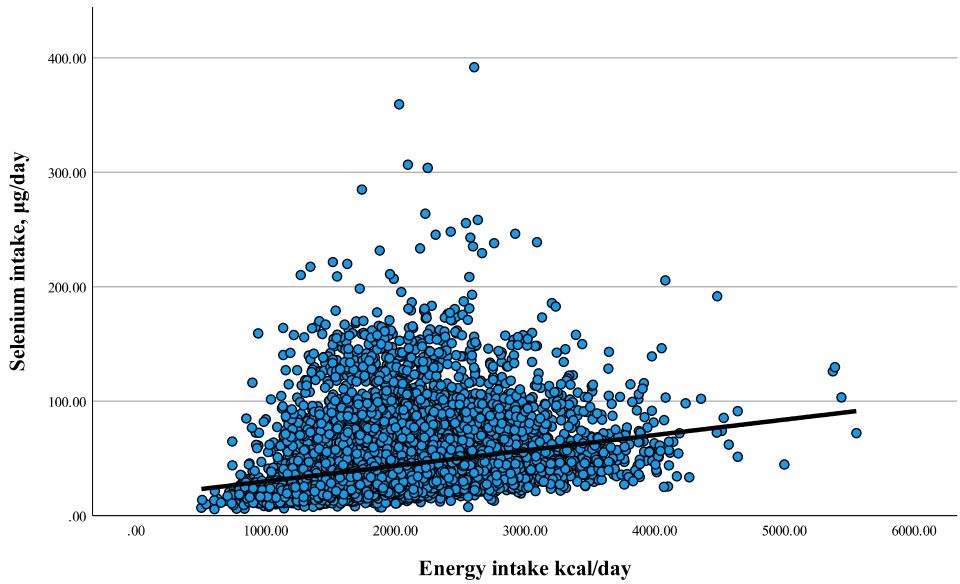
Four different exposure measurements were used in the papers included in this thesis: serum selenium, dietary selenium intake, genetic score of selenium exposure and intra-tumor expression of THRα-2. There were different ways to handle these data statistically.

Serum selenium was analyzed both as a continuous variable (paper I) and in tertiles (paper IV) and quartiles (papers I and II). Adjustment for potential confounders was then performed in the statistical models.

Another approach was used regarding the dietary intake of selenium in paper IV. The total daily selenium intake was adjusted for total energy intake and season of dietary data collection before any analyses. This was done by plotting total selenium intake versus total energy intake and assigning each individual their residual value

(distance to regression line). The data was then split into tertiles based on rank, which was performed separately for each season (spring, summer, autumn, winter).

Figure 12. Plot of selenium intake vs energy intake. The residual value is the distance between the individual value (blue dot) and the regression line (black).



The allele score tertiles used in paper IV were created in several steps. First, we assigned a weight for all included SNPs. The weight reflects the SNP's effect on serum selenium per allele. That figure is based on external data from the GWAS studies that identified the SNPs association with serum selenium. We applied the same weight as Papadimitriou et al. (2021) and collected the values from their Supplementary Table 1.[126] All SNPs were then harmonized so that an increase in the number of alleles was always an instrument for increased serum selenium. Each individual was then given a weighted allele score by adding together all alleles multiplied by their weights, and then dividing by the total weight of all seven SNPs. The allele score was then split into tertiles (low, intermediate or high) by rank, and the tertiles were used in the statistical analyses.

Table 5. The included SNPs and their weight (per allele) for the weighted allele score. Each individual can have 0, 1 or 2 of a specific allele. An example of how the unweighted and weighted allele score was calculated is presented.

SNP	Baseline allele	Effect allele	Weight	Example individual		
				Allele setup	Number of effect alleles (Unweighted)	Number of effect alleles (Weighted)
rs921943	C	T	0.25	T/T	2	0.50
rs567754	T	C	0.17	T/T	0	0
rs3797535	C	T	0.21	C/T	1	0.21
rs11951068	G	A	0.21	A/A	2	0.42
rs705415	T	C	0.23	T/C	1	0.23
rs6586282	T	C	0.12	T/T	0	0
rs1789953	C	T	0.12	T/T	0	0
Total		7	1.31		6	1.36
Calculation					6/7	1.36/1.31
Allele score					0.86	1.04

In this example, the individual has six effect alleles out of 14 possible for increased serum selenium, resulting in an unweighted allele score of 0.86, where the minimum is 0 (0/7) and the maximum is 2 (14/7). However, when her effect alleles are weighted she receives a relatively higher allele score of 1.04 (1.36/1.31) since she has alleles with a stronger effect on serum selenium. The minimum for the weighted allele score is still 0 (0/1.31) and the maximum is 2 (2.62/1.31).

The THRα-2 expression in paper III was handled by multiplying intensity (0-3) by fraction (0-4) of cells stained. The data was then split into low expression (0-7, 47% of tumors) and high expression (8-12, 53% of tumors) for the main analyses and into tertiles for sensitivity analyses.

Missing indicator method and multiple imputation

In papers I and II all missing values were replaced with a missing category (missing indicator method). However, as discussed under methodological considerations in this thesis, the missing indicator method is easily performed but has a few disadvantages. Multiple imputation by chained equations was used to handle missing data in papers III and IV. The method replaces missing values with estimates of what it most likely should be, based on available data from other variables and other individuals in the study. Each calculated estimation is repeated a number of times to increase precision. When the estimates of all missing values are complete, a new, imputed dataset is created, including all previously known values and all newly imputed values. This process is repeated to create several data sets, which explains the name, 'multiple imputation'. All new datasets are then included in the analysis, and a separate result will be presented for each dataset, as well as a pooled result, combining the results from all datasets. We imputed 25 new datasets both for papers III and study IV.

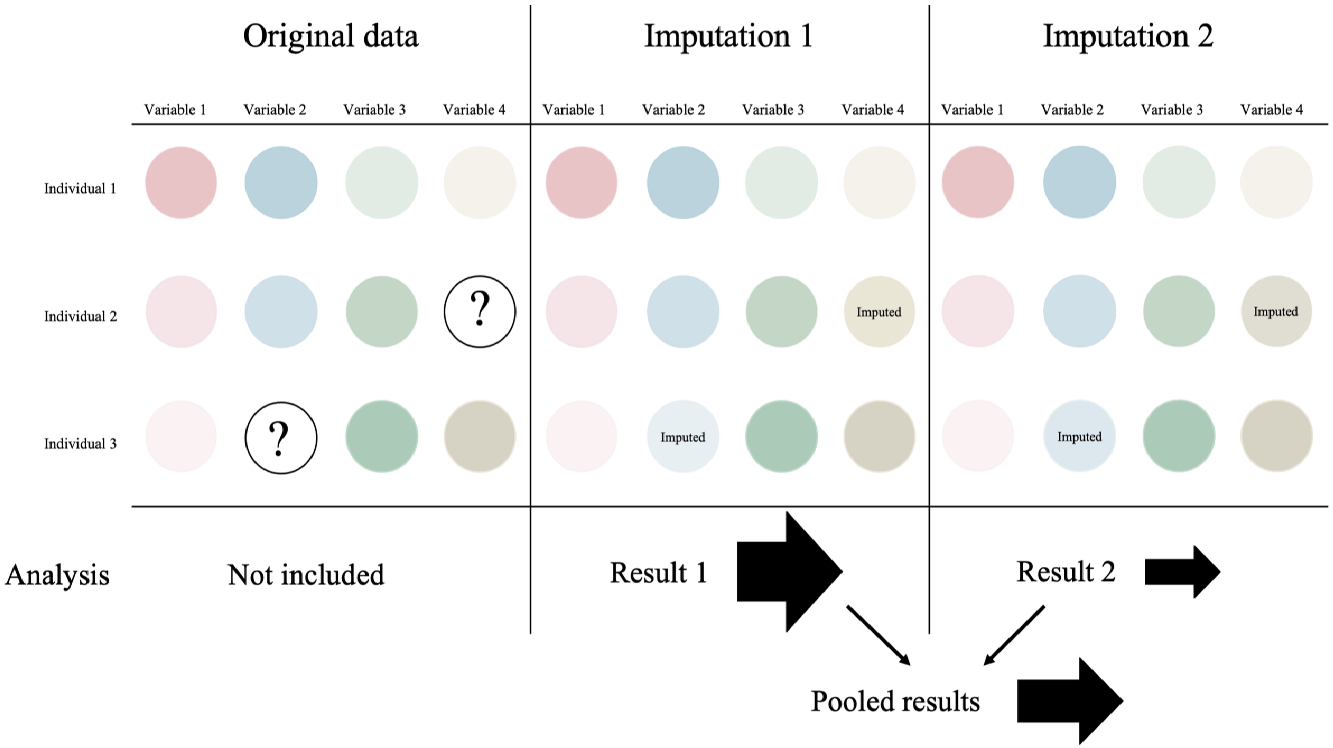
All variables included in the final analysis model should be included in the multiple imputation model. These include exposure, outcome and covariates. Additional factors can also be included if they are associated with variables that are

missing. We included both outcome and exposure variables, but individuals missing those variables were not included. Consequently those exposure and outcome variables were never imputed, just used as support to impute other data.

Table 6. Included variables in the multiple imputation models.

Paper	Exposure	Outcome	Covariates	Additional factors
III	THRα-2 expression	Breast cancer-specific death, time from diagnosis to censoring	BMI, Age at diagnosis, tumor size, HER2-, ER-, and PgR receptor status, Ki67, histological grade, lymph node invasion	Time period of diagnosis (1991-2004, 2005-2007, 2008-)
IV	Selenium intake (adjusted for energy intake and season of intake)	Breast cancer diagnosis, time from baseline to diagnosis	BMI, Age at diagnosis, menarche and menopause, HRT use, alcohol intake, oophorectomy, age at first childbirth socioeconomic index, marital status, education, SNPs	None

Figure 13. A simplifed schematic picture of multiple imputation. Each color represents a different variable, such as age or tumor size, while the intensity of the color represents the individual value in that variable, such as 63 years or 20mm. The differently sized arrows represent different point estimates of the results, such as odds ratio 1.2 and odds ratio 1.6.



Individual 3 has missing information in variable 2 and individual 2 in variable 4. As variable 1 (pink) and 2 (blue) are associated and variable 3 (green) and 4 (brown) are associated, the missing values can be estimated from the individuals with complete data in these variables. The estimation comes with a grade of uncertainty, and is performed multiple times to increase precison. The datasets are then analysed separately, yielding different results. Finally, those results are pooled, and the pooled results are used.

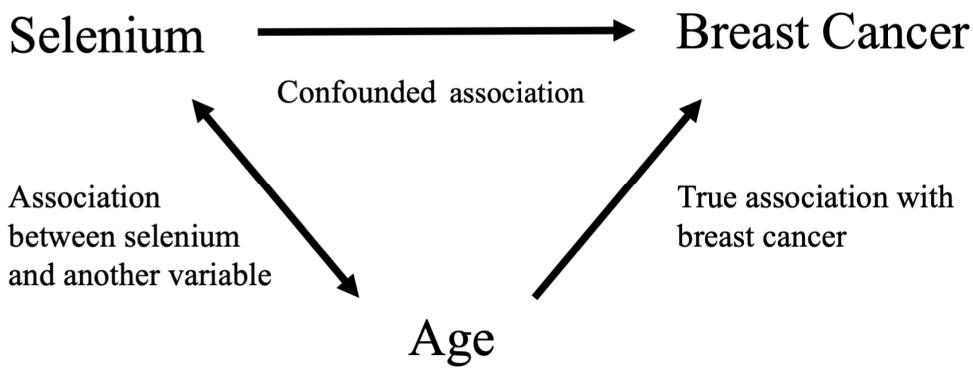
Methodological considerations

In observational studies it is seldom possible to have full control over the factors being studied. Instead, it is necessary to be aware of possible limitations, and if possible, avoid them; otherwise, they should be presented and discussed. Basic concepts include to adjust for confounding, handle missing data, and be aware of how accurately exposure and outcome are measured, and whether that information represents what one actually wants to know. Such considerations are discussed in this section.

Confounding

Confounding is possible when the exposure and outcome are both associated with a third variable, a confounder.[122] A relevant example is seen in paper I (Table 1 and Table 2) where age is associated both with selenium levels and with breast cancer. This means that an association seen between selenium levels and breast cancer risk might be due to an age difference between the cases and controls in the study, and if that age difference is adjusted for, the association could disappear or, more commonly, change in strength. However, confounders are not always known and measured, and can then not be adjusted for. This is called residual confounding and is a challenge in observational studies. However, that type of confounding can sometimes be controlled in the study design by diminishing differences in possible confounding factors between cases and controls. Most commonly by randomization or matching.[122]

Figure 14. Schematic picture of confounding.



Interaction

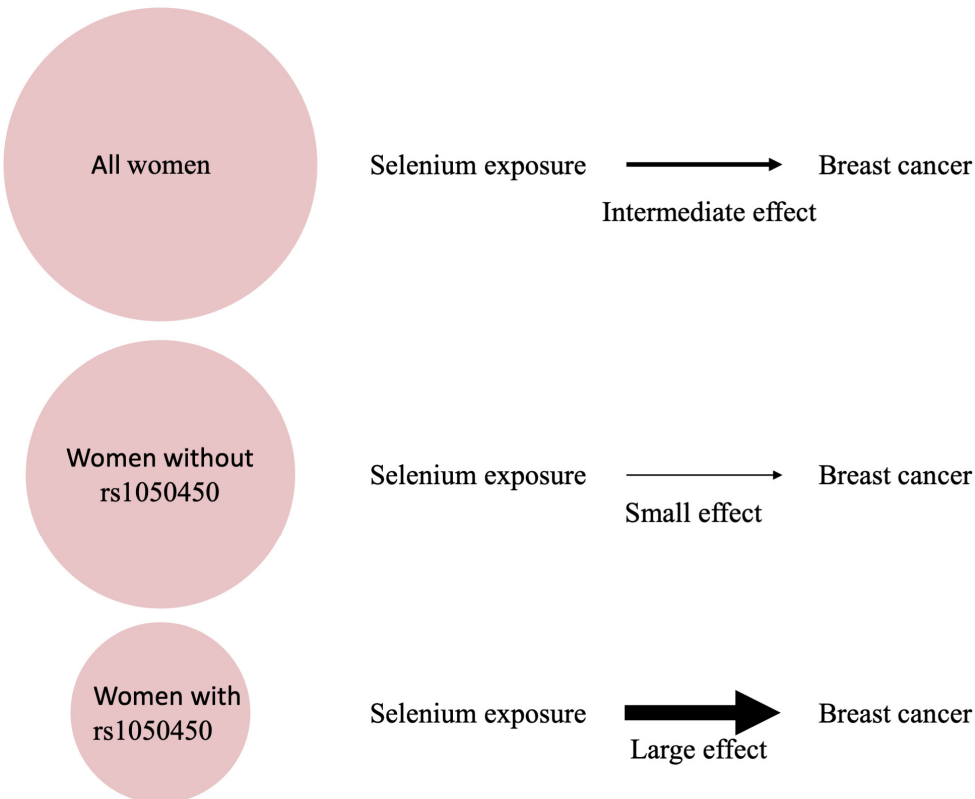
Interaction, also called effect modification, is a concept when an exposure has a different effect on the outcome depending on a third variable. A famous example in cancer research is asbestos and smoking, two risk factors for lung cancer. The risk increase from asbestos exposure differs between smokers and non-smokers, so the risks are not just additive, and nor can they just be multiplied. So there is interaction from smoking regarding the risk of lung cancer from asbestos exposure. [164] This means that a risk factor can be more important for some people than for others.

Women with a high BMI have lower selenium levels than women with normal BMI, and so do smokers compared with non-smokers.[64-66] Selenium has been shown to have different effect in smokers and non-smokers regarding the risk of bladder cancer.[165] Since BMI is a well-established risk factor for breast cancer and is also related to dietary intake, both smoking and BMI were interesting to investigate regarding a possible interaction between selenium and breast cancer.

Several reviews have highlighted the importance of possible genetic interactions in regards to the effect of selenium on cancer development.[166, 167] Individual studies have focused on SNPs in selenoprotein-related genes. The GPx-1 gene is often highlighted in such circumstances since loss of heterozygosity in the GPx-1 gene is common, both in breast cancer as well as in other cancers.[86] The SNP rs1050450 gives rise to two different functional variants of GPx-1 with either proline or leucin at codon 198 in the GPx-1 protein.[118] Although the exact biological effect of the different variants is not fully understood, one study suggests that the rarer variant with leucin at codon 198 increases the proportion of cytosolic GPx-1 and therefore increases the oxidative stress in the mitochondria, where GPx-1 is also present.[145] Other SNPs in selenoprotein genes, such as rs387789 and rs7579 in selenoprotein P and rs713041 in GPx-4, have also been studied regarding possible interactions. Women with A/A alleles in rs387789 have been shown to have higher levels of

selenium in breast tissue and a lower breast cancer risk.[120, 168] The rs7579 has been shown to affect the proportion of Selenoprotein P isotypes.[169] Women with A/A alleles in rs713041 that develop breast cancer have lower antioxidative activity in erythrocytes and an increased mortality from breast cancer. [120, 170] Some authors also stress the possibility of the interaction from non-selenoproteins. SOD-2 is one of the most important antioxidative enzymes and is closely connected to GPx-1. [171, 172] The functional SNP variation rs4880, gives rise either to valine (T allele) or alanine (C allele), in codon 16 in SOD-2.[173] The SOD-2 with valine is less efficiently transported to the mitochondria and its mRNA is less stable, reducing SOD-2 activity in vitro.[174, 175]. In prostate cancer, rs4880 has been shown to interact with selenium status regarding both risk and prognosis. [121, 176]

Figure 15. Schematic figure of a theoretical interaction by rs1050450 on breast cancer risk.



Exposure measurement

Humans are exposed to selenium mainly through diet, but also through drinking water and cigarette smoking. However, measuring the exposure to selenium has challenges.

Dietary measurements

To measure the dietary intake seems like a logical start. However, the intake of specific nutrients is strongly correlated to total energy intake and also other nutrients. Thus, when looking at an association between selenium intake and breast cancer, how can we be sure that the association is not the effect of another associated nutrient, or total energy intake? Energy intake is also associated with life style factors such as physical activity that can potentially bias the association with breast cancer. To overcome these limitations, adjustment for energy intake is needed and we choose to do so with the residual method, as described under the method section in paper IV. However, even energy-adjusted dietary intake suffers from further limitations, including the imprecise collection of data. To measure what someone eats, weighted food records are often used as a reference. That method is usually not feasible in large cohorts such as MDCS. Instead, as described above, MDCS combined information from a dietary questionnaire where participants were asked to consider the previous year's food intake, and a seven-day record of food intake. This method has been compared to the golden standard, weighted food records, and is regarded to be a suitable alternative, at least when ranking the intake of the study participants.[131, 132] We have used ranking to compare women with low vs intermediate and high selenium intake, arguably the most valid way to use the available data. However, a great strength of the dietary data method in MDCS, which is also the reason the method was used, is that data was systematically collected in all participants and thus there are no individuals with missing data and the statistical power in analyses is high.

Another factor to consider is that selenium can be ingested in different chemical forms. Selenomethionine constitutes the majority of the selenium in ingested food, while the inorganic selenite is more common in supplements. Selenomethionine has approximately twice the bioavailability of selenite.[177] Information regarding what chemical form of selenium that was ingested was not available in the MDCS data.

Biological samples

Measuring selenium in a biological sample does reduce some of the uncertainty of dietary measurements. However, different biological samples might reflect exposure to selenium with varying validity, and might not necessarily reflect the biological effect of selenium. We have used serum selenium for exposure

measurements in papers I, II and IV. It is affected by short-term selenium intake, but ranking is also maintained also over longer periods of time.[177, 178] Serum selenium also correlates to Selenoprotein P and GPx levels, but not equally well to other selenium specifications.[90, 179] Serum samples were also easily available to analyze in MDCS. However, different methods have been used in other studies, some examples are selenium levels in toenails and breast tissue or levels and activity of specific selenoproteins in the blood.[120, 168, 180, 181] However, different challenges exist with the different methods. Hair and toenails are markers of long-term selenium exposure, but products such as anti-dandruff shampoos and treatment against dermatologic mycoses contain selenium and could skew measurements.[182] During the last decade, intake recommendations have been based on when levels of Selenoprotein P and GPx-3 plateau in the blood, which is approximately when total serum selenium is 90ng/ml.[183, 184] GPx-1 and GPx-3 measured in erythrocytes and lymphocytes in the blood seem to plateau at around 70µg/day while Selenoprotein P continues to increase up to an intake of 80-100µg/day.[177, 183, 184]

Allele score

In paper IV we had a study design including allele score tertiles, as a way of reducing the risk of residual bias in a similar way as an MR study design. However, regarding the type of statistical method, it was not an MR. The design has both advantages and limitations. For a SNP to be valid as an instrumental variable it needs to conform to three assumptions:

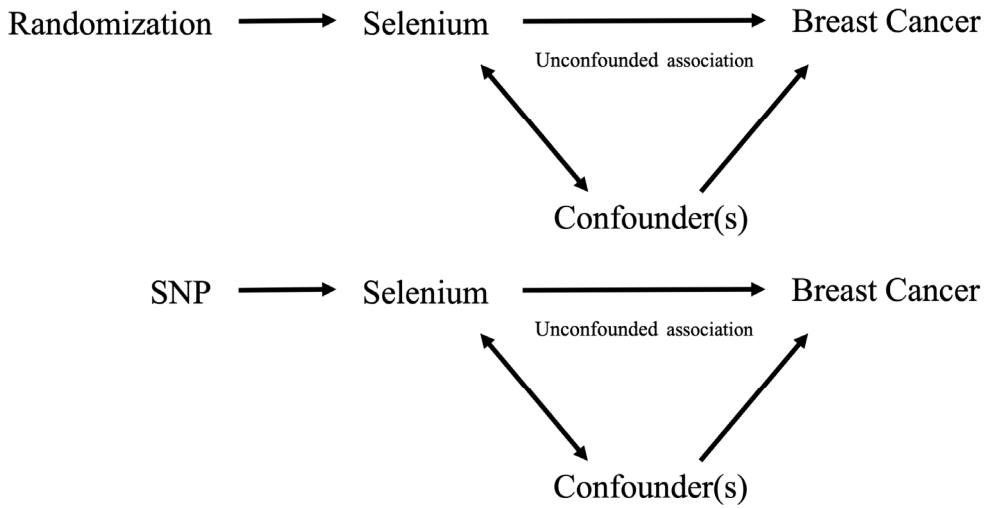
1. The SNP is strongly associated with serum selenium levels
2. The SNP only affects breast cancer through selenium
3. No association exists between the SNP and possible confounders

Assumption 1 was tested in the GWAS, since only SNPs with an association of $p=5x10^{-8}$ or stronger with serum selenium were selected. Assumption 2 is a reasonable assumption based on the genes involved, since the locus of the SNPs was focused around genes coding for enzymes in the metabolism of selenium-containing amino acids, it is reasonable that any possible effect would come from changes in selenium exposure.[141] The last assumption is based both on the same reasoning as assumption 2, but also on previous literature and that there was no difference in risk factors for breast cancer when comparing tertiles of allele score (Supplementary Table S2, paper IV). However, rs921943 has previously been associated with adult attained height, which is a risk factor for breast cancer, and could thus violate that assumption. The assumption was tested by Papadimitriou et al. (2021) in a 'leave one out' analysis, and they concluded it does not change the association of the genetic instrument with breast cancer, thus the correlation with height is unlikely to have any considerable confounding effect in the weighted allele score instrument.[126]

However, it is problematic that the genetic variations only account for a small portion of the selenium levels. In the MR of Papadimitriou et al. (2021), the

correlation between the weighted allele score and serum selenium was calculated to be 3%.[126] Therefore, a low statistical power might be an issue, at least when there is a limited population size, as in MDCS. The MR of Papadimitriou et al. (2021) was based on data from a breast cancer consortium combining several large population cohorts from Europe, Asia and America, with a total of 122,977 cases and 105,974 controls, and did not find any association between selenium and breast cancer risk.[126] Furthermore, we did not perform a classic MR study. Instead, we studied the tertiles of allele score relative to each other, as a way to triangulate three different exposure measurements in a comparable way (diet, serum and genetic score), and we believe that was a more feasible option in the MDCS population.

Figure 16. SNPs can be used in an MR to avoid confounding in a similar way as in a randomized control trial.



Immunohistologic evaluation of THRα-2

The breast cancer TMA in the MDCS population was initially constructed to analyse receptor status in breast cancer tumors diagnosed from 1991 to 2004, and has since been a valuable asset in the cohort and has also been updated to include tumors up until 2010.[150, 152, 154] Efficient evaluation is an obvious advantage with TMA compared to whole tumor slides. However, since only a small volume of the tumor is evaluated (two slices 3-4µm thick and 0.6-1.0 mm in diameter), there is a risk of missing heterogeneity within the tumor. This is a major issue with some factors, such as Ki67, while other factors, e.g. the estrogen receptor, are homogenously expressed in most tumors. To limit this problem, the two 1 mm cores are collected from two separate representative areas of the tumor. The concordance between TMA and whole slides is slightly lower regarding PgR expression than ER and more

so if the tumor shows heterogeneity between the two TMA cores, although concordance in general is good.[185]

Insufficient antibody validation has been debated as a major problem in breast cancer research e.g. regarding antibodies targeting ER-beta.[186] The antibodies used are optimally both specific and sensitive to what one wishes to detect, however those numbers are not always reported by the industry. In study III, antibodies specific to THRα-2 were used (MAI-4676 from Thermo Fischer Scientific), and the manufacturer did not report the specificity or sensitivity. However, we choose to use the same antibodies as another similar study to be able to compare our findings in a reliable way, with no further antibody validation performed by us.[112] Loss of antigenicity in stored breast tumors is another issue reported and could affect immunohistological evaluation in cohort studies.[187] Older tumors could then be systematically underscored regarding expression parameters compared with more newly diagnosed tumors that have been stored for a shorter time. However, in a study from 2018 the authors concluded that ER and PgR receptors were stable for up to 40 years in stored tumor material.[188] Although THRα-2 has not specifically been studied, there is no obvious reason that it would differ from ER and PgR receptors regarding loss of antigenicity. However, storage time is a possible confounder in paper III since it can possibly be associated both with risk of death and THRα-2 expression.

The microscopic evaluation was performed using digital pathology. That means that the TMA glasses were scanned and available for reading and scoring manually in a computer program. This was performed twice, and blinded for patient ID and characteristics, for higher validity. Tumors that differed between the two readings were identified and evaluated a third time. All readings were done by one researcher (me). Letting two researchers conduct separate readings might increase the generalizability of the results, since it might reduce skewness or bias that may be in my method of reading the THRα-2 expression. However, as described in paper III, if there were any issues with scoring a tumor, experienced colleagues were involved and consulted.

Endpoint measurement

The Swedish Cancer Register and the Swedish Cause of Death Register used for endpoint data collection have high quality regarding completeness.[189, 190] Thus it is unlikely that any breast cancer diagnoses or deaths were missed. One study evaluated the agreement between the registered cause of death and the expected cause of death from case summaries, and found an overall agreement of 77% but over 90% for malignancies.[191] Both breast cancer-specific death and overall death can be identified in the Swedish Cause of Death Register, but not information regarding relapse in breast cancer, and this was also not available from any other

source. According to a study in 2017, >99% of all deaths registered in 2015 had a specific cause of death in the registry.[190] The use of different endpoints in breast cancer literature has been discussed extensively, and since the definition of relapse can vary between studies, results can be hard to interpret or compare to other studies.[192, 193] We decided to present both breast cancer-specific mortality and all-cause mortality in paper II and III, and the results were similar for both endpoints.

Missing data

Missing data is more or less always present when working with cohorts. There can be several reasons, e.g. skipped questions in the questionnaire, a missed entry from the data collector, small tumors that complicate TMA construction, or the stored blood may have already been used up due to inclusion in other studies or lost to follow-up due to moving abroad. Either people have missing values in exposure (selenium, TMA expression), endpoint (tumor characteristics, vital status) or other variables (weight, smoking status, socioeconomic index etc.). The best possible method is of course to identify missing values and try to collect them, although that is in many cases not practically possible.

By excluding individuals with missing data, the statistical power decreases and there is also a risk of bias when analyzing only complete cases, since they might differ in their characteristics compared to individuals with missing data. The missing indicator method means that individuals with a missing value in a variable are given a shared categorical value. The strengths of this approach are that all individuals will be included in all analyses, it is reasonably robust, and it is uncomplicated to perform. However, introducing additional categories might make the statistical model less stable and there is also a risk of introducing an unpredictable bias when using the missing indicator model.[194] Thus we abandoned that model after the first two papers to instead use multiple imputation by chained equations. Multiple imputation is a model that uses all available data to make a "best guess" for the value that should replace a missing value, based on all other values in the model.[195] As long as there are no important unmeasured or unknown factors that cause the missingness, this is indeed a superior way to handle missing data. No individual will be lost in the analyses, and bias can be avoided. One good example comes from paper III, when Ki67, a proliferation marker, was missing for 22% of the included individuals. Ki67 is strongly associated with histological grade, which was missing among only 1.4%. The missing indicator model would label all 22% in the same category, while multiple imputation gives a good estimate of Ki67 from known data, like the histological grade and other parameters that also correlate with Ki67. While multiple imputation is a valid model to replace missing values in outcome, exposure and covariate variables, we have taken a fairly defensive approach in paper III and IV by not imputing values for the exposure (selenium

levels, genetic data and THRα-2 expression). The outcome regarding tumor characteristics was imputed in paper III, but not mortality or incidence.

Tumor characteristics

The data regarding tumor characteristics in MDCS were collected with different methods during different time periods, as mentioned above. This might lead to differences within variables during different time periods. One example is the PgR variable that during the years between 1991 and 2004 was collected from TMA data and later from clinical data. Initially, the PgR and ER data was based on another TMA evaluation between 2005 and 2007, but issues regarding the quality of this data was identified, believed to relate to poor antibody specificity. That led to a complementary data collection from medical records for these variables for paper II. Still, there were large differences between these time periods in the PgR variable. In paper III 40.7% of the women had ≤10% expression in the PgR variable, while other studies have reported around 24%.[196] In fact, between the years 1991 and 2004 that figure was 55.8%, while in 2005-2007 it was 26.9% and in 2008-2010 it was 16.9%. A slightly higher ER negativity was also seen in 1991-2004 with 14.5% compared to 2005-07 (9.0%) and 2008-10 (6.2%). One possible reason for these differences is that the TMA analysis in the period 1991 to 2004 underestimated the expression of the hormonal receptors compared to the clinical examination which is performed on whole tissue sections. There were no notable difference between the time periods for HER2, histological grade, KI67, tumor size or lymph node positivity.

Surrogate intrinsic subtypes

The breast tumors' intrinsic subtypes were not available in the MDCS data set, and considering it includes tumors diagnosed as early as 1991, clinical information regarding subtype would not be available for a majority of the patients. However, as described above, surrogate intrinsic subtypes can be derived from their immunohistochemical expression. For paper II we did extensive work to figure out which method would be best to apply. The Swedish guidelines included histological grade to separate luminal A-like tumors from luminal B-like tumors. That was not an international standard, since many instead used the definition established by expert consensus at the St Gallen 2013 meeting that defined luminal A and B based on Ki67.[41] However, the histological grade has been proved to correlate better with prognosis, so we decided to use the same method as in the Swedish guidelines.[197, 198] Another issue was the loss of statistical strength and robustness of the analyses when dividing the tumors into several small groups. Only around 7% of the included individuals had HER2+ tumors, although almost 20% had missing information

regarding HER2 status. We therefore decided to combine luminal HER2+ and non-luminal HER2+, defining four different surrogate intrinsic subtypes instead of five for the MDCS dataset, as presented in Table 7. In paper III, we further refined the surrogate intrinsic subtype by using multiple imputation for the missing data.

Table 7. Surrogate intrinsic subtypes as defined in papers II and III, with a slight variation of the St Gallen 2013 and the Swedish guidelines.[22, 41]

Surrogate intrinsic subtype	ER	HER2	Histological grade*	Ki67	PgR
Luminal A-like	+	-	1	Any	Any
	+	-	2	Low	Any
	+	-	2	Intermediate	+
Luminal B-like	+	-	3	Any	Any
	+	-	2	High	Any
	+	-	2	Intermediate	-
HER2+	Any	+	Any	Any	Any
Triple-negative breast cancer	-	-	Any	Any	-

Ethical considerations

In the four papers included in the present thesis the ethical considerations are similar, but not the same. The participants in MDCS provided written consent at the time of their inclusion in MDCS that the information they left at baseline and data collected later on by researchers would be used and published in future research. However, it is important to care for that consent respectfully and not cause any harm to the participants as well as to produce meaningful and high quality research for the harm or risk of harm already caused. Since no new tests, samples or examinations were conducted with the participants the possible harm lay essentially in how their integrity was handled. All data I have been handling is anonymized by using a participation number in MDCS instead of a personal identity number. And when the data is published, no individual level data is used. The MDCS has a central data manager that has a key between personal identity number and participation number in MDCS. In this way, the data manager can link the MDCS participants with the national registries we have used for breast cancer diagnosis, cause of death and also to collect clinical data. And before the data is sent to me or other researchers, it is anonymized again.

Specific considerations in the different articles include the use of biological samples with limited quantity. Those analyses conducted for my research automatically mean that other analyses cannot be performed. Serum selenium was analyzed from stored frozen serum. However, the serum selenium levels were used in papers I, II and IV as well as in studies not included in the thesis to maximize the utility of the available serum. THRα-2 tumor expression was evaluated using a TMA that was previously created from stored tumor material. The data I collected is planned to be used in future projects as well.

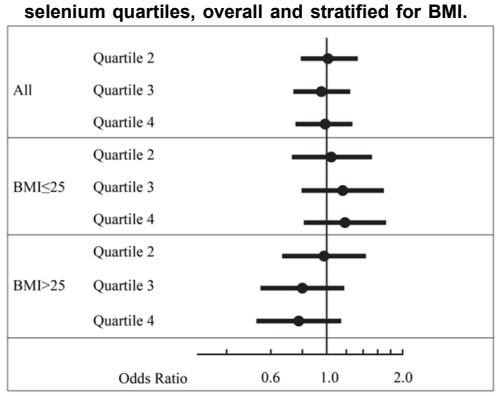
The doctoral thesis is a part of a larger project called “Breast cancer in regards to thyroid hormones, selenium and iodine; studies of how serum levels, receptors and genetic polymorphisms affect risk and prognosis”. That project includes all studies in the present thesis and was approved by the regional ethics board in Lund, DNR 2015/283. The original MDCS was also approved by the regional ethics board, DNR LU 51/90.

Results and discussion

Selenium and risk of breast cancer

In the results presented in paper I, there was no evidence of an effect on overall breast cancer risk from serum selenium. Null results were seen both when comparing selenium quartiles and when comparing risk increase for each 10ng/ml increase of selenium (OR 1.00 (95% CI 1.00-1.01)). In contrast to our results, a recent meta-analysis concluded that serum and toenail selenium, but not selenium in hair, was associated with an increased risk of breast cancer.[199] That meta-analysis included a mix of studies with pre- and post-diagnostic exposure measurements. Due to large between-study variance, most included studies were assigned similar weights under the random effects model, even though there were large differences in the number of included individuals. Thus, the effect seen in their results could be an effect of reverse causation from the included case-control studies, which were given a proportionally high weight despite a low number of participants. Indeed, another meta-analysis from the Cochrane institute investigated pre-diagnostic selenium levels and breast cancer risk, and concluded that there was no evidence of an overall effect.[80]

Figure 17. Odds ratio and 95% confidence intervals of breast cancer comparing serum selenium quartiles, overall and stratified for BMI.



When serum selenium and breast cancer risk was evaluated separately for women in different BMI groups and depending on their smoking status we found no evidence of a difference. Even though the point estimates differ between the BMI groups, the confidence intervals crosses 1 by some margin and my conclusion is that there is no evidence to support that this observed difference in point estimates is not due to chance. Similarly we found no evidence that serum selenium would specifically increase the risk of a breast cancer with certain characteristics such as ER+, different tumor size or HER2+, as presented in paper II.

In paper IV overall breast cancer risk was compared over tertiles of selenium intake, serum selenium and genetic score. Results from MDCS were previously published regarding serum selenium (Paper I, no difference between quartiles) and dietary intake (Bengtsson Y, Sandsveden M, Manjer J, (2021), weak evidence of a U-shaped association, suggesting that women with intermediate selenium intake have the lowest risk of breast cancer), and similar results were seen as expected in paper IV. [1, 200] The genetic score was previously not evaluated. However, no evidence of a difference in breast cancer risk was seen when comparing tertiles of genetic score. Although 1,956 women with breast cancer and 16,429 women in total were included in the analysis, there is a risk that the statistical power was to small to detect any difference. The reason is that the effect we study in this analysis is based only on the portion of serum selenium which depends on the genetic variation in our calculated allele score, as discussed above in methodological considerations. However, as similar results were seen in an MR study with a larger study population, it can be concluded that the known portion of selenium that varies with SNPs does not affect breast cancer risk.[126] So if there is an effect on breast cancer risk from selenium exposure, it is likely to be caused by factors that are modifiable.

Table 8. Exposure of selenium in diet, serum and genetically elevated and breast cancer risk. Presented as hazard ratios comparing tertiles, with tertile 1 as reference. Results from paper IV.

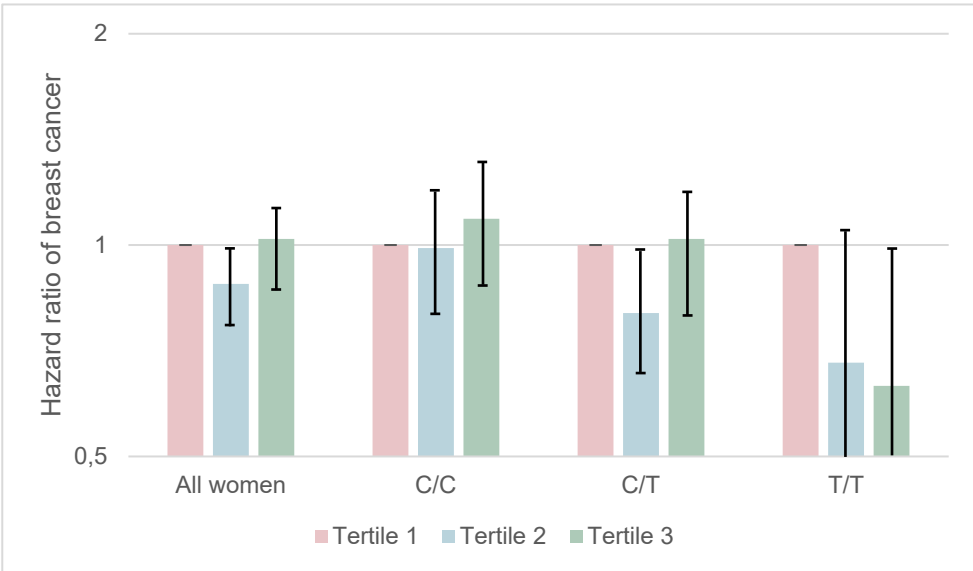
Tertile of selenium exposure	Diet	Serum	Weighted allele score
	HR[1]	HR[1]	HR
T1	1	1	1
T2	0.86 (0.76-0.98)	1.05 (0.90-1.21)	0.97 (0.87-1.08)
T3	0.97 (0.85-1.10)	0.91 (0.78-1.06)	1.00 (0.84-1.15)

[1]Adjusted for age at baseline, education, socioeconomic index, marital status, age at menarche, age at menopause, number of children, age at childbirth, use of oral contraceptives, oophorectomy, BMI, hormone replacement therapy and alcohol intake.

Regarding the U-shaped association with breast cancer risk and selenium intake, several risk factors do not fit the classic linear risk model. Both Waters and Chiang (2018) as well as Rayman (2012) argue that both too low and too high selenium exposure could be harmful, as selenium is protective in the right amounts but toxic in higher concentrations, but they do not present any specific evidence to support that theory in regards to breast cancer risk.[201, 202] However, when selenium has been studied as a potential therapeutic agent in cancer, the effect of high dose selenium seems to be that after selenoprotein levels are saturated, excess selenium will create metabolites that increase the oxidative stress.[77] So it is probable that a protective effect from selenium would reach a plateau at an intake around 70-100µg/day when the levels of selenoproteins reach their peak, and beyond that there could be a risk of increased oxidative stress. In paper IV, the tertiles are not based exclusively on total selenium intake. As described in the method section they are adjusted both for total energy intake and season of dietary measurement by using the residual method and ranking, and thus the total intake of selenium might overlap in the three tertiles. The mean intake however was 26, 35 and 69 µg/day respectively for the tertiles. If

the observed difference in breast cancer risk is in fact due to the difference in selenium intake, selenium reaches toxic levels at lower intake levels than when selenoproteins are saturated in serum, which is not in line with other literature. Arguably, it is more likely that the selenium intake in MDCS is associated with breast cancer via an unmeasured confounder or effect modifier, which explains at least a part of the u-curve. Thus, although the analyses were adjusted for several breast cancer risk factors (Table 8), the group of women with intermediate intake of selenium might differ from those with a low or a high intake. These results indeed highlight the challenges both with dietary measurements as an exposure variable and with observational studies.

Figure 18. Hazard ratios of breast cancer and 95% confidence intervals among women with different SNP alleles in the GPx-1 gene.



A U-shaped association between selenium intake and breast cancer risk was seen when studying all women and among women with C/T in rs1050450. There was no difference between selenium intake tertiles among women with C/C and it was an inverse relationship with selenium intake and breast cancer risk among women with T/T in rs1050450.

One unmeasured factor in the previous article from MDCS and in our overall analysis, is genetic variation. Indeed, we found an interaction from a SNP in the GPx-1 gene, rs1050450, regarding selenium intake and breast cancer risk. When investigating only women with two alternative alleles (T/T) in rs1050450, the mean intake was similar over tertiles as among all women (24, 36, 68 µg/day respectively). However, instead of a U-shape, there was a dose-response pattern between dietary intake and breast cancer risk, while among women with no alternative alleles (C/C) in rs1050450 there was no evidence of a difference between tertiles of selenium intake (Figure 18). Similar findings have been reported by others as well. Either one or two alternative alleles (C/T or T/T) have been associated with an increased risk

of breast cancer.[119] Another study did see weak evidence of a lower incidence of ductal breast cancer, but a higher risk of non-ductal breast cancer for T/T carriers.[120]

A weakness of our analysis is that only around 9% of the MDCS population had T/T in rs1050450, reducing the statistical strength and robustness of the analysis. Because of that, the analyses were only adjusted for age at baseline. However, as seen in Supplementary tables S3-S5 in paper IV, there were only small differences between the fully adjusted models and those adjusted only for age. It is unlikely that the results would change dramatically due to adjustment for additional risk factors.

However, there was no similar interaction from rs1050450 regarding serum selenium or allele score. It thus becomes an isolated finding in only one exposure measurement, and could be due to chance or bias in that exposure measurement. However, as already discussed, there might be a power problem regarding the allele score, and an additional power problem when investigating alternative alleles (T/T) of rs1050450 due to low effect size compared with the sample size. The combination of serum selenium and genetic data was available for 1047 cases and 990 controls, but less than 100 cases and controls were T/T carriers.

We also found a few other interactions from the five investigated SNPs regarding serum selenium or allele score. Women with no alternative alleles (G/G) in the SNP rs7579 in Selenoprotein P, had a higher risk of breast cancer if they had a high allele score, HR 1.20 (1.02-1.41). And women with one alternative allele (G/A) in rs7579 instead had a lower risk of breast cancer if they had a high allele score, HR 0.85 (0.73-1.00). However, these results seem to be isolated findings that follow no logical pattern, and could be chance findings due to multiple testing in this population. When evaluating the SNPs overall effect on breast cancer risk, women with T/T in rs1050450 had a lower risk of breast cancer, but none of the other SNPs showed a similar effect. This is also in line with a previously published GWAS.[117]

As shown by our research group previously, women with both high selenium and iodine levels in serum have a lower incidence of breast cancer, but there is no difference in risk when only comparing iodine levels.[103] Thus, there might be other factors interacting with selenium, not investigated in the scope of this thesis.

The SELINA trial was registered in 2019 (NCT04014283). This is a randomized trial with several arms randomizing Polish women with hereditary risk of breast cancer to selenium supplementation, placebo or dietary adjustments. The main outcome it is planned to study is the incidence of any cancer, while breast cancer incidence will be a secondary outcome.[203] No results have yet been published, but the results could give further high quality evidence of selenium intake in regards to breast cancer risk.

Selenium and survival in breast cancer

Our results indicate that women with low levels of selenium have a higher mortality. In paper II we compared the highest versus the lowest quartile of serum selenium among women later diagnosed with breast cancer and found a lower mortality among the women with the highest levels. In our results, there is also a dose-response relationship between serum selenium and mortality, although there is statistically weak evidence for any comparison between quartiles other than the highest versus the lowest. Results were similar for breast cancer-specific and all-cause mortality.

Table 9. Overall mortality among women with breast cancer, depending on pre-diagnostic serum selenium.

Quartile of selenium	Mortality	HR (95% CI)	HR[1] (95% CI)
1	35.14	1.00	1.00
2	32.83	0.94 (0.69-1.28)	0.91 (0.66-1.25)
3	28.94	0.82 (0.60-1.13)	0.73 (0.53-1.02)
4	25.42	0.71 (0.51-1.00)	0.62 (0.43-0.88)

[1] Adjusted for age at diagnosis, lymph node status, tumor size, intrinsic subtype, BMI, age at baseline, year and season the sample was taken.

Similar results regarding mortality have been seen in two recently published studies, both for overall serum selenium and for specific selenoproteins (GPx-3 and Selenoprotein P).[84, 181] Demircan et al. (2021) measured selenium at time of diagnosis and argue that selenium status outperforms established prognostic factors in predicting breast cancer outcome. And indeed, they show that it outperforms tumor size, lymph node status and histological grade. The only individual predictor with a stronger prognostic value of death in their study is age at diagnosis.[181]

We investigated pre-diagnostic serum selenium levels. By doing so, we reduced the risk of reverse causation, e.g. that an advanced breast cancer disease would be the cause of lower selenium levels and not the other way around. Furthermore, the mortality difference is not likely to be explained by a higher incidence of breast cancer since we only compare women later diagnosed with breast cancer, and also considering the results above that serum selenium does not seem to be associated with breast cancer risk. The difference is not likely to be explained by a more aggressive breast cancer defined by known prognostic factors, since neither we nor other authors found such an association. Thus there is likely to be another pathway for this association.

Interestingly, we did find a lower mortality among controls in the highest serum selenium quartile compared with those in the lowest quartile, HR 0.68 (0.48-0.95). And indeed, another Swedish study found an increased overall relative risk of death among elderly individuals with lower serum selenium levels, with selenium levels in the same range as in the MDCS population.[204] In theory, selenium could be a marker of frailty or poor health in general, identifying individuals at a higher risk of death. It is also possible that selenium is involved in a more common mechanism in cancer, not restricted to breast cancer, which would explain the increased mortality.

The Swedish study did not see such an association with cancer mortality, but Bleys et al. did find a lower mortality in cancer-related death, though not in cardiovascular death, in the highest selenium quartile compared to the lowest among 13,887 Americans.[205]

The available evidence comes from observational data and it cannot be concluded that selenium is involved in a causal mechanism regarding death in breast cancer. But regardless of that, there is overall strong evidence that an association exists between serum selenium and breast cancer outcome, whether it is causal or not.

THRα-2 and breast cancer prognosis

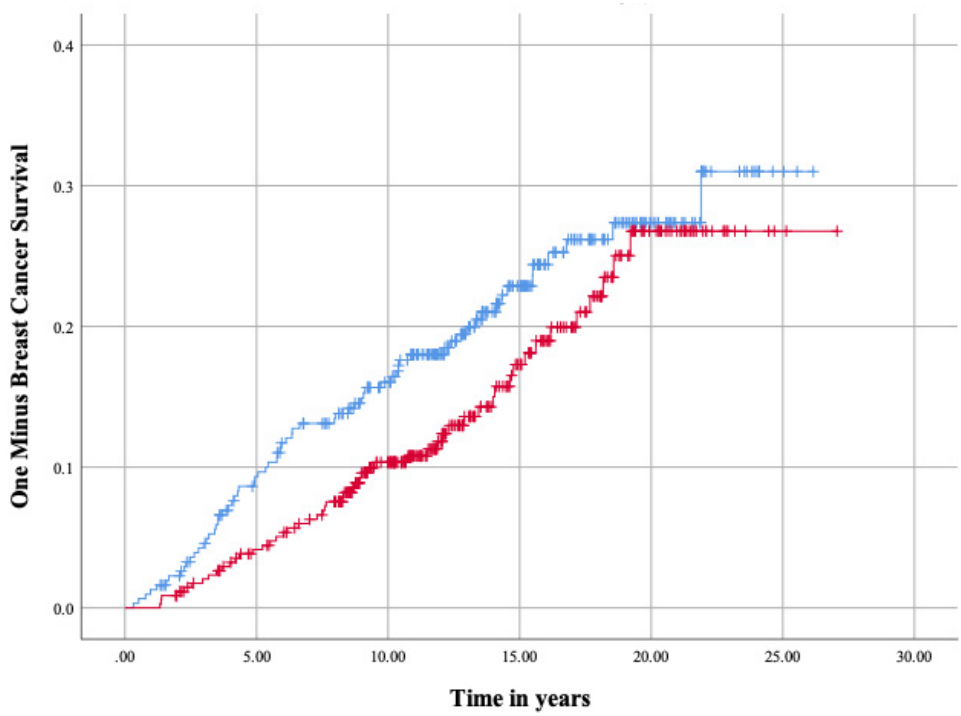
The results from paper III support the theory that tumor expression of THRα-2 is associated with favorable tumor characteristics and lower mortality in breast cancer (Figure 19 and Table 10). Similar results have been seen in three other studies.[111-113] Zehni et al. (2021) investigated the expression among 319 breast cancer patients and found that expression of THRα-1 had the opposite effect to THRα-2, with an inverse correlation to disease-free survival and a positive correlation to distant metastasis in the TNM-staging.[113]

Table 10. A high expression of THRα-2 is correlated to prognostically favorable tumor features in breast cancer. Results from paper III.

Tumor characteristic		Low	High	OR (95% CI)
Surrogate intrinsic subtype	Luminal A-like	43.7	66.0	1.00
	Luminal B-like	29.0	22.0	1.99 (1.34-2.97)
	HER2+	12.0	7.5	2.42 (1.38-4.27)
	Triple-negative	15.3	4.5	5.10 (2.70-9.65)
Tumor size	≤10 mm	16.8	22.5	1.00
	11-20 mm	43.7	53.2	1.10 (0.73-1.67)
	21-50 mm	35.6	21.6	2.20 (1.39-3.49)
	>50 mm	3.9	2.6	1.98 (0.78-5.02)
Lymph node invasion	No	60.0	70.4	1.00
	Yes	39.4	29.6	1.55 (1.11-2.15)

Although these findings are consistent, they do not provide enough evidence to say there is a causal relationship between THRα-2 expression and outcome. THRα-2 might just be correlated with other already established prognostic markers. However, as presented in Table 2 in paper III, there seems to be a dose-response relationship between both the intensity and fraction of cells stained and prognostic markers. Moreover, as shown in Table 10, the same applies when the expression is dichotomized. The evidence suggests that THRα-2 counteracts THRα-1, and thereby counteracts THRα-1's mediation of estrogen-like effects on breast cancer cells. [92, 94] This could be a mechanistic pathway in which THRα-2 affects breast cancer.

Figure 19. There is a higher breast cancer mortality among women with low intra-tumor expression of THRα-2 (blue) compared to high (red). Also seen in Figure 3a in paper III.



Generalizability of results

The results in this thesis come from a population cohort in the Swedish city of Malmö. At least some skewness regarding the selection of the population is common in cohorts and somewhat reduces the generalizability of the results, although the internal validity of the results is not affected by selection. As discussed above, although MDCS only had a participation rate of around 40%, it is likely to represent the background population of Malmö reasonably well, since the characteristics of the study participants were similar to another study with a 74.6% participation rate.[129] However, non-participants had a higher mortality and a disease spectrum indicative of a lower socioeconomic class.[129]

Sweden is a country with a low intake of selenium, which is in line with what we found in the MDCS population.[52] Other Swedish studies have reported a similar dietary intake and serum selenium levels as in the MDCS.[83, 204] As discussed in the thesis, the effect of selenium might plateau when the expression of selenoproteins is saturated. Thus, the results in this thesis are more likely to be valid in a population with a low exposure to selenium.

Strengths and limitations

Table 11. Strengths and limitations in short, in addition to what has been discussed above.

Paper	Strengths	Limitations
I	High number of cases. Data regarding many confounders. Pre-diagnostic serum selenium measurement. Long follow-up.	No genetic information. Risk of residual confounding. Risk of bias from missing data.
II	High number of cases. Data regarding many confounders. Tumor-specific characteristics and prognostic data. Pre-diagnostic serum selenium measurement. Long follow-up.	No genetic information. Risk of residual confounding.
III	High number of cases. Intra-tumor THRα -2 expression. Tumor-specific characteristics and prognostic data. Multiple imputation regarding missing data.	Evaluation on TMA instead of whole tumor slides. Risk of residual confounding. Thyroid hormone levels not analyzed.
IV	Several measurements of exposure. Cohort design. Genetic data available. Multiple imputation regarding missing data.	Low statistical power in allele score analyses. Risk of residual confounding in diet- and serum analyses.
Overall	Prospective population cohort. Low selenium setting. High quality endpoint data.	Observational studies with risk of residual confounding. Single measurement of selenium.

Conclusions

The general conclusion of this thesis is that there is no overall protective effect of selenium regarding breast cancer risk. However, it is likely that low selenium levels in serum and low THRα-2 expression in breast cancer tumors are associated with a worse breast cancer prognosis. The results of this thesis are likely to be applicable to a population with low selenium exposure. Specific conclusions of this thesis are that;

- Overall breast cancer risk is not likely to be influenced by selenium exposure. Although we found a lower risk of breast cancer for women with an intermediate intake of selenium compared to those with a low or a high intake, there is a high degree of uncertainty regarding those results.
- Clinically relevant prognostic or treatment-predictive factors are not affected by selenium exposure.
- Higher selenium exposure, measured as selenium levels in serum, is likely to be associated with a lower mortality in breast cancer. However, there is not enough evidence to conclude that a causal relationship exists.
- BMI or smoking do not seem to affect the association between selenium and breast cancer. However, selenium intake might be protective against breast cancer among women with alternative alleles (T/T) in the SNP rs1050450 in the gene coding for the selenoprotein GPx-1. A protective effect was seen among women with that variation and an intermediate or high intake of selenium, but not among those with a low intake, and not among women with standard alleles. These findings need to be replicated in another cohort.
- A variation in the selenoprotein gene GPx-1, the SNP rs1050450, might be associated with an overall lower risk of breast cancer. Since our results suggest that women with the same genetic variation also could have a protective effect from selenium exposure, GPx-1 might be mechanistically involved in the development of breast cancer.
- Low expression of THRα-2 in breast cancer tumors is associated with higher mortality. This might at least in part be explained by its inverse correlation with clinically unfavorable tumor and treatment predictive characteristics. Thus, THRα-2 might be a prognostic marker, but not independent from other known prognostic markers.

Future perspectives

Concluding that serum selenium is associated with breast cancer mortality, future studies should focus on whether it serves just as a marker for increased mortality or if it is involved in a causal mechanism. A similar approach is applicable for THRα-2 expression in breast cancer tumors. We know that it is associated with other prognostic factors and with mortality, but is it involved in a causal mechanism? If serum selenium or THRα-2 expression is indeed in a causal pathway, they might open up possibilities for new treatment options, while if they are not, they might serve as prognostic markers. Our results also suggest that the SNP rs1050450 in the GPx-1 gene affects breast cancer risk on its own and perhaps through interaction with selenium exposure. Studying GPx-1 in relation to mortality is a logical next step, since selenium exposure seems more important in prognosis than in incidence, and both the findings in this thesis and in previous literature indicate that GPx-1 could be involved in breast cancer development.

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