Trace Elements and Breast Cancer: Selenium, Zinc and Copper in Relation to Risk and Prognosis

Bengtsson, Ylva

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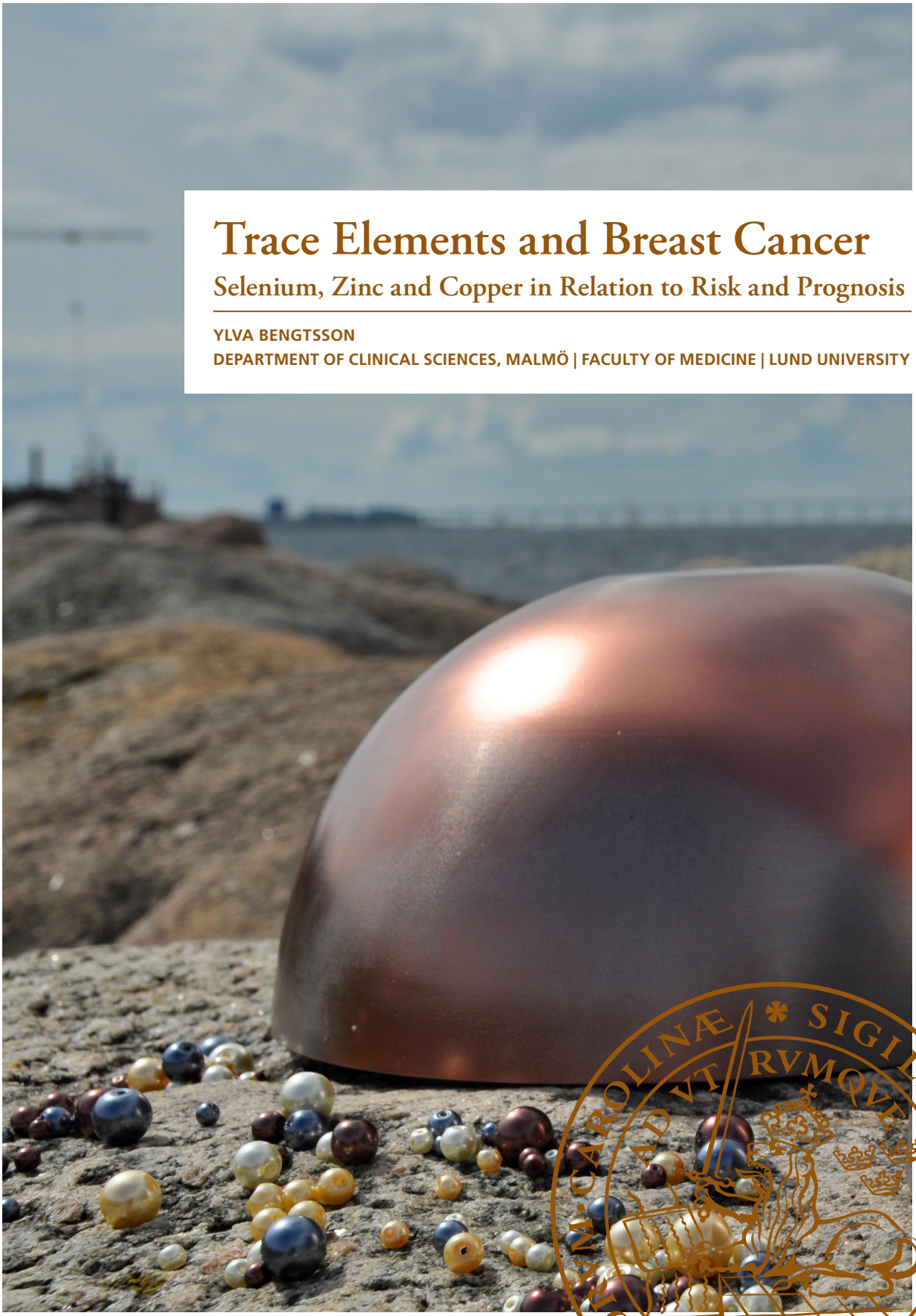
YLVA BENGTSSON
DEPARTMENT OF CLINICAL SCIENCES, MALMÖ | FACULTY OF MEDICINE | LUND UNIVERSITY



Trace Elements and Breast Cancer

Selenium, Zinc and Copper in Relation to Risk and Prognosis

Ylva Bengtsson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Faculty opponent
Professor Fredrik Wärnberg
Department of Surgery
Sahlgrenska Academy, University of Gothenburg, Sweden

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Background
Pre-clinical studies suggest that selenium, zinc and copper may play a role in breast cancer incidence and survival. However, epidemiological evidence on this topic is scarce. This thesis aims to investigate the associations between different levels of selenium, zinc and copper in serum and diet, and the risk and prognosis of breast cancer.

Methods
Epidemiological methods and data from two large prospective population-based cohorts were used: the Malmö Diet and Cancer Study (MDCS) and the Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B). The MDCS included 17,035 women who underwent baseline assessments during 1991-1996, including dietary assessments, serum sampling and a lifestyle questionnaire. The SCAN-B involves multiple participating hospitals in Sweden, and serum levels of copper and zinc was analyzed at time of diagnosis for 1998 breast cancer patients. Information on breast cancer diagnosis, death and recurrences was collected from the Swedish Cancer Registry, the Swedish Cause of Death Registry and medical records.

Results
In the MDCS, no strong relationships were found between selenium levels and breast cancer risk. Similarly, neither dietary nor serum zinc levels showed any association with the risk of breast cancer or different breast cancer subgroups. Additionally, serum and dietary zinc levels demonstrated no overall associations with breast cancer survival. However, among women with high phosphorus intake, concurrent low zinc intake was associated with poor overall survival. Furthermore, poor agreement was observed between serum selenium and selenium intake, as well as serum zinc and zinc intake. In the SCAN-B cohort, no strong overall associations were found between serum copper or zinc levels and breast cancer survival, but there was a tendency toward lower breast cancer survival with higher copper levels and lower zinc levels. Nonetheless, a higher copper/zinc ratio was associated with poor overall survival after breast cancer diagnosis.

Conclusion
Our findings provide evidence for the serum copper/zinc ratio as an independent prognostic indicator of breast cancer survival. No strong associations were found between selenium or zinc levels and breast cancer risk, or between zinc or copper levels and breast cancer prognosis. However, among women with high phosphorus intake, concurrent low zinc intake was associated with a poor overall survival. Further studies are needed to assess the clinical utility of the serum copper/zinc ratio as a prognostic marker in breast cancer.

Key words: Breast Cancer, Selenium, Zinc, Copper, Copper/Zinc Ratio, Risk, Prognosis

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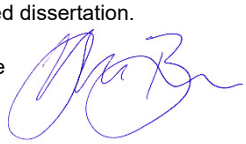
Signature  Date 2023-08-24

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Ylva Bengtsson

LUND
UNIVERSITY

Faculty of Medicine
Department of Clinical Sciences, Malmö

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Till min farfar,

som alltid har varit intresserad av min forskning
och som lovat att leva tills jag disputerat

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Papers included in this thesis

Paper I

Bengtsson Y, Sandsveden M, Manjer J. Risk of breast cancer in relation to dietary intake of selenium and serum selenium as a marker of dietary intake: a prospective cohort study within The Malmö Diet and Cancer Study. Cancer Causes Control. 2021;32(8):815-826.

Paper II

Bengtsson Y, Sandsveden M, Borgquist S, Manjer J. Serum zinc and dietary intake of zinc in relation to risk of different breast cancer subgroups and serum levels as a marker of intake: a prospective nested case-control study. Breast Cancer Research and Treatment. 2021;189(2):571-83.

Paper III

Bengtsson Y, Demircan K, Rosendahl AH, Borgquist S, Sandsveden M, Manjer J. Zinc and Breast Cancer Survival: A Prospective Cohort Study of Dietary Intake and Serum Levels. Nutrients. 2022;14(13):2575.

Paper IV

Bengtsson Y, Demircan K, Vallon-Christersson J, Malmberg M, Saal LH, Rydén L, et al. Serum copper, zinc and copper/zinc ratio in relation to survival after breast cancer diagnosis: A prospective multicenter cohort study. Redox Biology. 2023;63:102728.

Published papers not included in this thesis

Demircan K, Sun Q, **Bengtsson Y**, Seemann P, Vallon-Christersson J, Malmberg M, Saal LH, Rydén L, Minich WB, Borg Å, Manjer J, Schomburg L. Autoimmunity to selenoprotein P predicts breast cancer recurrence. Redox Biology. 2022;53:102346.

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. Nutrients. 2022;14(4):826.

Demircan K, **Bengtsson Y**, Sun Q, Brange A, Vallon-Christersson J, Rijntjes E, Malmberg M, Saal LH, Rydén L, Borg Å, Manjer J, Schomburg L. Serum selenium, selenoprotein P and glutathione peroxidase 3 as predictors of mortality and recurrence following breast cancer diagnosis: A multicentre cohort study. Redox biology, 2021;47, 102145.

Thesis at a glance

Paper and cohort	Research questions	Materials and methods	Results and conclusions
I **"MDCS"**	Are there any associations between selenium levels (in diet or a combination of diet and serum) and breast cancer risk? Could this potential associations be influenced by BMI or smoking? What is the level of agreement between serum selenium and selenium intake?	Pre-diagnostic selenium intake in relation to breast cancer risk was examined in 17,035 women using multivariate Cox regression analysis. Odds ratios were estimated for 1186 cases and controls based on combined intake and serum selenium levels. Stratified analyses were performed considering smoking and BMI.	No overall association between selenium intake, or a combination of intake and serum levels, and breast cancer risk was found. The level of agreement between serum selenium and selenium intake was relatively low.
II **"MDCS"**	Are there any associations between zinc levels (in serum or diet) and the risk of breast cancer or specific subtypes of breast cancer? What is the level of agreement between serum zinc and zinc intake?	Pre-diagnostic zinc levels in the diet and serum were analyzed using multivariate logistic regression to assess their association with the risk of breast cancer and breast cancer subgroups. The study included 1186 women and an equal number of controls.	No associations were observed between serum or dietary zinc and the risk of breast cancer or different breast cancer subgroups. Poor agreement was found between serum zinc levels and zinc intake.
III **"MDCS"**	Are there any associations between zinc levels (in serum and diet) and breast cancer survival? Could phosphorus or selenium levels impact these associations?	The relationship between pre-diagnostic zinc levels in the diet and serum and breast cancer survival was examined in 1062 invasive breast cancer cases using multivariate Cox regression. The analyses were stratified by phosphorus and selenium levels.	No overall associations were seen between zinc levels and recurrence-free, breast cancer-specific and overall survival. However, among women with high phosphorus intake, concurrent low zinc intake was associated with poor breast cancer survival.
IV **"SCAN-B"**	Are there any associations between serum levels of copper and zinc, as well as their ratio at the time of breast cancer diagnosis, and survival in breast cancer patients?	In 1998 patients diagnosed with primary invasive breast cancer, serum levels of copper and zinc at diagnosis, of copper and zinc, along with their ratio, were analyzed to investigate their association with breast cancer survival using multivariate Cox regression.	A higher serum copper/zinc ratio at breast cancer diagnosis is associated with poor survival after breast cancer diagnosis. There is a tendency for worse breast cancer survival with higher serum copper levels and lower serum zinc levels.

BMI = Body Mass Index, MDCS = Malmö Diet and Cancer Study, SCAN-B = Sweden Cancerome Analysis Network – Breast Initiative

Graphical abstract

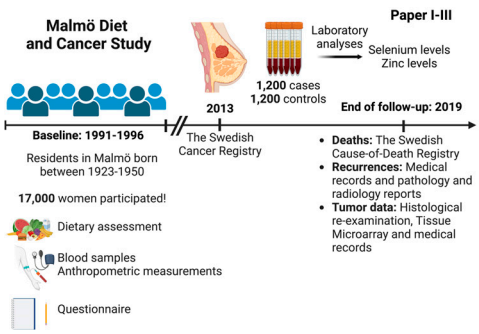
Trace Elements and Breast Cancer
Selenium, Zinc and Copper in Relation to Risk and Prognosis

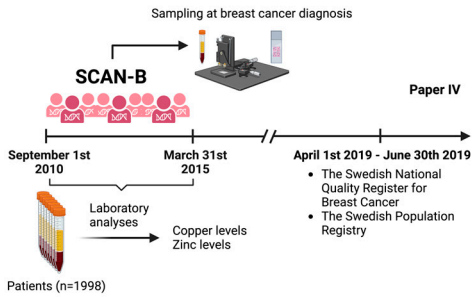
LUND UNIVERSITY

AIM

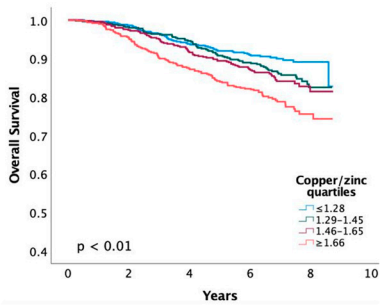
This thesis aims to investigate the associations between different levels of selenium, zinc and copper in serum and diet , and the risk and prognosis of breast cancer.

METHODS

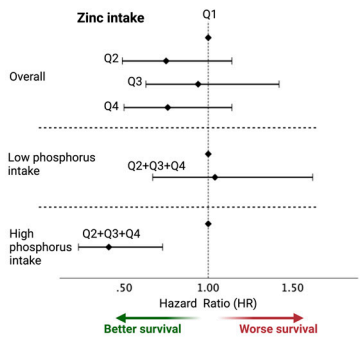




RESULTS



A higher copper/zinc ratio was associated with decreased overall survival after breast cancer diagnosis; adjusted HR Q4 vs. Q1: 1.58 (1.11-2.25) (P_{trend}=0.01).



Among women with a high phosphorus intake, higher breast cancer survival was seen in zinc intake Q2 to Q4 compared to Q1; the adjusted HR was 0.41 (0.23–0.73).

CONCLUSION

Our findings provide evidence for the serum copper/zinc ratio as an independent prognostic indicator of breast cancer survival. No strong associations were found between selenium or zinc levels and breast cancer risk, or between zinc or copper levels and breast cancer prognosis. However, among women with high phosphorus intake, concurrent low zinc intake was associated with a poor overall survival.

Populärvetenskaplig sammanfattning (in Swedish)

Bröstcancer är den vanligaste cancerformen hos kvinnor, både i Sverige och globalt. Ungefär en av nio svenska kvinnor kommer att drabbas av sjukdomen under sin livstid. Trots stora medicinska framsteg fortsätter bröstcancer att orsaka ett stort antal dödsfall årligen. Det är därför viktigt att identifiera individer med en ökad risk för att utveckla bröstcancer och som skulle ha nytta av effektivare preventiva åtgärder. Det är även viktigt att identifiera individer med en ökad risk för sämre prognos, eftersom dessa kvinnor skulle kunna behöva en mer intensiv tilläggsbehandling efter operation.

I denna avhandling undersöks potentiella samband mellan spårämnena selen, zink och koppar, och bröstcancer. Det har tidigare föreslagits att mängden av dessa spårämnen i kroppen kan ha en effekt på utvecklingen och förloppet av vissa cancerformer, inklusive bröstcancer. Vi får i oss dessa livsviktiga mineraler främst genom kosten och de behövs i mycket små mängder för att underhålla många av kroppens funktioner. Selen finns exempelvis i fisk och skaldjur och fungerar som en antioxidant som skyddar våra celler från skador. Zink finns bland annat i kött, skaldjur och nötter och är involverat i hundratals enzymreaktioner. Koppar får vi i oss från livsmedel som lever, nötter/frön och dricksvatten och ingår bland annat i enzymer som hjälper ämnesomsättningen samt omsättningen av järn i kroppen.

Tidigare djurförsök och studier på bröstcancerceller har föreslagit att låga nivåer av selen och zink och höga nivåer av koppar kan öka risken att insjukna och dö i bröstcancer. Resultaten från epidemiologiska studier har dock varit motstridiga samtidigt som många av studierna har varit små och mätt mineralerna efter diagnos och behandling. Detta gör att klara orsakssamband inte har kunnat fastställas och understryker behovet av fortsatt forskning där nivåerna av selen, zink och koppar mäts innan kvinnorna har hunnit utveckla bröstcancer eller fått behandling.

För att studera selen, zink och koppar i relation till bröstcancerrisk och prognos har vi använt oss av data från två stora svenska kohorter: Malmö Kost Cancer (MKC) och Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B). Vi har analyserat spårämnenas nivåer i kroppen genom att mäta koncentrationerna av selen, zink och koppar i blodet, samt genom att utvärdera kostintaget av dessa ämnen.

I MKC deltog 17,000 kvinnor, födda mellan 1923 och 1950. Inklusionen skedde mellan 1991 och 1996. Deltagarna registrerade sitt födointag i kostdagböcker, fyllde i formulär angående sina kostvanor och livsstil, genomgick intervjuer och lämnade blodprover. Vi följde därefter kvinnorna för att identifiera vilka som utvecklade bröstcancer, vilka som fick återfall eller vilka som avled till följd av sin bröstcancer. Vi samlade in data om tumörernas egenskaper från olika källor och använde cancer- och dödsorsaksregistret för att fastställa antalet kvinnor som diagnostiserades med bröstcancer samt antalet dödsfall. Vi analyserade nivåerna av selen och zink i de

sparade blodproven hos 1,200 bröstcancerpatienter, samt hos lika många deltagare som inte utvecklade bröstcancer. Information om mängden selen och zink i deltagarnas kost kunde sammanställas med hjälp av informationen från kostdagboken, frågeformuläret och intervjuerna.

SCAN-B är en stor pågående svensk studie. Från och med hösten 2010 har nydiagnostiserade bröstcancerpatienter som behandlats på sjukhus runt om i Sverige deltagit i studien. Vi har undersökt nivåerna av zink och koppar i blodprov som är taget innan behandling hos 2,000 patienter som deltog i studien. Information om patienterna, deras tumörer och vem som avlidit har vi inhämtat från det nationella registret för bröstcancer och dödsorsaksregistret.

I MKC kunde vi inte fastställa någon generell skillnad i bröstcancerrisk bland de kvinnor som exponerats för höga eller låga halter av selen eller zink, oberoende av om det mättes i blodet eller kosten. Vi kunde inte heller påvisa något samband mellan zinknivåer, vare sig i blodet eller i kosten, och risken att få en bröstcancerdiagnos med dålig prognos, eller risken för att få ett återfall eller dö till följd av sjukdomen. Vi mätte även fosforintaget via kosten eftersom fosfor i formen av fytat minskar absorptionen av zink i tarmen. Inom denna del av studien såg vi att bröstcancerpatienter som hade ett lågt zinkintag och samtidigt ett högt fosforintag, tenderade att ha en sämre överlevnad.

I SCAN-B såg vi att bröstcancerpatienter som hade en hög koppar/zink kvot i blodet, det vill säga höga nivåer av koppar i förhållande till zink, hade en sämre överlevnad. Mängden koppar och zink var för sig påverkade inte överlevnaden i bröstcancer.

Resultaten från denna doktorsavhandling visar på att koppar/zink kvoten i blodet potentiellt kan vara en oberoende markör för överlevnadsprognos hos bröstcancerpatienter. Vår studie är den första som undersöker sambandet mellan koppar/zink kvoten i blodet och bröstcanceröverlevnad, vilket gör resultaten både nya och betydelsefulla. Vi har också visat att kvinnor med särskilt lågt zinkintag, i synnerhet de med ett samtidigt högt fosforintag, verkar ha en sämre överlevnad efter en bröstcancerdiagnos. Denna observation behöver dock bekräftas i andra stora kohorter. Vidare tyder våra resultat på att det inte finns något samband mellan mängden selen i kosten och risken att utveckla bröstcancer.

Eftersom mätning av koppar och zink i blodet kan utföras relativt enkelt, kan denna information potentiellt tillämpas praktiskt inom hälso- och sjukvården. För att fullt ut klargöra de underliggande mekanismerna och fastställa ett säkert orsakssamband, krävs det ytterligare forskning. Framtida studier kan utforska om bröstcancerpatienter med höga koppar/zink kvoter skulle kunna dra nytta av zinktillskott eller läkemedel som minskar kopparnivåerna. Dessa forskningsinsatser skulle kunna resultera i nya insikter om sambandet mellan spårämnen och bröstcancer, och potentiellt öppna upp för nya behandlingsstrategier.

Abbreviations

AUC	Area Under the Curve
BBD	Benign Breast Disease
BCSS	Breast Cancer-Specific Survival
BMI	Body Mass Index
CI	Confidence Interval
DAG	Directed Acyclic Graph
EPIC	European Prospective Investigation into Diet and Cancer
ER	Estrogen Receptor
GPx-1	Glutathione Peroxidase 1
GPx-3	Glutathione Peroxidase 3
HER2	Human Epidermal growth factor Receptor 2
HR	Hazard Ratio
MAR	Missing At Random
MCAR	Missing Completely At Random
MDCS	Malmö Diet and Cancer Study
MICE	Multiple Imputation by Chained Equations
MKC	Malmö Kost Cancer
MNAR	Missing Not At Random
OS	Overall Survival
PgR	Progesterone Receptor
RCT	Randomized Controlled Trial
RFS	Recurrence Free Survival
ROC	Receiver Operating Characteristic
SCAN-B	Sweden Cancerome Analysis Network – Breast Initiative
SOD	Superoxide Dismutase
TNBC	Triple-Negative Breast Cancer
TNM	Tumor, Node, Metastases
TMA	Tissue Microarray

Introduction

The past few years have been dominated by the global pandemic. Suddenly, the term "epidemiology" found its way into everyday vocabulary. In this field, we use large amounts of data, seeking hidden patterns and connections. While infectious diseases have been the primary focus lately, the importance of chronic diseases such as cancer cannot be understated. A key area of focus in this regard is breast cancer, the most common cancer among women and a leading cause of cancer-related deaths worldwide.

Through the lens of epidemiology, we aim to identify new factors that can help us understand the risks associated with developing breast cancer and predict how the disease may progress in patients. Understanding these risk factors enables early detection and prevention of breast cancer. Moreover, insights into factors that predict the course of the disease guide treatment decisions and promote personalized care, potentially improving patient outcomes.

Emerging evidence from pre-clinical studies suggests a potential protective role of essential trace elements, specifically selenium and zinc, in preventing the onset and progression of several cancers, including breast cancer [1-3]. The chemo preventive properties of selenium are often ascribed to the antioxidant properties of certain selenoproteins [4], while zinc is proposed to prevent cancer onset and progression through diverse mechanisms, such as apoptosis regulation, involvement in the antioxidant defense system and the modulation of cell signaling pathways [5, 6]. Conversely, copper, and the balance between copper and zinc (copper/zinc ratio) may influence tumor growth and progression [7-9]. For instance, copper has been shown to activate several angiogenic factors, leading to increased angiogenesis and a pro-tumorigenic microenvironment [8]. Furthermore, an imbalance in these trace elements, manifested as low zinc and high copper levels, may enhance oxidative stress and disrupt the function of crucial enzymes [10].

At the start of this project, there was a noticeable absence of high-quality epidemiological research on the associations between selenium, zinc and copper levels, and breast cancer risk and prognosis. For instance, no prospective studies had been conducted to investigate the relationship between selenium intake and breast cancer risk in selenium-poor regions, such as Sweden. Furthermore, previous studies examining the associations between zinc levels (both in serum and diet) and breast cancer risk had inconsistent results. These studies were limited by their small

sample sizes and their methodology, which involved assessing zinc levels post-diagnosis rather than pre-diagnosis [11-14]. In addition, no epidemiological study had specifically investigated the role of copper, zinc and copper/zinc levels in breast cancer survival. Given all this and considering the large amount of evidence from pre-clinical studies, there was a clear need for more large-scale epidemiological research on selenium, zinc and copper in relation to breast cancer risk and prognosis.

This doctoral thesis, therefore, seeks to fill this knowledge gap by examining the associations between different levels of selenium, zinc and copper, in serum and diet, with breast cancer risk and prognosis. The papers included in this thesis use data from two large prospective cohorts, the Malmö Diet and Cancer Study (MDCS) and the Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B).

Background

Breast cancer

Anatomy of the female breast

The breasts consist of 15 to 20 milk-producing lobules that connect to ducts that can lead the milk out to the nipple. The lobules and ducts are lined by two layers of epithelial cells: an inner layer of luminal cells and an outer layer of myoepithelial cells. The lobular and ductal structure is surrounded by fibroadipose tissue, ligaments, arteries, veins, nerves and lymphatics [15] (Figure 1).

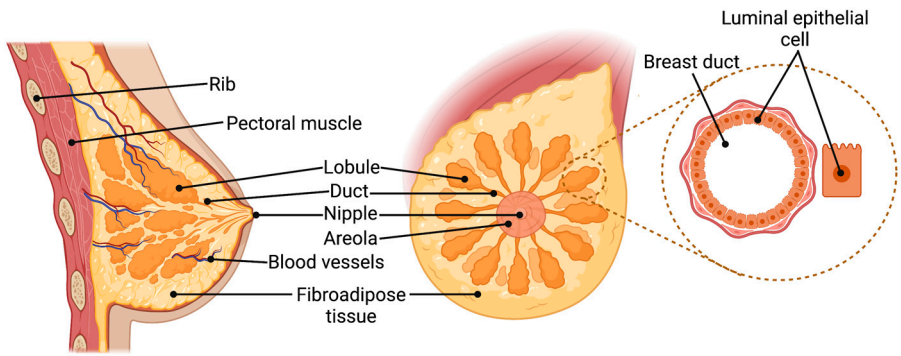


Figure 1. Anatomy of the female breast. Created using Biorender.com.

Breast carcinogenesis

The development of breast cancer is a complex biological process involving numerous genes and signaling pathways. Breast cancer initiation is typically driven by genetic and epigenetic alterations that enable cells to proliferate uncontrollably, evading mechanisms that normally regulate their survival and migration [16].

Breast cancers share common features with other types of cancer. These features, known as the 'hallmarks of cancer', were first identified in an important publication in 2000 that listed six such hallmarks [17]. In 2011, the list was updated to include four more features [18]. In line with our evolving understanding of cancer, two new

emerging hallmarks and two enabling characteristics were suggested in 2022 [19] (see Figure 2 for more details).

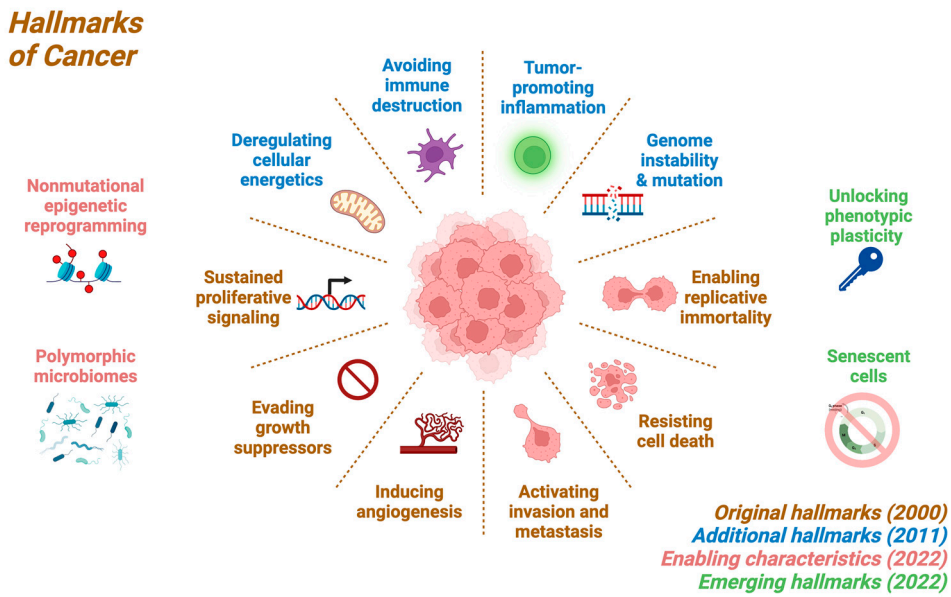


Figure 2. The hallmarks of cancer. Adapted from "Hallmarks of Cancer: Circle", by Biorender.com. The figure provides a visual representation of the key biological characteristics and processes that are commonly associated with cancer development.

The vast majority of breast cancers are carcinomas originating from the epithelial cells lining the lobules and ducts. These carcinomas are categorized into histological subtypes based on the structural organization of the tumor [20]. The carcinoma of no special type, previously referred to as ductal carcinoma, is the most prevalent subtype, accounting for approximately 70% of cases. This is followed by lobular carcinoma, representing around 20% of cases. The corresponding non-invasive versions of these carcinomas are known as ductal carcinoma *in situ* and lobular carcinoma *in situ*, respectively [20, 21]. The remaining subtypes, including tubular, mucinous, papillary and cribriform carcinomas, are comparatively rare, each constituting about 1-2% of cases [20, 21].

Incidence, mortality and survival

Breast cancer is the most common form of cancer among women worldwide. In 2020, approximately 2.3 million cases were diagnosed globally [22], with 10,893 incident cases reported in Sweden [23]. Globally, it causes more deaths among women than any other cancer, with a total estimated number of 685,000 deaths in 2020 [22]. In Sweden, there were 1,330 reported deaths [24]. However, despite

causing many deaths, the survival rates for breast cancer are steadily increasing [25]. In Sweden, the 10-year survival rate has improved from approximately 60% in the 1970s-1980s to over 86% at present [26].

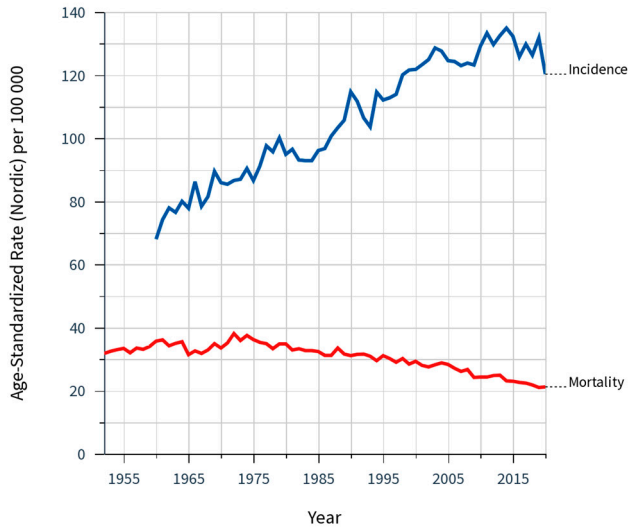


Figure 3. Trends in the age-standardized incidence and mortality rates of breast cancer in females in Sweden. The data is retrieved from NORDCAN database, provided by the International Agency for Research on Cancer [26]. The rates are presented per 100,000 individuals. The figure demonstrates an increase in breast cancer incidence from 1960 to 2000. In contrast, mortality has been consistently declining since the mid-1990s.

Risk factors

Risk factors for breast cancer are commonly categorized into determinants (e.g. sex, age and genetics), markers (e.g. socioeconomic status, education and geographics) and modifiable (e.g. alcohol, obesity, physical activity and hormone use), and the last-mentioned could potentially be affected by preventive actions [20].

Two of the strongest risk factors for breast cancer are female sex (99% of breast cancers are diagnosed in women) and advancing age [20]. Another important risk factor is genetics. Approximately 15-20% of breast cancer cases are considered familiar, meaning that affected women have one or more first- or second-degree relatives who have previously been diagnosed with breast cancer [27]. Specific genes, including *BRCA1, BRCA2, PTEN, TP53, STK11* and *CDH1*, are associated with a high individual risk of breast cancer and collectively account for about 20% of the familial risk [27]. The cumulative risk of developing breast cancer by age 70-80 years among *BRCA-1* and *BRCA-2* mutation carriers has been estimated to be 57-72% and 49-69%, respectively [28, 29]. Beyond high- or moderate-risk breast cancer genes, advances in genomic technologies have enabled Genome-Wide

Association Studies (GWAS). GWAS have identified a multitude of common, low-risk genetic variants known as Single Nucleotide Polymorphisms that also contribute to breast cancer risk. However, despite these advancements, most of the genetic basis in familial breast cancer has not yet been fully characterized [27].

Personal previous history of benign breast disease (BBD) increases the risk of breast cancer [30]. Compared with women without BBD, the risk of developing breast cancer is almost double for women with proliferative changes without atypia and threefold to fivefold higher for women with atypical hyperplasia [16, 30].

Exposure to high levels of endogenous estrogen hormone increases the risk of breast cancer, especially estrogen receptor (ER) positive breast cancer. Factors associated with high lifetime exposure to endogenous hormones include low age at menarche and high age at menopause [31]. Other reproductive risk factors include low parity, older age at first full-term pregnancy and short-term breastfeeding [32]. It is not only endogenous hormones that are associated with breast cancer risk; use of hormonal replacement therapy after menopause also increases the risk of developing breast cancer [33]. In addition, combined estrogen-progestin oral contraceptives have been shown to slightly increase breast cancer risk during use, while there is no excess risk of being diagnosed with breast cancer ≥10 years after stopping use [34].

Another risk factor for breast cancer is high mammographic density, which reflects the relationship between fibroglandular tissue and fat in a mammogram. Increased mammographic density is both an independent risk factor for developing breast cancer and decreases the sensitivity of screening mammograms [35].

Lifestyle factors associated with an increased breast cancer risk include alcohol consumption and physical inactivity. In addition, high socioeconomic status and long education increase the risk of developing breast cancer, partly due to reproductive factors and exogenous hormone use [20]. Another well-known risk factor for breast cancer is body fatness, often described by body mass index (BMI) or waist-hip ratio. High body fatness has been associated with an increased risk of postmenopausal breast cancer and, in many studies, is associated with a decreased risk of premenopausal breast cancer [20, 36].

Regarding diet and breast cancer incidence, numerous studies have evaluated the association between specific foods and breast cancer development. Some epidemiological studies indicate that certain foods, e.g. saturated fat and red and processed meat increase the risk of breast cancer [36-39]. Conversely, some foods, e.g. fiber, soy products and some types of dairy products have been suggested to have a protective role against breast cancer [37, 40, 41]. However, except for alcohol intake, no strong and consistent association has been found, as supported by the World Cancer Research Fund's Report on Nutrition and Physical Activity [42]. Although a considerable amount of research has focused on evaluating the impact of macronutrients and food groups on breast cancer risk, data concerning micronutrients remain comparatively sparse [42].

Breast cancer diagnosis

The population-based mammography screening program has been fully implemented in Sweden since 1997 [43]. Currently, all women in Sweden between 40 to 74 years receive an invitation to participate in breast cancer screening every 18 to 24 months [44]. The overall attendance rate in Sweden is just over 80%, and around 60% of all breast cancer cases among women aged 50 to 74 years are screening-detected [45].

In addition to imaging, the triple assessment concept includes clinical examination and biopsy, which are considered standard practices when a patient is selected from mammography screening due to suspicious findings. Triple assessment is also the standard approach when someone presents with clinical symptoms [44].

Prognostic and predictive factors

Breast cancer is a heterogeneous disease, and several tumor and patient characteristics have been found to be useful in predicting the risk of recurrence and/or death after a breast cancer diagnosis. In addition, predictive and prognostic factors guide decisions on treatment and care planning [20].

Age

Age is not only a strong risk factor for developing breast cancer but is also used for recommendations regarding adjuvant treatment [44]. Breast cancer onset at a young age (<40 years) is associated with unfavorable tumor characteristics and a poor prognosis. These patients are therefore more likely to receive more intense adjuvant treatments [46]. In contrast, a very high age at diagnosis (>80 years) may lead to a poor prognosis independent of tumor stage [47], and might co-vary with comorbid health problems, which can limit treatment options [48].

TNM Classification

TNM classification can be used to stage breast cancer tumors according to their anatomical features. T defines tumor size, N lymph node status and M distant metastases. Based on these characteristics, the breast cancer can be given a stage between I to IV [49]. The risk of death from breast cancer increases as the cancer progresses to more advanced stages. For instance, the five-year survival rate in the US is 99% for localized cancer, 86% for regional spread to lymph nodes and 30% for metastatic disease [50]. However, while the TNM classification system provides prognostic information, it does not reflect the biology of the breast tumor and needs to be used in combination with other factors to guide treatment decisions [20].

Histological grade

The Nottingham histological grade is a well-established grading system that quantifies the level of tumor cell differentiation under a microscope. Tumors are divided into three categories (I-III) based on the numerical scores of three morphological features: nuclear atypia, tubular formation and mitotic index. A higher grade indicates less differentiated cells and a more aggressive form of the disease [51]. The histological grade is used, for instance, to discern ER-positive cases that have a low or high risk of recurrence [44].

Estrogen and progesterone receptor

The majority of breast cancers overexpress the ER and progesterone receptor (PgR). ER is the leading growth and proliferative stimulus for ER+ breast cancers and the main target of endocrine therapies. Given that PgR is regulated by ER, its presence could be indicative of a functional and intact estrogen-ER pathway. High ER expression, particularly when paired with a proliferation marker, may point toward a favorable prognosis. However, a risk of recurrence can persist over an extended period [20, 52].

HER2

Human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor. Amplification or overexpression of HER2 occurs in approximately 15-30% of breast cancer cases and is a marker or poor prognosis in breast cancer. However, with the introduction of monoclonal HER2-targeting antibodies, the clinical outcome for HER2-positive patients has improved dramatically [20, 53].

Ki67

Ki67 is a nuclear protein that is involved in cell cycle regulation and is present in mammalian cells undergoing active proliferation. The Ki67 index, or the percentage of positively stained cells within the total number of malignant cells scored, is used as a measure of cell proliferation. In Sweden, the evaluation of Ki67 is standard practice in the clinical management of breast cancer patients [51, 54].

Intrinsic subtypes

In 2000, Sørlie and Perou identified distinctive gene expression patterns that categorize breast cancers into subtypes. Initially, five main intrinsic subtypes were identified: luminal A, luminal B, HER2+, basal-like and normal breast-like subtypes [55, 56]. These subtypes have been associated with different tumor characteristics and clinical outcomes. The luminal subtypes are genetically closer to luminal breast epithelium and are associated with the expression of ER. HER2+ tumors are characterized by the overexpression of the HER2 gene. Basal-like tumors are genetically closer to basal/myoepithelium and do not express ER, PgR or HER2.

The application of genetic analysis in daily clinical practice is both time-consuming and expensive. Therefore, the St Gallen international expert consensus on the primary therapy of early breast cancer (2013) described a surrogate molecular subtype categorization based on hormone receptor expression, the proliferation marker Ki67 and HER2 status. To separate luminal A-like from luminal B-like tumors, Ki67 (high/low) and PgR (high/low) were used [57]. In 2014, Maisonneuve et al. presented a new surrogate definition for the luminal intrinsic subtypes by introducing an intermediate Ki67 group. In addition, they demonstrated that PgR was a differentiator for prognosis only in tumors with intermediate Ki67 [58]. Later, tumor grade in addition to Ki67, were used to better discriminate between the subtypes luminal A-like and luminal B-like and is now recommended by Swedish national guidelines [44, 59].

The triple-negative breast cancer group (TNBC) represents a subtype that is particularly challenging to treat. TNBC, characterized by a lack of ER, PgR and HER receptor expression, is often associated with a poorer prognosis compared to other types of breast cancer [60].

The current definition of the specific subtypes based on the Swedish national guidelines is presented in Figure 4. In Sweden, there has been a gradual integration of genetic analyses recently. A notable initiative in this direction is SCAN-B, which will be described in more detail later.

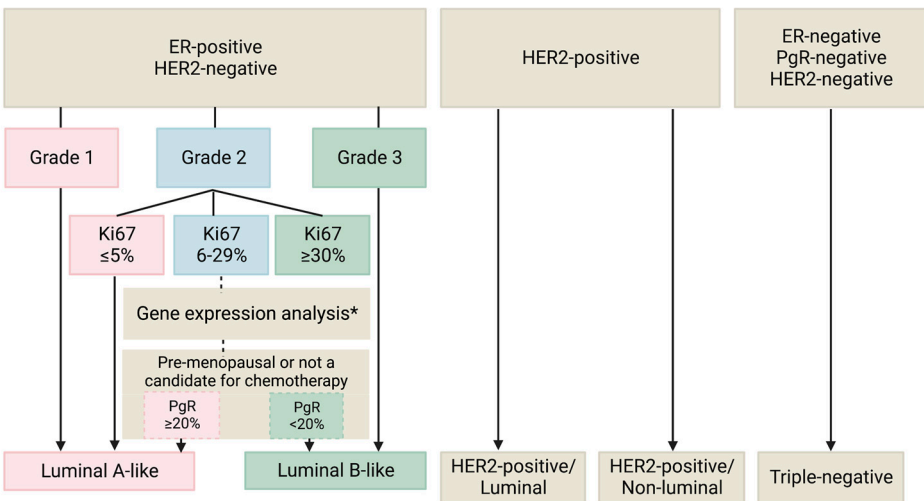


Figure 4. Surrogate intrinsic subtypes according to the Swedish national guidelines. Adapted from the Swedish national guidelines with permission [44]. The subtypes include Luminal A-like, Luminal B-like, Her2-positive/luminal, HER2-positive/non-luminal and Triple-negative.
*Gene expression analysis should be conducted in postmenopausal women with ER-positive/HER2-negative breast cancer where there is uncertainty about the tumor risk categorization prior to the selection of chemotherapy.

Treatment

Decisions regarding treatment are based on evidence-based clinical guidelines, i.e., the Swedish national guidelines [44]. In brief, early breast cancer is considered treatable and possibly curable with modern multidisciplinary management, unlike advanced breast cancer with distant organ metastases [61]. Treatment strategies differ according to the histological and molecular characteristics of the tumor. The two main components of breast cancer treatment include loco-regional (surgical intervention and radiation therapy) and systemic therapy approaches [20, 61].

The principal therapeutic approach for most women diagnosed with early breast cancer without metastases is surgical intervention, including breast conservation surgery or mastectomy. Surgical intervention typically involves a sentinel node biopsy, performed routinely for all invasive cancers without known metastases at diagnosis. Currently, sentinel node results guide decisions on adjuvant treatment, rather than determining axillary clearance. The approach to sentinel node evaluation and axillary dissection has been refined in line with current guidelines, reflecting a more targeted and individualized strategy in patient care. However, for the majority of patients included in this thesis, who were diagnosed several years ago, axillary dissection was common in cases of known metastases. Patients undergoing breast conservation surgery usually receive radiation therapy to reduce the risk of loco-regional recurrences [44, 61].

Systemic therapies, such as chemotherapy, endocrine therapy for hormone receptor-positive breast cancer, HER2-targeted therapy may be given before surgery (neoadjuvant therapy) or/and after surgery (adjuvant therapy) [44, 61]. It is worth noting that neoadjuvant therapy has become increasingly common, though it was less prevalent during the period when the patients included in this thesis, specifically those within the MDCS, were diagnosed.

Trace elements

Trace elements (or trace minerals) are usually defined as minerals that are required in small quantities of 1 to 100 mg/day by adults or make up less than 0.01% of the total body weight [62, 63]. Humans are mainly exposed to trace elements via food and water, but also through supplementation, topical creams, breathing air and inhalation of fumes such as tobacco smoke [63]. Many trace elements play important roles in various biological processes such as enzyme activation, DNA synthesis, immune responses, oxidation-reduction reactions and hormone production [64].

The World Health Organization has classified trace elements based on their nutritional significance. Trace elements are divided into three groups: essential

elements (e.g. selenium, zinc and copper), probably essential elements (e.g. nickel, boron and silicon) and potentially toxic elements (e.g. aluminum, lead and mercury) [62].

Selenium

Selenium primarily exists in the environment in two forms: organic (selenomethionine and selenocysteine) and inorganic (selenate and selenite). The inorganic forms, selenate and selenite, are found in soils, and plants accumulate and convert them into organic forms [64].

In humans, the biological functions of selenium are attained by 25 selenoproteins that have the amino acid selenocysteine at their active site. Selenoproteins have important functions, e.g. in antioxidant defense, redox signaling and thyroid hormone metabolism [65]. The adequate intake of selenium for women aged 50-71 years in Sweden is 75 µg [66]. Severe selenium deficiency can lead to issues such as cardiomyopathy, and excessive intake can cause toxic symptoms [64] (Table 2).

The intake of selenium varies greatly worldwide. Intakes are high in the US, Canada, Japan and Venezuela, and much lower in some parts of Europe, including Sweden [65]. Indeed, selenium in vegetables, cereals and livestock is related to the amount in the soil, and the distribution of selenium in the soil is different from one region to the next [67]. In addition, other factors affect selenium concentration in foods, including soil pH, selenium speciation and the presence of ions that can form complexes with selenium [67].

In Sweden selenium is usually added to animal feed to increase the intake from meat and dairy products. Other important dietary sources of selenium in Sweden are fish, seafood and eggs. Vegetables and cereal products grown in Sweden have low selenium content, while wheat imported from North America has high selenium content [66]. It is worth noting that factors other than selenium intake, such as obesity or smoking, can lower selenium levels in the blood and affect the overall selenium status in the body [68-70].

Selenium is mainly absorbed in the small intestine and is then transported to the liver where it is incorporated into selenoproteins. A selenoprotein called selenoprotein P transports selenium to other tissues and organs. Selenium is primarily excreted through urine [64].

Table 1. Overview of the trace elements selenium, zinc and copper

	Selenium	Zinc	Copper	Reference
Examples of food sources in Sweden	Brazil nuts, offal, fish, seafood, meat and eggs	Oysters, wheatgerm, meat, nuts/seeds and whole grains	Liver, seafood, nuts, cacao and tap water	Livsmedelsverket [66]
RI (mg/day) 51-70 years	-	Women: 9.50[a] Men: 12.4[a]	Women: 0.90 Men: 0.90	Livsmedelsverket [66]
AI (mg/day) 51-70 years	Women: 0.075 Men: 0.09	-	-	Livsmedelsverket [66]
UL (mg/day)	0.255	25.00	5.00	Livsmedelsverket [66]
Reference range in serum (µmol/L)	0.7-1.2	Women: 9-15 Men: 9-18	11-23	Analysportalen [71-73]
Effects of deficiency	Keshan disease Kashin-Beck disease Problematic reproduction Thyroid autoimmune disease etc.	Children: Diarrhea, alopecia, delayed growth and frequent infections. Adults: Delayed wound healing and impaired cognitive and physiological functions	Anemia, hypopigmentation hypercholesterolemia, connective tissue disorders, osteoporosis etc.	NIH [74-76]
Effects of excess	Nausea, vomiting, nail discoloration, hair loss, fatigue, irritability and breath odor	May induce vomiting but is considered non-toxic	Liver damage, abdominal pain, cramps, nausea, diarrhea and vomiting	NIH [74-76] Livsmedelsverket [66]
Total amount in the body	3-15 mg	Women: 1.5 g Men: 2.5 g	0.05-0.12 g	Lanham-New (2019) [64] NIH [75, 76]
Storage in the body	Muscles, liver, kidney and heart	Muscles and bones	Liver, muscles, brain, plasma and erythrocytes	Lanham-New (2019) [64]
Transport protein in blood	Selenoprotein P, albumin and transferrin	Albumin and alpha 2-macroglobulin	Ceruloplasmin	Lanham-New (2019) [64]

RI = Recommended intake.
AI = Adequate intake. Used when RI cannot be determined. Adequate intake based on observed intakes in healthy people or approximations from experimental studies.
UL = Upper limit. Maximum levels of usual intakes judged to be unlikely to pose a risk of adverse health effects in humans.
aAssuming a mixed animal/vegetable diet with a phytic acid intake of around 600 mg/day.
NIH = National Institute of Health

Zinc

Zinc is the most abundant trace element in the human body. It acts as a cofactor for more than 300 different enzymes, and approximately 2500 transcription factors rely on zinc for their structural integrity [77]. Moreover, zinc regulates many genes through the metal response element–binding transcription factor-1. In addition, zinc can control several cellular signaling pathways by modulating phosphorylase and kinase activities. Consequently, zinc influences numerous cellular processes, including cell growth, differentiation and apoptosis [64].

Marginal or moderate zinc deficiency might be relatively common all over the world, while overt zinc deficiency is generally restricted to low-income countries. Children, infants and adolescents have higher zinc requirements compared to adults, which increases their risk of zinc depletion [75]. The clinical manifestations of severe zinc deficiency include stunted growth, delayed sexual maturation, characteristic skin rashes, chronic diarrhea, impaired wound healing and immune system deficiencies. The effects of moderate and mild zinc deficiency are still unclear [64].

Good food sources of zinc are oysters, meat, nuts and seeds and whole grain [66]. However, vegetarian sources of zinc typically have lower bioavailability due to the presence of phytates in these foods. Phytates, the storage form of phosphorus in plants, bind zinc in the gastrointestinal tract and form an insoluble complex that hinders the absorption of zinc [64]. Individuals who restrict animal products in their diet, such as vegans, are at potential risk of zinc inadequacy unless they incorporate supplements or fortified food into their meals [66]. Excessive intake of selenium can also interfere with zinc bioavailability [64].

In addition, factors unrelated to diet, such as infection and inflammation, time of day and levels of albumin, have been identified to potentially impact serum zinc concentration [78-80].

The total amount of zinc in the body is around 1.5 grams in women and 2.5 grams in men, and approximately 90% is found in the muscles and bones [75]. Oral uptake of zinc is absorbed in the small intestine. Subsequently, distribution occurs via the plasma, where it predominantly exists bound to proteins such as albumin and alpha 2-macroglobulin [77]. Plasma zinc represents 0.1% of total body zinc. Zinc is excreted mainly through the gastrointestinal tract [64]. Strong homeostatic mechanisms exist through changes in excretion and absorption to keep whole-body zinc content stable oven a wide range of intakes [78, 81]. Despite these mechanisms, as previously noted, zinc deficiency can still occur.

Copper

Copper is an essential trace element in humans. It is unique in its ability to cycle between two oxidation states, copper (I) and copper (II), which enables its primary role in facilitating oxidation-reduction reactions. Additionally, copper acts as a cofactor for several enzymes, including ceruloplasmin (copper transporter and iron oxidase), zinc-copper superoxide dismutase (oxidative stress defense), cytochrome c oxidase (electron transfer), lysyl oxidase (cross-linking of collagen fibers and formation of bones), dopamine-mono-oxygenase (neurotransmitter production), tyrosinase (melanin synthesis) and dopamine beta-hydrolase (pigmentation of the skin) [63].

Copper is primarily absorbed in the small intestine. Only a small quantity of copper is usually stored in the body, primarily in the liver and muscles, and the total body content of an average adult is 50 mg to 120 mg [76]. The majority of copper in plasma is transported as ceruloplasmin, a protein that is synthesized in the liver. Copper homeostasis is maintained by both absorption and excretion, particularly through the bile [64].

Although copper is essential for normal physiological functions in humans, excessive amounts of copper can result in acute toxicity, with symptoms such as nausea, vomiting, gastric pain and diarrhea. Conversely, copper deficiency, though rare in humans, can lead to various health issues [76] (Table 2). Consuming excessive amounts of zinc over extended periods is one way to develop copper deficiency, as zinc disrupts the absorption of copper [10, 64]. Therefore, maintaining a proper balance of copper and zinc intake is crucial to avoid potential health issues associated with their deficiencies or excess [64]. It is also worth noting that a genetic predisposition to hepatic copper accumulation can lead to severe chronic liver diseases such as Wilson's disease and idiopathic copper toxicosis, while congenital Menkes disease is characterized by a severe deficiency of copper [64].

The richest dietary copper sources include liver and other offal, shellfish, seeds and nuts, wholegrain products and cacao. In addition, copper can be found in tap water, as it can leach into the water from copper pipes, fittings and plumbing fixtures [66].

Selenium, zinc and copper in relation to breast cancer

Selenium and breast cancer

Extensive pre-clinical evidence supports a protective role of selenium against breast cancer [1-3]. However, at the outset of this doctoral thesis, evidence from observational studies was inconclusive and intervention studies were rare.

The chemo-preventive effects of selenium are often attributed to the antioxidant function of certain selenoproteins [4]. One such selenoprotein, glutathione peroxidase 1 (GPx-1), is known to convert intracellular toxic peroxidases into non-toxic hydroxyl compounds, thereby protecting cells from oxidative damage [82]. Indeed, both overexpression of GPx-1 and selenium supplementation have demonstrated protection for mammalian cells against chromosomal damage in cell cultures [83]. In addition, loss of heterozygosity at the GPx-1 locus is a common event in the development of various cancer types, including breast cancer [2, 84]. Furthermore, breast cancer has been linked to the downregulation of glutathione peroxidase 3 (GPx-3), and its reduced levels have been reported to indicate a negative prognosis [85, 86]. As reviewed by Ferguson et al., selenium may also prevent DNA or chromosome breakage during early stages of breast carcinogenesis through mechanisms other than antioxidation [1]. Additionally, studies conducted on mouse models have shown that reduced levels of selenoproteins may increase the risk of developing mammary tumors [87].

Consequently, selenium intake has been identified as a potential factor that could influence the development of breast cancer. However, the two largest randomized control trials (RCT) investigating the role of selenium in chemoprevention, namely Nutritional Prevention of Cancer and the Selenium and Vitamin E Cancer Prevention Trial (SELECT), have mainly or exclusively enrolled male subjects and focused on prostate cancer [88-90]. Unfortunately, this limits our understanding of the potential impact of selenium on common cancers in the female population, such as breast cancer. To date, only one small-scale trial examining the effects of selenium supplementation on breast cancer has been initiated. In this study, 1135 BRCA-1 positive women were randomly assigned to receive 250 μg selenite per day or placebo. However, the trial has only published a meeting abstract, which reports 60 incident breast cancers in the supplementation arm and 45 in the placebo arm [91].

Several observational studies have investigated the relationship between selenium levels and breast cancer risk. These studies have used various indicators of selenium status, such as dietary intake, serum/plasma levels and concentrations in hair and toenails. However, the results of these studies have been inconclusive [92-97]. The largest study on selenium intake and breast cancer risk to date, which enrolled

145,033 postmenopausal women in the US, found no association between selenium intake and breast cancer incidence [95].

As mentioned previously, the soil in the US is rich in selenium, and the average selenium intake in the study mentioned above was 103 μg/day. However, it is worth noting that the activity of most selenoproteins reaches maximal saturation levels at a selenium intake of around 50-60 μg/day [66, 98]. Therefore, it can be hypothesized that an inverse association between selenium and breast cancer risk can only be seen in populations with a low selenium intake. When we initiated this project, no prospective studies had been conducted on selenium intake and breast cancer risk in a selenium-poor region, such as Sweden.

Copper, zinc and their ratio in relation to breast cancer

While there are several studies on the potential link between selenium and breast cancer, the relationship between breast cancer and copper or zinc has not been as extensively studied.

A growing body of pre-clinical research has shown that copper plays a crucial role in tumor growth, the progression of metastatic cancer and the development of the tumor microenvironment and pre-metastatic niche [7-9]. For instance, copper can activate several angiogenic factors, leading to an increased angiogenesis and a pro tumorigenic microenvironment [8]. In addition, decreased copper bioavailability reduces levels of cellular ATP and induces cellular oxidative stress [99]. Specific to breast cancer, copper has been shown to increase the expression and transcription of estrogen-regulated genes and to induce the proliferation of cells in estrogen-dependent breast cancer [100].

Studies in rodent models have shown that copper supplementation enhances breast cancer progression through an increase of microsatellite instability and decreased activity of the antioxidant catalase [7, 101, 102]. Conversely, Tetrathiomolybdate (TM), a copper chelator used to treat Wilson's disease, has been shown in pre-clinical studies to suppress tumor growth and angiogenesis through copper depletion [103, 104]. Furthermore, treatment with TM after completion of adjuvant/standard therapies in a phase II study of women with high-risk breast cancer showed promising overall survival, especially for TNBC [105].

Zinc has been suggested to prevent the onset and progression of cancer through a wide range of mechanisms, including participation in the antioxidant defense system, regulation of apoptosis, modulation of immune function and regulation of cell signaling pathways [5, 6]. Indeed, experimental studies in rat models have shown that low zinc intake reduces the risk of mammary tumorigenesis, and dietary intake of zinc nanoparticles inhibits breast tumor growth [106, 107]. It has also been demonstrated that different breast cancer subtypes exhibit unique profiles of zinc distribution and the zinc transporting network [108, 109]. Chandler et al. reported a

hyper-accumulation of zinc at the margins of luminal breast tumors while zinc was distributed more evenly throughout TNBC [108]. Moreover, overexpression of the zinc transporter ZIP 6 has been observed in ER-positive subtypes and has been linked to less aggressive tumors [110].

Maintaining a proper balance between copper and zinc seems to be essential for optimal health and well-being. An imbalance in these trace elements, with low zinc and high copper, can lead to increased oxidative stress and impaired function of important enzymes [10]. The copper/zinc ratio has been suggested as a better prognostic marker for health status, carcinogenesis and cancer progression compared to assessing each mineral alone [111-114].

Prior to the publication of Paper II, the association between zinc levels (in serum and diet) and breast cancer risk had been investigated in only a few case-control studies [11-14]. However, these studies yielded inconsistent results and were limited by their small sample sizes, as well as the fact that they assessed zinc levels after diagnosis instead of before it. Furthermore, no epidemiological study had previously examined the relationship between zinc levels and the risk of different breast cancer subtypes with varying tumor characteristics. The lack of prospective studies and the potential association between zinc and specific breast cancer subtypes motivated further and larger studies.

Similarly, before the publication of Papers III and IV, no epidemiological study had specifically investigated the role of copper, zinc and copper/zinc levels in breast cancer survival. While a few studies had examined the relationship between these trace elements and cancer survival more broadly, their findings were inconclusive. For instance, some studies suggested that high levels of serum copper and a high serum copper/zinc ratio might be associated with lower cancer survival rates [111, 115, 116]. However, the relationship between zinc levels (in serum and diet) and cancer survival was not as clear, with some studies reporting an inverse association and others finding no association at all [111, 115-118]. Considering the large body of pre-clinical evidence, further epidemiological studies were needed to investigate the association between copper, zinc and their ratio and breast cancer survival.

Rationale for the thesis

Breast cancer remains the most common cancer among women and a leading cause of cancer-related deaths worldwide. Therefore, identifying new risk and prognostic factors is crucial to improve patient outcomes. Although pre-clinical studies suggest a role for selenium, zinc and copper in breast cancer incidence and survival, high-quality epidemiological evidence is lacking.

Aims

The overall aim of this thesis was to investigate the associations between different levels of selenium, zinc and copper, in serum and diet, and breast cancer risk and prognosis using data from two large prospective cohorts: the Malmö Diet and Cancer Study (MDCS) and the Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B).

The specific aims of the papers were as follows:

Paper I

To examine the relationship between pre-diagnostic selenium levels, both in the diet and in groups combining dietary intake and serum levels, and breast cancer risk within the MDCS. An additional aim was to investigate the agreement between dietary selenium intake and serum selenium levels.

Paper II

To examine pre-diagnostic levels of serum zinc and dietary zinc intake and the risk of breast tumors with different biological characteristics within the MDCS. An additional aim was to study serum zinc as a marker of dietary intake of zinc.

Paper III

To investigate the potential effect of pre-diagnostic levels of zinc, in serum and diet, on breast cancer survival as measured by recurrence-free survival, breast cancer-specific survival and overall survival, overall and stratified for phosphorus and selenium levels, within the MDCS.

Paper IV

To investigate the association between serum levels of copper and zinc, as well as their ratio at the time of breast cancer diagnosis, and survival after breast cancer within the SCAN-B cohort.

Methods

Methodology overview

This doctoral thesis employs epidemiological study designs using two cohorts - the Malmö Diet and Cancer Study (MDCS) and the Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B) – to investigate our research questions. Cohort studies are "natural experiments" in which the researcher does not intervene, and the exposure is observed to determine how it affects the outcome at its natural pace [119]. In addition to using data from the cohorts, we have incorporated information from Swedish national registries, patients' medical records and re-examined tumor material. We analyzed serum selenium, zinc and copper at laboratories located in Luleå, Sweden, and Berlin, Germany. We then applied various statistical methods to investigate the potential relationship between selenium, zinc and copper (in both diet and serum) and breast cancer risk or prognosis). The following section provides a more detailed description of the materials and methods used in this thesis.

Cohorts

The Malmö Diet and Cancer Study

MDCS is a prospective population-based cohort study conducted in Malmö, Sweden, and is a part of the larger European Prospective Investigation into Diet and Cancer (EPIC). EPIC aims to investigate the association between diet and the risk of cancer and other chronic diseases, and has recruited over half a million participants across 10 European countries [120].

Between 1991 and 1996, the MDCS recruited a total of 17,035 women (born 1923-1950) and 11,063 men (born 1923-1945), with a participation rate of 42.6% for women and 38.3% for men [121, 122]. Eligible individuals had to be living in Malmö and have Swedish reading and writing skills, while those with limited mental capacity who were not able to fulfill the baseline examinations were excluded. Further details about the recruitment process are available elsewhere [123].

The baseline examinations included a dietary assessment, a self-administered questionnaire about lifestyle, socioeconomic status, reproductive and medical history, as well as anthropometric measurements and blood samples [122].

The participants selected for Papers I-III were chosen from the population of 17,035 women enrolled in the MDCS. Across all three studies, women with breast cancer were identified by record linkage to the Swedish National Cancer Registry. However, there were variations in the final study population due to different study designs across the papers. For more information on the inclusion and exclusion criteria applied in each study, please see the corresponding methodology section in the papers.

SCAN-B

The SCAN-B (ClinicalTrials.gov ID NCT02306096) is a population-based multicenter study that aims to collect patient material and perform genomic and molecular analyses in order to identify and validate useful biomarkers for treatment of breast cancer. The initiative, officially named the Sweden Cancerome Analysis Network – Breast Initiative, has been recruiting patients since autumn 2010, and as of March 2023, had enrolled 19,718 patients from hospitals in Lund, Malmö, Helsingborg, Kristianstad, Halmstad, Varberg, Ljungby, Växjö, Karlskrona, Jönköping, Borås and Uppsala [124]. Further details about the infrastructure of SCAN-B can be found elsewhere [125-127].

The inclusion and exclusion criteria for patients in Paper IV are described in detail in the methodological section of the paper. In summary, patients with a new diagnosis of primary invasive breast cancer without distant metastases were included in the study prior to treatment. Patients were excluded if they had a history of contralateral breast cancer, no planned treatment or an unknown treatment status. Out of 5,417 patients registered in SCAN-B between September 1st 2010, and March 31st 2015, 2,903 consecutive cases were selected for the study, with a target of 2000 patients. A total of 905 were excluded due to missing serum, resulting in a final study population of 1,998 patients (see flowchart in Paper IV).

Data collection

Dietary data

The MDCS used a modified diet history method consisting of three parts. Firstly, a seven-day food diary was used to register prepared meals (typically lunch and dinner), cold drinks, nutrient supplements, drugs and natural remedies. Secondly, a 168-item diet history questionnaire was used to gather information about the overall meal pattern and the frequency of regularly consumed foods (such as hot beverages,

breakfast and snacks). Portion sizes were estimated from a booklet with photographs. Thirdly, a 45-60-minute interview was conducted with trained personnel, during which portion sizes and cooking preparations in the food diary were specified in more detail. The interviewer also checked for any duplications between the food diary and the food frequency questionnaire. The intakes recorded in the food diary and the questionnaire were then combined into an average daily intake. Subsequently, the food data was converted into nutrient intake data by using a combination of interactive computer software and the Swedish Food Database PC KOST2-93 from the Swedish National Food Administration [128, 129] (Figure 5). In Papers I-III, the dietary intake of each nutrient is expressed as the sum of food intake and supplemental intake of the nutrient.

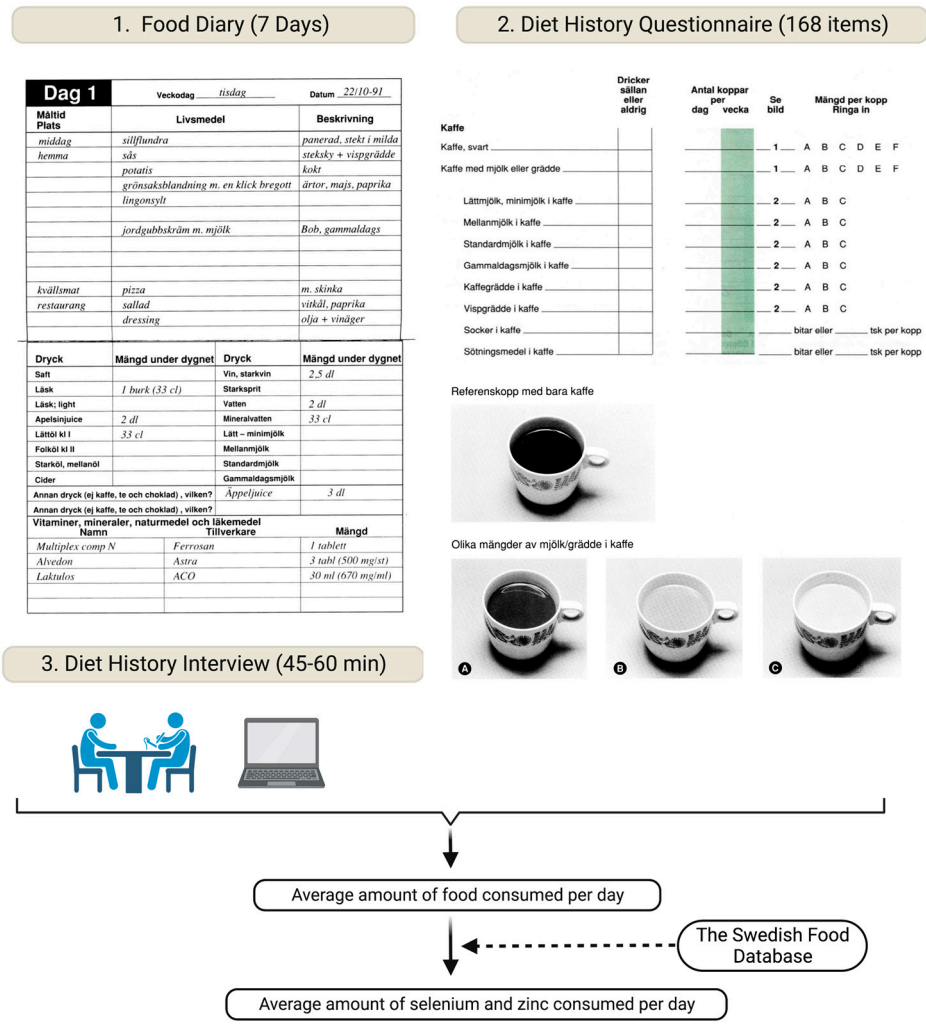


Figure 5. Dietary assessment method used in the Malmö Diet and Cancer Study.

Serum analyses

MDCS

During the baseline examination, blood samples (45 mL) were drawn from non-fasting participants and their blood components were separated, frozen and stored in the Biological Biobank at −80 °C [122]. In Papers I-III, a total of 1,186 women with breast cancer and 1,186 controls were selected for serum analysis of selenium and zinc levels. The analyses were conducted in October 2015 by ALS Scandinavia AB in Sweden. To carry out the analysis, 0.15 mL of serum was diluted with an alkalic liquid containing 0.1% NH_3 and 0.005% EDTA/Triton-X to obtain a 10 mL solution. The samples were then analyzed using ICP-SFMS (Thermo Element 2) with single-element standards traceable to the National Institute of Standards and Technology. Moreover, a reference material, SeronormTM (Trace Elements Serum level 1, lot 0608414) was used and analyzed along with the samples. In Papers II and III, we adjusted and stratified our main analysis for serum albumin and phosphorus levels. We used the albumin and phosphorus levels that were analyzed for a subsample comprising 694 breast cancer cases and 788 controls, as part of another study described in detail in Almquist et al. [130]. The inter-assay coefficients of variation were 3.3% for zinc, 3.4% for selenium, 3.0% for phosphorus and 4% for albumin.

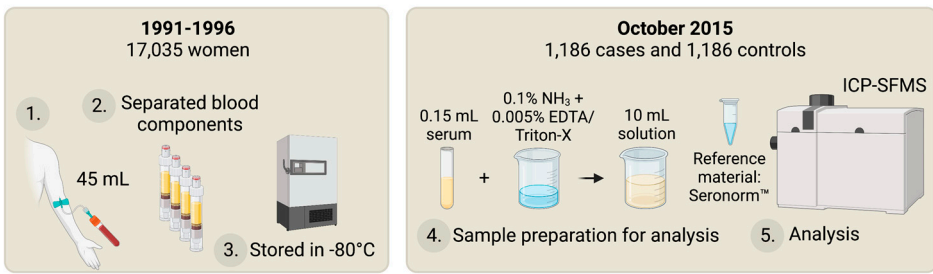


Figure 6. Analysis of serum selenium and zinc levels in breast cancer cases and controls in the Malmö Diet and Cancer Study.

SCAN-B

At the time of diagnosis before initiation of treatment, blood sampling was conducted, and 200 µL serum aliquots were prepared and stored at −80 °C in the Department of Clinical Chemistry at Skåne University Hospital. The saved serum samples were later analyzed in an off-site laboratory located in Berlin, Germany. To ensure unbiased analysis, all samples were randomized based on their storage time, and clinical data was blinded for scientists running the laboratory analyses and linked when the laboratory measurements were completed.

To analyze the serum copper and zinc levels, a dilution of patient serum was made by mixing it with a standard solution containing gallium (1000 µg/L) at a ratio of 1:2. An aliquot of the diluted solution (8 µL) was then applied to a quartz glass slide (Bruker Nano GmbH, Berlin, Germany) and left to dry overnight. The analysis was conducted using total reflection X-ray fluorescence spectroscopy with an ultratrace element analysis system (S4 T-STAR, Bruker nano, Berlin, Germany). Reference samples (Seronorm; Sero AS, Billingstad Norway) were included in all batches to ensure accuracy. The intra-assay coefficients of variation were 3.6% for copper and 16.9% for zinc, while the mean inter-assay coefficients were 1.2% for copper and 1.5% for zinc.

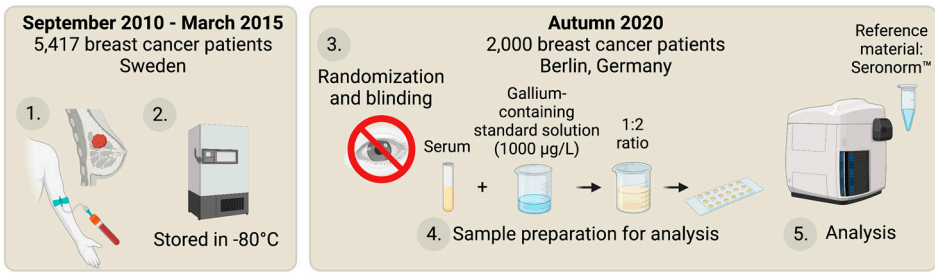


Figure 7. Analysis of serum copper and zinc levels in 2000 breast cancer patients in the SCAN-B.

Tumor data

MDCS

Figure 8 illustrates the collection of data on tumor characteristics in Papers II and III, which occurred during three different periods. For patients diagnosed from 1991 until the end of 2004, receptor status and Ki67 were re-evaluated using a tissue microarray (TMA), while the histological grade was reassessed through histological examination, as described in more detail in two previous studies [131, 132]. Likewise, from 2005 to 2007, TMA was used to reassess hormone receptor status and Ki67. However, due to potential quality concerns regarding PgR data in that period, supplementary data from medical records were gathered for ER and PgR status. From 2005 onwards, except for HER2 and Ki67, data on tumor characteristics were collected solely from medical records.

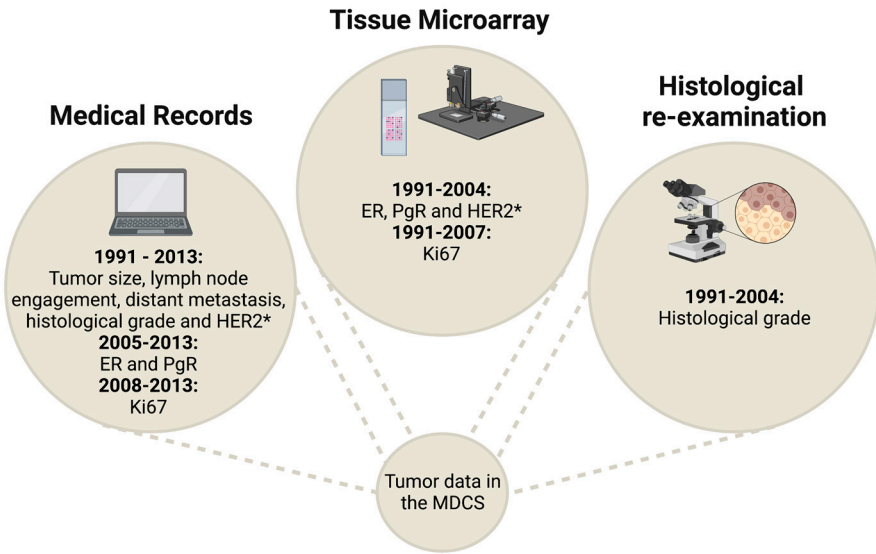


Figure 8. Methods used to collect data on tumor characteristics in the Malmö Diet and Cancer Study. *Data on HER2 was gathered from national registries. If such data was unavailable there, information from clinical records and TMA was used. TMA data for HER2 was only available until 2005.

HER2 status was primarily collected from national registries, and if not available there, it was obtained from medical records. If it was still missing, the HER2 status was retrieved from the TMA [133].

ER and PgR were dichotomized into positive (>10% nuclear staining intensity) and negative (≤10% nuclear staining intensity). Information on Ki67 was based on TMA up until 2007. Tumors were categorized into tertiles (low, intermediate and high) based on their Ki67 expression levels. This categorization was carried out separately for three different periods: 1991-2004, 2005-2007 and 2008-2013.

Throughout all periods, medical records were used to retrieve information on breast and axillary surgery type, lymph node status and planned adjuvant treatment.

SCAN-B

Clinical data, patient-related data and information on tumor characteristics and stage were extracted from the Swedish National Quality Register for Breast Cancer (NKBC) [134]. The local pathology department reported the Ki67 status (low, intermediate or high) based on the department's individual cut-off values at the time of examination.

Surrogate intrinsic subtypes in MDCS and SCAN-B

Information on surrogate intrinsic subtypes was not collected with tumor data in Papers II-IV. Instead, a surrogate intrinsic subtype variable was constructed from the available data on tumor characteristics in the MDCS and the SCAN-B. The categorization of the tumors was mainly based on the Swedish national guidelines regarding the classification of breast tumors [44], as previously described in Figure 3. Due to a small number of cases with HER2+ tumors, luminal HER2+ and nonluminal HER2+ were merged into one category, defining four surrogate intrinsic subtypes: luminal A-like, luminal B-like, HER2-positive (all tumors regarded as HER2+ tumors) and triple-negative (TNBC).

Endpoint retrieval

To identify women with breast cancer and those who died from breast cancer, the Swedish identity number was used to link MDCS and SCAN-B participants with the Swedish Cancer Registry and the Swedish Cause of Death Registry [23, 135]. In Paper III, breast cancer-specific death was defined as a death in which breast cancer was either the cause or a contributing factor. In Paper III, data on recurrent disease were collected from medical records and pathology and radiology reports by registered nurses who were experienced in monitoring clinical studies. When necessary, any unclear findings were discussed and classified in collaboration with a senior consultant in breast surgery. Recurrent disease was defined as a local, regional or distant recurrence. Contralateral breast cancer was reported as distant metastasis, regardless of whether it was defined as a new cancer or not.

Selection of controls in the MDCS

The controls in Papers I and II were selected using two different methods. The first set of controls within the MDCS was chosen based on a previous study conducted by Almquist et al. This study used a method known as incidence density matching, where factors such as age, menopausal status and the time of inclusion were considered in the matching process. The study by Almquist et al. included breast cancer cases diagnosed up to 31st December 2006 [130]. All unique controls from that study who remained free from breast cancer until December 31, 2013, were included as controls in Papers I and II. The remaining controls were randomized from the female population of the cardiovascular (CV) subcohort within the MDCS. The reasoning behind these methods of control selection originates from the context of a broader project, of which my thesis is a component. Although not explicitly addressed in this doctoral thesis, some aspects of the project investigate thyroid hormones (as analyzed in the Almquist et al. study) and genetic data (available within the CV subcohort). For more detailed information about the selection process of controls, please see the flowcharts in Papers I and II.

Statistical methods

This doctoral thesis used a variety of statistical methods to investigate the research questions. In all four papers, regression analysis was applied to examine the relationship between the dependent variable (outcome) — such as the risk of developing breast cancer, relapse or death — and one or more independent variables (exposures), including factors like levels of selenium, zinc and copper. Table 2 summarizes the statistical methods used in each paper. All statistical analyses were conducted using IBM SPSS Statistics (versions 25 and 28), with the exception of the AUCt curves and Schoenfeld residual plots in Paper IV. For those analyses, the R software (the R Foundation, version 4.0.4) with the risksetROC, survival and survminer packages were used [136-138].

Table 2. Overview of statistical methods for each study

Study	I	II	III	IV
Descriptive Statistics	X	X	X	X
Cohen's Kappa	X	X		
Binary Logistic Regression	X	X		
Cox Regression	X		X	X
Time-specific ROC curves				X
Missing Indicator	X	X		
Multiple Imputation			X	X

Descriptive statistics

In all four studies, we used descriptive statistics to report the basic characteristics of our study participants and their exposures. Descriptive tables are often used to reduce a large amount of data into a simpler summary and to help the readers to evaluate the generalizability of the findings [139]. Though common, we chose to avoid using significance tests in descriptive tables, in line with the *Strengthening the Reporting of Observational Studies in Epidemiology (STROBE)* guidelines [139]. This decision was primarily driven by the understanding that significance tests can lead to misinterpretation and inappropriate comparisons, diverting focus from the main research question, and thereby not directly answering the scientific objectives of the study.

Cohen's Kappa

Cohen's Kappa is a statistical measure that we used in Papers I and II to analyze the agreement between quartiles of serum selenium and zinc, and quartiles of dietary intake of selenium and zinc, respectively. We created a cross table and applied Cohen's Kappa to determine the level of agreement among the classifiers, while considering the possibility of agreement occurring by chance. A kappa value of 1 indicates complete agreement, while a value of 0 indicates the level of agreement expected that would be expected by chance alone. A value less than 0 indicates less agreement than what would be expected by chance [140].

Logistic regression

In the analysis of case-control data, logistic regression is often applied, with binary logistic regression being the preferred approach when the outcome is dichotomous [141]. In Paper II, our outcome was binary (breast cancer or no breast cancer), and the study design was a nested case-control study. Therefore, binary logistic regression was an appropriate choice for our statistical analyses. This model also allowed us to adjust for potential confounding variables, which are variables associated with both the exposure and outcome but are not part of the causal pathway between them. The use of logistic regression in SPSS software provided us with odds ratios (OR) and 95% confidence intervals (CI) [141].

Survival analysis

Survival analysis, also known as time-to-event analysis, is useful in follow-up studies where participants are observed until they experience the event of interest [142]. Although it is called survival analysis, the event does not have to be death; it can be any event of interest, such as breast cancer occurrence or recurrence. In this thesis, we used survival analysis in Studies I, III and IV. Initially, we used a non-parametric technique known as Kaplan-Meier plots to graphically display the survival rate of patients in different groups, such as varying levels of zinc intake or serum zinc. To determine if there was a statistical difference between groups in their time-to-event, we used the Log-Rank test [141, 142]. We then implemented univariable and multivariable Cox proportional hazard regression models. The multivariable Cox regression allowed us to adjust for multiple confounding factors. It is worth noting that the Cox model relies on the assumption of proportional hazards over time. This means that the ratio of hazards (i.e., the likelihood of the event occurring at any given time) for any two groups stays constant over time. These are several methods for testing this assumption, and in Paper IV, we tested it using Schoenfeld residual plots [141, 142].

The residual method

Consuming a larger amount of a single dietary component is often associated with a higher overall energy intake [143]. However, individuals who eat more food also tend to differ in important ways, such as body composition and size, making it difficult to isolate the effect of one nutrient on a disease. Therefore, overall energy intake can be used as a proxy to address confounding variables such as body size, physical activity and metabolic efficiency [143].

To adjust for total energy intake in Papers I-III, we used the residual method [144]. In this method, the adjusted nutrient intake is calculated as the residual value obtained from a linear regression model where the total energy intake is the independent variable and absolute nutrient intake is the dependent variable (Figure 9). The residual is the difference between the actual nutrient intake and the predicted nutrient intake based on the regression model. The residual value can be seen as a measure of the relative richness or density of the diet with respect to the nutrient [144].

There are also other ways to adjust for overall energy intake, as discussed in the methodological consideration section.

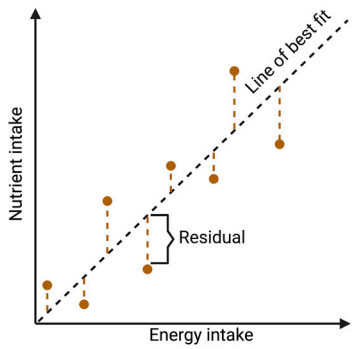


Figure 9. A linear regression model with total energy intake as the independent variable and nutrient intake as the dependent variable. The residual value is calculated as the difference between the observed nutrient intake and the predicted nutrient intake based on the regression model.

Time-dependent receiver operating characteristic

In Paper IV, we used time-dependent receiver operating characteristic (ROC) curves to assess the predictive value of serum copper, zinc and copper/zinc levels for breast cancer survival over time [145]. The ROC curve is a commonly used tool in medicine that quantifies the accuracy of a diagnostic test for a disease by plotting the true positive rate against the false positive rate at different threshold settings. The area under the ROC curve (AUC) is a measure of how well the model can distinguish between two classes [146].

The traditional approach to ROC curve analysis considers event status to be fixed over time, and it does not take censoring into account. In contrast, the time-dependent ROC curve method introduced by Heagerty P.J. et al. allows for a more dynamic analysis. It extends the classical ROC curve analysis for binary data to time-to-event data, accommodating the fact that each ROC is calculated at multiple times, i.e., each time of an event [145]. The area under the time-specific ROC curves (AUC(t)) can then be extracted and plotted as a function of time to illustrate temporal changes in accuracy [145] (Figure 10).

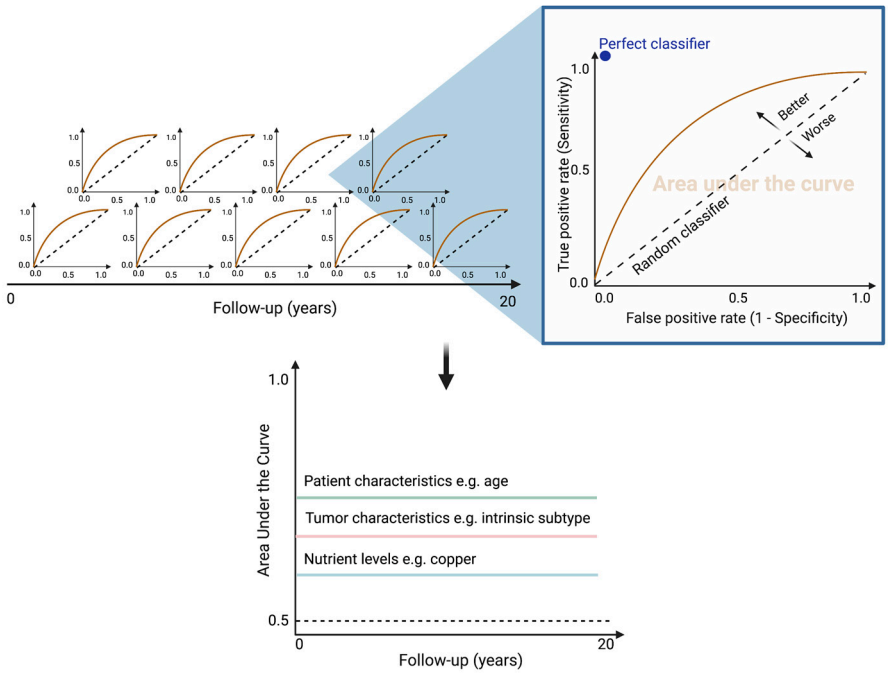


Figure 10. Time-dependent ROC curves used to estimate the predictive value of patient or tumor characteristics on breast cancer survival over time. The AUC(t) is extracted and plotted as a function of time. The green, pink and blue lines are exemplary lines representing AUC(t) values for patient and tumor characteristics and nutrient levels and do not represent any true values.

Strategies for managing missing data

Missing indicator method

Missing data is a prevalent issue in epidemiological and clinical research. In Papers I and II, we addressed missingness using the missing indicator method. This approach involves replacing all missing values with a single fixed value, thereby minimizing the loss of statistical power [147]. However, this method has certain limitations, as further discussed in the methodological consideration section.

Multiple imputation

In Papers III and IV, we used a method called multiple imputation by chain equations (MICE) to address the issue of missing data in our datasets. The MICE method involves replacing missing values with estimates based on available data from other variables and individuals in the study. Depending on the nature of each variable, specific imputation methods are selected; for example, predictive mean matching for continuous variables or logistic regression for binary variables, among other techniques. This process is iteratively repeated until the calculations stabilize.

Multiple datasets are created using this method, and the results from each are then combined into a pooled result. This combination follows Rubin's rules, which provide a structured way to synthesize the results. The resulting single set of estimates captures the uncertainty inherent in the imputation process [148] (Figure 11).

In Papers III and IV, we generated 25 new datasets, using 10 iterations for each. We chose not to impute missing values in the exposure and outcome variables but included them in the model to assist in imputing other variables. Specific details about the variables included in our imputation models are outlined in the papers.

It is important to emphasize that all variables present in the final analysis model must be included in the multiple imputation model, including exposure variables, outcome variables and covariates. In addition, auxiliary variables can be integrated, as these assist in estimating the missing data but are not part of the primary analysis [148]. In the imputation process, variables with the fewest missing values were imputed first. Finally, to assess the robustness of the imputation models, we compared the regression results from Papers III and IV with complete case analyses.

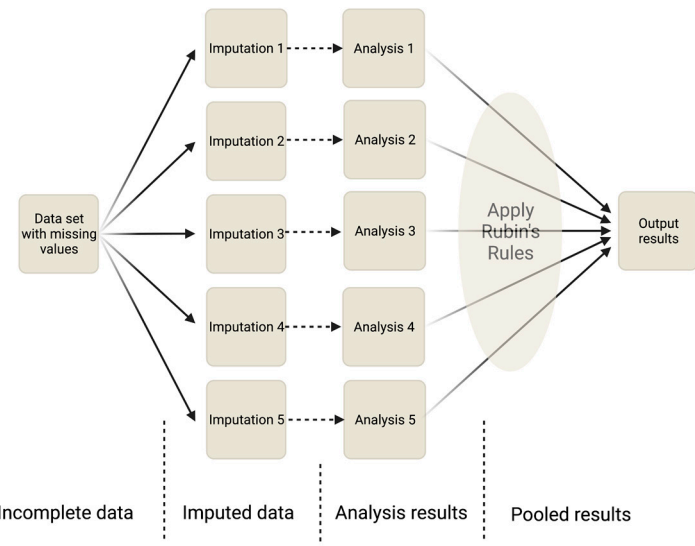


Figure 11. Schematic representation of multiple imputations

Ethical considerations

In this doctoral thesis, several ethical considerations need to be considered. When handling personal data, you must ensure confidentiality and protect personal integrity. In both the MDCS and the SCAN-B, all data is handled according to the Personal Data Act. As researchers, we only have access to coded material, which minimizes the risk of privacy violations.

Participants in the MDCS and SCAN-B studies provided their consent to participate and be linked to relevant registries at baseline. In the case of SCAN-B, physicians and nurses at the breast clinics provide written and oral information to participants and collect their consent forms. The written consent is filed either in the patient's medical record or in a separate ledger, and a copy is given to the patient. Before the initiation of the current studies in the MDCS, information material was distributed via the media, allowing individuals to opt out if desired.

The risks for individuals in our current studies within the MDCS are minimal. Our analysis relies solely on previously collected material such as blood samples, dietary data and tumor samples. Furthermore, the analysis of tumor markers has already been conducted in parallel projects. No additional biological material was collected, and there was no direct feedback to participants.

In SCAN-B, data collection is still ongoing. The additional blood samples taken have a negligible risk of complications. However, there is a minor risk that patients undergoing investigation for breast cancer about participating may have concerns about the exposure of their personal information. Nevertheless, we believe that, in the vast majority of cases, this risk is marginal and outweighed by the positive aspect of contributing to research work.

Participants in the MDCS and SCAN-B should not expect personal benefits beyond the satisfaction of contributing to the development of better treatments for future breast cancer patients.

All studies included in this doctoral thesis adhere to the Helsinki declarations and their subsequent revisions. The regional ethics board in Lund approved Papers I-III under the reference DNR 2015/283 and the original application for the MDCS baseline examinations under the reference DNR LU 51/90. For Paper IV, ethical clearance was obtained from the Regional Ethical Review Board of Lund (diary numbers 2007/155, 2009/658, 2009/659, 2014/8), the county governmental biobank center and the Swedish Data Inspection group (diary number 364–2010).

Results and discussion

Paper I

Selenium and breast cancer risk

We found no overall association between pre-diagnostic selenium intake, or a combination of intake and serum levels, and breast cancer risk. Moreover, there were no interactions between selenium intake and BMI or smoking status.

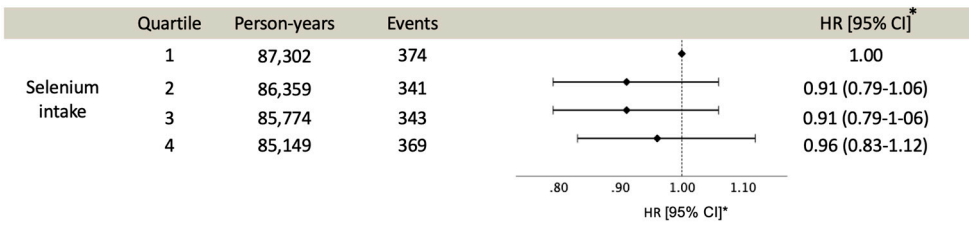


Figure 12. Selenium intake in relation to breast cancer incidence.
*Adjusted for age, socioeconomic index, education, marriage, number of children, age at first childbirth, age at menarche, use of oral contraceptives, hormone replacement therapy, menopausal status, oophorectomy, smoking, BMI, alcohol consumption, season and year of inclusion.
HR = Hazard Ratio; CI = Confidence Interval

Our results align with several recent large prospective studies that examined the association between selenium levels and the risk of breast cancer. These studies analyzed selenium levels measured in the diet [95], serum [96] or used Mendelian randomization to evaluate genetically elevated levels of circulating selenium [149]. Similarly, a Cochrane analysis conducted by Vinceti et al. (2018) also reported no association between selenium intake, supplemental selenium, serum/plasma and toenail selenium, and breast cancer incidence [93]. Contrary to the aforementioned results, a meta-analysis conducted by Zhu et al. (2021) demonstrated an inverse relationship between serum and toenail selenium levels and breast cancer risk [97]. However, the result from that study needs to be interpreted with caution due to the presence of significant between-study variance and the inclusion of post-diagnostic exposure measurements, which introduce the possibility of reverse causality.

While not specifically studied in this doctoral thesis, it is important to mention that various independent studies have reported dose-dependent associations between low selenium levels and poor prognosis in breast cancer [150-154]. Indeed, our research group has conducted two studies within the MDCS and the SCAN-B, which demonstrate an association between higher serum selenium levels and improved breast cancer survival [150, 151]. The underlying biological mechanisms for this association are not yet fully understood, but several in vivo and in vitro studies suggest that selenium may impact cancer survival by influencing cellular processes such as proliferation, apoptosis and immunity [65, 85, 155, 156]. Furthermore, it has also been suggested that selenium compounds, when combined with anticancer drugs, can reduce the proliferative activity of cancer cells [157, 158].

Serum selenium as a marker of dietary intake

Our results reveal a relatively low agreement between serum selenium and dietary intake of selenium (kappa value = 0.10, $p < 0.01$).

Our findings are in line with existing knowledge regarding the connection between dietary selenium intake and serum selenium concentrations, particularly when taking into account the complex physiological processes such as absorption, distribution, metabolism and excretion [159]. These processes play a crucial role as they determine how selenium form our diet is transformed into a form that our body can use and can be measured in the serum. For instance, the response of serum selenium to dietary intake is influenced by the chemical form of selenium in the diet. This means that different selenium compounds can be absorbed and metabolized differently, leading to variations in serum selenium concentrations [159]. In a supplementation trial conducted by Burk et al., selenium-replete individuals were administered either a placebo or selenium doses of 0.20 mg or 0.60 mg/day as selenomethionine or selenite for 16 weeks. Both forms of supplementation affected plasma selenium levels. However, based on urinary excretion, selenomethionine exhibited higher bioavailability compared to selenite [160]. Furthermore, accurately assessing selenium intake using food composition databases is challenging due to variations in soil selenium concentration, which affect both the selenium content in crops and the animals that graze these lands. Despite the limitation as a marker for dietary intake, serum selenium remains one of the most informative biomarkers for assessing selenium status at both individual and population levels [65, 98, 159].

Paper II

Zinc and risk of breast tumors with different biological characteristics

Our findings indicate no associations between pre-diagnostic levels of zinc measured in diet or serum and overall risk of breast cancer or the risk of different breast cancer subgroups.

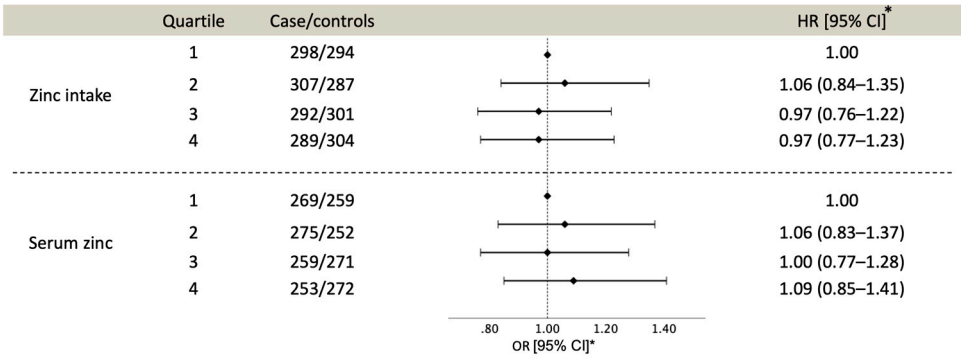


Figure 13. Zinc levels, in diet and serum, in relation to breast cancer incidence.
*Adjusted for age, socioeconomic index, use of oral contraceptives, hormone replacement therapy, menopausal status and year of inclusion.
OR = Odds Ratio; CI = Confidence Interval

Notably, our study was the first to use pre-diagnostic levels of serum and dietary zinc and evaluate their association with the risk of various breast cancer subtypes based on tumor characteristics. Previous studies examining zinc levels in serum and diet, measured after diagnosis, have reported inconsistent results [11-14]. In accordance with our findings, a recent Mendelian randomization study found no associations between genetically elevated levels of circulating zinc and breast cancer risk [149]. However, shortly after our study was published, Pala et al. published a study indicating that high pre-diagnostic zinc levels were associated with decreased risk of breast cancer when zinc was measured in urine or a combination of urine and plasma, although the association was not as strong for zinc measured solely in plasma [161]. The differences observed in the findings of urine zinc levels and plasma zinc levels may be attributed to the use of plasma/serum zinc as an indicator of zinc status, which will be discussed later. In addition, disparities in research findings might also be the result of differing population characteristics or varying study designs. The strengths and limitations of our study will be addressed in the methodological consideration section.

Regarding zinc and breast cancer subtypes, previous research has demonstrated a subgroup-dependent pattern of zinc distribution and zinc transporter expression [108, 109]. However, consistent with our study, Pala et al. found no variation in

breast cancer risk when analyzing plasma plus urine zinc levels in relation to ER, PR or HER2 status [161].

Serum zinc as a marker of dietary intake

Our results revealed a low agreement between serum zinc levels and dietary intake of zinc (kappa value = 0.03, p = 0.02). This finding aligns with previous knowledge regarding serum zinc as a marker for dietary intake [79, 162]. It is well-known that the plasma pool of zinc is relatively small and that plasma/serum zinc concentration can decrease in response to factors unrelated to zinc intake, such as inflammation, infections, stress or trauma [81]. On the contrary, during periods of starvation, tissue catabolism can release zinc into the circulation, resulting in a temporary increase in circulating zinc levels. Moreover, the level of zinc absorption, which can be inhibited by factors like phytate intake, also affects serum zinc levels [79].

Consistent with our findings, other observational studies have shown a lack of association between plasma/serum zinc and zinc intake. For instance, an Italian cohort study including 992 women [161] and the National Health and Nutrition Examination Survey 2011-1014, involving 4347 individuals in the US [78], found no correlations between dietary zinc intake and serum zinc concentration. A meta-analysis by Lowe et al., which included RCTs and observational studies, revealed that doubling of zinc intake in adults only increased plasma/serum concentrations by 6% [162]. However, the association was slightly stronger when considering only the RCTs, as observational studies suffer from measurement errors regarding zinc intake [163]. Furthermore, considerable heterogeneity was seen among the studies, as they used different designs, zinc doses, follow-up times and populations. Notably, other studies have shown that serum zinc levels do respond to zinc supplementation or severe dietary zinc restriction [79].

Even though serum zinc concentration may not necessarily reflect dietary intake accurately, it can still serve as a proxy for overall zinc status. In fact, both plasma/serum zinc levels and dietary intake are recommended as biomarkers of zinc status by the Biomarkers of Nutrition for Development (BOND) Zinc Expert Panel. However, the search for a more reliable biomarker continues [79].

Paper III

Zinc and breast cancer survival

No overall associations between pre-diagnostic zinc, measured in diet and serum, and recurrence-free survival (RFS), breast cancer-specific survival (BCSS) or overall survival (OS) were seen. However, in the group with high phosphorus intake, better BCSS and OS were seen among women with intermediate/high zinc intake.

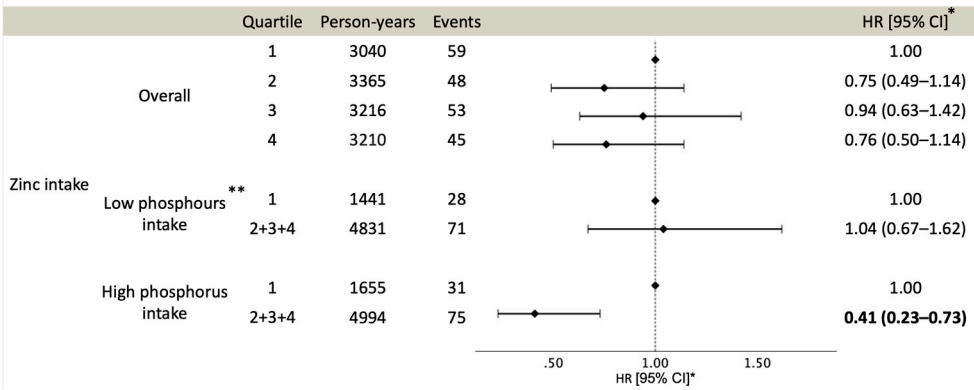


Figure 14. Selenium intake in relation to breast cancer-specific survival.
*Adjusted for age at baseline, age at diagnosis, baseline year, tumor size, lymph node status, distant metastasis status and intrinsic subtype.
**The cut-off is set at the median
HR = Hazard Ratio; CI = Confidence Interval

To the best of our knowledge, this study is the first to assess the potential association between zinc levels and breast cancer survival. Previous research on overall cancer survival has yielded inconclusive results. For instance, the Second National Health and Nutrition Examination Study, which included over 6000 US adults, found a non-linear relationship between serum zinc levels and cancer survival [116]. Similarly, a Swedish study involving 525 men diagnosed with prostate cancer found that a high dietary intake of zinc was associated with higher prostate cancer survival [164]. However, Fang et al. (2018) found no overall associations between serum zinc levels and liver cancer-specific survival in the Guangdong Liver Cancer Cohort [111]. In contrast, Shi et al. (2017), found an association between higher zinc intake and worse all-cause and cancer-specific survival in a Chinese cohort. Nonetheless, the authors discuss whether this association may be a result of confounding factors, such as participants with high zinc intake typically belonging to wealthy families or residing in the south, or covariation with lead or iron intake [165]. Taken together, in line with our findings, previous research does not provide conclusive evidence of an association between zinc, measured in serum or diet, and cancer survival.

Our results demonstrated that women with intermediate/high zinc intake in the group with high phosphorus intake had better BCSS and OS. It is widely recognized that phosphorus, in the form of phytate, significantly hinders zinc absorption from composite meals [166]. For instance, a meta-analysis by Bel-Serrat et al. (2014), comprising 30 studies, revealed an overall 45% reduction in fractional zinc absorption compared to control meals when the phytate/zinc molar ratio of the diet exceeded 15 [167]. Consequently, our study potentially identifies a subgroup with considerably low zinc intake, in which zinc may have a beneficial effect on breast cancer survival. The impact of zinc on breast cancer survival might therefore primarily be observed in populations with generally lower zinc levels or in the presence of external factors that lead to reduced zinc levels. Given that our study was the first to account for phosphorus intake while evaluating the association between zinc and breast cancer survival, future studies should consider the potential interaction between zinc and phosphorus, as well as other factors that influence zinc levels.

Paper IV

Copper, zinc and their ratio in breast cancer survival

We found evidence of an association between a higher serum copper/zinc ratio at breast cancer diagnosis and poor OS. No statistical evidence of overall associations between serum copper and zinc levels on their own and survival after breast cancer diagnosis was seen, although a tendency toward worse breast cancer survival was seen in cases with higher copper levels and lower zinc levels.

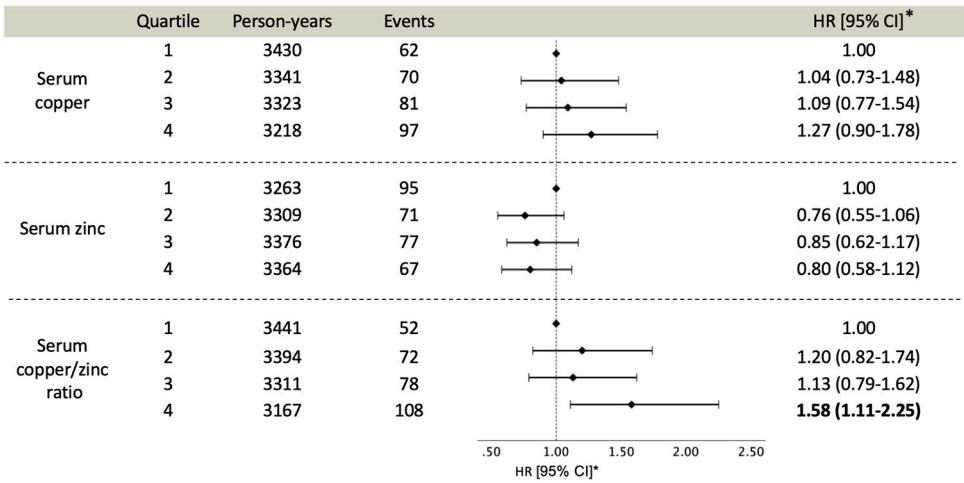


Figure 15. Serum levels of copper, zinc and copper/zinc ratio in relation to OS after breast cancer diagnosis.
*Adjusted for age at diagnosis, menopausal status, mode of breast cancer detection, histological type, tumor size, lymph node involvement and intrinsic subtype.
HR = Hazard Ratio; CI = Confidence Interval

Concerning zinc levels and survival following a breast cancer diagnosis, our findings hint at a potential threshold effect, suggesting that intermediate or high serum zinc levels might correspond to better survival rates. However, after adjusting for potential confounding factors, the observed differences in OS became less pronounced. These findings align with results in the MDCS (Paper III), where we also observed a relatively high BCSS for women with intermediate/high serum zinc levels. In statistical terms, the adjusted hazard ratio (HR) for Q2-Q4 vs Q1 was 0.79 (0.56-1.12) [168]. Nevertheless, it is worth noting that both studies had relatively wide and overlapping confidence intervals, which prevents us from drawing definitive conclusions about the true effect.

Although this thesis did not specifically investigate copper or copper/zinc levels in relation to breast cancer risk, previous studies have suggested a potential relationship between high copper/zinc levels and increased risk of breast cancer. For instance, a recent meta-analysis by Feng et al., which analyzed 16 case-control studies, found that women with breast cancer generally had higher serum copper and copper/zinc levels compared to healthy controls [112]. Similarly, the only study to date that investigated pre-diagnostic serum copper and copper/zinc levels reported a higher breast cancer risk in women with elevated plasma and urine copper/zinc levels. However, the evidence regarding the association between plasma and urine copper levels and breast cancer risk was not as conclusive, despite observing a tendency toward increased risk among women with higher copper levels in urine and plasma [161]. In contrast, a meta-analysis by Jouybari et al., incorporating 22 studies, did not support a relationship between copper levels in blood, breast tissue or hair obtained from newly diagnosed breast cancer patients and controls without breast cancer. Nonetheless, the results should be interpreted cautiously due to significant heterogeneity in the findings [169].

Our findings, combined with previous research, suggest that a high serum ratio of copper to zinc could serve as a more reliable marker for poor breast cancer survival than each mineral independently. The mechanisms underlying this association are still under investigation, but several theories have been proposed. An elevated copper/zinc ratio has been suggested as a potential indicator of systemic inflammation [10, 113, 167]. It is well-documented that the host's inflammatory response during cancerogenesis correlates with the risk and degree of metastatic spread across various cancer types. Inflammatory mediators are believed to increase the permeability of blood vessels, support the infiltration of cancer cells and facilitate their adhesion to the endothelium and invasion at metastatic sites [18, 170, 171]. Moreover, an imbalance between copper and zinc levels could trigger oxidative stress, which in turn may enhance carcinogenesis and cancer progression [102, 172, 173]. Furthermore, an altered copper and zinc balance could impair the function of the crucial enzyme copper/zinc superoxide dismutase (CuZnSOD), which plays an important role in preserving DNA stability and integrity to prevent carcinogenesis [174]. Experimental in vitro and in vivo studies have demonstrated that the overexpression of CuZnSOD can suppress breast cancer growth and decrease metastasis [175, 176]. However, more research is needed to fully understand the biological processes that link high copper/zinc levels in serum to poor outcomes in breast cancer survival.

Methodological considerations

Study design

RCTs are widely regarded as the best method for investigating causal relationships between exposures and outcomes due to their ability to reduce confounding. RCTs focusing on single micronutrients, such as selenium, zinc and copper are generally easier to conduct compared to studies involving macronutrients or dietary patterns. Micronutrients can be administered in the form of identical pills along with a placebo, allowing for true blinding of participants. However, these studies face some challenges that need to be considered [177]. Firstly, it is difficult to control the background intake of the specific nutrient of interest. Variations in background intake can potentially influence the response being studied, making it difficult to isolate the effects of the specific nutrient under investigation. Unlike drug trials where the absence of the drug is typically assumed at baseline, nutrient studies must account for the participant's baseline nutrient status. Some nutrients exhibit threshold intakes, meaning that if the baseline level of the nutrient is already sufficient, minimal or no change in the outcome can be expected. Moreover, recruiting participants who are known to have low or deficient nutrient levels could raise ethical concerns if the trial involves withholding needed supplementation from these individuals [177].

Another study design option would be to use animal models, which has the advantage of allowing researchers to implement long-term and fully controlled diet designs. However, it is important to note that no animal model can perfectly replicate human diseases [178].

In my doctoral thesis, we used an observational design to study the relationship between breast cancer and selenium, copper and zinc. Indeed, observational designs are valuable in this setting where ethical or practical considerations may limit experimental manipulation. We conducted cohort studies (Papers I, II and IV) and a nested case-control study (Paper II). In all these studies, we measured the exposure (trace elements in diet and serum) before the occurrence, recurrence or death from breast cancer. This design enabled us to minimize the risk of reverse causality, wherein the disease process itself affects dietary patterns, metabolism and biomarkers. However, it is important to acknowledge that an observational design has inherent limitations due to its reliance on natural course and lack of full control over factors of interest. As a result, residual confounding factors can never be ruled out [119]. In addition, it is challenging to measure diet in epidemiological studies, particularly since they often involve a large number of participants. As a result, dietary assessment methods need to be not only relatively accurate but also cost-effective and practical [179]. Common challenges faced by epidemiolocal studies and how we addressed them are described below.

Validity, reliability and bias

When evaluating the relationship between an exposure and an outcome, it is crucial to assess the internal and external validity of the results. Internal validity involves the process of eliminating alternative explanations for the observed relationship. To enhance internal validity, one must minimize the risk of bias, meaning the risk of systematic errors in the study design that can lead to inaccurate results. Moreover, it is important to ensure external validity, which involves the generalizability of the findings. Researchers should carefully consider whether the study sample is representative of the population to which one intends to generalize the results [119, 180].

In terms of the generalizability of our findings, the SCAN-B study provides a broad representation as it includes multiple participating hospitals in Sweden and has a high inclusion rate of approximately 85% [125]. On the other hand, the MDCS had a lower participation rate, specifically 42.6% among women. However, the participants in the MDCS had similar socioeconomic characteristics and prevalence of smoking and obesity when compared to individuals who took part in a mailed health survey within the same population, where the participation rate was 74.6 % [121].

Bias is defined as a systematic error that results in an incorrect estimate of the association between exposure and outcome. There are many different types of bias, but three primary categories are often distinguished: selection bias, information bias and confounding [180]. Selection bias occurs when a systematic difference exists between the participants and non-participants in a study, affecting the generalizability of the findings, as discussed earlier. It can also appear in the selection of controls in a case-control study. Controls should represent the population of individuals who would have been cases if they had developed the disease [180]. Our controls in Papers I and II were selected using two different methods, incidence density matching, which involves matching on menopausal status, time of inclusion and age (n=694), as well as random selection (n=492). More details on the control selection process can be found in Papers I and II. As the matching factors were adjusted for in the analyses, we believe that the use of two control selection methods should not significantly impact the results.

Information bias occurs when the data collected and used in a study are either measured or recorded inaccurately [180]. Concerning our main endpoints, incidence breast cancer and breast cancer-specific or overall death, we used the Swedish Cancer Registry and the Swedish Cause of Death Registry, both known to have high validity and completeness [134, 135, 181, 182]. For instance, the Swedish Cause of Death Registry is a virtually complete record of death events, with a specific underlying cause of death recorded for 96% of the individuals in the registry [135]. Furthermore, the registry has been proven accurate in approximately 90% of cases where malignant neoplasms were determined to be the cause of death [182]. In

addition, the modified diet history method used in the MDCS was validated against a reference method using 18-day weighted food records collected over one year. The energy-adjusted correlation coefficients for selenium and zinc were 0.44 and 0.44, respectively [183]. Indeed, using diet as an exposure presents many challenges, as will be discussed later. Regarding the serum analyses, it is a limitation of our studies that serum sampling was performed only once. Ideally, multiple serum samples from each participant at different time points would allow for accounting for potential fluctuations in selenium, copper and zinc levels. However, one-time serum sampling is still considered a valuable proxy for long-term levels. Moreover, these trace elements tend to be relatively stable over time due to the presence of homeostatic mechanisms that help maintain serum levels despite temporary fluctuations in dietary intake [64, 74-76].

Confounding factors are an important consideration in research and are discussed in a separate section below.

Another methodological consideration to address is reliability. Reliability means that the results are trustworthy and can be replicated in different situations. It is therefore important to use standardized measurement methods and to make sure that the research process is consistent and repeatable [119]. Regarding the dietary assessment in the MDCS, the reproducibility of the method has been previously published [184]. The average energy-adjusted Pearson's correlation coefficients, obtained when the same method was used one year apart, ranged from 0.50 to 0.80 for most nutrients. Furthermore, the laboratory analyses in both the MDCS and SCAN-B were conducted in a standardized manner. The laboratory procedures were blinded for both the receivers of the samples and the technicians and scientists performing the laboratory analyses. In addition, the laboratory analyses were conducted at separate remote sites from the clinics and biobank, specifically in Luleå, Sweden, and Berlin, Germany. In the MDCS, the inter-assay coefficients of variation were 3.3% for zinc, 3.4% for selenium, and 3.0% for phosphorus. Moreover, in SCAN-B, the mean inter-assay coefficients of variation were 1.2% for copper and 1.5% for zinc. Considering all these factors, one can argue that our measurements for the main exposures showed good reliability.

Selection of confounders and interaction testing

When it comes to selecting which variables to adjust for in a model, several approaches can be used. One frequently used method involves using statistical significance to identify potential confounders. However, leading experts, such as the American Statistical Association, strongly advise against relying solely on an arbitrary p-value cut-off of 0.05 for determining which variables to adjust. Indeed, even subtle variations in a confounder can be of great importance in determining the appropriate model [185, 186]. Instead, it is better to consider prior knowledge, experience and relevant theories in the field. Constructing causal diagrams, such as directed acyclic graphs (DAGs), can be useful in this regard [119].

It is also important to aim for balance when selecting confounders. One should include an appropriate number to minimize bias while avoiding unnecessary and collinear variables that could introduce instability into the model. There is a rule of thumb suggesting that logistic and Cox models should ideally have a minimum of 10 events per predictor variable. However, this guideline can be relaxed to at least half of that threshold in certain situations [187].

In addition to considering confounders, interaction analyses can be valuable in statistical modeling. While confounders, if not properly controlled, may distort the association between an independent variable and an outcome, interaction refers to a situation where the relationship between a predictor and the outcome varies depending on the level of a third variable. By analyzing these interactions, we can achieve a more nuanced understanding of complex relationships, allowing for a more precise representation of real-world phenomena [119].

Most of the variables included in our models are either prognostic or risk factors for breast cancer, or factors affecting selenium, zinc or copper levels. We have followed the aforementioned reasoning when selecting variables to adjust for in our models. However, it is important to note that even though we tried to adjust for many potential confounders, there could still be some known and unknown factors that we did not include in our models, reflecting the inherent complexity of biological systems and the limitations of available data. Additionally, we have tested for interactions when there is a biological or theoretical basis to believe that the effect of one variable may change based on another. The list of variables included in the multivariable models, as well as those examined for potential interactions, can be found in Papers I-IV.

Diet as an exposure

Diet is a complex exposure for many reasons. Diet is an integral part of an individual's overall lifestyle and culture, leading dietary factors to co-vary with many socioeconomic and lifestyle factors. Moreover, food is composed of a great number of bio-active components, and our diets consist of combinations of different foods in varying frequencies and amounts. The interconnection of nutrients and dietary components within foods often leads to covariation between different dietary variables. As a result, studying the independent effects of single nutrients, such as selenium, zinc and copper, becomes challenging. In addition, self-reported data is most often used in nutritional epidemiology, which unavoidable introduces measurement errors [179].

Despite the challenges associated with using diet as an exposure, we have taken several measures to ensure the robustness and validity of our findings. Firstly, the dietary data collection in the MDCS is comprehensive and highly detailed, providing a solid foundation for our analysis. In addition, the inclusion of both dietary data and serum levels is unique and adds a broader dimension to the

underlying nutrient of interest. Furthermore, we have adjusted for many potential confounding factors, such as socioeconomic and lifestyle factors. Sensitivity analyses have also been conducted to test the robustness of our models. For instance, in Paper II, we repeated all analyses after excluding women who reported substantial diet changes prior to the baseline assessment. Taken together, while acknowledging the challenges associated with working with dietary data, we have made efforts to address these concerns through rigorous data collection and careful analyses.

We used the residual method to adjust for total energy intake, as previously described. Alternative techniques to adjust for energy intake include the standard multivariate approach and the nutrient density approach. The standard multivariate approach involves incorporating both energy and nutrient intake into the same model. However, this approach is considered conservative as it overlooks the correlation between energy and nutrient intake, often leading to overadjustments. Moreover, it assumes a linear relationship between energy intake and the outcome variable, but this relationship may not always exist. Nevertheless, the nutrient density approach calculates nutrient densities by dividing the nutrient intake by energy intake. However, this method may introduce correlation and still require adjustment for energy intake. In contrast, the residual approach interprets the residuals as variation in nutrient intake that is independent of energy intake. This approach is the most widely employed method in the literature due to its practicality and effectiveness [143, 144, 179].

Missing data

Missing data, though unavoidable in epidemiolocal and clinical research, have often been overlooked in terms of their potential to undermine the validity of research findings. Missing data can occur for many reasons, including loss to follow-up, missed medical appointments, non-response to questionnaires, incomplete measurements, and errors during the transfer of data from paper records to electronic databases [119, 188].

Missing data are often classified into three categories: missing completely at random (MCAR), missing at random (MAR) and missing not at random (MNAR). MCAR means that there is no association between the missingness of the data and any observed or missing values. An example of MCAR could be when individuals have no weight recorded due to a broken scale. MAR occurs when the missingness is dependent on information that has already been observed. For instance, in a weight study, data on weight are less likely to be recorded for younger individuals since they tend to visit healthcare facilities less frequently than older individuals. MNAR appears when the missingness is systematically associated with unobserved data. For example, individuals who are overweight or underweight may be more likely to have their weight measured compared to individuals with normal weight, even after accounting for age [188].

Several methods have been developed to address the issue of missing data. Complete case analysis is the most commonly used method. In this method, individuals with missing data for at least one variable are excluded from the analysis. This method is favored by many practitioners because it is easy to implement and is the default option in most statistical software. However, it may result in biased estimates of association because missing data are assumed to be MCAR, which is not often the case. In addition, it affects the statistical power if a large proportion of subjects are discarded [188].

In Papers I and II we used the missing indicator, as previously described. This method enables the inclusion of all subjects in the analysis, thus preserving statistical power. However, in non-randomized studies, it may result in biased estimates, regardless of whether the missing data are MCAR or MAR. The direction and size of the bias depend on the underlying reason for missingness [147].

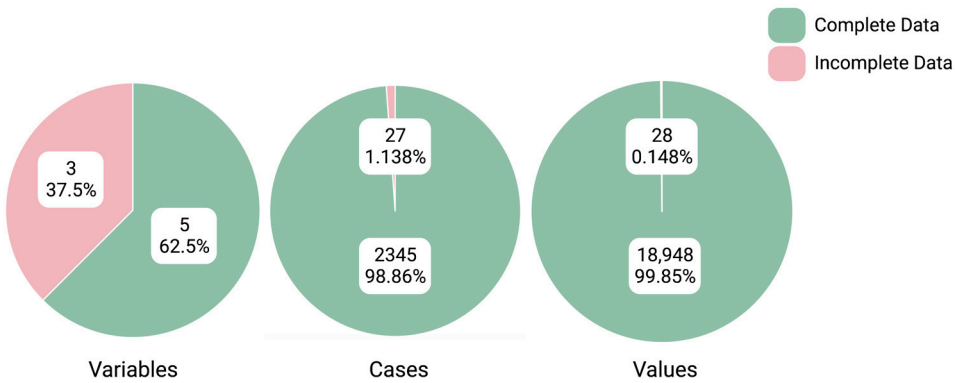


Figure 16. Pie charts illustrating the distribution of missing data within the fully adjusted model presented in Paper II. The first pie chart displays information on all variables, such as zinc intake, with the red section representing the variables that have at least one missing value. The second chart shows information on all cases, where the red part highlights the number of individual subjects with at least one missing value for a variable. The third pie chart focuses on all values, representing the specific measurements for each variable and case. In this model, zinc intake is used as an indicator of zinc status, and thus, it does not include information on missing values related to serum zinc levels.

As seen in Figure 16, the percentage of cases with at least one missing value for a variable in the fully adjusted main model using zinc intake in Paper II was 1.14%. Consequently, there was a low level of missingness for the variables included in the model. As a result, both complete case analyses and the missing indicator method can be regarded as acceptable approaches to handling the missing data. However, it should be noted that there is a higher amount of missing data in the models that include either serum zinc levels or breast cancer subtypes.

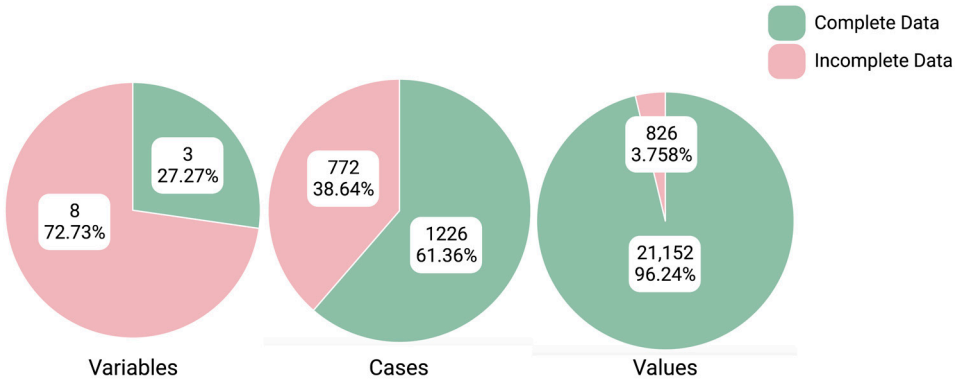


Figure 17. Pie charts illustrating the distribution of missing data for all variables, cases and values included in the fully adjusted model presented in Paper IV.

In Paper IV, we had a higher percentage of missing values, affecting 39% of all cases, as shown in Figure 17. To maintain our sample size, a complete case analysis was not a good option. We analyzed the patterns of missingness among the variables through visual inspection. Given the context of our study and the nature of the variables, we found that the missingness was probably dependent on information that had already been observed, leading us to conclude that the missing data was MAR. Multiple imputation, as previously described, is a suitable approach for analyzing MAR data. A good example illustrating the usefulness of multiple imputation is the variable with the highest proportion of missing values in the model: surrogate intrinsic subtype, with 34% missing values. This variable correlates with other variables related to tumor characteristics and treatment information, which had a lower percentage of missing values and were included in the imputation model. This correlation highlights the value of multiple imputations, particularly when variables with many missing are closely associated with other variables included in the imputation model.

Conclusions

This doctoral thesis examined the associations between different levels of selenium, zinc and copper, in serum and diet, and breast cancer risk and prognosis. To do so, data from two large prospective cohorts was used: the Malmö Diet and Cancer Study (MDCS) and the Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B).

Paper I

No overall association was found between selenium intake or a combination of intake and serum levels, and breast cancer risk in the MDCS. The agreement between serum selenium and selenium intake was relatively low.

Paper II

No associations were observed between serum or dietary zinc and the risk of breast cancer or different breast cancer subgroups within the MDCS. Poor agreement was found between serum zinc levels and zinc intake.

Paper III

No overall associations were found between zinc levels and recurrence-free, breast cancer-specific and overall survival within the MDCS. However, among women with high phosphorus intake, concurrent low zinc intake was associated with poor overall survival.

Paper IV

A higher serum copper/zinc ratio at breast cancer diagnosis was found to be associated with poor overall survival in the SCAN-B. Although there was no statistical evidence of associations between serum copper and zinc levels individually and survival after a breast cancer diagnosis, there was a tendency toward better breast cancer survival in patients with lower copper levels and higher zinc levels.

Future perspectives

The findings presented in this doctoral thesis propose that the serum copper/zinc ratio is a potential biomarker for breast cancer survival. The translatability of these findings into clinical practice is supported by the fact that serum copper and zinc levels can be reliably obtained from routine blood tests. However, additional research is needed to understand the biological mechanisms and to establish a causal relationship between the copper/zinc ratio and breast cancer survival. Confirming causality could pave the way for interventional studies to investigate whether breast cancer patients with a high copper/zinc ratio could improve their prognosis through zinc supplementation or the use of copper chelators.

The second key finding from this thesis is that within the group of women consuming high levels of phosphorus, better breast cancer survival was seen for women with intermediate or high zinc intake. This may imply the existence of a subgroup with generally low zinc intake, in which dietary zinc could have a beneficial effect on breast cancer survival. Further verification of this finding is necessary, which could be achieved through replication in larger cohorts such as other EPIC cohorts. Future research should explore the impact of zinc on breast cancer survival in populations with generally lower zinc levels or under circumstances of external factors that contribute to reduced zinc bioavailability.

Lastly, our research highlights the need to enhance the biomarkers for selenium, zinc and copper status. Metabolomics, the comprehensive study of small molecules within cells, tissues or biofluids, is a promising area for future research, potentially offering a deeper understanding of the status and interactions of these trace elements. Furthermore, advancements in genetic research, particularly Mendelian randomization, may help determine whether an observed association between a risk factor and an outcome is likely to be causal. With the evolution of technology and research methodologies, we might develop better and more effective ways to analyze people's dietary habits. The introduction of more user-friendly methods to gather dietary data, including mobile applications, online platforms and remote testing, could potentially increase participation rates and simplify the process of taking repeated measurements of exposures, covariates and outcomes throughout follow-up periods. Such advancements in data collection could also enhance the practicality of conducting extended RCTs to investigate the causal relationship between trace elements and breast cancer risk and prognosis.

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YLVA BENGTSSON was born in Lund, Sweden, in 1995. She received her medical degree and began her PhD studies at Lund University in 2020. She completed her medical internship at Skåne University Hospital in 2022.

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