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DETERMINANTS AND INFLUENCE OF MAMMOGRAPHIC FEATURES ON BREAST CANCER RISK

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Stockholm 2020

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2020

ISBN 978-91-8016-015-5
Cover illustration: Created by Mrs. *Azadeh Afifi*; a graphic designer, a breast cancer survivor & an advocate.

Determinants and Influence of Mammographic Features on Breast Cancer Risk

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents & my love for always believing in me

& all women affected by breast cancer.

“It's not only the question, but the way you try to solve it.”

Maryam Mirzakhani*, 1977 – 2017

*First woman awarded a Fields Medal in Mathematics, died due to the breast cancer.

ABSTRACT

Mammographic density and mammographic microcalcifications are the key imaging features in mammography examination. Mammographic density is known as a strong risk factor for breast cancer and is the radiographic appearance of epithelial and fibrous tissue which appears white on a mammogram. While, the dark part of a mammogram represents the fatty tissue. Mammographic microcalcifications appear as small deposits of calcium and they are one of the earliest sign of breast cancer. Malignant microcalcifications are seen in both in situ and invasive lesions. In this thesis we used the data from the prospective KARMA cohort to study the association between established breast cancer risk factors with mammographic density change over time (***Study I***), to examine the association between annual mammographic density change and risk of breast cancer (***Study II***), to investigate the association between established risk factors for breast cancer and microcalcification clusters and their asymmetry (***Study III***), and finally to elucidate the association between microcalcification clusters, their asymmetry, and risk of overall and subtype specific breast cancer (***Study IV***). The lifestyle and reproductive factors were assessed using web-based questionnaires. Average mammographic density and total microcalcification clusters were measured using a Computer Aided Detection system (CAD) and the STRATUS method, respectively.

In ***Study I***, the average yearly dense area change was -1.0 cm^2. Body mass index (BMI) and physical activity were statistically associated with density change. Beside age, lean and physically active women had the largest decrease in mammographic density per year. In ***Study II,*** overall, 563 women were diagnosed with breast cancer and annual mammographic density change did not seem to influence the risk of breast cancer. Furthermore, density change does not seem to modify the association between baseline density and risk of breast cancer. In ***Study III***, age, mammographic density, genetic factors related to breast cancer, having more children, longer duration of breast-feeding were significantly associated with increased risk of presence of microcalcification clusters. In ***Study IV***, 676 women were diagnosed with breast cancer. Further, women with ≥3 microcalcification clusters had 2 times higher risk of breast cancer compared to women with no clusters. Microcalcification clusters were associated with both in situ and invasive breast cancer. Finally, during postmenopausal period, microcalcification clusters influence risk of breast cancer to the similar extend as baseline mammographic density.

In conclusion, we have identified novel determinants of mammographic density changes and potential predictors of suspicious mammographic microcalcification clusters. Further, our results suggested that annual mammographic density change does not influence breast cancer risk, while presence of suspicious microcalcification clusters was strongly associated with breast cancer risk.

LIST OF PUBLICATIONS

I. **Determinants of Mammographic Density Change**
Shadi Azam, Arvid Sjölander, Mikael Eriksson, Marike Gabrielson, Kamila Czene, and Per Hall.
Journal of National Cancer Institute Cancer Spectrum. *2019 Mar; 3 (1). DOI: 10.1093/jncics/pkz004*

II. **Mammographic Density Change and Risk of Breast Cancer**
Shadi Azam, Mikael Eriksson[1], Arvid Sjölander, Roxanna Hellgren, Marike Gabrielson, Kamila Czene, Per Hall.
Journal of National Cancer Institute*. Volume 112, Issue 4, April 2020, Pages 391–399. DOI: 10.1093/jnci/djz149*

III. **Predictors of Mammographic Microcalcifications**
Shadi Azam, Mikael Eriksson, Arvid Sjölander, Marike Gabrielson, Roxanna Hellgren, Kamila Czene, Per Hall.
International Journal of Cancer. 2020 Sep 19. Online ahead of print.
DOI: 10.1002/ijc.33302

IV. **Mammographic Microcalcifications and Risk of Breast Cancer**
Shadi Azam, Mikael Eriksson[1], Arvid Sjölander, Roxanna Hellgren, Marike Gabrielson, Kamila Czene, Per Hall.
Submitted

[1] Shared first authorship

PUBBLICATIONS NOT INCLUDED IN THIS THESIS

Hormonal Determinants of Mammographic Density and Density Change

Marike Gabrielson, **Shadi Azam,** Elina Hardell, Madeleine Holm, Kumari A. Ubhayasekera, Mikael Eriksson, Magnus Bäcklund, Jonas Bergquist, Kamila Czene, Per Hall.

Breast Cancer Research. *2020 Aug 26;22(1):95.*

DOI: 10.1186/s13058-020-01332-4

Heritability of Mammographic Breast Density, Density Change, Microcalcifications, and Masses

Natalie Holowko, Mikael Eriksson, Ralf Kuja-Halkola, **Shadi Azam**, Wei He, Per Hall, Kamila Czene.

Cancer Research*. 2020 Apr 1;80(7):1590-1600.*

DOI: 10.1158/0008-5472.CAN-19-2455

CONTENTS

LIST OF ABBREVIATIONS

ACR	American College of Radiology
AI	Aromatase inhibitor
AJCC	American Joint Committee on Cancer
BI-RADS	Breast Imaging-Reporting and Data System
BMI	Body mass index
BRCA	Breast Cancer gene
CAD	Computer Aided Detection
CC	Craniocaudal
CI	Confidence interval
DBT	Digital breast tomosynthesis
DCIS	Ductal carcinoma in situ
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FDA	Food and Drug Administration
FSH	Follicle stimulating hormone
GWAS	Genome-wide association studies
HR	Hazard ratio
INCA	Information for cancer care
KARMA	Karolinska Mammography Project for Risk Prediction of Breast Cancer
MET	Metabolic equivalent of task
MHT	Menopausal hormone therapy
MRI	Magnetic resonance imaging
NHS	Nottingham Histologic Score System
OR	Odds ratio
PR	Progesterone receptor
SNP	Single nucleotide polymorphisms
TDLU	Terminal ductal lobular units
WHI	Women's Health Initiative

1 BACKGROUND

THE BREAST

Anatomy

The breast is part of the female reproductive system and located above the pectoralis major muscles. The breast is rich in blood and lymphatic vessels, which play important roles in spreading of cancer. The breast consists of adipose tissues, glandular tissue (epithelium), and connective tissues (stroma). The glandular tissue is a minor compartment of the breast, whereas the majority of the breast is composed of stroma (collagen elastin) and adipose tissue [1]. The glandular tissue consists of 15-25 lobes which are covered and separated by connective and adipose tissue. Within lobes, there are smaller structures called lobule which is known as the milk-producing and milk-secreting unit of the breast. Each breast lobe is drained by a collecting duct which is responsible for delivering the milk to the surface of the skin and out of the small pores in the nipple [1]. The collecting duct has several branches which ends in a terminal ductal-lobular unit (TDLU). TDLU is the basic functional and histopathological unit of the breast and it is known as a primary anatomical source of most breast cancer [1].

1. Chest wall
2. Pectoralis muscles
3. Lobules
4. Nipple
5. Areola (pigmented area)
6. Milk ducts
7. Fatty tissue
8. Skin

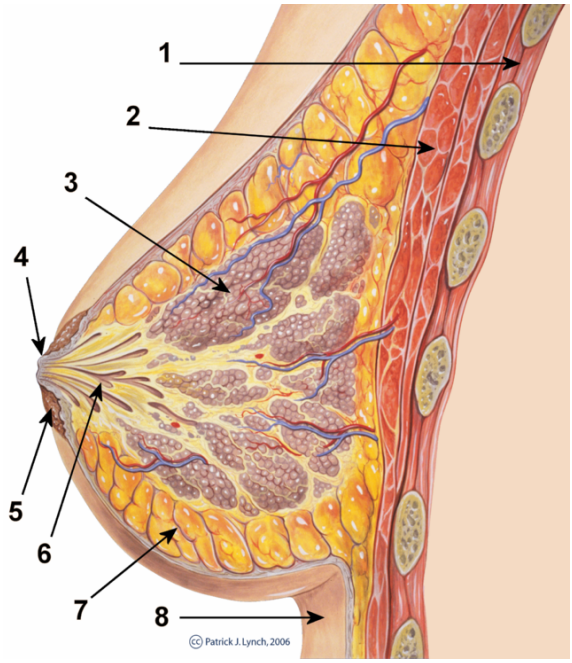


Figure 1. Anatomy of the female breast. Illustration was produced by ©Patrick J. Lynch.

Development

Development of the breast starts at about the 5th week of embryonic life and is determined by several placental hormones, such as prolactin, estrogen, and progesterone. The latter two hormones influence the physiological changes of breast tissue during a woman's premenopausal lifespan. The development of the female breast accelerates during puberty and mature after full-term pregnancy. Estrogen stimulates the formation of connective tissue, blood vessels, and ducts through hormone receptors in the breast and later progesterone contributes in formation of lobular structures [1, 2].

Normal changes over time

The mammary glands undergo normal physiological changes throughout a woman's life. The major changes are seen during puberty, pregnancy and lactation, menopause, and under the menstrual cycle [3, 4].

Puberty

Prior to puberty, the male and female breasts are structurally similar. In contrast to the male breast, the female breast undergoes physiological changes in response to hormonal stimulus during puberty. At puberty, ovaries start to produce and release estrogen and progesterone which resulting in maturation of the breast cells begins and the duct and lobular structures continue to grow and proliferate. The rate at which breast cells proliferate are different for each young woman [5].

Menstrual cycle

The breast tissue responds to the periodic hormonal changes during the menstrual cycle. The phases of the menstrual cycle in women are regulated by fluctuations in the pituitary gland hormones, follicle-stimulating hormone and luteinizing hormone, and ovarian hormones (estrogen and progesterone) [6]. The proportion of proliferating (dividing) cells and cells in apoptosis (cell death) is highly dependent on the stage of the menstrual cycle. The menstrual cycle may be divided into two main phases: 1) follicular or proliferative phase, and 2) the luteal or secretory phase. The follicular phase begins from the first day of the menstruation until ovulation. Follicular phase is regulated by the hypothalamus, the pituitary gland relapses follicle stimulating hormone (FSH). This hormone stimulates the ovary to produce follicles. One of the follicles becomes dominant and within it develops a single mature egg. The

maturing follicle produces estrogen which increases over the follicular phase and peaks days prior to ovulation.

It has been shown that the main proliferative phase is the mid-luteal phase, during the time which progesterone and estrogen are both at a high level and new lobular structures (alveolar buds) are formed. Progesterone is suggested to be a key hormone in regulating mammary gland development and regression during the menstrual cycle. Conversely, during the late luteal phase (the time between ovulation and before the start of menstruation, progesterone is produced, peaks, and then drops) or at the menstruation phase (the period – shedding of uterine lining. Levels of estrogen and progesterone are at low) the newly formed alveolar buds undergo apoptosis and tissue remodeling and the mammary gland return back to its basic state and is ready for another menstrual cycle [3].

Pregnancy and Lactation

During each pregnancy the breast tissue undergoes cycles of proliferation, differentiation, secretion, and cell death. The female breast attains its maximum development during pregnancy. The breasts are not considered fully mature until a woman has given birth and breast-fed. Changes in the breast are normally one of the earliest signs of pregnancy. During pregnancy, the breasts enlarge in response to stimulus of several hormones particularly, by the influence of estrogen resulting in remarkable blood vessels growth and proliferation of lobular structure. Additionally, secretion of other hormones including progesterone, prolactin, epidermal growth factor, fibroblast growth factor, insulin-like growth factor, and parathyroid hormone-related protein resulting in both increase number and size of lobular structures and extension and branching of the ductal system during the first half of the pregnancy. The proportion of the breast development during pregnancy varies among women ranging from little or no increase to a substantial increase in size [5, 7, 8]. By the third trimester, the adipose tissue of the breast is remarkably reduced and replaced by glandular tissue. During late pregnancy, under the influence of the prolactin and oxytocin hormones, epithelial cells differentiate further to enable milk production. All these changes in the breasts are in preparation for breast feeding after birth. Following delivery, prolactin and nerve stimulation from suckling establish the lactation [8].

On cessation of lactation, wearing of the infant induces post-lactation involution. Reduction in prolactin levels is considered to be the main factor in the process of involution, resulting the majority of epithelial cells rapidly undergo programmed cell death and remodeling the

glandular structure to resemble the prepregnant state. Post-lactational involution is considered as an important mechanism for removing unnecessary epithelial cells under a highly controlled process. Imperfect post-lactational involution may result in formation of benign breast cysts [5, 8, 9]

Menopause

Menopause is initiated when ovaries cease to produce estrogen and progesterone. Decline in production of these hormone leads to the age-related breast involution. During menopause the process of involution is associated with reduced size and number of lobular structures, resulting in a breast composed of mainly connective tissue and fat by the end of menopause [4]. Since majority of breast cancers are originated in the lobules, the reduction in size and number of cells decreases the risk of cancer formation. Previous studies showed that a significant reduction in risk of breast cancer among women whose breast tissue had undergone extensive and complete lobular involution [10-12]. Additionally, the involution of breast glandular structures due to menopause, may be reflected in mammographic density, which is an independent risk factor for breast cancer [13].

BREAST CANCER

Symptoms

Breast cancer symptoms vary widely among women. A common sign of breast cancer is the appearance of a lump or mass in the breast tissue. A lump that is painless, hard, and has uneven edges is more likely to be cancerous. Other possible breast cancer symptoms includes; general pain and discomfort in/on any part of the breast, change in size or shape of the one or both breasts and nipples, clear or bloody discharge from either of nipples, and change in breast color [14]. However, it should be emphasized that in countries with national breast cancer screening programs the most common mode of detection is a screening detected cancer without any apparent symptoms [15].

Triple diagnostic

The gold standard approach to examine any breast abnormalities is the triple diagnostic assessment; physical examination of the breast, imaging, and biopsy. Each of these diagnostic modalities complements the other, and together improve the likelihood of diagnosing a breast cancer [16, 17] .

Breast examination

Breast Self-Examination

For many years, women have been taught to practice methods of breast self-examination in order to find the breast tumors at an earlier stage which is expected to lead to a better treatment and decrease the mortality. Breast self-examination is appealing as a patient-centered, noninvasive procedure that allows women to be aware of changes in their breasts [18]. However, to date evidence show that routine breast self-examination has not been an effective method in improving breast cancer survival rate. Therefore, breast self-examination should not be promoted as a primary screening procedure [19, 20]. Nevertheless, women should, of course, be aware of changes in their breasts.

Clinical Breast-Examination

Clinical breast examination seeks to detect breast abnormalities or to assess the patient report of symptoms for breast cancer at an early stage of progression. Clinical breast examination is performed by a healthcare professional who is trained to recognize different type of breast abnormalities. The clinical examination includes both physical examination (palpation of the breasts and lymph nodes and visual inspection of the breasts and surrounding skin for any physical changes) and review of the clinical patient history (family history of breast cancer, menstrual status, previous breast diseases or surgeries, previous cancers, endocrine therapy, and previous symptoms) [21]. Clinical breast examination may be important for some women who do not receive regular mammography (i.e., women aged 40 in some countries and/or younger women), however, the effectiveness of clinical breast examination in improving the survival from breast cancer in comparison with mammography screening has not been shown in well-design clinical trials [22]. Therefore, to date many organizational guidelines (The U.S. Preventive Services Task Force [23, 24], the American Cancer Society [25], the U.K. National Health Services [26], and the World Health Organization [27]) removed clinical breast examination from their recommendations for the routine breast cancer screening.

Breast imaging

Mammography

Mammography is known as the primary imaging technique for diagnosis of breast cancer. Mammography could be used for screening (used women who have no symptoms or signs of breast cancer) or diagnostics (used for women who have breast cancer symptoms or to

evaluate abnormalities detected in a screening mammogram). It is a radiographic imaging of the breast which uses low-dose X-ray to detect asymptomatic cancers early when it is most treatable [28]. For a long time, screen-film mammography has been used as the standard method for breast cancer screening programs and all important randomized controlled trials in population-based breast cancer screening programs were conducted using analog screen-film mammography [29-31]. However, in recent years screen-film mammography has been replaced by full-filed digital mammography in most Western breast cancer screening programs since it first gained U.S. Food and Drug Administration (FDA) approval in 2000 [32].

Full-filed digital mammography offers several potential benefits as compared to screen-film mammography including a better image quality and dose optimization, easier and more efficient archiving, image transfer capacities, improved diagnostic accuracy, especially for women with high mammographic density due to higher contrast resolution, and implementation of advanced technologies such as Computer Aided Detection (CAD) [33]. Additionally, full-field digital mammography shows improved image quality with higher reliability in characterizing microcalcifications compared with screen-film mammography [34, 35]. To increase the accuracy of mammography, the screening examination includes two views; a mediolateral oblique (MLO) view in which the breast is compressed along an oblique line passing through the nipple towards the axillary region to follow the pectoral muscles and the glandular tissue and a craniocaudal (CC) view in which the breast is compressed horizontally [36].

Sensitivity and specificity

Sensitivity and specificity are a way of describing the performance of an examination. Sensitivity is defined as the proportion of people with the disease who have the positive test. Overall, the *sensitivity* of mammography ranges from 75-90% which means that using mammography it is possible to detect 75% to 90% of women who truly have breast cancer [37]. *False negative* test result is defining as the people with the disease who incorrectly identified as healthy. The sensitivity of a mammogram depends on the composition of the breast tissue [38]. Sensitivity of a mammogram is higher in women with low dense breast compared to women with high dense breast [39]. In a large study by Carney et al., showed that the sensitivity of mammograms reduced from 87% in women with fatty breasts to 63% for women with extremely dense breasts [40]. Therefore, additional imaging tests such as

digital breast tomosynthesis, ultrasound, and breast magnetic resonance imaging (MRI) are recommended for women with dense breasts.

Specificity is defined as the proportion of people without the disease who have a negative test. Specificity of mammography range from 90-95% which means that 90% to 95% of women who do not have breast cancer are correctly identified as not having cancer, *true negatives,* but the rest of women without breast cancer are incorrectly identified as having the breast cancer known as *false positive* [41]. *False positive* is defined as healthy people incorrectly identified as sick.

High mammographic density influences both sensitivity and specificity. As density increases sensitivity decreases which means that there will be more *false negative* examinations. Specificity also decreases (thereby increasing the proportion of *false positives*) but normally not to the same extent as with sensitivity. [42].

Digital Breast Tomosynthesis (DBT)

Standard mammography technique creates a standard two-dimensional view of the breast, whereas an advanced form of mammography develops a three-dimensional view of the breast by capturing multiple images. This technique is known as digital breast tomosynthesis and was approved by the FDA in 2011 [43]. Tomosynthesis is not yet considered as the standard care for breast cancer screening due to limited availability of the technology in hospitals. The accuracy of digital breast tomosynthesis has been shown to be higher than digital mammography [44] and two-view digital breast tomosynthesis in combination with two-view digital mammography has been shown to increased cancer detection compared to two-view digital mammography [44-46]. A major population-based screening study in Sweden (Malmö Breast Tomosynthesis Screening Trial) showed that digital breast tomosynthesis detects over 30% more cancers compared to digital mammography with a majority of the detected tumors were invasive cancers [47]. Additionally, the study found that tomosynthesis has the potential to reduce the radiation dose and compression force to the breast during examination [47].

Ultrasound

Breast ultrasound is an imaging technique that sends high-frequency sound waves through breast and creates images of the breast tissue without emitting radiation. Breast ultrasound is widely used in clinical setting and it can complement mammography, particularly in women

with dense breast. An advantage of using ultrasound is the ability to evaluate the tissue without ionizing radiation exposure. Ultrasound is therefore used when evaluating breast abnormalities in women who are pregnant and / or lactating [48]. Breast ultrasound has excellent contrast resolution and is an effective diagnostic method in differentiating solid breast abnormalities (such as benign fibroadenoma, masses, or cancerous lumps) from the fluid-field abnormalities (such as cysts) [49]. One of the weaknesses with breast ultrasound is very that it is operator-dependent meaning that the test value depends on the operator's level of skill and experience and it is more time consuming than mammography, making it less suitable for breast cancer screening.

Magnetic Resonance Imaging (MRI)

The basis for MRI emerges from the interaction between radiofrequency signals generated by the MRI scanner and the body's natural magnetic properties to produce detailed images from any part of the body. Compared with mammography, breast MRI has a higher sensitivity for the detection of breast cancer and it is not influenced by breast density [50]. Breast MRI is usually performed 1) to identify breast cancer in high-risk women due to breast cancer gene (BRCA) mutation carriership, family history of breast cancer or previous radiotherapy towards the chest wall, 2) to determine the extent of breast cancer that has been already diagnosed on mammography/ultrasound examination, 3) to determine whether the artificial breast implants is intact, and 4) used as a supplemental imaging method for women whom the results of mammography screening and biopsy are inconclusive [50].

Despite the high sensitivity of MRI in detecting early breast tumors, it is not recommended as a screening tool for breast cancer due to higher cost than mammography and poor specificity, resulting in significantly increase of false-positive findings which lead to additional diagnostic costs and medical procedures [51]. Furthermore, MRI can also take longer than mammography.

Mammography screening

Screening for breast cancer with mammography has been the most common screening method for many decades [52] and women older than 50 years in several of European countries are participating in screening activities [53].
The primary goal of breast cancer screening is to reduce breast cancer mortality by early detection. Results from randomized control trials, first from Sweden, showed that screening with mammography could reduce breast cancer mortality [54]. A review of the randomized

controlled trials showed that screening delivers a 20% reduction in mortality rate [55]. A meta-analysis of screening trials found that mortality reduction due to breast cancer screening is varying across all ages, with 12% reduction in women aged 39 to 49, 14% in women aged 50 to 59 years, 33% in women aged 60 to 69 years, and 20% in women aged 70 to 74 years [24]. Finally, a recent observational study showed screening for breast cancer using mammography has a clear long-term beneficial effect with a 20% reduction in breast cancer associated mortality among invited women [56].

The first breast cancer screening program in Europe was introduced in parts of Sweden in 1986 [31, 57]. From 1986 to 1995 the Swedish National Board of Health and Welfare issued recommendations to all county councils in Sweden to initiate population-based mammography screening programs for women 40–74 years of age. The recommendations were based on the results of the Two-County study published in Lancet in 1985, which showed a 31% reduction in mortality among participating women [31]. Following the results from Malmö Mammography Screening Trial in 1988 which did not achieve the same cancer mortality reduction as did the Two-Country study, especially not in younger women [58] , Swedish National Board of Health and Welfare altered the screening recommendations and focused on women aged 50-69 years. Accordingly, Stockholm introduced screening in 1989, inviting all women 50-69 years of age to mammography every 24 months [59, 60]. Interestingly, more research has shown a favorable effect of screening in younger age group [61], thus since July 2005 women aged 40 – 49 years have also been invited in the screening program in Sweden. As a result of high participation rate throughout the years, since 2013, invitations are also issued to women aged 70-74. Today, the Swedish national guideline recommendation is to invite women from 40 to 74 years old, with an interval of 18 to 24 months and 70-80% of the invited women participate in the screening program [62]. Currently, all breast cancer screening recommendations are mainly age based.

Whilst the benefit of systematic breast cancer screening is reduced mortality, overdiagnosis is acknowledged as the major harm of mammography screening. Overdiagnosis occurs when screen-detected cancer is either non-growing or slow-growing which never causes medical and health problems if left alone. The rate of overdiagnosis has been estimated at 10% [63-66]. In case of overdiagnosis, medical treatments are unnecessary, costly, and have psychological side effects such as anxiety.

Biopsy

Although clinical examination and imaging could locate the breast abnormality, a biopsy is the only possibility to get a final diagnosis before surgery. A biopsy is conducted when a clinical examination and / or a breast imaging modality reveal an abnormality. There are three main types of breast biopsies: fine-needle aspiration, core-needle biopsy, and surgical biopsy.

Fine-needle aspiration

A fine needle aspiration involves passing a thin needle though the skin to sample fluid or tissue from a cyst or a solid mass. The sample of the cellular material is taken and sent to pathology for cytopathology analysis. Sometimes, an ultrasound is used to help the physician in order to guide the needle to the exact site. A fine needle aspiration is an effective, simple, and quick tool in evaluating and diagnosing of suspicious lumps or masses. However, it is not possible to determine the invasiveness of a tumor by cytology analysis, therefore a needle aspiration biopsy has no ability to differentiate between in situ and invasive breast cancer [67, 68].

Core-needle Biopsy

Core needle biopsy is similar to a fine needle aspiration, expect that a larger needle is used. A core needle biopsy removes a small amount of suspicious tissue from the breast while the patient is under local anesthesia. During the procedure the physician may use specialized imaging equipment to guide the needle to the desired site. Core needle biopsy provides histopathological analysis of the suspicious breast tissue and unlike the fine needle aspiration biopsy has a strong ability to specifically diagnose benign lesions and ability to differentiate between in situ and invasive breast cancer [69].
It is important to mention that the main difference between a fine needle and core biopsy is that a fine needle biopsy only provides cells whereas core needle biopsy provides tissue for further pathology analysis.

Surgical Biopsy

Similar to core needle biopsy, surgical biopsy is performed while the patient is under local anesthesia. During a surgical biopsy, the surgeon makes a cut on the breast and removes a portion of the breast mass for examination or removes all or part of the abnormal lump. Surgical biopsy provides accurate and detailed information on the tumor characteristics

including the tumor size, type, grade, and molecular classification which help the patients' treatment plan.

Tumor characteristics

After the tumor has been surgically removed it is characterized by a pathologist in a number of ways. Several techniques are used such as immunohistochemistry, in situ hybridization, gene expression analyses and morphology, that is, looking at the tumor in a microscope.

Non-Invasive and Invasive

Based on the cellular origin of the cancer cells, breast cancer can be divided into different types. The most common types are ductal carcinoma in situ, invasive ductal carcinoma, and invasive lobular carcinoma. About 1 in 5 new breast cancers are ductal carcinoma in situ (DCIS). DCIS is characterized by proliferation of the epithelial cells confined to the breast ductal structure that has not yet spread into the surrounding breast tissue. DCIS is the most common type of non-invasive breast cancer and is typically a non-palpable and asymptomatic breast lesion. DCIS is primarily diagnosed in a routine mammographic screening followed by needle biopsy [70]. It has been shown that DCIS have the potential to become invasive [70, 71]. Because of concerns that a small proportion of DCIS could become invasive, nearly all women diagnosed with DCIS receive various form of treatments.

The most common histological types of invasive breast cancers are ductal carcinoma and lobular carcinoma. Invasive ductal carcinoma is the most common type of invasive breast cancer and about 80% of all breast cancer are invasive ductal carcinomas. As the name indicates, invasive ductal carcinoma refers to a cancer where malignant cells have broken though the ductal membrane and invaded the surrounding tissue. Invasive ductal carcinoma has the ability to metastasize through the bloodstream and/or lymphatic system to the other parts of the body [72].

Invasive lobular carcinoma is the second most common type of breast cancer after invasive ductal carcinoma. About 10-15% of all invasive breast cancers are invasive lobular carcinomas. Invasive lobular carcinoma refers to the cancer that has broken through the wall of the lobule and begun to invade the tissue of the breast and tends to spread to the lymph nodes and possibly to other parts of the body. As compared with invasive ductal carcinoma, the diagnosis of invasive lobular carcinoma through mammography is more difficult. Other

imaging tools such as MRI and ultrasound maybe used to assess the area of the concern followed by biopsy [73, 74].

Tumor stage

Tumor stage is one of the most important prognostic factors in breast cancer. Prognostic factor defines as a variable that estimates the chance of recovery from a disease, or disease relapse. The staging system is called the TNM system, and was developed in the 1970s. The TNM system was recently revised and the American Joint Committee on Cancer (AJCC) published the 8th version in 2018 [75]. The TNM system classify breast cancer into different stages based on the *tumor size* "T", *lymph node involvement* "N" and *metastasis* "M". Breast cancer stage is usually expressed as a number on a scale of 0 to IV. Stage 0 is representing non-invasive cancers and stage IV is a metastatic breast cancer [76].

Numerous studies have been conducted aiming at understanding the influence of tumor size and lymph node involvement on effect of treatment recommendations and prognosis of breast cancer patients. The tumor is measured in two dimensions on its maximum diameter and reported in either millimeters or centimeters. Tumor size is considered as one of the key factors to predict the risk of cancer metastasis and recurrence. Increase in tumor size has been associated with increased breast cancer mortality [77]. Additionally, several studies concluded that there is a positive association between tumor size and number of lymph node involvement [78-80].

Breast cancer first spreads to the lymph nodes of the axilla. In current clinical practice, sentinel lymph node biopsy is commonly used to test for lymph node involvement. The lymph node or group of lymph nodes that the cancer reaches first is called sentinel lymph node. Sentinel lymph node biopsy involves injection of either a special blue dye or radioactive isotope (in some cases both) into the tumor which work as a tracer material that helps the surgeon find the sentinel nodes during surgery [81]. According to St Gallen guidelines, axillary lymph node involvement is the most important prognostic indicator for primary breast cancer, especially among patients with four or more lymph nodes which are involved [82]. Distant metastasis is an indicator of poor prognosis with a median survival time of 2 – 3 years [83]. The most common metastatic sites are the skeleton, liver, lungs and brain.

Histological grade

The histological grade of breast cancer is an important prognostic factor which represent the "aggressiveness potential" of the tumor and clinician use the histological grade information to guide the treatment options for patients. In general, low grad cancers tend to be less aggressive than the high-grade tumors. In breast cancer low grade tumor (grade I) usually means the cancer is slow-growing and less likely to spread. Grade I tumors are *well differentiated* meaning that cancer cells look like normal cells. High grade tumor (grade III) refers to the cancer that grow and spread fast and has a worse prognosis than grade I. Grade III tumors are *poorly differentiated* which means that the cancer cells look very different from the normal cells. One of the most validated scoring systems for determining the grade of a breast cancer is Nottingham Histologic Score System (NHS) also known as Elston-Ellis grading system. In this scoring system, there are three main factors that pathologists take into consideration: 1) mammary cell formation (or differentiation), 2) nuclear features, and 3) mitotic activity which represents the proliferation of the tumor cells [84].

Hormone receptors

Estrogen receptor (ER) positive breast cancer means that breast cancer cells have receptors that are influenced by estrogen. ER is expressed in the majority of invasive breast cancers and it is known as an important therapy predictive and prognostic marker. About 75% of breast cancers are ER positive at diagnosis [85] and results from previous studies showed that the 5-year survival of patients with ER positive breast cancers is about 10% better than those with ER negative tumors [86, 87]. All invasive and recurrent breast cancers are tested for ER expression by immunohistochemistry staining.

Progesterone is another female hormone that has an important role in breast development. The breast cancer is PR positive if at least 1% of the cells tested have progesterone receptors meaning that the cancer cells receive signals from progesterone to proliferate [88]. Analysis from observational studies showed, the absence of PR expression was associated with worse prognosis among ER+ breast cancer [89-92].

The HER-2 gene is encoding for the HER-2 protein which forms the HER-2 receptor in breast cells. HER-2 receptor is associated with cell proliferation, growth, repair and apoptosis. Overexpression of HER-2 in breast cancer cells occurs primarily through amplification of HER-2 gene. HER-2 overexpression is detected in 10-20% of all breast cancers and is

associated with high grade tumors, lymph node involvement, a higher rate of disease recurrence and mortality [93].

Proliferation marker

Ki-67 is known as the cell proliferation marker. Healthy breast tissue expresses low levels of Ki-67 and high level of Ki-67 expression is a sign of uncontrolled proliferation which is a hallmark of malignancy [94]. Evidence showed that tumors with higher levels of Ki-67 have a worse overall survival than tumors with lower levels. However, tumors with a high level of Ki-67 may respond well to chemotherapy as chemotherapy is designed to target cells with a high proliferation rate [95].

Molecular subtypes

Based on the breast tumor gene expression profiling, breast cancer can be divided into distinct molecular subtypes including luminal A, luminal B, HER-2 enriched, and basal-like cancer. *Luminal A* tumors are the most common breast cancer subtype with about 30-70% of all breast cancers are luminal A. Luminal A breast cancer is hormone receptor positive (estrogen receptor and/or progesterone receptor positive), HER-2 negative, and has low levels of Ki-67 protein. Luminal A tumors tend to have the best prognosis, with high survival rates and low recurrence rates [96].

Luminal B breast cancer is hormone receptor positive (estrogen receptor and/or progesterone receptor positive), and either HER-2 positive or HER-2 negative with high levels of Ki-67. About 10-20% of breast cancers are of luminal B subtype. Women with luminal B tumors are often diagnosed at a younger age than those with luminal A and have a significantly worse prognosis than the luminal A subtype [97].

In *HER-2 enriched* breast cancer, the tumor cells are hormone receptor negative (estrogen receptor and progesterone receptor negative) and HER-2 positive. About 10-20% of breast cancers are HER-2-positive subtype tumors. Women with HER-2 enriched tumors may be diagnosed at a younger age than those with luminal A and luminal B tumors. HER-2 enriched tumors are highly proliferative and have high tumor grade and high rate of metastasis [98].

Triple-negative/basal-like breast cancers are hormone receptor negative (estrogen receptor and progesterone receptor negative) and HER-2 negative. About 15-20% of breast cancers

are triple negative/basal-like and these tumors tend to occur among young women, women with African ancestry, and women with BRCA1/2 gene mutation. Triple-negative/basal-like tumors are aggressive and have a poorer prognosis compared to luminal A and luminal B tumors [99, 100].

Treatments

Treatments for breast cancer patients are divided into a) local treatments (surgery, radiotherapy) and b) systemic treatments (chemotherapy, endocrine therapy, targeted therapy).

Local treatment

The main goal of therapy for non-metastatic breast cancer is eradicating tumor by breast cancer surgery. Breast surgery is a key component of breast cancer treatment that involves removal of the entire breast known as mastectomy or removal of the tumor or other abnormal tissue from breast known as lumpectomy/breast-conserving surgery. The use of these surgical techniques varies depending on age of the patient, size, location, and distribution of the tumor [101].

Mastectomy may be recommended to patients with multicentric tumors, large tumors, in case of skin or chest wall involvement, patients with BRCA1/2 gene mutation, and patient with recurrent breast cancer who underwent previous treatment with lumpectomy and radiation therapy. Elderly patients that will have difficulties coping with the post-surgical radiotherapy after a lumpectomy, will be suggested mastectomy. Mastectomy is sometimes accomplished by an axillary lymph node dissection which is a procedure in which the surgeon removes the affect the lymph nodes [101, 102]. Lymphedema of the arm is reported as the primary complication of axillary lymph node dissection. There are several strategies for prevention and risk reduction prior to the onset of lymphedema, one such a technique is sentinel lymph node biopsy. Other complications involve numbness of arm and chest wall, restriction movement of arm, pain and infection in arm and chest wall [103, 104].

Lumpectomy is mainly recommended to women with early-stage breast cancers followed by radiation therapy. The main advantages of lumpectomy are; it could preserve much of appearance and sensation of the breast and it is less invasive compared to mastectomy, thus the recovery time is shorter and easier [101]. Evidence showed that women with small tumors

(<4 cm) treated with lumpectomy followed by radiation therapy had a similar survival rate as women who underwent mastectomy [105].

The main purpose of the *radiation therapy* followed lumpectomy or mastectomy is to eradicate the remaining tumor cells and reducing the local recurrence. In radiation therapy, high doses of radiation are used to destroy tumor cells or slow their growth by targeting the tumor cell DNA. When the DNA in cancer cell is damaged it stops proliferating and it will eventually undergo the apoptosis. [106]. In Sweden, radiation therapy is recommended to almost all early-stage breast cancer patients operated with lumpectomy and breast cancer radiation therapy accounts for a third of all radiation therapies given in Sweden [107]. Results of a Swedish randomized trial showed that, radiation therapy after lumpectomy reduced the risk of breast cancer recurrence by approximately 15% [108]. The common side effects of radiation therapy are fatigue, swelling, breast pain, and localized skin changes (rash, burning, dry skin and etc.). Late adverse health effects from radiotherapy includes cardio-vascular events such as myocardial infarction [109].

Systemic treatment

Systemic treatment for breast cancer is recommended to patients with primary invasive breast cancer with high risk of recurrence and distance metastasis. Systemic treatments are in forms of chemotherapy, endocrine therapy, and targeted therapy. The decision to use systemic therapy is highly dependent on factors such as patients' age and menopausal status, tumor size, and tumor molecular subtype.

Chemotherapy has been an important treatment option for treating breast cancer for decades with the aim of interrupting tumor cell proliferation and inducing apoptosis. Chemotherapy is available in forms of *neoadjuvant* and *adjuvant*.

Neoadjuvant chemotherapy is provided before surgery and the aims are to decrease tumor burden which may enable patients to undergo curative surgical resection and to reduce the risk of recurrence. [110, 111].

Adjuvant chemotherapy is given to destroy microscopic cells that maybe present after tumor is removed by surgery. The aim of adjuvant chemotherapy is to prevent cancer recurrence. It is offered to high risk breast cancer patients that physician accept the associated toxic effects. [112]. Chemotherapy agents interrupt cell proliferation, therefore normal cells with high proliferation activity are also affected; causing side effects. Most chemotherapy related side effects are acute and include neutropenia, anemia, diarrhea and nausea. Long-term toxic

effects are premature ovarian failure, heart failure, postmenopausal symptoms such as hot flashes, chills, mood changes, weight gain, and cognitive impairment in some women [112].

Endocrine therapy is a main treatment for patients with invasive breast carcinoma that is positive for ER receptor expression. Endocrine therapy reduces the risk of recurrence and it is either used alone or in combination with chemotherapy. Tamoxifen is normally prescribed to premenopausal women and aromatase inhibitors (AIs) to postmenopausal women [113].
Tamoxifen is a nonsteroidal antiestrogen drug that received FDA approval in 1970s and currently is prescribe to women with ER+ breast cancers for the period of 5-10 years[114]. Results from a large randomized trial showed that using tamoxifen for 10 years reduce the risk of breast cancer recurrence and mortality more than taking tamoxifen for 5 years [115].
Aromatase inhibitors interfere with the body's ability to produce estrogen from androgens by suppressing aromatase enzyme activity. In postmenopausal women, estrogen is no longer produced by ovaries and aromatase in fat and muscle may be responsible for much of the circulating estrogen [116]. Common side effects of aromatase inhibitions include heart problems, bone loss (osteoporosis) which may cause bone fractures, joint stiffness, and joint pain [116].

Targeted therapy for breast cancer involves substances that blocks the growth of cancer cells by interfering with molecules that are responsible for tumor cell proliferation and survival. The main targeted therapy drug used for breast cancer treatment is trastuzumab (Herceptin). *Trastuzumab* is a monoclonal antibody that was approved by the FDA [117]. Trastuzumab may be combined with another monoclonal antibody targeted therapy drug called *pertuzumab (Perjeta)* and a chemotherapy drug in HER-2 positive breast cancer patients with high risk of recurrence [118]. Cardiac toxicity is reported as a serious adverse effect of trastuzumab in 1% to 4% of patients treated with the drug [119].
There are a number of other targeted therapies, most of them still in clinical trials that will add to the systemic therapies used in both the neoadjuvant and adjuvant setting.

Epidemiology

Breast cancer remains the most common cancer among women worldwide. Each year 2.1 million women are diagnosed with breast cancer [120]. In 2018, it is estimated that 627,000 women died from breast cancer which represents approximately 15% of all cancer death among women [121]. Breast cancer incidence is still increasing, both in developing and developed countries. According to report by Global Burden of Disease in 2016, breast cancer

incidence was higher in high income countries (1 in 10), compared to low income countries (1 in 50) [120]. There are several explanations to this discrepancy. The differences in reproductive patterns, lifestyle factors, and environmental exposures are the primary contributors of different risks of breast cancer among high and low income countries [122]. The other suggested reasons for this geographical variation may be access to mammography screening, and the quality of cancer registrations [123].

Although breast cancer incidence has increased in high income countries, the age-standardized mortalities are more heterogeneously scattered over the globe. In low income countries breast cancer is the most common cause of cancer death in women, whereas in high income countries it is the second most common, after lung cancer. This could be due to a better screening and treatments in these regions.

In Sweden, one woman in eight will be diagnosed with breast cancer during her lifetime which corresponding to one woman being diagnosed with breast cancer every hour [124]. In 2016, breast cancer accounted for 29.2% of all female cancers and 13.0% of all cancer mortalities among females [125]. Annually in Sweden, around 7,800 women are diagnosed with breast cancer and around 1,400 die from the diseases [125]. In 2018, Sweden were among the top twenty counties with the highest incidence rate of breast cancer in women [126]. Despite the increase in breast cancer incidence, breast cancer mortality has been decreasing in Sweden for the past decades [127]. The trend in decreasing mortality may be due to early detection through nationwide mammography screening program and improvements in treatment strategies. The overall 5-year survival of Swedish breast cancer patients 88% in 2013 to 92% in 2016 [127]. As can be seen in **Figure 2** below, there has been a long-term trend of increasing incidence, decreasing mortality, and improvement in survival rate of patients with breast cancer in Sweden.

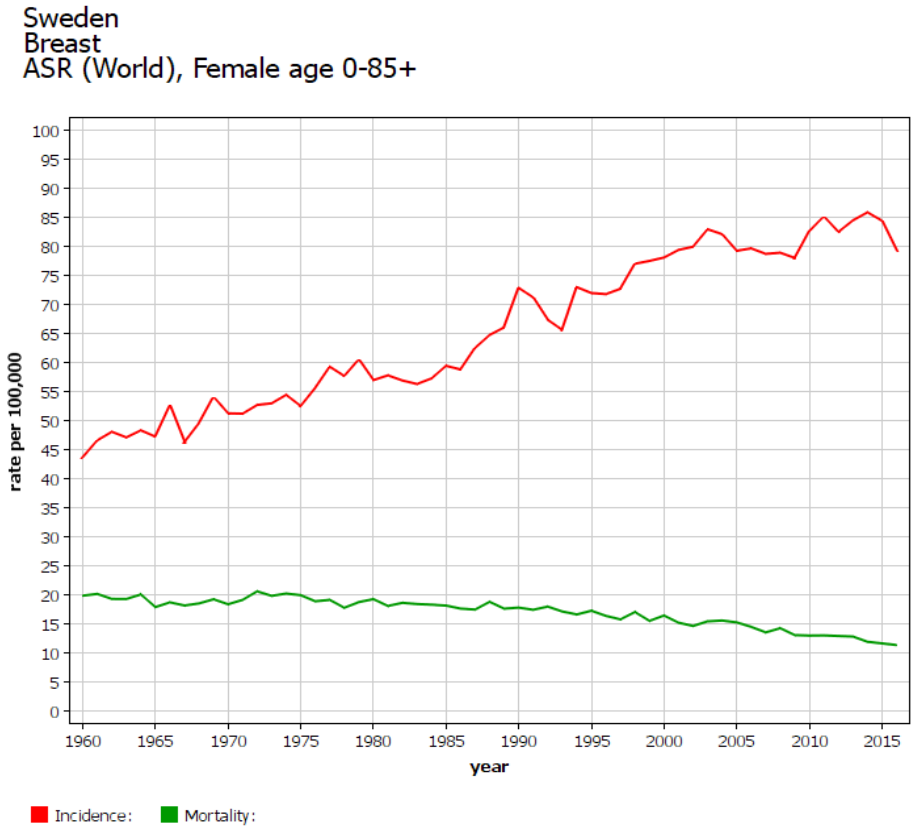


Figure 2. Above: **Time trends in age-standardized incidence and mortality per 100,000-person years in Sweden.** Source: NORDCAN

Risk factors

Age and Female Sex

Breast cancer occurs in both sexes, however it is a very rare cancer among males [128]. It is estimate that 1 in every 1,000 men will be diagnosed with the disease during a life-time [128]. Together with female sex, high age are the strongest risk factors for breast cancer. Approximately, 25% of breast cancer cases are diagnosed among women below age of 50 years and 50% are between 50-69 years and the remaining 25% of cases among women aged 70 years or older. In Sweden the median age at diagnosis was 66 years in 2016 [127]. **Figure 3** illustrates a rapid increase of breast cancer incidence in Nordic countries after 50 years of age until menopause when the rate of increase become weaker.

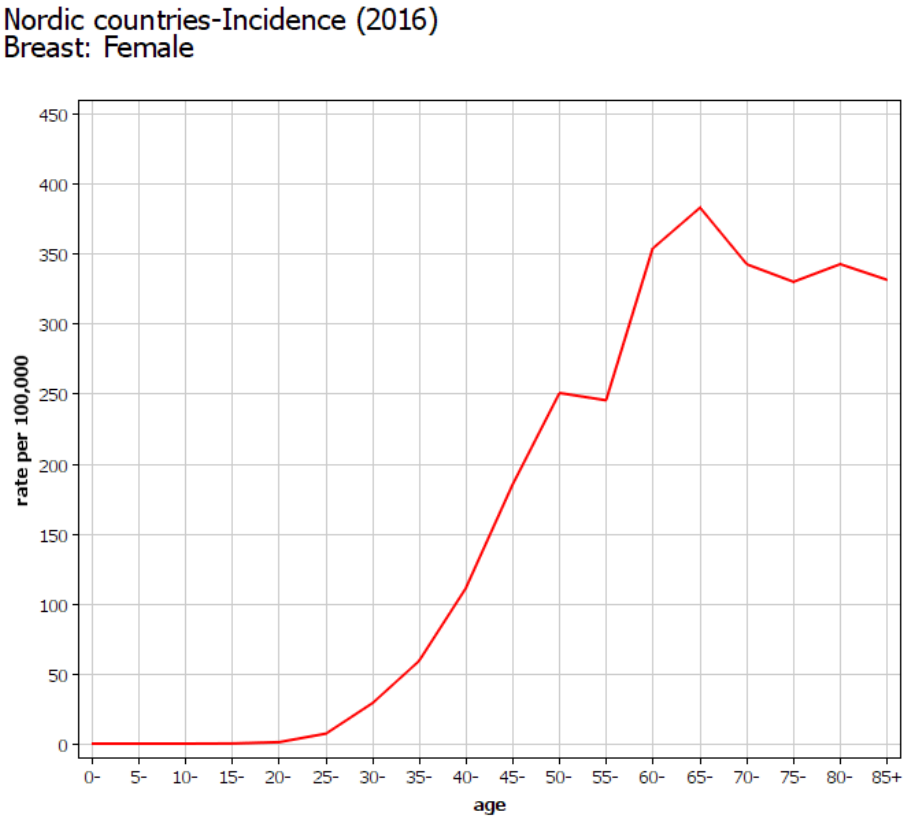


Figure 3. Age-specific breast cancer incidence in Nordic countries in 2016. Source: NORDCAN

Mammographic density

High mammographic density is among the strongest risk factors for breast cancer [129]. Women with mammograms where the dense tissue occupies more than 75% of a mammogram have a four to six times greater risk of breast cancer compared to women with dense tissue occupying less than 5% [129, 130].

Family history of breast cancer and genetics

Breast cancer is a heritable trait and hereditary breast cancer is responsible for 5-10% of all breast cancers. Women with one first-degree relatives (sister, mother, and daughter) with the disease have a 2-fold increased risk of breast cancer [131]. Comparing women with no family history of breast cancer, women with two and more than two affected first-degree relatives increase the risk of breast cancer by twice and three times, respectively [132]. The age of onset of first-degree relatives with breast cancer is also an important factor. The risk of breast cancer is more pronounced in the relatives with age of onset below 50 versus ≥ 50 years of age [133].

Mutations in well-known rare but high-penetrance genes *BRCA1* and *BRCA2* account for 15-20% of breast cancers in women with a family history, and no more than 5-10% of all female breast cancers [134]. In addition to the risk of breast cancer, women with *BRCA1* mutation have an increased risk of ovarian cancer [134]. Mutations in other genes such as *P53*, *PTEN*, *STK11*, *CDH1*, *CHEK2* and *PALB2* are responsible for approximately 2.3-5% of breast cancers.

Genome-wide association studies (GWAS) have identified multiple common, low-risk variants (single nucleotide polymorphisms [SNPs]) associated with breast cancer. The majority of these SNPs are associated with estrogen receptor status, proliferation and cell cycle control [135-137].

Polygenic Risk Score

Over the last decade, researchers using GWAS have identified hundreds of genetic variants (or SNPs) that are linked to a disease. Although single genetic variant associated with a disease provide valuable information such as a biological pathway in relation to the disease, but most genetic variants have been associated with small individual effects which is not informative for assessing the risk of a polygenic disease. In a polygenic disease, more than one gene is associated with the disease. Therefore, combining multiple variants into a single score may be more informative, known as polygenic risk score. Polygenic risk score is a weighted sum of the number of risk alleles that an individual carriers [138]. In a recent study, Mavaddat and colleagues developed a 313-SNP polygenic risk score for breast cancer based on the largest available GWAS dataset [139].

Reproductive factors

The breast tissue is highly sensitive to estrogens and progesterone exposure; thus, a woman's risk of breast cancer is strongly associated with several reproductive factors. Early age at menarche and delayed menopause are known as important reproductive factors associated with increased risk of breast cancer [131, 138]. Previous studies found that women with onset of menstruation at age ≤ 12 had 23% higher risk than those with age at menarche ≥ 15 years [139, 140]. Additionally, a result of a meta-analysis of data from 117 studies showed that for every year older a woman was at menopause, her breast cancer risk increased by about 3% [141]. The production of steroid hormones mainly estrogens and progesterone start at around menarche time and decreases rapidly at around menopause time. The increased risk

associated with early age at menarche and late age at menopause suggests that the longer exposure to sex steroid hormones during reproductive years, the higher risk of breast cancer.

Nulliparity is a well-established risk factor for breast cancer when compared with the risk among parous women who gave birth at relatively young age [142, 143]. Overall, nulliparous women have a 20-40% higher risk of breast cancer after menopause compare to parous women who have their first child before age of 25 years [144]. Additionally, parous women, benefits from a protective effect of breast-feeding [143, 145]. Further, pregnancy has a dual effect on the risk of breast cancer. It is associated with a transient increased risk of breast cancer but reduces the risk in later years. A plausible biological interpretation for the short-term increase risk of breast cancer by pregnancy is that during pregnancy hormone levels increase and that may stimulate the growth of premalignant cells. The long-term protective effect of pregnancy on breast cancer risk has been suggested to be due to an early differentiation of mammary stem cells that have the potential of carcinogenic transformation [146]. Nevertheless, it is important to mention that women who give birth for the first time above the age of 30 years have an increased risk of breast cancer compared to nulliparous women [147].

Exogenous hormones

For the past decades, exogenous hormones in form of menopausal hormone replacement therapy (MHT) was used as an effective treatment or alleviating the climacteric symptoms of menopause such as hot flushes, sleeping disturbance, depressive mood, muscle and joint pain. In 2002, the Women's Health Initiative (WHI) study showed that postmenopausal women who received combined estrogen plus progestin therapy significantly increased the incidence of breast cancer within a 5-year period compared to non-MHT users [148]. Additionally, the study found that the frequency of suspicious findings in mammograms was higher in estrogen plus progesterone group than non-MHT users [148]. This could be partially explained by the increase of mammographic density as a result of combination of estrogen and progestin use. Several studies showed that postmenopausal women who began using MHT especially the combination of estrogens plus progestin experienced mammographic density increase [149-152]. Later, increase in mammographic density as the result of MHT use found to be associated with reduction in both the sensitivity and specificity of the mammography [153]. Interestingly, after cessation of MHT the increased risk of breast cancer diminishes within 2 years, suggesting that the harmful impact of MHT use on breast cancer risk is likely to be reversible [154]. It has been also suggested that the effects of MHT

on mammographic density should be reversible, since the specificity and sensitivity of former MHT users were similar to never users [155].

Current and recent use of hormonal contraception is associated with higher risk of breast cancer compared to the risk among women who had never used hormonal contraceptives. Also, the risk is depended on the duration of use [156]. Interestingly, previous findings showed a similar pattern as seen with MHT use that the excess risk of breast cancer disappears after 5-10 years cessation, depending of the duration of the hormonal contraceptive use [156, 157]. Also, it has been found that initiation of use before age of 20 years old maybe associated with higher risk of breast cancer compared to those who start at the late age [156].

Lifestyle factors

There are several important lifestyle factors related to risk of breast cancer. Body mass index (BMI) which is defined as body weight in kilogram divided by the square of height in meters, is one of the most commonly used anthropometric measurement. The association between BMI and breast cancer risk has been studied extensively over the past years. An inverse association between BMI and risk of breast cancer among premenopausal women was observed in majority of studies. The increased incidence of breast cancer in lean women is found to be strongest among younger women [158-161]. However, high BMI and obesity is significantly associated with an increased risk of breast cancer in postmenopausal women [158, 162]. The exact biological mechanism for relationship between BMI and risk of breast cancer among pre and postmenopausal women is not well understood, however, there are some potential hypotheses. Exposure to estrogen and progesterone elevates risk of breast cancer. The positive association between BMI and breast cancer risk in postmenopausal women speculated to be the result from increased level of estrogen derived from the conversion of androgenic precursors to estrogen through the peripheral aromatization in adipose tissue [158].

Prospective studies have investigated the association between physical activity and breast cancer risk [163-166]. Overall, the results show regular physical activity lowers breast cancer risk by 10-20% and the benefit is seen most clearly among postmenopausal women [165] and longer duration of physical activity provides most benefits, and such activity need not be strenuous [163]. The proposed biological mechanism by which physical activity exerts a protective effect against breast cancer especially in postmenopausal women could be in

relation to the decrease in sex steroid hormones, decrease in adiposity, increase in immune system function, reduce in inflammatory markers, and reduce in levels of insulin-related factors (insulin-like growth factors) [167]. Assessment method of physical activity varies across studies include using the self-reported questionnaire, pedometers, and heart rate monitors. However, in an attempt to improve the comparability of findings across studies on physical activity, the concept of metabolic equivalent of task (MET) score developed and introduced. MET is a unit that estimates the amount of energy used by the body during physical activity, as compared to resting metabolism. In other words, MET indicates the intensity of the activity and one MET per hour is defined as 1kcal/kg/hour which it is approximately equal to the energy spend during one hour of quiet sitting [168]. Physical activity assessment method used in the studies included in this thesis was based on the MET-hour score and it has been developed and used in previous publication by our group [169].

Extensive epidemiological data have shown the association between alcohol consumption and risk of breast cancer [170-173]. A meta-analysis that combined the results of 98 studies found women who drank alcohol were had 11% higher risk of breast cancer than non-drinkers. [170]. Alcohol consumption is found to be related with increase of estrogen levels and this may in turn be partially responsible for the association between alcohol consumption and risk of breast cancer. Another hypothesis is that alcohol is considered as carcinogenic substance, meaning that alcohol metabolism can damage the DNA of the cells, thus inducing tumor development [174].

The carcinogenic potential of tobacco smoke in relation to breast cancer is investigated. A large cohort study of 102,927 of women age 16 years and older from United Kingdom showed that women who had ever smoked were 14% at higher risk of developing breast cancer than those who had never smoked [175]. Interestingly, this study highlighted that women who started smoking at adultescent age before 17 years had a 24% increased risk of breast cancer while those who began smoking between age 17-19 years had 15% increased risk of the disease [175]. It has been suggested that at puberty, the breast is mainly consists of undifferentiated ductal and lobular structures which makes it more sensitive to carcinogenesis chemical exposure [176]. Other studies also found that an increased risk of breast cancer when smoking started in adolescence [177, 178] or close to menarche [179, 180]. Finally, it is been also found that the relative risk of the breast cancer associated with smoking is greater in women with family history of breast cancer [175].

Benign breast diseases

Benign breast diseases lead to unusual growths or other changes in the breast tissue that not cancerous. Although many benign breast diseases may not develop to cancer, some can increase the risk of breast cancer. Benign breast conditions are classified as either non-proliferative or proliferative. Proliferative breast diseases linked to a moderate increase risk of breast cancer. The most common type of proliferative breast condition is hyperplasia [181, 182]. Two types of hyperplasia including usual hyperplasia and atypical hyperplasia exist. Women with usual type of hyperplasia have about 2-fold increased risk of breast cancer compared to women without a proliferate breast condition [181, 182]. Whereas, women with atypical hyperplasia have about 3-5 times higher risk of breast cancer than women without proliferative breast conditions [181-183].

Ionizing radiation

Ionizing radiation is an established risk factor for breast cancer and there is a dose-response relationship. The knowledge on radiation-related breast cancer risk derives mainly from epidemiological studies of patients who have been exposed to diagnostic or therapeutic medical radiation and Japanese atomic bomb survivors. The age of exposure is found to be an important effect modifier, the highest risk is seen among girls exposed before age of 20 years and minimal risk detected in women exposed after menopause [184].

Race and Ethnicity

Incidence rate of breast cancer vary by race and ethnicity. In the United State, white, African Americans have the highest overall incidence rates for breast cancer among racial/ethnic groups, while American Indians/Alaskan Natives women have the lowest incidence rates [185, 186]. In addition, African American women tend to be diagnosed with breast cancer at a more advance stage [185], to be high grades tumors [187], to have larger tumors that are more likely be estrogen receptor negative than those in white women [188]. African American women have also higher mortality rate than white women [189]. Socioeconomic disadvantages, language and communication barriers, low education status among African American women create obvious barriers to healthcare access, resulting in more advance stages of the diseases at the time of diagnosis and increased mortality rate. Other factors such as ethnicity-related variation in tumor biology, variations in lifestyle behaviors, environmental exposures, nutritional factors, and hormonal metabolism may partly explain

the differences in the rate of breast cancer incidence and mortality among racial/ethnic groups [189].

In the United States, there is evidence that Ashkenazi Jewish heritage women have slightly higher risk of breast cancer then other women [190]. This increased risk is likely due to the high prevalence of BRCA1/2 gene mutations in Jewish women of Eastern European descent (Ashkenazi Jews). About 8-10% of Ashkenazi Jewish women diagnosed with breast cancer in the United States have a BRCA1/2 mutations [191].

MAMMOGRAPHIC DENSITY

Definition and assessment

Mammographic density reflects the tissue composition of a breast. The epithelial and fibrous tissue, appears white on a mammogram, while fatty tissue appears darker [192]. The denser the breast, the more epithelial and fibrous tissue. In 1976, John Wolfe observed and publish the association between breast tissue and occurrence of breast cancer. Mammographic density could be measured both qualitatively and quantitatively and the first qualitative classification of mammographic density based on breast tissue pattern was suggested by Wolfe (known as Wolfe grades) [193]. Wolfe assigned the mammograms to four parenchymal patterns (N1, P1, P2 and DY).

- **N1:** the breast consists mainly of fat
- **P1:** this pattern includes fat as well as linear densities (enlarged ducts) occupying no more than 25% of the breast
- **P2:** linear densities (from enlarged ducts) occupying more than 25% of the breast. They are prominently in the upper outer quadrant but may be distributed throughout the breast (P=prominent ducts)
- **DY:** dense, radiopaque breast (DY=dysplasia); these patterns are again subdivided into low-risk (N1 and P1) and high-risk (P2 and DY) patterns

A modification of Wolfe´s classification was developed by Lázló Tabár in 1997. In Tabár pattern-based model of breast density assessment, N1 category was divided into two sub-categories [194]. An alternative, semi-quantitative method proposed by Norman Boyd which attempt to quantify density visually using a six-category classification (A (0% density), B (0 to <10%), C (10% to <25%), D (25% to <50%), E (50% to < 75%) and F (≥75%)) [195]. Another visual method is the Breast Imaging-Reporting and Data System (BI-RADS)

developed by the American College of Radiology (ACR) [196] and commonly used in the clinical practice in the United States, **Figure 4** . According to the fifth edition of the BI-RADS guidelines the density definition follows from the least to most dense:

- **A**: the breasts are almost entirely fatty
- **B**: there are scattered areas of fibroglandular density
- **C** the breasts are heterogeneously dense, which may obscure small masses
- **D**: the breasts are extremely dense, which lowers the sensitivity of mammography

Visual-based mammographic density assessment is a crude measurement and it has suffered from a lack of reproducibility. Over the years, there are several semi-automated and fully automated computer-assisted techniques developed to quantitively assess mammographic density. Cumulus as the first semi-automated computerized measure of dense area developed by Boyd and collogues at the University of Toronto and has been the gold standard for qualitative density measurement for many years [197]. Cumulus uses reader-based thresholds to define the breast edge and regions of density on a mammogram. The main disadvantage of this measure is that it is reader-dependent and it takes 2-3 minutes to measure density in one single mammogram. Additionally, Cumulus is a software program which the observer visually selects what are considered to be dense areas for each mammogram, it limits it use in large-scaled research studies.

A fully automated density measurement method developed in our group known as an ImageJ based method with the aim of mimicking Cumulus [198, 199]. Later it introduced under the name of STRATUS. STRATUS is a fully automated tool developed to analyze digital and analogue images using an algorithm that measures density on all types of images regardless of vendor, **Figure 5**. STRATUS measures mammographic density as absolute dense area (cm^2) and percent density [200].

Other methods such as Volpara^{TM}, Libra, Quantra and Single X-ray Absorptiometry, estimate volumetric density. The automated Volpara system has been commonly used as a research tool and it estimates the breast volume and the volume of dense tissue on raw mammograms [201]. The weakness of Volpara is that it operates on raw mammograms and in the clinical setting, normally processed mammograms are stored.

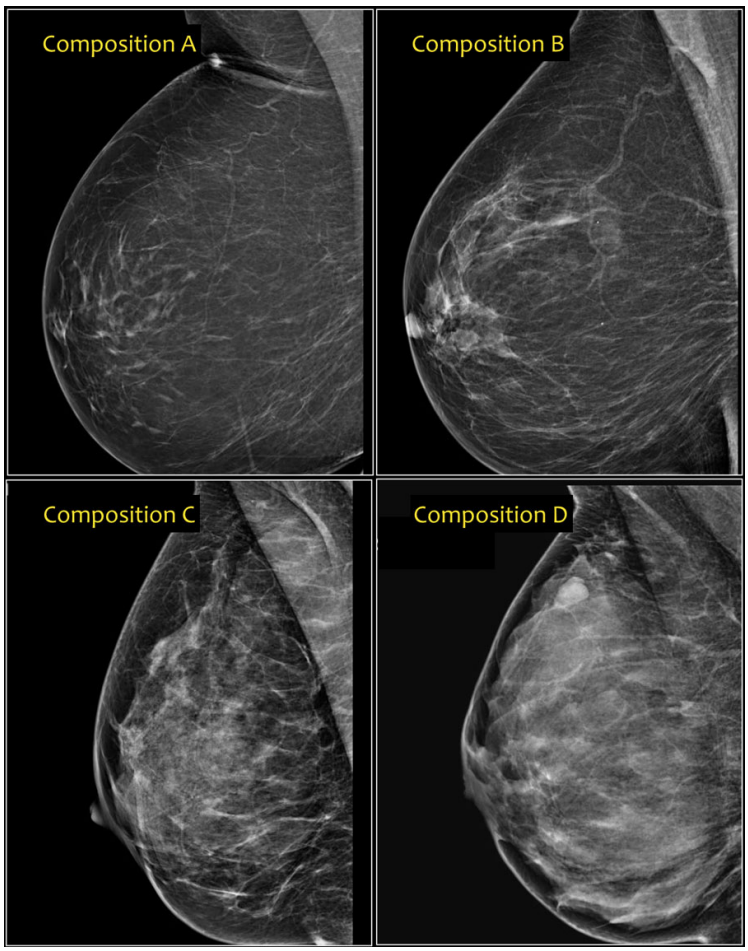


Figure 4. BI-RADS 5th edition categories for mammographic density.
Source: *Radiology Assistant*

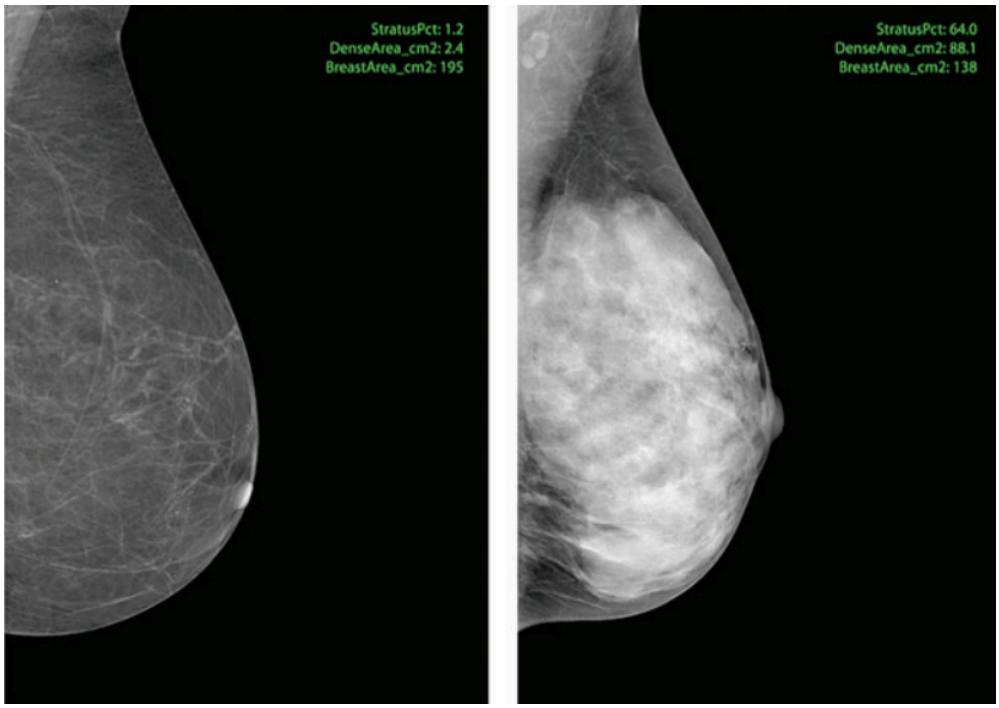


Figure 5. Examples of STRATUS mammographic density measurement on processed mammograms. Percent density (proportion of the entire breast that is dense) was measured in the left (1.2%) and right (63.8%) image using STRATUS

Mammographic density as a risk factor

High mammographic density induces two major problems. Firstly, high mammographic density decreases the sensitivity of a mammogram. Secondly, mammographic density is a strong and independent risk factor for breast cancer.

Masking-effect

As it is mentioned earlier mammography still remains the most widely used methods for breast cancer screening. However, a number of studies have shown that the sensitivity and specificity of the mammography decrease with increasing mammographic density. The epithelial and fibrous tissue, and most tumors appears white on a mammogram. Therefore, the lack of contrast between cancer and breast dense tissue makes it difficult for radiologists to detect breast cancer on a mammogram with dense breasts [129]. In other words, masking is defined as a tumor being hidden in a mammogram due to similar appearances of tumor and fibroglandular tissue.

Specificity which is an indicator of mammography performance is also decreased in women with dense breasts. Previous findings showed that the specificity of mammography is changing from 93.5-96.5% in women with entirely fatty breasts to 89.6-89.9% in women with extremely dense breasts [40]. This means that women with dense breasts are more likely to experience both false-negatives results and false-positive recalls of mammography screening [202]. Data obtained by Dutch screening agency from 2003 to 2011 showed that false-positive recalls increased gradually with increasing mammographic density from 11.2% in women with mostly fatty breasts to 23.8% in women with extremely dense breasts [203].

Additionally, masking-effect leads to an increase in the incidence of interval breast cancers [129, 204, 205]. Interval breast cancers are those cancers which detected during the interval between recommended screens. Also, high mammographic density is found to be also associated with large tumor size, highly proliferative tumors and lymph node metastasis which could be as a result of masking-effect [206, 207]. These findings and increase in false-positive and false-negative rates as the result of high mammographic density supports the notion that having high dense breast tissue can interfere early detection of breast cancer by mammography and therefore the results of mammography for women with high dense breast tissue are usually inconclusive. Therefore, it is recommended that women with dense breasts require supplemental imaging such as using MRI or ultrasound. It has been shown in a

randomized controlled trial that use of supplemental MRI screening in asymptomatic women for breast cancer with extremely dense breast tissue resulted in the diagnosis of fewer interval cancer than using only mammography [208].

Mammographic density as an independent risk factor for breast cancer

Apart from the masking-effect, mammographic density is recognized as an independent risk factors for breast cancer. It is postulated that mammographic density may reflect the number and proliferative activity of breast epithelial cells as well as the quantity of stromal tissue [209]. Histological studies also confirmed that both epithelium and stroma contribute in formation of the dense part of the breast [210, 211]. In addition to these hypotheses and findings, it is been suggested that mammographic density reflects the degree of lobular involution of the breast cells (a biological process which epithelial cells undergo apoptosis and replace by adipose tissue). Previously, it has been shown that progressive lobular involution was statistically associated with reduced breast cancer risk [12]. The decrease in risk with complete involution may reflects fewer number of epithelial cells at risk of malignant transformation. The result of a study on association between lobular involution and mammographic density with breast cancer risk found that, majority (nearly 76%) of women with no involution of breast tissue had high breast density (P2, DY in Wolfe's density pattern) [212]. Interestingly, this study found that mammographic density and lobular involution were independently associated with breast cancer risk. However, women with extremely dense breast and no lobular involution had the highest risk of breast cancer compared to women with non-dense breasts and complete lobular involution [212].

Few studies have proposed that extracellular matrix (a three-dimensional network of extracellular molecules that provide structural and biochemical support to surrounding cells) and tissue rigidity or matrix stiffness (which could be the result of changes in the stromal collagen), and stromal-epithelial interaction may partly explain the association between mammographic density and breast cancer risk [213, 214]. It has been found that tissue stiffness could increase the formation of abnormal 3-dimentional groups of cells, and elevate certain enzyme levels and signaling that promotes epithelial growth, proliferation and produce a less normal and more cancerous epithelial cell type [214]. Another hypothesis is related to changes in mechanical or structural prosperities of the breast tissue that may be related to breast cancer risk [215]. Results of previous studies showed that loss of tissue mechanical homeostasis as a result of matrix stiffness is a hallmark of a disease and it is not

surprising that multiple diseases including cancer is characterized by changes in normal mechanical stress and homeostasis [215, 216].

Determinants of mammographic density

Heritability and genetic factors

Mammographic density is a highly heritable trait. Results of a study comparing percent mammographic density among monozygotic and dizygotic twins suggested that the correlation coefficient between percent mammographic density was about twice as high in monozygotic twins (correlation coefficient 0.63) compared to dizygotic twins (correlation coefficient 0.27) [217]. Another study confirmed that higher percent and absolute mammographic densities in monozygotic twins (correlation coefficient 0.74) compared to dizygotic twins (correlation coefficient 0.38) [218]. These findings suggest that genetic factors explain up to 60-70% of mammographic density variations [218]. Also, in our recent publication we have shown that approximately 60% heritability in mammographic density using both dense area and percent density at baseline [219].
Additionally, some studies found that mammographic density is influenced by candidate genes which were previously identified to be associated with breast cancer [220-222]. Other studies have shown relations between mammographic density and genes that regulates steroid hormones, synthesis and metabolism, hormone receptor and proliferation pathways [223, 224].

Lifestyle factors

Apart from genetic factors, mammographic density is also influenced by well-established risk factors for breast cancer. In contrast to risk of breast cancer, previous studies showed, high BMI is inversely associated with percentage of mammographic density. This is because body weight reflects a greater quantity of fat (non-dense tissue) in the breast [13, 225, 226]. Previous results also showed the similar inverse association between high BMI and mammographic dense area [227].

Previous studies on the association between physical activity and mammographic density showed conflicting results. The majority of studies found no associations between physical activity and mammographic density [228-231] but few suggested that physical activity may be inversely associated with mammographic density [232-234]. In a cross-sectional study of

38,913 women, Trinh et al found that women with high physical activity had lower breast absolute dense volume compare to women with the lowest physical activity level [234]. Previous studies have shown that physical activity may reduce circulating level of and exposure to sex steroid hormones during premenopausal and reduce the estrogen levels among postmenopausal women, in part by affecting adipose tissue reduction [167, 235, 236]. The majority of studies suggested a modest positive association between alcohol intake and mammographic density [237-240]. The positive association between alcohol consumption and higher mammographic density could be explained by increase in circulating estrogen levels.

The association between tobacco smoking and mammographic density has been evaluated in a number of epidemiological studies. These studies found that smoking was associated with lower mammographic density [238, 241-243], however, few studies reported a null associations [230, 237, 244]. In contrast to alcohol consumption, smoking has been reported to have anti-estrogenic effects which will lead to lower circulation estrogen levels, thus women who smoke may have low mammographic density [245].

The association between diet and mammographic density has been investigated extensively [240, 246-252]. Some studies found the association between saturated fat and protein intake with higher mammographic density [247], whereas increased fiber and carotenoid consumption were associated with lower mammographic density [248]. It is hypothesized that dietary factors could affect mammographic density through their effect on sex hormones therefore the influence might differ by menopausal status. In an intervention study by Boyd et al, the low-fat, high carbohydrate diet reduced mammographic density among premenopausal but not postmenopausal women [246]. Vachon et al, in another study found that saturated fat and polyunsaturated fat were significantly associated with percent density in premenopausal women but not in postmenopausal women [240].

Reproductive factors

A number of reproductive factors have been studied in relation to mammographic density. Lower mammographic density has been associated with parity versus nulliparous, younger age at first birth, and having more children [253-256]. Previously, it has been reported that short after pregnancy and during lactation mammographic density increases, this could be explained by the differentiation of the terminal duct lobular units which referred as the epithelial structures of the breasts that produce milk during lactation. In pregnancy, estrogen

and progesterone levels increase and promote ductal proliferation and differentiation. During lactation, plasma prolactin (hormone that promote lactation) concentration increases which causes breast tissue to grow and develop. These changes in the level of circulating hormones during pregnancy and lactation also result in a short-term increase in mammographic density [257, 258].

Results from previous studies evaluating age and menarche and mammographic density were inconsistent. Some studies reported no association between age and menarche and mammographic density [244, 259, 260] while Tehranifar et al. reported older age at menarche was associated with lower mammographic density [261]. Further, it is been well-established that postmenopausal status has been also strongly associated with decrease in mammographic density [262].

Exogenous hormones

There is a strong evidence that use of MHT increases mammographic density and women who used the combined estrogen and progesterone formulas may have the most pronounced effects[151, 263-265]. A result from a nested cases-control study showed that for every 1% increase in mammography density among women who have used estrogen plus progesterone breast cancer risk increase by 3.4% [266]. Increase in mammography density and risk of breast cancer among current MHT users especially those who use the combined estrogen plus progesterone should raise concern and consideration. The increase of mammographic density as a result of MHT use supports the evidence that breast tissue is highly responsive to hormonal interventions and therapies. Further, Cuzick and collogues in a nested cases-control study, found that women receiving tamoxifen and had a 10% reduction in mammographic density were experienced a 63% reduction in breast cancer risk. whereas those who were taking tamoxifen but experienced less than 10% or no reduction in mammographic density had no decrease in breast cancer risk [267].

Mammographic density change

Most studies regarding the association of established risk factors for breast cancer and mammographic density and its influence on the risk of breast cancer have involved only a single measure of density. However, mammographic density is a dynamic trait, meaning that the extend of breast density change by several factors. Use of MHT is associated with increased density, whereas use of tamoxifen and increase in age are associated with reduction

in density [263, 268-270]. Mammographic density decreases over age; reducing the amount of epithelial and stroma tissues, this is known as involution [210, 271]. The paradox is that mammographic density is a strong risk factor for breast cancer that decreases over age while breast cancer incidence increases. An explanation to this enigma could be that mammographic density change, rather than a mammographic density measure at single point in time might be a better measure of breast cancer risk.

The few studies have tried to identify determinants of mammographic density change [272-274] and evaluate the association between mammographic density change and risk of breast cancer [274-277], however, the results are conflicting and all previous studies have important limitations such as using retrospective case-control sampling using out of date imaging technique (screen-film mammography instead of full-field mammography); and using qualitative and crude measurement of mammographic density (BI-RADS score).

MAMMOGRAPHIC MICROCALCIFICATIONS

Definition and assessment

Calcification is a process of the accumulation of calcium in the body tissue. Breast calcifications are common findings on mammography among women over 50 years of age and on about 1 in 10 mammograms of women under 50 years old. Calcification can be in form of both benign and malignant, they appear as macro- or micro- calcifications. Macrocalcifications appear large and round and they are not usually related to cancer. Microcalcification refers to calcifications with diameter ranging from 0.1 millimeter (mm) to 1 mm and they are present as either single calcifications or form clusters [278] Evidence showed that microcalcification clusters are more likely a sign of malignancy as compared to single microcalcifications [279, 280] . The presence of microcalcifications was first reported in 1913 by a German surgeon, Albert Salomon, who conducted a radiographic examination on over 3000 mastectomy specimen and described the association between presence of microcalcifications with breast cancer [281]. In 1949, another radiologist, Raul Leborgne, proposed that the presence of microcalcifications maybe the only manifestation of breast carcinoma. Leborgne was a pioneer of differentiating diagnosis between benign and malignant microcalcifications [282]. Since then microcalcifications are becoming one of the most important diagnostic markers of breast lesions.

Improvements in mammography screening using high-resolution imaging techniques and magnification views allowed better detection of microcalcifications. In 1986, Stickles

classified microcalcification into *"benign" (requiring no further intervention)*, *"probably benign" (managed by periodic mammography)* and *"suggestive of malignancy" (requiring biopsy)* based on their visual structures on a mammogram [280]. Later microcalcifications described and characterized according to their *morphology* (*appearance), distribution*, and by their *physical* and *chemical properties*. BI-RADS category system developed by the American College of Radiology is currently used worldwide as a standard method to describe and classify microcalcifications [283]. Using BI-RADS category system, microcalcifications are categorized into benign, suspicious, and malignant based on their morphology.

Benign microcalcifications

Approximately 50% of women have benign breast calcification and they are common findings of mammography. *Vascular microcalcifications* found in the middle layer of the mammary arteries and increases with increasing age and it is a common findings in women over 50 years old. *Round and punctate calcifications* are called round if they are greater than 0.5 mm and punctate when they are smaller than 0.5 mm. Both these types of calcifications are considered typically benign; however, distribution of these calcifications should be carefully evaluated. When these calcifications are grouped, or appear recently compare to previous examinations should be evaluated further [283]. *Dystrophic calcifications* are common after surgery, post radiation therapy or following a trauma. They are usually greater than 1mm, rough and irregular "lava-shaped" [283]. *Coarse or "Popcorn-like" calcifications* are seen within degenerating fibroadenomas. They are usually dense, thick and large. If these calcifications appear small and numerous in a fibroadenoma, they may be a sign of malignant-type calcification and need a biopsy [284]. *Rim calcifications* are called "eggshell" and "radiolucent centered". They can occur anywhere in the breast, but mostly in superficial location. These calcifications are thin, round shape, and usually associated with a history of surgery, trauma, or fat necrosis [278].

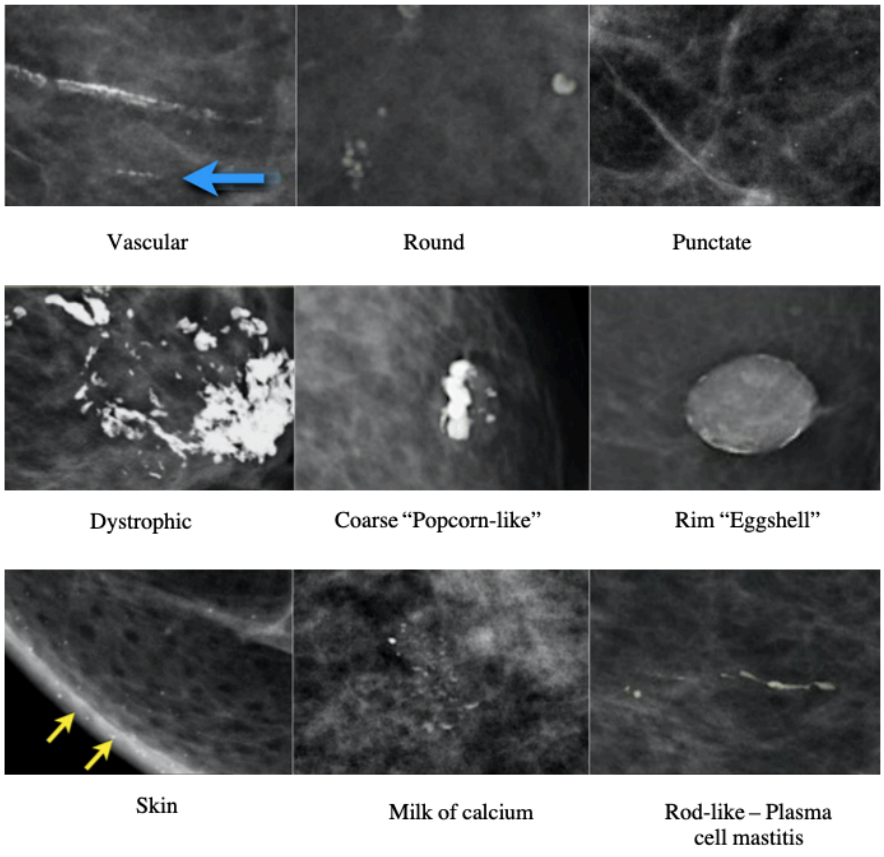
Skin calcifications are small calcifications and usually involves in inflammatory process. They usually form in dermal sweat glands and their morphology is round, with a radiolucent center. They are typically benign calcifications [285]. *Milk of calcium calcification* are found in variety of cystic structures including macro- or micro cysts. They are seen more frequent in the peri and postmenopausal women and also called "teacup" calcifications [283]. *Large Rod-like, Plasma cell mastitis calcifications* form continuous rods that may be branching and are more common in over 60 years old women. These calcifications follow a ductal distribution and radiating toward the nipple and are present usually bilaterally [286]. *Suture*

calcifications are usually seen in patients after lumpectomy and/or radiation therapy. Tissue damage that induced by radiation therapy delays the resorption of the suture and this provides matrix for calcium deposit. They are seen as linear or tubular with calcified knots [287].

Suspicious and malignant microcalcifications

Coarse heterogeneous calcifications are rough, heterogeneous and irregular. They may locate in the breast stroma or ducts and most of these calcifications are originated in benign lesions such as fibro adenoma, fibrosis, or the area of trauma due to fat necrosis but may also be found in malignant lesions [278]. *Fine linear and branched calcifications* are corresponding to small calcifications (<0.5 mm), very thin, linear with irregular edges. Among the suspicious calcifications, these are the ones that have the highest positive predictive value for malignancy up to 70% [278]. *Amorphous calcifications* are known as the "powder-like" calcifications. They are small calcifications (<0.1mm) and it is not possible to count them nor determine their shapes. Therefore, they are called "amorphous" (shapeless). About 20% of amorphous calcifications turn to be malignant and usually are found DCIS [278] . Fine pleomorphic calcifications may form clusters in specific area of the breast and they are most likely a signal of the presence of DCIS.

Benign microcalcifications



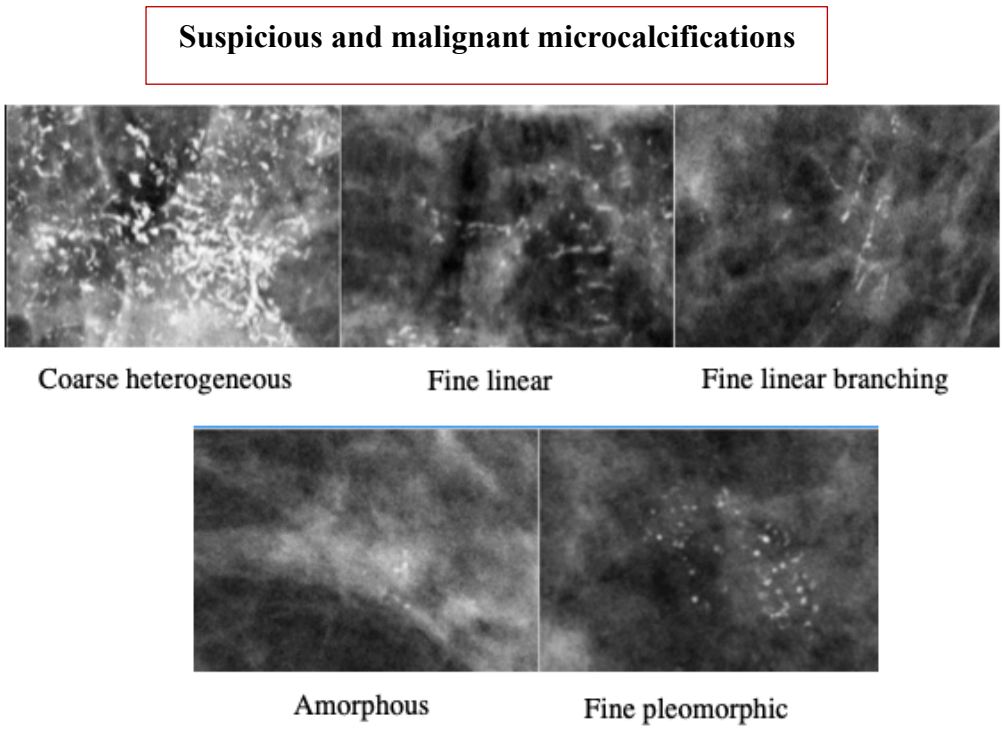


Figure 6. Benign and malignant microcalcifications. Source: *Radiology Assistant*

According to BI-RADS category system, the arrangement of microcalcifications inside the breast may also suggest the probability of malignancy and it is as important as the morphology.

Diffuse distribution is also known as "scattered", these calcifications that are randomly distributed in the breast tissue. Microcalcifications with diffuse distribution are usually sign of benign lesions, especially if they are bilateral. *Regional distribution* pattern describes microcalcifications are seen in a large volume and are not typically associated with malignancy. *Grouped or Clustered distribution* is usually five or more than five calcifications seen in a small area of 1 cm^3 and are most often suspicions for malignancy of the breast. *Segmental distribution* pattern suggests the calcium deposits in the ducts and it branches of a segment or the breast lobe and are topically suspicious microcalcifications. *Linear distribution calcifications* are arranged in a linear path that can suggest calcium deposits within a duct. These microcalcifications are the most common sign of malignancy in the ductal structures.

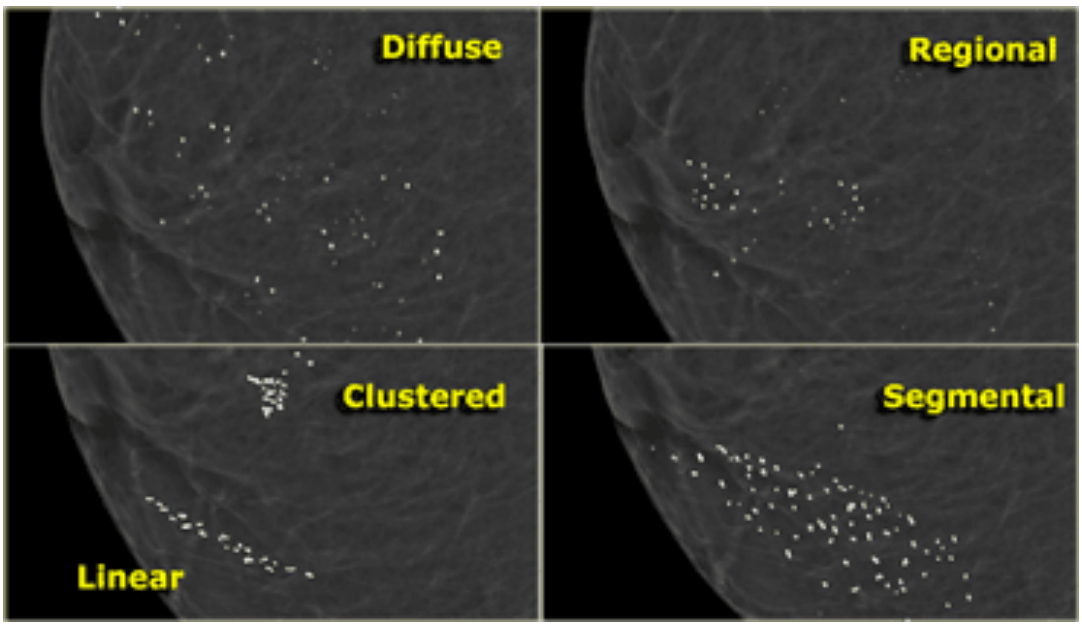


Figure 7. Distribution of microcalcifications according to the BI-RADS category
Source: *Radiology Assistant*

Based on the *chemical properties*, microcalcifications are classified into two different types. Type I microcalcifications consist of calcium oxalate, partially transparent and form pyramidal structures with relatively planar surface. Type I are seen mostly frequently in benign ductal lesions and are rarely associated with breast cancer [288]. Type II microcalcifications are composed of calcium phosphate, mainly hydroxyapatite and they are grey-white, opaque with ovoid or fusiform shapes and have irregular surface. Type II are found in both benign and malignant lesions, but mostly in proliferative lesions including breast cancer [289].

Computer Aided Detection (CAD)

More recently, computer programs have been developed and approved for use in clinical practice to help radiologists in detecting suspicious abnormalities on radiology exams. This application is known as computer-aided detection commonly referred to as CAD. The primary goal of CAD system is to increase the detection of disease by reducing the false negative rate due to overlooked abnormalities. Additionally, fully automated CAD systems have the potential to provide efficiency in the radiology workflow. CAD algorithms are developed to mainly search for the same mammographic features that a radiologist looks for while assessing a mammogram. Therefore, CAD algorithms for breast cancer search for microcalcifications, masses, architectural distortions, and asymmetric findings [290, 291].

In the study III and IV, the CAD system used for identification of microcalcification (iCAD; M-Vu iCAD®, Nashua, USA) [292] is an FDA approved class 3 device (PMA number P010038) with an accuracy of 92% [293]. The algorithm of the system identifies suspicious microcalcification clusters that corresponds to microcalcifications with malignant morphology as defined by the BI-RADS 3-5 scores [293, 294]. Previous results showed that the iCAD software is achieving a sensitivity of 92% in detecting suspicious microcalcification clusters [292, 293] with average specificity of 87% [295]. Clusters were defined according to a well-established neural network algorithm implemented by the iCAD software [293, 296]. The software marks regions of interest on standard mammographic views to bring them to the attention of the radiologist.

Nevertheless, it is important to mention that the main disadvantage of CAD systems is the high rate of false-positive marks. Thus, reduction of false-positive findings by CAD systems in future is a desirable and objective target for improvement of these applications [297].

Microcalcifications as a risk factor

The biological mechanism by which calcium deposition occurs in the soft tissue is not clearly understood. There could be several mechanisms behind the formation of microcalcifications. One such mechanism is epithelial-mesenchymal transition (EMT) [298]. EMT is a biological process which allows polarized epithelial cells which normally attached to the basement membrane to undergo several biochemical changes that enable them to gain several properties of mesenchymal cells including the migratory capacity, invasiveness, elevated resistance to apoptosis, and increased in production of extracellular matrix (ECM) components. As the result of the EMT process, cells lose the polarity, gain the spindle morphology and enhance the migratory capacity [298]. It is important to mention that the cellular changes and rearrangements that occur with EMT are extensive and it is estimated that in human cells EMT changes the expression of about 4000 genes which represents approximately 10% of the entire genome [299]. It has been hypothesized that epithelial cells that acquire mesenchymal characteristics become capable of producing breast microcalcifications [298]. In contrast, the formation of benign microcalcifications are considered to be explained by cell necrosis and debris which could be explained by previous injury or surgery to the breast (fat necrosis), previous radiation to the breast, skin (dermal) or blood vessel (vascular) trauma of the breast [300].

Predictors of microcalcifications

The knowledge on predictors of mammographic microcalcification is limited. There are few studies investigated predictors of microcalcifications in the breast tissue [301-308]. However, most of these studies were only focused on the presence of breast arterial breast calcifications. arterial calcifications [302, 304, 307, 308]. Breast arterial calcifications are potential surrogate marker of atherosclerotic cardiovascular disease but are not associated with breast cancer [309]. These studies found higher age was associated with increased number of arterial calcifications. Additionally, current smoking [301, 304, 307, 310] and other factors that decreases the prevalence of arterial breast calcifications were higher age at first birth, few children and shorter period of breast feeding [301, 302, 304, 307, 308]. Finally, three case-report studies showed post-lactational increases of suspicious microcalcifications [303, 305, 306]. In Study III, we investigated the association between established risk factors for breast cancer and suspicious microcalcification clusters and their asymmetry.

RISK-BASED SCREENING

Early breast cancer detection decreases the risk of breast cancer mortality. All women are at substantial risk for breast cancer, however, several factors including genetic predisposition, number of affected first-degree relatives, and having dense breasts further increase a women's risk for breast cancer. For mammography screening to be effective, it is important that it identifies women at increased risk. In current population-based screening systems all women of a certain age are invited to screening and which normally means that both the same time interval between screenings and the same breast imaging method is used. Recently there is more concern on moving from a general one-size-fits-all approach screening to a more personalized, risk-based one that is tailored to the individual woman's risk, while accounting for cost, potential harms, and benefits. [311, 312].

Previously, risk prediction models such as Gail [313] and Tyrer-Cuzick models [314] were introduced with the aim of identifying women at high risk of developing a future breast cancer. Gail model which developed from the National Cancer Institute produces estimate of breast cancer risk in the next 5 years and lifetime risk using an eight-question tool namely age, first-degree history of breast cancer, history of breast diseases, reproductive and hormonal factors (age at first menstrual cycle and age at first birth of child), and ethnicity [313]. The Tyrer-Cuzick model is another commonly used model for the risk prediction of breast cancer and it estimates the likelihood of a woman developing breast cancer in 10 years and over the course of her lifetime. In the Tyrer-Cuzick model, similar factors from the Gail

model are using with addition of personal and genetic factors, including the BRCA1/2 genes [314]. A limitation of these risk prediction models is that neither of them included mammographic density in their models even though it is known as a strong risk factor for breast cancer. However, more recently it is been demonstrated that the combination of breast density with either of the Gail or the Tyrer-Cuzick model resulted a better breast cancer risk assessment [315]. Additionally, to date only one risk prediction model for breast cancer which developed in our group has included all mammographic features including mammographic density, microcalcifications and masses [316]. Since mammographic density is a dynamic trait, including mammographic density change in conjugation with other mammographic features and established breast cancer risk factors might increase prediction power of future risk prediction models.

2 AIMS

There are two major aims of this thesis. Firstly, to identify determinants of mammographic density *change* and its influence on breast cancer risk. Secondly, to identify predictors of mammographic microcalcifications and its impact on breast cancer risk.

The specific aims of my four papers were:
Study I: To study the association between established risk factors of breast cancer and mammographic density change.

Study II: To study the association between mammographic density change and risk of breast cancer.

Study III: To study the association between established risk factors for breast cancer and breast microcalcification.

Study IV: To study the association between breast microcalcifications and breast cancer risk.

3 MATERIALS AND METHODS

STUDY POPULATION

This thesis is based on data from the KARolinska Mammography Project for Risk Prediction of Breast Cancer (KARMA; www.karmastudy.org). The KARMA cohort is a populational-based prospective screening cohort comprising women attending mammography screening or clinical mammography in four hospitals in Sweden (Stockholm South Hospital, Helsingborg Hospital, Skåne University Hospital, Lund and Landskorna Hospital between January 2011 to March 2013. A total of 210,233 women were invited to participate to KARMA study and more than 70,000 women consented (consent rate = 34%). The highest proportion of consent rate was seen among women with age group of 65-69 years old (39%) and lowest among women in the age group 40-44 years (30%). Upon enrollment, all study participants were asked to respond to a detailed online questionnaire that contains approximately 250 questions related to breast cancer risk factors such as reproductive factors, hormonal, lifestyle factors, family history of breast cancer, benign breast diseases, medical history, and quality of life questions. BMI was calculated using self-reported height and weight. Processed and unprocessed (raw) full-field digital mammograms have been collected continuously every time KARMA participants underwent mammography at mentioned hospitals. Current rate is approximately 25,000 image per month. Further, upon study entry, all KARMA participants donated blood after they consented. Approximately 20,000 KARMA participants have been genotyped with iCOGS or Illumina Infinium OncoArray assay as a part of a collaboration with the Breast Cancer Association Consortium [317].

In ***study I***, the study base was made up 70,874 women who completed the baseline questionnaire in KARMA cohort and had the baseline mammogram. Women were excluded from the study if they not given the informed consent (n = 34), had missing information in age and/or BMI (n = 4,044), had previous breast cancer (n = 4,111), had other cancers (n = 3,432), had breast enlargement and/or breast reduction (n = 3,340), had other breast surgeries (n = 1,160), were younger than 40 and older than 70 years old (n = 3,192), women with < 3 mammography examinations (n = 18,588), and had screening interval outside 12 – 36 months (n = 1,191). A total of 31,782 women included in the final analyses.

The study base of ***study II*** is similar as study I, included 70,874 women with a baseline mammogram. Exclusion criteria were no informed consent (n = 34), missing information on age and/or BMI (n = 4,044), prevalent breast cancer (n = 2,934), women with previous

cancers (n = 3,478), breast enlargement and/or breast reduction (n = 3,377), other breast surgeries (n = 1,195), women with < 2 mammography examinations (n = 12,002). Thus, 43,810 women remained for analyses.

The base of ***study III*** consisted of similar number of women as study I and II. We included 70,874 women with a baseline mammogram. Exclusion criteria were no informed consent (n = 34), women without mammographic feature measurements (7,511), missing information in age/or BMI (n = 3,500), women with previous cancers (n= 3,316), breast enlargement and/or breast reduction (n = 2,115), other breast surgeries (n = 1,125). The final study group included 53,273 women.

In the ***study IV***, similar number of women as previous studies were included as the study base. Exclusion criteria were no informed consent (n = 34), women without mammographic feature measurements (n = 7,511), missing information in age/or BMI (n = 3,500), women with previous cancers (n = 3,316), breast enlargement and/or breast reduction (n = 2,115), other breast surgeries (n = 1,125). The final study group included 53,273 women.

Table 1. Overview of data source and study design used in each study

Study	Data source	Study design	Exposure	Outcome
Study I	KARMA	Prospective cohort	Lifestyle and reproductive factors, Exogenous hormones, family history of breast cancer	Baseline MD MD change
Study II	KARMA	Prospective cohort	Lifestyle and reproductive factors, Exogenous hormones, family history, baseline MD, MD change	Breast cancer risk
Study III	KARMA	Prospective cohort	Lifestyle and reproductive factors, Exogenous hormones, family history of breast cancer, polygenic risk score	Mammographic microcalcification clusters and their asymmetry
Study IV	KARMA	Prospective cohort	Mammographic microcalcification clusters and their asymmetry	Breast cancer risk

Abbreviations: MD = mammographic density

DATA COLLECTION

Questionnaire data

All women entered the KARMA study completed a self-administered web-based questionnaire that covers detailed information on established risk factors for breast cancer including, reproductive factors; age at menarche, age at first birth, number of children, breast-feeding duration, oral contraceptive use, MHT use, family history of breast cancer, previous breast diseases, and surgeries, and lifestyle factors; cigarette smoking, alcohol consumption and physical activity.

The KARMA questionnaire for alcohol consumption is based on the validated food frequency questionnaire MiniMeal-Q. The alcoholic beverages including in the questionnaire are in different type of beer, cider, wine and spirits [318]. Women reported the frequency and amount of the consumed beverages at least once per months during the months prior to completion of the questionnaire. Later, the frequency and the amount of each beverage were used to report the volume consumed per day. The volume was then multiplied by the ethanol concentration of each beverage to find the alcohol consumed per day and reported as gram per day (g/day). Further, data on physical activity was collected using Active-Q questionnaire and it includes; daily occupation, leisure time activity, sport, transportation from and to work, as well as duration of sleep [319]. Participants were also asked to report the frequency and duration of each activity during a day in order to calculate physical activity hour per day. MET value later calculated using the information provided by the participants multiple by the MET value for each activity obtain daily in order to get the MET-h/day. The calculation of alcohol gram/day and MET-h/day has been performed by the previous PhD candidate in our group (Thang Trinh) and used in previous publications [169, 320].

BMI (kg/m^2) was calculated using self-reported height and weight at study enrolment. Women reporting no natural menstruation over the past 12 months before the study entry or no menstruation due to oophorectomy were considered postmenopausal. Women with missing information on menstruation status or having no menstruation due to gynecological surgeries other than oophorectomy were considered premenopausal if they were age 50 years or younger and postmenopausal if older than 50 years.

Mammographic density data

Full-field digital processed mammograms from MLO and CC views of left and right breasts were collected to measure mammographic density. We used mammograms from MLO view, which is a routine projection during mammography screening in Sweden. Previous study using KARMA data reported a high correlation between mammographic density from MLO and CC views [321]. Mammographic density was measured using the area-based (cm^2) STRATUS method. For studies I – IV, we used average STRATUS over left and right breast dense area (cm^2). The percentage mammographic density was calculated by dividing the dense area by the total breast area. Briefly, STRATUS is a fully automated tool developed to analyze digital and analogue images using an algorithm that measures density on all type of images regardless of vendor. STRATUS uses machine learning techniques to estimates mammographic density [200].

In ***study I***, we used average STRATUS dense area (cm^2). In ***study II***, for women with breast cancer, mammogram from the contralateral breast (breast without having a tumor) and for women healthy we randomly selected mammograms from either left or right breast and followed mammographic density of the same breast to the of follow-up. We presented the results mainly using dense area, because as compared to percent density it is less influenced by BMI. However, in order to be in concordance with other published articles in measuring mammographic density, we also presented results using percent mammographic density.
Further, in ***study I*** and ***II***, we calculated mammographic density change across age and in studying repeated mammograms from the same woman, it is important to consider technical artefact between the mammograms into consideration. **Figure 8** illustrates that the same amount of breast tissue is not always found in two different mammograms from the same woman. To reduce this artefact, images should be aliened before density measurements are performed. The concept of alignment is described in detail in previous publication of our group [200].

Mammographic microcalcification data

Raw mammograms from the MLO and CC views of left and right breasts were collected. We used mammograms from MLO view and the CAD system used for identification of microcalcifications(iCAD; M-Vu iCAD®, Nashua, USA) [292] . iCAD is an FDA approved class 3 device (PMA number P010038) with an accuracy of 92% [293]. The algorithm of the system identifies suspicious microcalcification clusters that corresponds to

microcalcifications with a malignant morphology as defined by the BI-RADS 3-5 scores [293, 294]. The total number of microcalcification clusters of the breasts were calculated and the asymmetry was defined as the absolute difference between the numbers of microcalcification clusters between the breasts. **Figure 9** illustrates how microcalcification clusters are marked on the left and right MLO views using the iCAD software. We used microcalcification clusters rather than single microcalcifications since clusters are more likely a sign of cancer [279, 280]. For the ***study III*** and ***IV***, we used the total number of suspicious microcalcifications clusters and their asymmetry detected by iCAD.

Breast cancer diagnosis

Although the Swedish Cancer Register has overall responsibility and coverage for combining and reporting all cancer cases in Sweden, however, all detailed information for specific types is not included. The Swedish Cancer Register was found in 1958 and approximately it registered around 60,000 malignant cancer cases every year [322]. Since 1970s, six reginal registries situated at the major oncological centers were responsible for own breast cancer quality registers. In 2007 all these quality registers were combined and a national breast cancer quality register named INCA was launched. INCA register holds information on diagnosis of breast cancer such as date of diagnosis, invasiveness of the tumor, histological grade, ER status, and treatment of breast cancer in Sweden [323].

KARMA cohort holds breast cancer records for all KARMA participants who have diagnosed with breast cancer as a primary diagnosis, however, the information on breast cancer is restricted to information on first breast diagnosed with cancer, invasive and in situ cancers. To identify women with breast cancer we linked the records of women within the KARMA cohort using the Swedish personal identification number to the nation-wide cancer registry. In ***study II*** and ***IV***, the diagnosis of invasive breast cancer was ascertained through linkage to the Swedish INCA register.

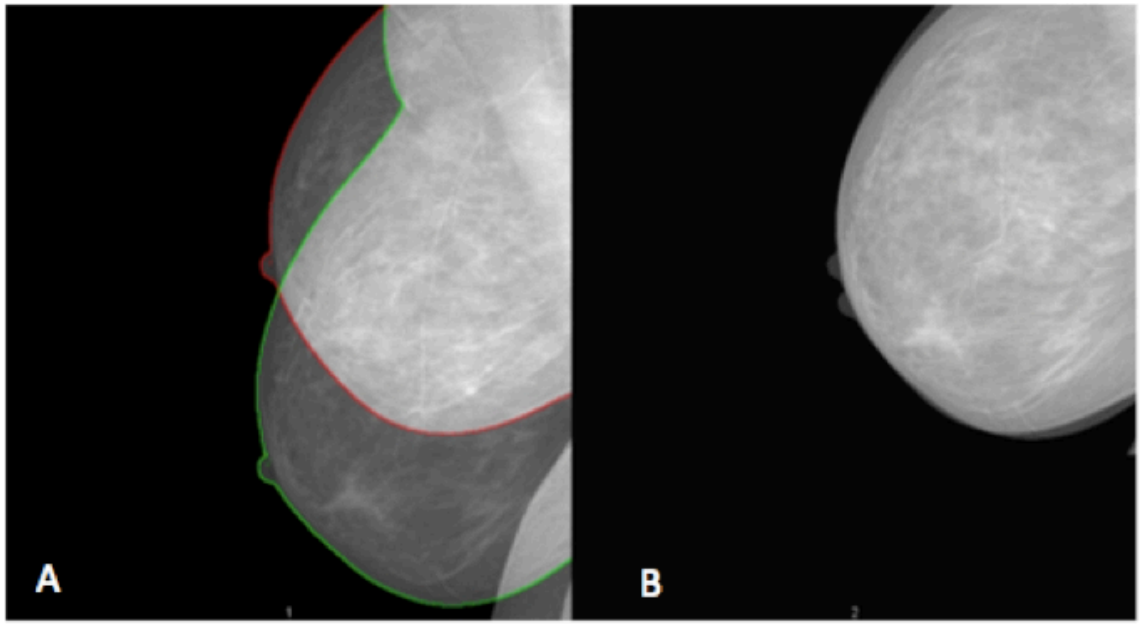


Figure 8. Two mammograms of the same breast were taken 2 minutes apart by the same radiographer. In Frame **A**, the mammograms were superimposed to show the difference in breast placement in the mammography machine. In Frame **B**, the two images were digitally aligned to the image showing the smallest breast size (outlined with red in Frame A) prior to density measurement

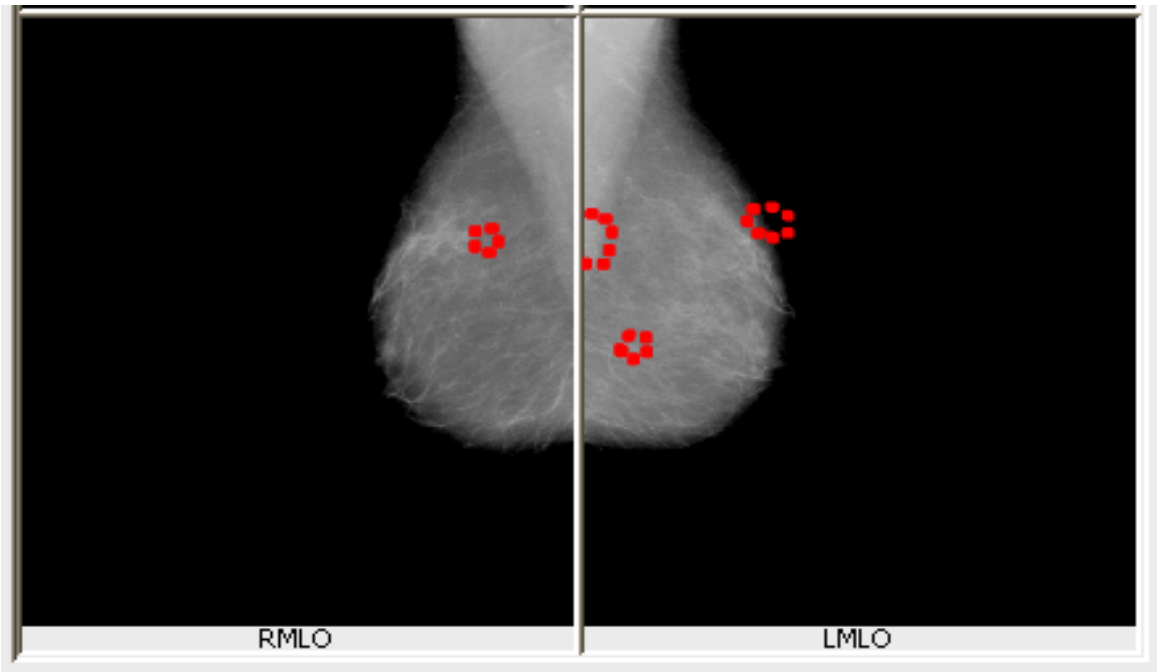


Figure 9. Illustrates suspicious microcalcification clusters s detected by a fully automated iCAD software on MLO views. *Source: iCAD*

STATISTICAL METHODS

All analyses in this thesis were performed using the statistical software R. The level of significance was less than 0.05, and all statistical tests were two-sided.

Linear regression model (Study I)

The linear regression model is generally used to examine the potential association between an exposure variable (X), which could be in form of either continuous, binary, or categorical with an outcome variable (Y), which is usually continuous. Univariable linear regression is used to investigate the association between the outcome variable Y and a single exposure variable X. The equation for simple linear regression model is based on the equation:

$$y = \beta 0 \ + \ \beta 1 x \ + \varepsilon$$

y is the outcome variable and x is the exposure variable, β_0 is the intercept (expected mean value of when x = 0). β_1 is the regression coefficient or slope, and interpreted as the mean difference in the outcome between two groups that differ with one unit in the exposure. Finally, ε is the error of the estimate, which is assumed to have a normal distribution with a mean 0 and represents the "residual variation" in the outcome, after conditioning on the exposure. An advantage of a regression model is that it can be used to adjust for covariates, or to consider multiple potential risk factor in one single model; the resulting model is referred to as "multivariable". When the 95% confidence interval (CI) for a coefficient in the linear regression model includes zero, it is statistically non-significant [324].

In ***study I***, linear regression models were used to estimate the association between established risk factors for breast cancer with baseline mean mammographic dense area (cm^2), together with 95% CIs. Mammographic density change was measured by one linear regression model that was fitted for each woman in the dataset, regressing all observed measurements of dense area during the follow-up. By doing this we obtained slopes that quantify the woman-specific dense area change. The obtained slopes were regressed on all established risk factors for breast cancer using one linear regression for each factor. These models were adjusted for age at first and last mammograms, baseline BMI, and baseline menopausal status. Interaction analyses were performed to examine if the menopausal status modify the association between determinants of mammographic density change.

We used local polynomial regression (LOESS curve) for qualitative assessment (visual) of how dense area changes as a function of established breast cancer risk factors. LOESS curve fitting is a method for fitting a smooth curve between an exposure and an outcome variable and it does not assume linearity.

In ***study I,*** after fitting a linear regression model, it is often desirable to use the fitted model to estimate the absolute mean of the outcome. This can be done with a method called "regression standardization" [325]. For a fixed value x of the exposure, the method uses the fitted model to create a predicted mean for each subject in the dataset. These predictions are averaged, to produce an estimate of the mean outcome under the hypothetical (counterfactual) scenario where everyone is exposed to level x.

Cox regression model (Study II and IV)

The Cox proportional hazard model is a common statistical technique for analyzing the effect of an exposure on a time-to-event variable. In other words, the Cox model is used for analyzing the length of time until the occurrence of a well-defined end point of interest for example, death or cancer diagnosis. The Cox model compares the ratio of the hazard rate of an event in the exposed group to the hazard rate in the unexposed group. Using this method, the model produces an estimated hazard ratio (HR). The Cox proportional hazards model relies on a fundamental assumption that the hazard ratio between the two groups remains constant over time. This assumption must always be checked using, for instance, the Schoenfeld residuals test. When analyzing time-to-event-data, typically not all study participants experience the event by the of the follow-up period; this is referred to as (right) censoring. A key assumption of survival analysis is "non-informative" censoring, conditional on the covariates that are adjusted for in the analysis. This means that the participants are lost censored due to reasons unrelated to the event of interest occurring [326], given the covariates.

In ***study II*****,** we used Cox proportional hazard regression with age as the underlying time scale to investigate the association between established risk factors for breast cancer and breast cancer risk. We reported hazard ratios (HRs) and 95% CIs adjusted for age and BMI at baseline. To investigate mammographic dense area change, we first calculated the relative mammographic dense area change per year between each consecutive pair of examinations. Relative dense area change was categorized as decrease (>10% decrease per year), stable (no change), and increase (>10% increase per year). Then we used Cox proportional hazard

regression to examine the association between relative density change with breast cancer risk, treating relative density change as a time-varying exposure. The associations were adjusted for age, BMI, and dense area at baseline. Finally, we used Cox proportional hazard regression to investigate whether adding dense area change could modify the association between baseline dense area and breast cancer risk. We tested the proportional-hazards assumptions using Schoenfeld residuals test and no major model violation was seen.

In ***study IV***, we used Cox proportional hazard regressions with age as underlying time scale to examine the association between microcalcification clusters and their asymmetry with the risk of breast cancer. Hazard ratios (HRs) and 95% CIs were reported. Microcalcification clusters asymmetry was coded as 0 (for women with no microcalcification clusters in any breast and/or women with symmetrical microcalcification cluster distribution between the breasts), 1-2 (for women with one to two) and ≥ 3 (for women with three or more asymmetric distribution of microcalcification clusters between the breasts). The Cox regression models were adjusted for BMI (continuous), baseline mammographic density (continuous), smoking status (categorical), alcohol consumption (continuous), age at menarche (continuous), age at first birth (continuous), number of children (continuous), breast feeding duration (continuous), oral contraceptive use (categorical), menopausal hormone therapy use (categorical), and family history of breast cancer (categorical). The proportional-hazards assumption was tested by using Schoenfeld residuals and no major model violation was observed. Finally, Cox proportional hazards regression was used allowing the interaction between microcalcification clusters and mammographic density on breast cancer risk. A global test was used to determine the presence of interaction.

<u>Logistic regression model (Study III, part of study IV)</u>

Logistic regression is a generalized linear model which works similar to linear regression; however, it deals with binary outcome (a binomial response variable). Clinical studies that evaluate the association of various exposure variables with a single binary outcome such as the presence or absence of a disease or death, often employ the method of logistic regression. Often, the regression coefficients in the logistic model are presented as the estimated Odds Ratio (OR) which are easier to understand and interpret than log odds ratio. An OR is a measure of association between an exposure and a binary outcome, and it is the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring the absence of that exposure. The OR takes values between "0" and infinity. One ("1") is the neutral value and means that there is no difference between the groups compared,

i.e. no association between the exposure and the outcome. So, if the 95% confidence interval of the OR includes 1, it means that the result is not statistically significant [327].

In ***study III***, we used logistic regression analyses to estimate the OR and to quantify the association between breast cancer risk factors with risk of having microcalcification clusters and their asymmetric distributions between the breasts. For this study, microcalcification clusters were coded as 0 (no microcalcification clusters in both breasts) and 1 (one or more microcalcification clusters in the breasts). Microcalcification clusters asymmetry was coded as 0 (for women with no microcalcification clusters in any breasts and/or women with symmetrical microcalcification cluster distribution between the breasts) and 1 (for women asymmetric distribution of microcalcification clusters between the breasts). All models were adjusted for age, BMI, and menopausal status at baseline.

In ***study IV***, we used logistic regression analyses to investigate the association between breast cancer tumour characteristics (in situ versus invasive and ER positive versus ER negative) and the presence of microcalcification clusters, among women who developed breast cancer during follow-up, while adjusting for potential confounders. For this analysis microcalcification clusters were coded as 0 and 1 similar to study III.

4 RESULTS

In this section, the main results from each individual study are presented. More detailed description of the results is available in the published articles and the manuscript.

STUDY I

In this study, we found statistically significant association between being at younger age and leaner with greater baseline mammographic density area compared with older and obese women. We also found a significant association between current smoking and lower baseline dense area, while the results showed that women drinking alcohol had greater baseline dense area. Finally, the results showed physical activity, lower age at first birth, having more children, lower age at menarche, more than 10 years since the last birth were significantly associated with lower mammographic dense area. Finally, current MHT user and women with family history of breast cancer had a greater mammographic dense area than those who did not use MHT and had not family history of the disease (Table 2 in Article 1).

We showed that the overall annual dense area change was -1.cm^2. Among lifestyle determinants, BMI was statistically associated with dense area change. Meaning that, lean women (BMI <20 kg/m^2) had a mean dense area change of -1.13 cm^2/y (95% CI = -1.25 to -1.02) compared with women with a BMI of 30 or more kg/m^2 -0.46 cm^2/y (95% CI = -0.57 to -0.35) (**Figure 10** A, the BMI-dependence difference in MD change is visible). Physical activity was another factor that showed to be associated with dense area change. Women with less than 40 MET-h energy expenditure per day to those with 50 or more MET-h/d, women with higher MET-h/day had an annual change in dense area that was -0.4 cm^2 larger than women with lower MET-h/day (**Figure 10 B**). Our results showed physically active women had a stronger decrease in annual dense area than less active women (Table 3 in Article 1).

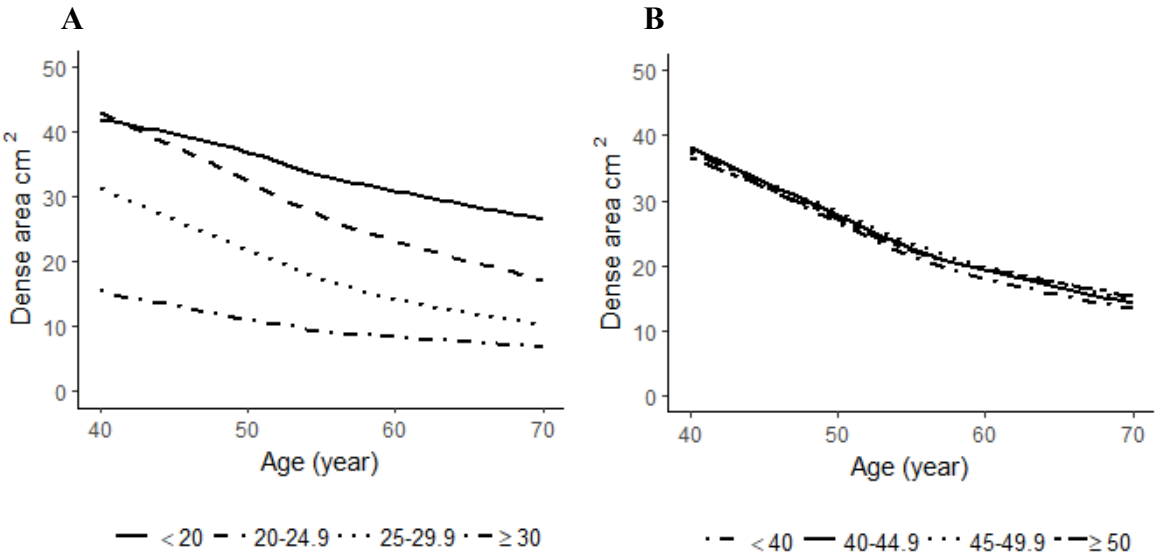


Figure 10. Mean baseline mammographic dense area (cm^2) as a function of age at mammography screening and established lifestyle factors including: **A**) body mass index (<20, 20–24.9, 25–29.9, ≥30 kg/m^2), **B)** physical activity (<40, 40–44, 45–49.9, ≥50 metabolic equivalent of task-h/d) at study entry.

Finally, other lifestyle factors, reproductive factors, oral contraceptive, MHT use, and family history of breast cancer did not seem to have a substantial effect of dense area change.

STUDY II

In study II, majority of women participated in the study (77.6%) had ≥3 round of mammography and some women had even up to five examinations. A total of 563 women were diagnosed with breast cancer during follow-up. The average follow-up was 5.4 years. We found that premenopausal women with high BMI (≥30 kg/m^2) had significantly lower risk of breast cancer (HR = 0.47, 95% CI = 0.24 to 0.92) compared to premenopausal women

with low BMI (<20 kg/m^2). No statistical association was found between postmenopausal BMI and risk of breast cancer. Women with older age, having the first child at older age, having family history of breast cancer, and postmenopausal women who were current MHT users had significantly higher risk of breast cancer (Table 2 in Article 2).

We found no difference in risk of breast cancer among women with stable dense area (HR = 1.01, 95% CI = 0.82 to 1.23, *P = 0.*90) and women with more than a 10% increase in dense area per year (HR = 0.98, 95% CI = 0.80 to 1.22, *P = 0.*90) as compared to women with an annual decrease of dense area 10%. Among premenopausal there was a weak but nonsignificant association between increase in annual dense area more than 10% and increase risk of breast cancer (Table 3 Article 2). Additionally, the results showed that only women age 40-49 years had a tendency of difference in risk in relation of dense area change. We found that women with an increase in annual dense area more than 10% had higher risk of breast cancer by 30% (HR = 1.30, 95% CI = 0.77 to 2.21) compared to perimenopausal women with greater than 10% annul dense area reduction, however, the results did not reach the statistical significance (Supplementary Table 2 Article 2).
Furthermore, we found that mammographic dense area change did not seem to influence the association between baseline mammographic density and risk of breast cancer. This showed that baseline mammographic density is an independent risk factor for breast cancer with no additional value of adding mammographic density change to the model (Table 4 Article 2).
Finally, we performed analyses to determine the correlation between mammographic dense area with BMI as well as the correlation between percent mammographic density with BMI. We found that both mammographic dense area and percent density were both negatively correlated with BMI, however, the correlation was stronger for percent density (ρ = -0.50) as compared to dense area (ρ = -0.30).

STUDY III

In study III, the majority of women included in the study had no microcalcification clusters (82.7%). Older women (age >60) had two times higher risk of having suspicious microcalcification clusters (OR = 2.51; 95% CI = 2.28-2.77) as compared to younger women. Lifestyle factors associated with lower risk of having suspicious microcalcification clusters were high BMI, former and current smoking and moderate alcohol consumption (0.1-10 g/day). We found that overweight (BMI, 25.0-29.9 kg/m^2) and obese women (≥30 kg/m^2) had nearly 20% lower risk compared to women with a normal BMI. Former and current smokers also had nearly 10% lower risk compared to never smokers. Women with moderate alcohol

consumption had lower risk of having suspicious microcalcification clusters (OR = 0.87; 95% CI = 0.82-0.92) compared to non-drinkers (Table 2 Article 3).

Among reproductive factors and exogenous hormonal factors, women with higher age at first birth >25 years had lower risk of suspicious microcalcification clusters. Higher age at menarche was also associated with 8% reduction in risk of having suspicious microcalcification clusters. Both oral contraceptive and MHT users had lower risk of having suspicious microcalcification clusters. In contrast, having >2 children and women who breast-fed more than 1 year had significantly increased risk of having microcalcification clusters than nulliparous women and women who never breast-fed.

Finally, women with high dense area (dense area >40 cm^2) had two times higher risk of having microcalcification clusters compared to women with low dense area (dense area <9.0 cm^2). Similarly, we found women with first-degree family history of breast cancer and women in the highest quintile of polygenic risk score for breast cancer had significantly higher risk of having microcalcification clusters.

Similar results as for the association between established risk factors for breast cancer and microcalcification clusters were seen when assessing the relationship between risk factors and asymmetry of microcalcification clusters.

STUDY IV

In study IV, a total of 676 women were diagnosed with breast cancer during the average of 5.4 years follow-up. Overall, we found that women with ≥ 3 microcalcification clusters had increased the risk of breast cancer by 2 times (HR = 2.17; 95% CI=1.57 to 3.01) compared to women with no clusters. The results were more pronounced among premenopausal women (HR = 2.93; 95%CI = 1.67 to 5.16). Similar results were seen when assessing the asymmetry of microcalcification clusters and risk of breast cancer. Additionally, we found that women at highest category of dense area had (dense area >40 cm^2) nearly 3 times higher risk of breast cancer compared to women at the lowest category (dense area <9.0 cm^2) while adjusting for potential confounders (HR = 2.87; 95%CI = 2.13 to 3.86). Similar to the association between microcalcification clusters and risk of breast cancer, the results for the association between dense area and breast cancer risk was more pronounced among premenopausal women (HR = 5.09; 95%CI = 2.28 to 11.41) (Table 2 Article 4).

We did not find an interaction between microcalcification clusters and baseline mammographic dense area with risk of breast cancer ($P_{interaction}$ = 0.50).

Finally, we examined the association between presence of microcalcification clusters and risk with breast cancer types. We found that presence of microcalcification clusters were significantly associated with both in situ (OR = 3.46; CI = 2.03 to 5.83) and invasive breast (OR = 1.84; CI = 1.47 to 2.30) cancer when comparing to women without breast cancer. Microcalcification clusters were not associated with ER status (Table 4 Article 4).

5 DISCUSSION

STUDY I

In this study, we examined the association between established risk factors for breast cancer with both baseline mammographic dense area and dense area change across age. We found that a number of factors to be associated with baseline dense area while few of these factors were associated with dense area change over time.

In agreement with previous findings we showed that baseline dense area is associated with age, lifestyle factors, reproductive and hormonal factors, except for oral contraceptive use which we did not see an association [241, 243, 274, 328, 329]. Surprisingly, we observed that longer duration of breast-feeding was associated with greater baseline dense area. This finding is in line with our previous findings which has been shown that breast-feeding is associated with greater proportion of epithelial tissue [330]. Interestingly, we also found that shorter time since last birth to be associated with greater baseline dense area. This finding has been observed previously by Gertig et al. that found significance increase in proportion of epithelial tissue nearly 10 years since last birth [331]. This might also explain partly the short-term increase risk of breast cancer after childbirth.

Further, we found that beside age, lean and physically active women had the greatest reduction in dense area. The greater decline in mammographic density among lean women was observed previously [273, 274] and it could be explained by several hormonal-related mechanisms including the lower conversion of androgenic precursors to estrogen through peripheral aromatization in adipose tissue during postmenopausal [332, 333]. The slower reduction in mammographic density over time which is seen among obese women could be also explained by the increase of insulin-like growth factor levels that is found to be associated with higher cell proliferation and increase risk of breast cancer [334].

There are also several hormonal-related mechanisms behind the association between physical activity and dense area. Physical activity is found to be associated with reduced level of and cumulative exposure to sex steroid hormones during premenopausal period. Additionally,

physical activity is showed to be related to reducing the amount of estrogen-producing adipose tissue. [167, 235]. These biological mechanism may partly explain the association between greater reduction in dense area among physically active women. A greater decrease in mammographic density may influence the risk of breast cancer and this should be investigated further in large prospective populational-based cohorts using longitudinal data.

STUDY II

In this study, we investigated the association between established risk factors for breast cancer and risk of breast cancer. Furthermore, we examined the association between mammographic dense area change with age as the underlying time scale and risk of breast cancer among both pre and postmenopausal women. Finally, we evaluated if adding mammographic dense area change to baseline dense area might be a better method to predict women's risk of breast cancer than only using a single measure of mammographic density.

Overall, we found that high premenopausal BMI to be associated with lower risk of breast cancer. Also, our results were in line with previous findings which showed that high baseline mammographic dense area, older age, high age at first birth, postmenopausal MHT use, and having family history of breast cancer were associated with increased risk of breast cancer [132, 154, 161, 335, 336]. However, the results for the association between postmenopausal BMI and breast cancer risk remained nonsignificant.

We found no evidence of association between annual mammographic dense area change and risk of breast cancer which agrees with previous findings [274, 277, 337]. However, studies which did not observed any association between density change and risk of breast cancer, suggested that reduction in density might occur at around menopause, thus perimenopausal age may be a critical period to investigate this association. Therefore, in a sensitivity analysis we tested the association between dense area change and risk of breast cancer only among women age 40-49 years considering as perimenopausal. We observed a tendency of a difference in risk related to dense area change meaning that women with an increased in annual dense area greater that 10% had 30% higher risk compared to perimenopausal women with greater 10% annual reduction, however, we did not reach the statistical significance due to small sample size.

Finally, we observed that change mammographic dense area did not seem to influence the strong association between baseline dense area and risk of breast cancer. This could be explained by the fact that majority of women at menopause experience the involution (a process that breast epithelial process gradually transformed to fatty tissue) and this transformation at around the rate of 10% per year might be of lesser importance as compared

to the risk of breast cancer associated with baseline dense area. Nevertheless, our study indicated that the change in mammographic density at younger age (perimenopausal age) might be of importance for evaluating the risk of breast cancer and it should be investigated further.

STUDY III

Presence of microcalcifications is one of the earliest mammographic sign of breast cancer. Previous findings showed that microcalcification are present in nearly 50% of nonpalpable breast cancers found by mammography [338]. However, the knowledge on the biological mechanisms and predictors of mammographic microcalcification is limited. In this study we examined the association between established risk factors for breast cancer and microcalcification clusters and their asymmetry. We found that older age, high baseline mammographic density, family history of breast cancer and high polygenic risk score for breast cancer, and having more children, and breast feeding more than 1 year are associated with increased risk of clusters microcalcification presentence.

The finding on the association between increase age and higher prevalence of microcalcification clusters supports the hypothesis of EMT process in formation of microcalcification clusters. Since previous studies suggested that there is an association between increase age and EMT process [339]. Additionally, the mechanisms regarding higher mammographic density and higher risk of microcalcification clusters presence is unclear, however, previous findings showed that there is an association between higher epithelial component with increased matrix rigidity and tissue stiffness which could promote EMT process [340, 341].

In contrast, we found that factors such as high BMI, smoking, alcohol consumption, late age at first birth, use of oral contraceptive and MHT were significantly associated with lower risk of microcalcification clusters. This association could be partly explained by the association between estrogen exposure and microcalcification presence. These results confirmed the previous findings on the inverse association between estrogen and microcalcifications [310, 342]. However, the majority of these studies included the breast arterial calcifications.

To conclude, our results suggested that the majority of lifestyle and reproductive risk factors for breast cancer *protect* against presence of microcalcification clusters, which reflects the complexity of biological mechanism behind microcalcifications. More research is required to understand mechanism in relation to formation of mammographic microcalcifications.

STUDY IV

In this study we examined the association between microcalcification clusters with risk of overall and subtype specific breast cancer. Additionally, we investigated if baseline mammographic density modifies the association between microcalcification clusters and risk of breast cancer.

We found that greater number of microcalcification clusters to be strongly associated with increased risk of breast cancer. The results were pronounced among premenopausal women as compared to postmenopausal women. Interestingly, we found in postmenopausal women microcalcification clusters and mammographic dense area influence the risk of breast cancer to the same extent. High baseline dense area was associated with risk of breast cancer and the result was more pronounced among premenopausal woman. We found no interactive effect of microcalcification clusters and mammographic dense area with risk of breast cancer. Finally, the results showed that microcalcification clusters were associated with both in situ and invasive breast cancer.

Despite a well-established association between microcalcification clusters and breast cancer, few studies have investigated the association between these two entities and they have several limitations such as inability to exclude microcalcifications without malignant potential[301], using crude, qualitative and reader-dependent measure of mammographic features (BI-RADS score) [343-346], inability to investigate the joint effect of mammographic density [301, 344-346], and not including invasive breast cancer [344, 345], not considering menopausal status [301], and using case-control designs rather prospective cohort design[301, 346]. In this study we addressed all the limitations mentioned regarding previous studies. More importantly to reduce subjectivity in measuring mammographic features, we used a CAD system which developed to mimic the BI-RADS classification and target microcalcifications as BI-RADS 3-5.

Our results suggested, it could be that microcalcification clusters will become a complementary risk factor for breast cancer, while mammographic density indicates a general risk of breast cancer, microcalcifications have the potential to indicate not only where the breast cancer will develop but also when it will emerge.

6 METHODOLOGICAL CONSIDERATIONS

STUDY DESIGN

Epidemiological study designs are typically grouped into *observational* and *experimental* studies. The majority of studies conducted are observational studies and each study design has their own strengths and weaknesses. In observational studies, the investigator is observing the natural relationship between the exposures and outcomes, without making interventions. Observational studies are useful for studying various research questions, particularly when an experimental study might be unethical or not feasible to conduct. In experimental study designs, the investigator tries to intervene at some point through the study, the most common and strongest experimental study design is a randomized controlled trial (RCT). The main difference between an RCT and an observational study is that the exposure of interest (intervention) is controlled by the investigator and study participants are allocated randomly to receive one of the clinical interventions. In the RCT design one intervention is usually the standard of comparison or control, which is known as placebo [347].

Our studies have all been observational in nature and we have used prospective cohort designs. The word "cohort" has been adopted into epidemiology from the Latin word *cohors* to define a set of people that have been followed over a period of time. A major advantage of the cohort study is the ability to evaluate multiple outcomes associated with a single or multiple exposures in a single study. Important disadvantages of cohort design are; maybe expensive to conduct and being time-consuming. Cohort design can be in form of prospective or retrospective. In prospective cohort study participants are enrolled before the outcome of interest has occurred and then followed for a period of time. In contrast, retrospective studies are conceived after both the exposure and the outcome has already occurred, meaning that some participants have already developed the outcome of interest. A prospective cohort study design is ranked higher in the hierarchy of evidence than retrospective design and this is because the outcome, predictors, and confounding variables can be better measured and controlled. Therefore, it provides strong evidence for temporality of exposure – outcome relationship (exposure must precede the onset of outcome)[348].

Our studies have several strengths related to the study design such as; the use of prospective cohort study, including large number of study participants, detailed information on the

established risk factors for breast cancer collected using a comprehensive web-based questionnaire. Further, using validated questionnaires; Active-Q and MiniMeal-Q to assess physical activity and alcohol consumption, respectively. Additional strengths are access to repeated and longitudinal measurements of mammographic density from a same women (Study I and II). The use of Swedish INCA Register and possibility of linkage to personal identification number, eliminate loss to follow-up which is the important strength of the study II and IV.

SELECTION BIAS

Selection bias is a systematic error and it comes from any error in selection of the study participants and/or from factors affecting the study population. Selection bias occurs when the participants in the study are not representative of the target population. The participants in all four studies are the attendees in the Swedish national mammography screening program and later agreed and consented to participate in KARMA cohort study. Previous results have shown that women with low socioeconomic status including low income or low educational level have a higher rate of non-attendance to mammography screening and non-attenders are at higher risk for advanced breast cancer [349]. Women participated to KARMA study were healthy with self-perceived high risk of breast cancer due to familial risk of breast cancer. Therefore, these women might be more prone to participate to the cohort, which is reflected by a higher incidence of breast cancer in the KARMA cohort than in nationwide cohort. Additionally, we observed that approximately 13.0% of KARMA women included in the studies had first-degree relative with breast cancer. This effect is known as the *volunteer bias* which occurs when the study participants who volunteer to participate in a study are different from the general population.

INFORMATION BIAS

Information bias is another major class of systematic error which arise from errors introduced in measuring the exposure, the outcome, or the covariates, and it is also referred to as misclassification bias. There are two types of misclassification; (i) misclassification of exposure which arises when the error occurs during data collection of the exposure and (ii) misclassification of outcome which derives from errors in collection of the outcome data. Misclassification can be in form of differential and non-differential. Differential misclassification happens when the information on the exposure is influenced by the outcome

of interest or when the information on the outcome is influenced or related to the exposure. If the misclassification is non-differential between exposed and un-exposed group it is likely to attenuate the exposure-outcome association towards the null value. If the misclassification is differential between the exposed and unexposed groups, it can either overestimate or underestimate the effect.

Assessment of established risk factors for breast cancer

In this thesis, the data on lifestyle, reproductive, and exogenous hormonal factors were collected using self-reporting questionnaire and therefore is prone to information bias. The bias may arise from social desirability in self-reported questionnaire, which means that the study participant chooses responses which are more socially desirable or acceptable rather than perfectly accurate. This can be seen in questions related to lifestyle factors such as smoking, alcohol consumption, or physical activity. Further, the bias in self-reporting questionnaire could also be related to recall bias, which happens when the study participant does not remember previous events or omit important details. This might be seen more among older participants with cognitive problems, for example, it may be difficult to recall the age of onset for the question regarding first-degree family history of breast cancer. In our studies, since study participants were not aware of their mammographic density, annual density change, and presence of microcalcification clusters as well as the potential associations between established risk factors with mammographic features and risk of breast cancer, differential misclassification of exposure is unlikely. If anything, the effects could therefore be expected to be diluted.

Measurement of mammographic features

In this thesis, for measuring mammographic density we used a fully automated tool, STRATUS which is based on machine learning algorithms to measure mammographic dense area and percent density. Using this method for measuring mammographic density reduces the risk of misclassification of density measures that might be present in studies using crude and subjective measures such as BI-RADS scores. Furthermore, we used a novel approach of aligning mammograms before density measurements were performed, this is important when measuring mammographic density change over time. Aligning mammograms is mostly important when analyzing factors that might influence the size of the breast, such as BMI and physical activity. Using aligning technique reduces the risk of information bias in measuring mammographic density change over time.

Finally, a novel CAD system used to detect suspicious microcalcification clusters. iCAD is an FDA approved with an accuracy of 92% that identifies suspicious microcalcification clusters corresponding to microcalcification with malignant morphology as defined by the BI-RADS 3-5 scores. Previous results have shown that the iCAD software is achieving a sensitivity of 92% in detecting suspicious microcalcification clusters [292, 293] with average specificity of 87% [295]. The software marks regions of interest on standard mammographic views to bring them to the attention of the radiologist. Using CAD system might reduce the risk of information bias; however, it is possible that some of the microcalcifications detected were breast arterial calcifications. Breast arterial calcifications are benign findings which are not associated with risk of breast cancer.

CONFOUNDING BIAS

A *confounder* is a risk factor that influence the relationship between an exposure and an outcome, thereby "distorting" the true exposure-outcome association. There are several ways of minimizing confounding in the design phase of a study using randomization, restricting the enrollment criteria, matching, as well as methods for adjustment during the analysis. However, there might be always unmeasured (aka residual) confounders in the study. For example, in our studies we have excluded women with previous breast surgeries. Previous breast surgeries affect the whole breast area which is an important factor when measuring mammographic density and density change and it could reflect previous benign diseases which might affect breast cancer risk. Thus, this exclusion was performed to eliminate the potential confounding effect.

At the stage of statistical analysis, we have always used multivariable regression models and stratification to adjust for potential confounders. As an example, to investigate the association between microcalcification clusters and risk of breast cancer we adjusted the analysis for baseline age. This is because older women have a greater number of microcalcification clusters and a higher risk of breast cancer compare to younger women. Thus, if age were not adjusted for, we could overestimate of the true association between microcalcification clusters and risk of breast cancer.

7 CONCLUSIONS

In this thesis, we have provided a better understanding of how mammographic density *changes* over time, identified potential determinants of mammographic density *change,* and, investigated whether density *change* may be a better method to predicts a woman's risk of breast cancer than a single measure of density. Further, we have tried to understand the potential predictors of another important mammographic feature; microcalcification clusters. Lastly, we have conducted the first comprehensive prospective cohort study to investigate the influence of suspicious microcalcification clusters on breast cancer risk while using an FDA approved CAD system to detect the suspicious microcalcification clusters. From each studies, few conclusions might be noted:

- On average mammographic dense area declines by 1.0 cm^2 per year. Beside age, BMI, and physical activity were the only determinants of mammographic density change. We observed that lean and physically active women had the largest reduction in mammographic density per year.

- Our results suggested no evidence of association between mammographic dense area change and risk of breast cancer. Nevertheless, our results indicated a weak and statistically nonsignificant association between density change and risk of breast cancer among perimenopausal women (age 40-49 years). This finding suggests that density change at a younger age might be of importance.

- Most established risk factors for breast cancer, with exception of age, mammographic density, first-degree relative with breast cancer, polygenic risk score of breast cancer, having more children, and breast-feeding more than 1 year might protect against presence of microcalcification clusters. However, it is important to mention that knowledge on possible mechanisms by which microcalcifications are formed in the breast tissue is still limited.

- Microcalcification clusters with a malignant potentials were an independent risk factor for breast cancer and had a similar effect as mammographic density on breast cancer risk, at least the association was observed during postmenopausal part of life.

- Taking all these studies together we achieved a better understanding on determinants of important mammographic features; *mammographic density* and *mammographic microcalcifications* and their influence on risk of breast cancer. Findings in this thesis could be used further in development of risk-based screening using information available in the mammogram images.

8 FUTURE PERSPECTIVE

In recent years, the incidence of breast cancer has been increased, it is currently the most common type of cancer among women worldwide. Changes in reproductive and lifestyle patterns are main factors contributing to this increase. However, breast cancer mortality rate began to decrease in many part of the world which reflects improvement in breast cancer treatment and possibly early detection via mammography screening. Overall, the routine (age-based) mammography screening has been shown to reduce mortality for women at average risk of breast cancer, however, for those women at increased risk the start age and screening interval remain still unclear. For mammography screening to be effective, it is important that it identifies women at increased risk, and this requires moving from the general age-based screening to a screening regimes which tailors to individual's risk.

Over the past years, given the strong association between mammographic features including mammographic density and microcalcifications with the risk of breast cancer, it has increased attention to design future breast cancer screening strategies (risk-based) while considering mammographic features. Implementing screening strategies based on women's levels of risk requires more complex interactions between healthcare providers and the target population. Therefore, further investigations on breast cancer risk perception and risk communication among both screening participants and professionals are required.

The association between high baseline mammographic density and presence of malignant microcalcifications with the risk of breast cancer is well documented. Breast density can be also potentially be used as a marker to observe normal *changes* of breast tissue known as *involution*. In future studies, integrating mammographic features with analysis of cellular composition changes, and molecular markers in blood (proteins and biomarkers) possibly identifying proteins in plasma which are associated with mammographic density and mammographic microcalcifications may provide a better understanding on the associations between mammographic features and risk of breast cancer. Applying findings from these studies may help to identify women with an increased risk and perhaps, prevent breast cancer. Additionally, future studies should investigate the molecular pathways interconnecting microcalcification genesis with breast tumorigenesis.

9 ACKNOWLEDGEMENTS

Here I would like to express my sincere gratefulness to every past and current MEBers, and especially to:

My brilliant and patient main supervisor **Per Hall,** for your endless support in both research and personal life. I cannot thank you enough for your guidance and encouragements during this memorable journey. Dear Pelle, I am so happy when you mentioned me as one of your *"children at work*". Thank you, for making this journey as perfect as it can be and I look forward to future collaboration.

Kamila Czene, my wonderful and kind co-supervisor, for your constructive feedback and support. You are truly a great inspiration for me and for all female researchers. I feel extremely lucky to have you as my co-supervisor and I look forward to future collaboration.

Arvid Sjölander, my amiable co-supervisor, I have learned a lot about biostatistics and statistical thinking from you. Thank you for your help and sharing your knowledge with me during these years. Beside all, I truly enjoyed biostatistics/epidemiology courses that organized by you. You are truly one of the best teachers I ever had during my education.

Marike Gabrielson, my "Big Sister"-like co-supervisor, for your willingness to share your insights and knowledge with others. I am so thankful to your constructive feedback and supports. I truly enjoyed our "biology discussions" in the corridor and will always remember them. I feel so fortunate to have you as my co-supervisor. I wish we can work more together in future!

My co-authors for the studies, including **Mikael Eriksson** and **Roxanna Hellgren**, for your wonderful comments and collaboration.

Many thanks to **Ami Rönnberg** and **Alessandra Nanni** for your kind support with all important administrative stuff.

I also want to acknowledge my officemate **TingTing Huang**, you have your place in my heart and mind. Thank you so much for being kind and caring. I learned a lot from you and I feel so lucky to know you.

Additionally, I want to mention other members (past and present) in our research group: **Matias Hammarström**, **Jose Tapia**, **Aki Tuuliainen, Haomin Yang**, **Wei He**, **Fredrik Strand**, **Emilio Ugalde-Morales, Erwei Zeng**, **Xinhe Mao**, **Jingmei Li,** and **Natalie Holowko**. Past and present Mebbers for creating a great research environment, **Jingru Yu**, **Maya Alsheh Ali**, **Fang Fang**, **Yudi Pawitan**, **Marie Reilly**, **Jonas Ludvigsson**, **Camailla Ahlqvist**, **Gunilla Sonnebering**, **Gunilla Nilsson Roos**, **Miriam Lashkarani**, and many more!

None of these works would have been possible without the availability of data. For this I wish to acknowledge: **All participants** in the KARMA study and **personnel** for this devoted work during data collection. Funding, KARMA was mainly funded by the substantial donation from **Märta and Hans Rausing's Initiative Against Breast Cancer**.

My previous supervisor and lifetime mentor Prof. **Zorana J. Andersen** and wonderful collaborators Prof. **Elsebeth Lynge** and Prof. **My von Euler-Chelpin** from Copenhagen University. Thank you dear Zorana for introducing me to the field of breast cancer epidemiology and mammographic density. I am so lucky to know you.

My friends, included but not limited to: **Sahel** & **Sara Zamani, Amineh Ghaderi**, **Marzieh Choupani**, **Arian Lundberg**, **Afsaneh Mahmoodnejad** & **Nima Pourmohamadi**, **Azadeh Afifi**, and **Dr. Maryam Tabatabai** – for your friendship, kindness, and fun moments together!

My neurosurgeon **Dr. Jula Pohjola**, you are truly a life-saver, I am forever grateful to you. My physiotherapist **Sonja Syngg**, words cannot describe how grateful I am for you. Thank you for taking such a great care of me.

My grandparents & my aunt, **Mehri, Moustafa,** & **Gita Vahdati**, I will forever be grateful for your love, kindness, and care.

**

To my parents, **Maryam** & **Jalal** for your continuous support and constant steadfastness throughout my life and education. Your love means everything, and I am so, so grateful for having you. Without you, I most certainly would not be where I am today. You are my everything!

To my little brother, **Alireza** for friendship and support.

Last but most important of all, to my love, my husband **Morteza** for being so patient and supportive. Thank you for being always there for me. You make my life fun and filled with love. ♥

**

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"When you do things from your soul, you feel a river moving in you, a joy".

— Rumi