Breast Cancer Prevention – lifestyle modifications or targeted interventions?

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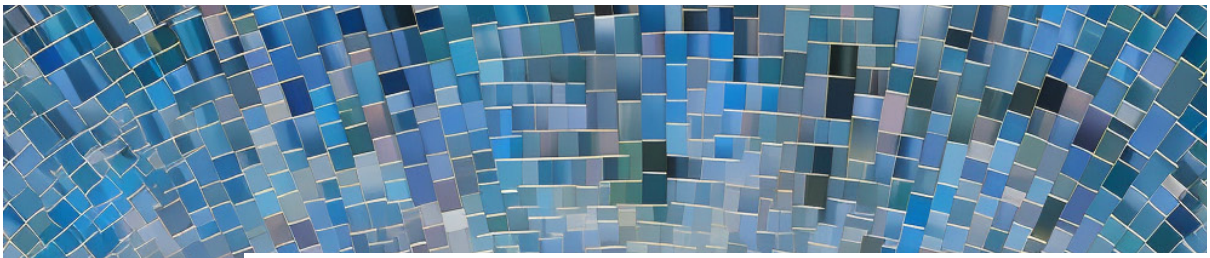

Breast Cancer Prevention

– lifestyle modifications or targeted interventions?

ÖYKÜ BORAKA
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Öykü Boraka

LUND
UNIVERSITY

DOCTORAL DISSERTATION

for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on February 6, 2024, at 09.00 in Segerfalksalen, BMC A10, Sölvegatan 17, Lund.

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Title and subtitle: Breast Cancer Prevention–lifestyle modifications or targeted interventions?

Abstract:

Breast cancer is now the most frequently diagnosed cancer in the world. Breast cancer prevention has gained increasing attention due to increasing breast cancer incidence rates in the West and can be actualized through lifestyle modifications or targeted interventions. Mammographic breast density (MBD) is a well-established independent breast cancer risk factor that is not well-understood on a molecular level. The aim of this thesis work was to offer further insight to breast cancer prevention with a special focus on mammographic breast density.

We first investigated physical activity as a lifestyle modification in studies I and II. In study I, we found that physical activity of at least 1 hour walking per day was associated with an overall breast cancer risk reduction by 23%. The risk reduction was predominantly observed for women who exercised during or after menopause and women who had lower-middle and upper-middle values of body composition. Study II examined physical activity in relation to MBD, mammographic appearances, and mode of breast cancer detection in breast cancer patients; we found no association for any of the mammographic features.

We also investigated FGF/FGFR1 system and tamoxifen responses as potential targets for intervention in studies III and IV. In study III, we showed that FGFR1 expression was upregulated in almost 60% of tumor tissues versus tumor-adjacent tissues from the same patients. We further showed that FGFR1 expression was associated with less favorable tumor characteristics. We noted associations between *FGF* ligand expression and MBD. Study IV showed that tamoxifen inhibited the proliferation, disrupted the cell cycle progression, and inhibited the ECM adhesion capacity of healthy breast epithelial cells.

In conclusion, we offer further evidence on physical activity as a breast cancer preventive measure with details on life stage and body composition. We studied mammographic appearances and mode of breast cancer detection in relation to physical activity for the first time and also proposed novel findings supporting a role for the FGF/FGFR1 system in MBD-driven breast carcinogenesis. Mechanistic insight for tamoxifen as a breast cancer preventive drug was also elucidated.

Key words: breast cancer, breast cancer prevention, physical activity, mammography, breast density

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Erkek egemen dünyaya inat, özünden kopmadan var olmaya çalışan bütün hemcinslerime,

En çok kendime,

Table of Contents

Abstract

Breast cancer is now the most frequently diagnosed cancer in the world. Breast cancer prevention has gained increasing attention due to increasing breast cancer incidence rates in the West and can be actualized through lifestyle modifications or targeted interventions. Mammographic breast density (MBD) is a well-established independent breast cancer risk factor that is not well-understood on a molecular level. The aim of this thesis work was to offer further insight to breast cancer prevention with a special focus on mammographic breast density.

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Popular science summary

One in every eight women get a breast cancer in her lifetime. If we understand breast cancer and prevent it from occurring in the first place, then we can save the lives of millions of women worldwide. There are many preventive measures that are readily available and being studied such as surgery, lifestyle modifications, or medications.

The recommended preventive measures differ depending on the breast cancer risk of each woman. Women with a very high risk for reasons like familial history can have preventive measures as drastic as full or partial removal of the breast. In other cases, such as a having a dense breast (which is a risk factor), drugs that reduce density can reduce risk, but these are not yet approved in Sweden. Lifestyle changes such as being physically active are breast cancer preventive measures recommended for everyone to maintain health.

In this thesis work, we investigated breast cancer prevention with a focus on breast density. In study I, we showed that women who engaged in physical activity equivalent to or more than one hour of walking per day had a breast cancer risk reduced by almost 25%. The risk was reduced mostly for women who exercised during or after menopause or women who had average bodies. Study II evaluated physical activity in relation to mammographic features that are important for the detection and course of the disease. We showed no relationship between these.

Study III investigated a biological pathway (the so-called fibroblast growth factor system) in relation to breast density and cancer. We showed that some factors in this pathway were increased in nearly 60% of breast tumors versus tumor-adjacent tissues of the breast or associated with breast density, thus indicating the cancer-promoting roles of this system, which needs further investigation. Study IV studied the effects of breast cancer treatment tamoxifen on healthy breast cells and brought potential explanations on the clinically observed breast density and cancer risk-reducing aspect of tamoxifen.

In conclusion, we reported strong evidence on physical activity as a breast cancer preventive measure and identified groups of women who benefited the most. We also offer new insight on physical activity in relation to mammography. We then proposed a biological pathway as a potential target to be further investigated for breast cancer prevention mediated by density. We also offer mechanistic insight on tamoxifen in support of clinical investigation as a preventive measure for women with dense breasts. Taken together, the findings of this thesis increase the knowledge about breast cancer prevention.

Populärvetenskaplig sammanfattning

En av åtta kvinnor får en bröstcancer under sin livstid. Om vi förstår bröstcancer och förhindrar att den uppstår i första hand, då kan vi rädda livet på miljontals kvinnor världen över. Det finns många förebyggande åtgärder som är lättillgängliga och som studeras, såsom kirurgi, livsstilsförändringar eller mediciner.

De rekommenderade förebyggande åtgärderna skiljer sig åt beroende på risken för bröstcancer för varje kvinna. Kvinnor med en mycket hög risk av skäl som familjehistoria kan ha förebyggande åtgärder så drastiska som helt eller partiellt avlägsnande av bröstet. I andra fall, som att ha ett tätt bröst (vilket är en riskfaktor), kan läkemedel som minskar densiteten minska risken, men dessa är ännu inte godkända i Sverige. Livsstilsförändringar som att vara fysiskt aktiv är bröstcancerförebyggande åtgärder som rekommenderas för alla för att behålla hälsan.

I detta avhandlingsarbete har vi undersökt bröstcancerprevention med särskilt fokus på brösttäthet. I studie I visade vi att kvinnor som ägnade sig åt fysisk aktivitet motsvarande, eller mer än, en timmes promenad per dag hade en minskad bröstcancerrisk med nästan 25%. Risken minskade mest för kvinnor som tränade under eller efter klimakteriet eller kvinnor som hade en genomsnittlig kropp. Studie II utvärderade fysisk aktivitet i relation till mammografiska egenskaper som är viktiga för upptäckt och förlopp av sjukdomen. Vi visade inget samband mellan dessa.

Studie III undersökte en biologisk väg (det så kallade fibroblasttillväxtfaktor-systemet) i relation till bröstdensitet och cancer. Vi visade att vissa faktorer i denna väg ökade i nästan 60% av brösttumörerna jämfört med tumörangränsande vävnader i bröstet eller associerade med bröstdensitet, vilket indikerar de cancerfrämjande rollerna för detta system, vilket behöver ytterligare utredning. Studie IV studerade effekterna av bröstcancerbehandling tamoxifen på friska bröstceller och gav potentiella förklaringar på den kliniskt observerade minskning av bröstdensiteten och cancerriskreducerande aspekten av tamoxifen.

Sammanfattningsvis rapporterade vi fördjupat stöd för fysisk aktivitet som en bröstcancerförebyggande åtgärd och identifierade grupper av kvinnor som hade mest nytta. Vi erbjuder också ny insikt om fysisk aktivitet i relation till mammografi. Vi föreslog sedan en biologisk väg som ett potentiellt mål som skulle undersökas ytterligare för förebyggande av bröstcancer förmedlad av täthet. Vi erbjuder också mekanistisk insikt om tamoxifen som stöd för klinisk undersökning som en förebyggande åtgärd för kvinnor med täta bröst. Sammantaget ökar resultaten i denna avhandling kunskapen om förebyggande av bröstcancer.

List of Papers

Paper I

Boraka Ö, Klintman M, Rosendahl AH. Physical Activity and Long-Term Risk of Breast Cancer, Associations with Time in Life and Body Composition in the Prospective Malmö Diet and Cancer Study. Cancers 2022; 14:1960, DOI 10.3390/cancers14081960

Paper II

Boraka Ö, Sartor H, Sturesdotter L, Hall P, Borgquist S, Zackrisson S, Rosendahl AH. WHO-recommended levels of physical activity in relation to mammographic breast density, mammographic tumor appearance and mode of detection of breast cancer. Manuscript 2023

Paper III

Boraka Ö, Klintman M, Vallon-Christersson J, Zackrisson S, Hall P, Borgquist S, Rosendahl AH. FGF/FGFR1 system in paired breast tumor-adjacent and tumor tissues, associations with mammographic breast density and tumor characteristics. Frontiers in Oncology 2023; 13:1230821, DOI 10.3389/fonc.2023.1230821

Paper IV

Puig Blasco L, **Boraka Ö**, Hall P, Zackrisson S, Borgquist S*, Rosendahl AH* *Joint senior authors. Inhibitory effects of tamoxifen treatment on non-tumorigenic breast epithelial cell proliferation and adhesion. Manuscript 2023

Abbreviations

BI-RADS	Breast Imaging Reporting and Data System
BMI	Body mass index
CI	Confidence interval
DCIS	Ductal carcinoma in situ
ECM	Extracellular matrix
ER	Estrogen receptor
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FN	Fibronectin
HER	Human epidermal growth factor receptor
HR	Hazard ratio
IF	Immunofluorescence
IHC	Immunohistochemistry
KARMA	Karolinska Mammography Project for Risk Prediction of Breast Cancer
KARISMA	KARMA Intervention Study
MBD	Mammographic breast density
MET	Metabolic equivalent of task
MDCS	Malmö Diet and Cancer Study
MRI	Magnetic resonance imaging
OR	Odds ratio
PR	Progesterone receptor
SCAN-B	Sweden Cancerome Analysis Network – Breast
TMA	Tissue microarray
WHO	World Health Organization

Introduction

Cancer

The most generic definition of cancer is constructed from a genetic standpoint: a disease characterized by the uncontrolled proliferation of cells that are burdened with sets of mutations and the subsequent spread from its origin to distant sites (1-3). Immunologically, cancer has long been likened to "a wound that never heals" (4). These definitions, however, fail to shed light on the causality of cancer *per se*. The completion of the Human Genome Project and the subsequent surge of studies that ambitiously aimed to identify the genetic causes or biomarkers of cancer has so far failed to provide conclusive explanations as to why or how cancers form and progress. The molecular, cellular, and spatiotemporal heterogeneity of cancers render the causality of cancers far more complex than a linear causation based on genetic mutations. Following this line of thought, cancer, can alternatively be defined as "a disease caused by the perturbation of the continuously ongoing systemic relations of an organism, of its natural reciprocal dynamism" (5).

The complexity of cancer is also reflected upon its terminology–or more so led by it in a feed-forward loop. The word "cancer" originates from the ancient Greek word for "crab" because of the visual resemblance between the two (6). The word "tumor" is simply derived from the ancient Greek word for "swelling" (7). The distinction is clear here: not all cancers present with tumors and not all tumors are cancers. More than 200 cancer types are known to exist (8) because cancers can arise from almost anywhere in the body. Cancers are classified according to the cell type in which they originate. Carcinomas are the most common cancer type and arise from epithelial cells (9). Tumors, on the other hand, are merely used to describe abnormally growing tissues that can be either benign (noncancerous) or malignant (cancerous) (10).

Cancers are believed to develop through the clonal expansion of mutated cells (11, 12) that inevitably and frequently occur in a multicellular organism and are normally suppressed by the organism´s repair mechanisms (13). However, lower rates of cancer risk in larger animals who technically would accumulate more mutations renders the somatic mutation theory of carcinogenesis paradoxical as previously forwarded by Peto (14). The etiology of the disease is yet to be resolved.

The breasts in females serve as the mammary glands. The mammary gland originates from the ectoderm germ layer and starts developing in the embryonic stage. It continues developing first throughout puberty, and then pregnancy. The mammary gland produces milk in the lobules, which exits the nipple through the ducts. Estrogen and progesterone are both secreted from the ovaries (until menopause) and are responsible for the growth of ducts and lobules, respectively (15). The breast contains 15–25 lobes surrounded by fibrous connective and adipose tissues (Figure 1) (16). Interestingly, humans are the only animals that maintain perennially enlarged breasts (17).

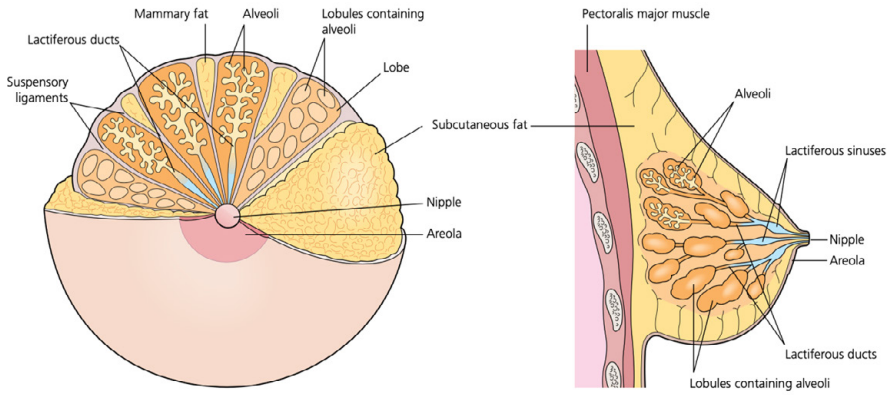

Figure 1. Anatomy of the breast (16).
Reprinted with permission from Wiley-Blackwell. Farhadieh, Ross D.; Bulstrode, Neil; Cugno, Sabrina. Plastic and Reconstructive Surgery: Approaches and Techniques: 2015;481.

Breast cancer

Breast cancer is a disease that originates from the breast and emerges mostly from the epithelial cells lining the milk ducts. Although, carcinomas are cancerous by definition, benign tumors of the breast contained inside the basement membrane are described as ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS); these comprise 15–30% of all newly diagnosed breast "cancers". DCIS comprise 80% of the *in situ* carcinomas (18) and 25% of all breast "cancers" (19). In Sweden, 11% of all diagnosed breast cancer cases were *in situ* between 2018–2021 (20). Around 50–70% of DCIS is estimated to never become invasive (21-23). Although, carcinomas are invasive by definition, the cancerous tumors of the breast are referred to as invasive ductal carcinoma and invasive lobular carcinoma and comprise 80% and 10-15% of all breast cancers, respectively (24) (Figure 2).

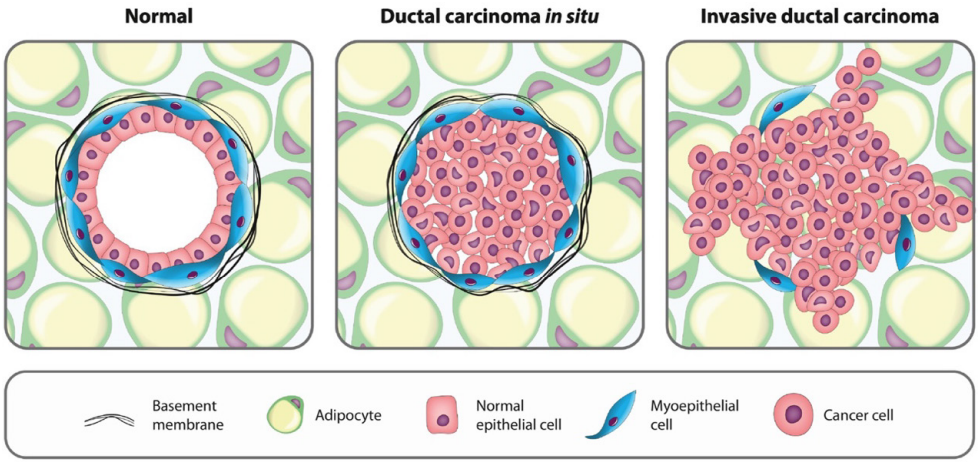

Figure 2. Breast cancer progresses to further invade the surrounding tissues when the basement membrane is breached, and the cancer cells are no longer confined within the ducts (25).
Reprinted from Elsevier. Gibson SV, Roozitalab RM, Allen MD, Jones JL, Carter EP, Grose RP. Everybody needs good neighbours: the progressive DCIS microenvironment. Trends Cancer. 2023;9(4):326-38.

Breast cancer, when taken as a metastasizing disease, is systemic (26). Local and systemic changes in the immune system, factors secreted from the primary tumor, and individual or clustered cancer cells disseminated to distant sites contribute to metastasis, rendering breast cancer a systemic disease that influences the entire body (27).

Breast cancer molecular mechanisms and subtypes

The etiology of breast cancer is not known for the most part. Inherited mutations of breast cancer type 1 (*BRCA1*) and 2 (*BRCA2*) susceptibility genes increase breast cancer risk substantially (by 44-78% for BRCA1 mutation carriers and 31–56% for BRCA2 mutation carriers) (28) and account for 3% of all breast cancers (29). BRCA1 and BRCA2 are structurally unrelated, however, both are involved in DNA repair (30). Molecular profiling of breast tumors has revealed the inter- and intra-tumor heterogeneity of breast cancers (31).

Breast cancer is most commonly classified into four main subtypes based on the immunohistochemical evaluation of three receptors: estrogen receptor positive (ER+), progesterone receptor positive (PR+), human epidermal growth factor receptor positive (HER2+), and triple negative (TNBC) breast cancer (32). The evaluation of these receptors along with the histological grade and Ki67 proliferative index is used to determine surrogate molecular subtypes of breast cancer (luminal A-like, luminal B-like, HER2+, triple negative) during clinical decision making for treatment (33, 34).

Most breast cancers are ER+, and the majority of ER+ cancers are also PR+ (32). ER/PR+ breast cancers are mainly driven by estrogen signaling (35). Both estrogen and progesterone are female sex hormones that are responsible for the development and regulation of female reproductive systems (16). Estrogen signaling may be triggered through nuclear receptors (ERα-β) and membrane receptors (G protein-coupled receptor 30 (GPR30), ER-X) (36). The classical estrogen signaling pathways in breast cancer are described through the nuclear receptors (37).

Receptor tyrosine-protein kinase erbB-2 (HER2) is a member of the human epidermal growth factor receptor family. Unlike the other members of this family, it must dimerize to activate. Interestingly, no ligand for HER2 has been identified to date (38). Binding to the respective receptors leads to the activation of the downstream signaling pathways. In ER/PR/HER2+ breast cancers, overexpression of ER/PR/HER2, and thus overactivation of estrogen/progesterone/HER2+ signaling leads to the proliferation of cancer cells (39-41). None of these apply for TNBC (42).

Genetic variations in breast cancers cluster in four distinct entities (described as intrinsic molecular subtypes): luminal A, luminal B, HER2-enriched, and basal-like. This categorization was coined by Perou upon their Prediction Analysis of Microarray assay that analyzed the expression of 50 genes (PAM50) in tumors (43); this is the most widely employed assay in the clinic (44).

Breast cancer can also be classified into different subtypes to predict risk of recurrence and tailor treatment strategies (45). Luminal A and B subtypes are burdened with ER and/or PR positivity, but the former has a low level of Ki67 proliferative index, and the latter has a high level. The HER2-enriched subtype accounts for HER2+ breast cancers and basal-like for TNBC. Luminal A breast cancers comprise 50% of all cancers and are followed by HER2-enriched (20%), luminal B (15%), and basal-like (10%) (46, 47). Breast cancer prognosis gets worse, and recurrence becomes more probable from luminal A to B and then to HER2-enriched and basal-like subtypes (48-50). The prevalence of luminal A tumors increases, but the prevalence of basal-like tumors decreases with age (51).

Finally, breast cancer is divided into two distinct entities based on menopausal status: premenopausal and postmenopausal breast cancer. The ovaries significantly reduce the production of estrogen and progesterone at menopause (52). Estrogen, however, is still produced in negligible amounts through the conversion of androgens in the adrenal glands or of testosterones in the ovaries by aromatases (53). This conversion could also locally take place in the breast adipose where the fibroblasts express aromatases and synthesize estrogens (54, 55) as well as the adipose tissue depots in the body. This explains why women with overweight/obesity have higher circulating estrogen levels than lean women (56).

Breast cancer incidence and risk factors

Breast cancer has been the most frequently diagnosed and prevalent cancer in the world since 2020. In 2020, breast cancer was diagnosed in 2.3 million women and caused the death of 685,000 women (Figure 3). There were 7.8 million women living with a breast cancer diagnosis in the past five years by the end of 2020 (57). Breast cancer has an age-standardized global incidence rate of 47.8/100,000 women (58). In Sweden, the incidence rate is 83.9/100,000 (59). Globally, premenopausal breast cancer cases comprise approximately 1/3 of the incidence rate (60) and have a case-fatality rate of 20%, while postmenopausal breast cancer cases have a case-fatality rate of 32% (61). The global increase in the premenopausal breast cancer incidence is mainly derived from high-income countries whereas it is the low-income countries that drive the increase for postmenopausal breast cancer incidence (60, 61).

The biggest risk factor for developing breast cancer is being female, which is followed by old age, family history of breast cancer, genetic predisposition (e.g., mutations of BRCA, PTEN, TP53), radiation exposure, and breast density (62). The incidence rates for both premenopausal and postmenopausal breast cancer in the West are disproportionately high versus the rest of the world and are partly attributed to the differences in reproductive and lifestyle factors (63) as well as the introduction of breast cancer screening programs that have increased the number of diagnosed cases (64). Reproductive factors associated with an increased breast cancer risk include early menarche (65), late menopause (66), as well as less and late reproduction (67). These factors are thought to contribute to breast carcinogenesis for their increasing effects on estrogen exposure (68), although, almost paradoxically, the majority of breast cancer cases occur among postmenopausal women.

Interestingly, while breast cancer incidence for females increased by 25% from 1975 to 2015, that of males (who are naturally exempt from the aforementioned reproductive factors) rose by 40% (69). Lifestyle factors associated with an increased breast cancer risk can be listed as, but not limited to, physical inactivity (70), having overweight (71), oral contraceptive use (72), hormone replacement therapy (73), and alcohol consumption (74). How or why the lifestyle changes such as physical inactivity or being overweight that have occurred in parallel to socioeconomical developments in the West seemingly have not exerted the same level of influence on the incidence rates of other cancer types such as liver or colon is also worth pondering. The cancer incidence data overall suggest that there may be other breast cancer risk factors that have not been discovered to date. The increase in female breast cancer incidence that is much higher compared to other cancer types thus warrants further investigation.

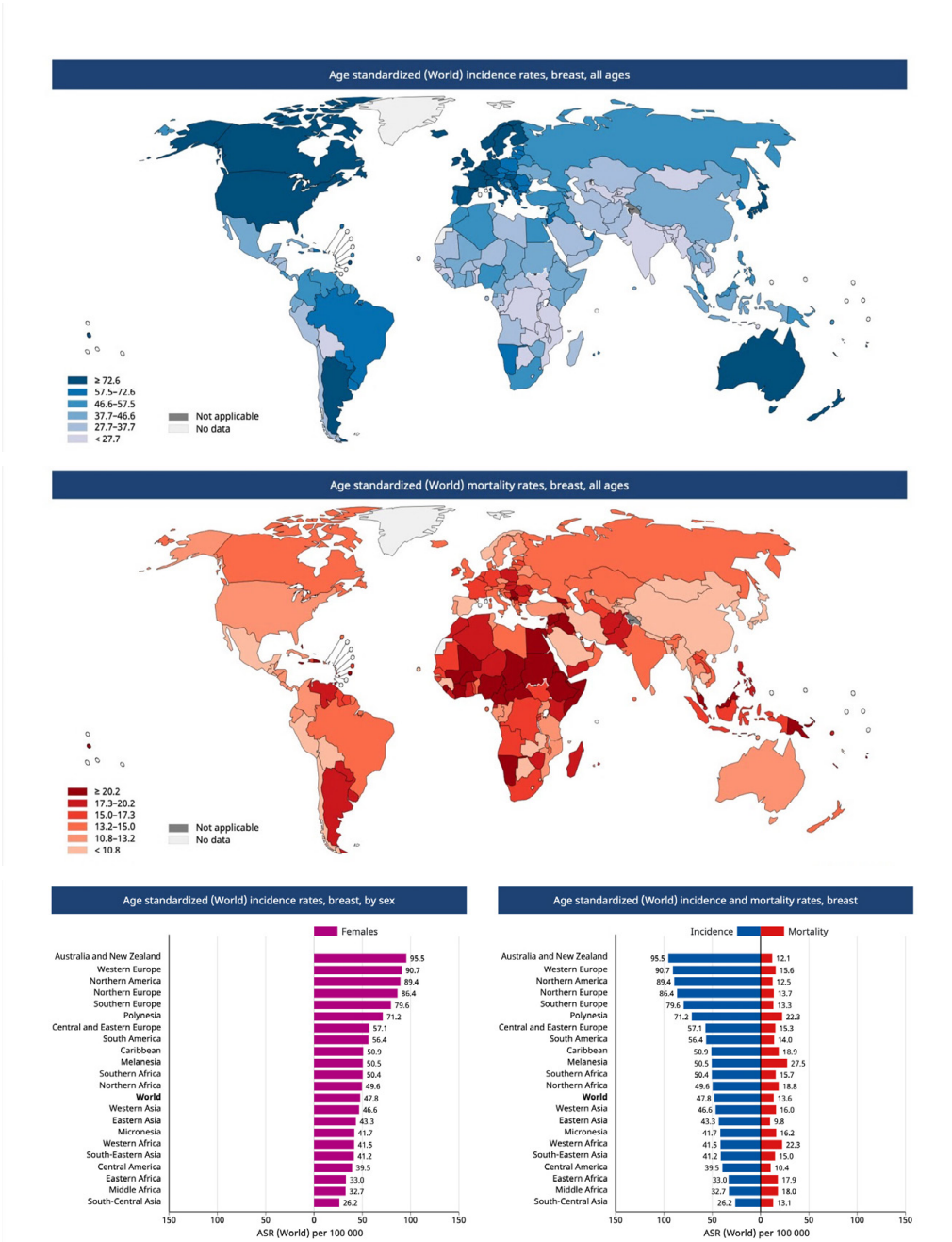

Figure 3. Age standardized breast cancer incidence and mortality rates (2020) (75).
Reprinted from the Breast factsheet by The Global Cancer Observatory, 2020.

Breast density

Breast density refers to the proportion of the fibroglandular areas in the breast parenchyma and is an independent breast cancer risk factor assessed via mammography (76, 77). The fibroglandular area is composed of non-cellular stroma and extracellular matrix along with the cellular compartment including stromal fibroblasts and epithelial cells. Collagen is the main density component by 30% when dense breasts are assessed histologically (78); the epithelial area comprises less than 5% (79). Breast density is evaluated either visually or quantitatively via the computation of mammograms (analogue or digital) or with relevant software such as Cumulus or Volpara (80). Dense tissue appears white (radio-opaque) on a mammogram whereas the non-dense area of the breast, which is merely the adipose tissue, is dark (radiolucent).

The Breast Imaging Reporting and Data System (BI-RADS) is the most commonly used visual assessment in the clinic and classifies breast density into four levels: almost entirely fatty (A, <25%), scattered areas of fibroglandular density (B, 25-50%), heterogeneously dense (C, 51-75%), and extremely dense (D, >75%) (Figure 4). While 10% of women have breasts with BI-RADS A density, 40% have BI-RADS B, 40% have BI-RADS C, and 10% have BI-RADS D. The 5th and latest version of BI-RADS classification system removed the density percentages and emphasized the masking effect of the dense tissues (81).

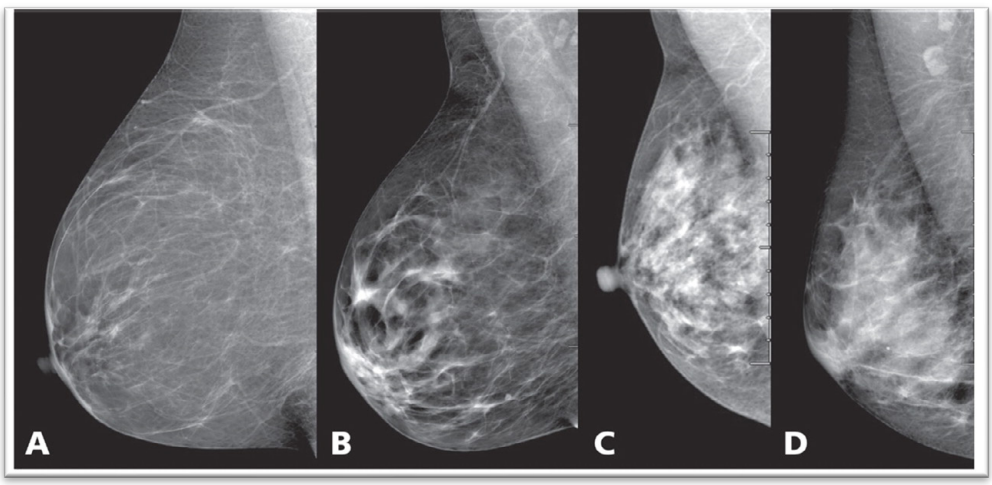

Figure 4. BI-RADS categories of breast density (82).
Reprinted with permission from Harborside Press. Pinsky RW, Helvie MA. Mammographic breast density: effect on imaging and breast cancer risk. J Natl Compr Canc Netw. 2010;8(10):1157-64; quiz 65.

Breast cancer risk increases 4- to 6-fold among women with the densest breasts versus women with the least dense breasts (83-85). The risk of missing cancer during screening also increases proportionally (86). A systematic review of nine studies recently showed a 1.83-fold increase in breast cancer risk from women with BI-RADS B to D breasts after adjusting for age and BMI (87).

Breast density is estimated to be heritable by 50–70% (88-90). Despite having a lower risk of breast cancer, the number of Asian women with dense breasts is significantly higher versus Caucasian women (91-94). Breast density is negatively associated with age, BMI, parity, but positively associated with late menarche, hormone replacement therapy (95), and alcohol consumption (96). Tamoxifen has been reported to reduce breast density in both high and low doses, especially in premenopausal women (97-100).

The molecular mechanisms that drive breast carcinogenesis in dense breasts have not yet been elucidated. Preclinical studies have been hampered by a lack of robust experimental model systems. Breast density has been found to be positively associated with the amount of stroma, stromal ER expression (79), stromal composition (101), collagen I expression (102), collagen remodeling (103), and IGF-I expression (77, 104), but negatively associated with CD36 expression (105). Its associations with epithelial proliferation (78, 106) and ER expression (79, 107) are rather mixed.

The high abundance of fibroglandular tissue, thus, fibroblasts in a dense breast are believed to impact breast density-mediated carcinogenesis (Figure 5) (108). Fibroblasts can be activated into cancer-associated fibroblasts (CAFs) via growth factors secreted from epithelial cells (109). Of these, transforming growth factor beta (TGF-β) is the most prominent one (110) with a wide range of effects on the extracellular matrix (ECM) (111) such as upregulation of collagen and fibronectin expression (112). CAFs, in turn, can affect epithelial cells by either direct cell-to-cell contact or in a paracrine manner by secreting factors such as matrix metallopeptidases (MMPs) (113) or fibroblast growth factors (FGFs) (114, 115) which ultimately lead to cellular proliferation. This crosstalk between fibroblasts, epithelial cells, and the surrounding stroma is thought to drive breast carcinogenesis forward in a positive feedback loop.

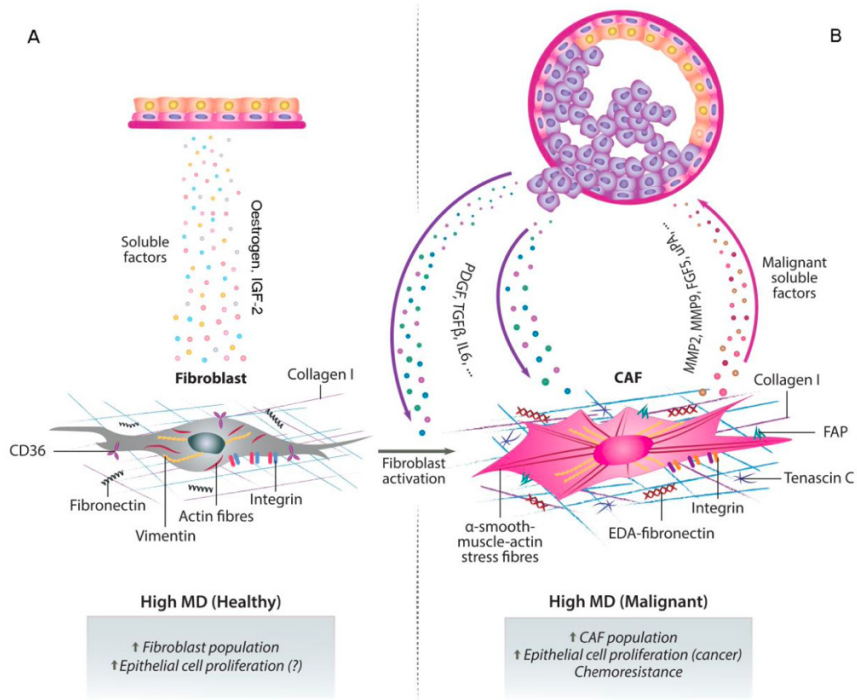

Figure 5. The crosstalk between the fibroblasts and epithelial cells in healthy and cancerous dense breasts demonstrating plausible mechanistic explanations of the MBD–breast cancer link (108).

Reprinted from MDPI. Fernandez-Nogueira P, Mancino M, Fuster G, Bragado P, Puig MP, Gascon P, et al. Breast Mammographic Density: Stromal Implications on Breast Cancer Detection and Therapy. J Clin Med. 2020;9(3).

Physical inactivity

Physical activity has long been investigated and acknowledged as a cancer preventive measure, and there is abundant research supporting its use. The World Health Organization (WHO) recommends 150–300 minutes of moderate intensity physical activity weekly, or 75–150 minutes of vigorous intensity physical activity, or an equivalent combination of both for health. Unfortunately, 25% of adults fail to meet these recommendations on a global scale (116, 117).

Physical activity, as a sole factor, was reported to reduce the risk for 13 different cancer types including breast cancer (118). A review of 33 observational studies reported an overall decrease in breast cancer risk by 25–30% with a dose-response effect in 28 of them (119). Another observational study reported an overall decrease by 6–10% (120) while another one reported 20% (121). The discrepancies in the

risk reduction rates are most likely derived from the variation in physical activity assessments. A Mendelian randomization study added further causal evidence for the breast cancer risk reducing effect of physical activity (122).

Physical activity most likely exerts its beneficial effects synergistically through different mechanisms. Physical activity reduces adiposity that may contribute to cancer risk through upregulated sex and metabolic hormone secretion, chronic inflammation, and alterations in adipokine secretion (123). Physical inactivity may also decrease insulin sensitivity (123, 124), which would in turn increase insulin levels. Insulin activates the insulin-like growth factor (IGF-1) signaling pathway involved in cell differentiation, proliferation, and apoptosis (125).

Breast cancer screening

Mammography uses low dose X-ray radiation to create an image of the breast and is the gold standard breast cancer screening method. The more solid the tissue is, the whiter it appears on a mammogram. Mammography has a false negative rate of 10% (126) and a false positive rate up to 50% for women who attend screening annually for 10 years (127, 128). In Sweden, 2.5 women per 100 receive a false positive report at a single screening round (129). There are several methods discussed to supplement or replace mammography in screening. Digital breast tomosynthesis (DBT) is 3-D mammography that reduces the impact of overlapping dense breast tissue and increases the visualization of breast structures including tumors. Screening trials with DBT indicate a ~30% increased breast cancer detection versus mammography with specific benefits for women with dense breasts (130-132). There is a conditional recommendation in the EU to use DBT in women with BIRADS C or D density in screening (133).

Breast cancer screening aims to reduce breast cancer specific mortality rates through early detection of breast cancer. Several randomized-controlled trials as well as many cohort and case-control studies have been conducted to assess the pros and cons of mammography screening. As a result, mammography screening has been shown to substantially reduce breast cancer mortality for women aged 50–69 and 70–74. However, the evidence for a clear benefit has persistently been insufficient for younger (<50) women (134). The high rate of false negatives in women with extremely dense breasts (BI-RADS D) underscore the need for advanced screening methods to replace or supplement mammography. The European Society of Breast Imaging (EUSOBI) recommends offering breast magnetic resonance imaging (MRI) to women with extremely dense breasts every 2–4 years (135) based on findings from The Dense Tissue and Early Breast Neoplasm Screening (DENSE) trial (136). Breast MRI is currently used for women who have a very high risk due to genetic susceptibility (137).

Mammography screening was gradually implemented in Sweden starting from 1986 and fully implemented by 1997 (138). The age intervals of women who were invited to screening differed based on the time and location of the invitation. In Malmö, the age intervals were as follows: 1990–1996, 50–69; 1997–2008, 50–74; and 2009–onwards, 40–74 years. Today in Sweden, all women aged 40–74 years are invited to screening biennially. The participation rate is around 85% (2021). Among women who attend breast cancer screening regularly, 60-70% of all breast cancer cases are detected through screening (139).

Mammographic tumor appearance

The appearances of tumors on a mammogram may provide prognostic insight. Spiculated tumors are found to be associated with good prognosis (140). Ill-defined masses are found to be associated with less favorable tumor characteristics (141). Microcalcifications are one of the breast cancer risk factors and associated with poor prognosis (142).

Mode of breast cancer detection

Breast cancer can be detected through screening or detected clinically when the patient starts feeling symptoms of the disease and/or a lump in the breast and seeks medical help. Cancers that are clinically detected in between two screenings are described as interval cancers which could be missed cancers or "real" cancers that proliferate fast. Clinically detected cancers are associated with less favorable tumor characteristics and worse prognosis compared to screening-detected cancers (143, 144).

Breast cancer diagnosis

Breast cancer is diagnosed by triple diagnostics that consists of physical examination and imaging of the breast and a tissue biopsy. This methodology is found to have a positive predictive value of 100% for breast cancer diagnosis with a 95.5% sensitivity (145). Biopsy is required to microscopically determine the cancer diagnosis and the type of tissue in question (e.g., DCIS, invasive lobular carcinoma), its stage (I–IV), histological grade (I–III), as well as to immunohistochemically examine its Ki67 proliferative index and ER/PR/HER2 status. Staging is determined based on tumor size (T), lymph node involvement (N) and potential metastatic disease (M). Very small tumors are defined as stage T0; tumors less than 2 cm are defined as stage T1, 2–5 cm as stage T2, >5 cm stage T3, and any size that has spread outside of the breast is stage T4. Lymph node involvement is classified into four categories: no involvement (N0), spread to 1–3 axillary lymph nodes (N1), spread to 4–9 axillary lymph nodes or cancer has spread to the internal mammary lymph nodes (N2), spread to 10> axillary lymph nodes and

one site is >2 mm or cancer has spread to the lymph nodes under the collarbone and one site is >2 mm (N3). Metastatic disease is either no metastasis (M0) or metastasis to distant organs (the bones, lungs, brain, or liver) (M1) (146). Histological grade is determined by the assessment of tubule/gland formation, nuclear pleomorphism, and mitotic frequency (147). Ki67 is a nuclear protein involved in cellular proliferation and ribosomal RNA transcription. It is used as a proliferation marker, although, the cut-offs that are employed have changed over time and is still debated on (148).

Breast cancer treatment

Breast cancer treatment decisions are made by a multidisciplinary team (MDT) consisting of a surgeon, an oncologist, a radiologist, a pathologist, and a nurse, and may consist of additional health care professionals such as a pharmacist, nutritionist, and therapist (149). Most women with breast cancer undergo surgery (breast conserving surgery or mastectomy), which is usually preceded and/or succeeded by radiotherapy and chemotherapy (e.g., doxorubicin, gemcitabine) in a neoadjuvant and/or adjuvant setting. Breast cancer patients are also treated with endocrine or anti-HER2 targeted therapy if tumors are proven susceptible to this therapy (150). An example of endocrine therapy is a gold standard drug used to treat ER+ breast cancers in both premenopausal and postmenopausal women: tamoxifen—a selective ER modulator (SERM) that competitively binds to ER and inhibits ER signaling in breast cancer cells in an antagonist fashion. Tamoxifen has been clinically employed for the last 40+ years and reduced breast cancer mortality by 30–40%—mostly among premenopausal breast cancer patients (151). Unfortunately, breast cancer patients who have been treated with tamoxifen may develop resistance to the drug at a rate of 20–30% (152). Furthermore, tamoxifen can agonistically act on ER in other tissues such as the endometrium where it increases endometrial cancer risk—especially in postmenopausal women (Figure 6) (153). Other endocrine therapies against breast cancer are aromatase inhibitors that act by blocking the conversion of androgens to estrogens. Aromatase inhibitors are only used to treat postmenopausal breast cancer patients for whom they exert more benefit than tamoxifen (154). Examples of anti-HER2 targeted therapy is the gold standard drugs for HER2+ breast cancers: trastuzumab and pertuzumab (155). During the past decade, the use of immune checkpoint inhibitors (IHIs) has proved to be an effective breast cancer treatment (156).

Breast cancer prevention

Preventive measures against breast cancer include bilateral mastectomy or salpingo-oophorectomy, lifestyle modifications, and medication. Bilateral mastectomy or salpingo-oophorectomy are also suggested for women at high risk (157).

Among lifestyle modifications, being physically active, maintaining a normal BMI, eating a healthy diet, and limiting alcohol consumption are the major breast cancer preventive measures. In combination with not smoking, these lifestyle modifications have been estimated to reduce overall breast cancer risk by 30% (158, 159).

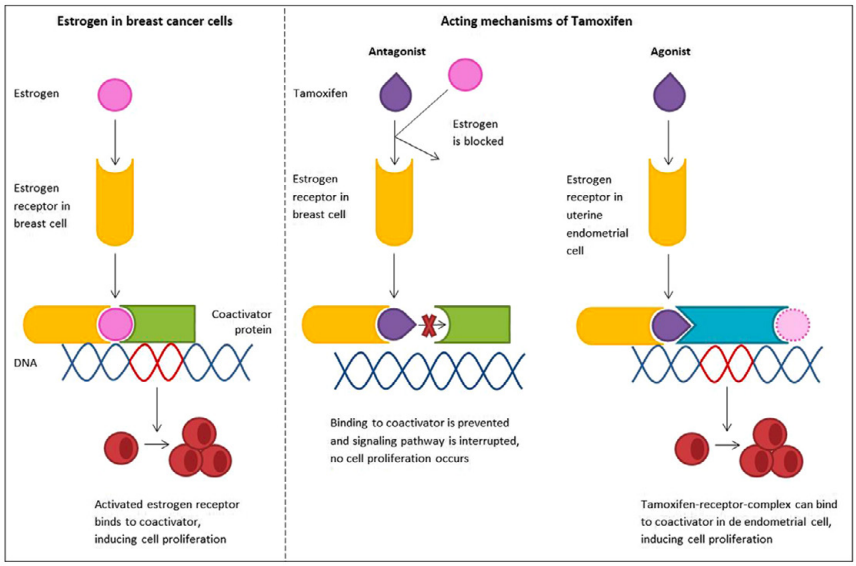

Figure 6. Tamoxifen´s mechanism of action in the breast and endometrium (153).
Reprinted with permission from Springer Nature. Schuurman TN, Witteveen PO, van der Wall E, Passier JLM, Huitema ADR, Amant F, et al. Tamoxifen and pregnancy: an absolute contraindication? Breast Cancer Res Treat. 2019;175(1):17-25. The figure is copyright protected and excluded from the open access licence.

Preventive medication is suggested in women who are at high risk but not keen on undergoing surgery. A well-studied breast cancer preventive medication for both premenopausal and postmenopausal women is tamoxifen. It was reported to cause a long-lasting reduction in breast cancer risk in premenopausal women by 33%, albeit with serious side effects (e.g., hot flushes, doubled risk of thrombosis) and an increased risk for endometrial cancer (160). A randomized control trial, KARISMA, showed that 2.5 mg tamoxifen could decrease breast density in a non-inferior magnitude versus the standard dose in breast cancer treatment, 20 mg, in healthy premenopausal women (100). In a follow-up analysis, premenopausal women experienced fewer pronounced side effects upon treatment with 2.5–5 mg tamoxifen versus 10–20 mg (161). Low dose tamoxifen treatment was further shown to decrease epithelial areas in healthy premenopausal women in the KARISMA trial (162). Another SERM, raloxifene, was tested on postmenopausal women and reported to be less beneficial than tamoxifen in terms of breast cancer risk reduction.

However, there were fewer thrombosis cases and a lower risk for endometrial cancer (163).

Fibroblast growth factor/fibroblast growth factor receptor (FGF/FGFR) system

FGFs are a family of growth factors with diverse biological activities that were discovered in the 1970s after an experiment showed its proliferative effects on fibroblasts. FGFs are found in many organisms ranging from *Caenorhabditis elegans* (a nematode that can display hyperproliferative phenotypes but cannot form malignant tumors) to vertebrates; FGF has no activity in unicellular organisms. To date, 22 FGF ligands have been discovered in vertebrates that activate FGF receptors, a receptor tyrosine kinase (RTKs) family of 4: FGFR1–4. Across vertebrate species, FGFs are largely conserved in gene and amino acid sequences. Based on sequence homology and phylogeny, FGFs in humans are classified into five paracrine subfamilies (FGF1, 4, 7 8, 9 subfamilies), one endocrine subfamily (FGF9 subfamily), and one intracrine subfamily (FGF11 subfamily) (Figure 7) (164). FGFRs share sequence identity at a rate of 56–71% despite being encoded by four separate genes. Like other RTKs, FGFRs are also dimerized and activated through autophosphorylation upon ligand binding.

Activated FGFRs further trigger a wide range of downstream intracellular signaling pathways depending on the cellular environment and developmental stage. The spatiotemporal expression of FGFs and FGFRs tightly regulate FGF/FGFR signaling, thus enabling this diversity of pathways (165). On a functional level, the FGF/FGFR system is involved in the development of almost all organs such as lung, heart, urinary system, brain, skeleton, muscle, skin, and breast as well as angiogenesis and lymphangiogenesis. The FGF/FGFR system also participates in tissue repair, regeneration, and inflammation. Erroneous FGF/FGFR signaling is found in many diseases ranging from genetic diseases of the skeleton to respiratory diseases as well as from cardiac arrest to cancer. In a cancer context, FGF/FGFR has a diverse set of roles from tumor initiation and progression to metastasis, quite expectedly (166).

FGF/FGFR system in the breast

In the breast, the FGF/FGFR system is crucial for mammary gland development. FGFR2b signaling drives the formation of mammary placodes in the embryo that later give rise to mammary buds and finally glands (167). Fgfr2b knockout models

in mice were shown to fail in mammary placode development (168). FGF10/FGFR2b signaling is further involved in branching of the mammary epithelial tree (169). Fgfr1 deletion, on the other hand, has a minor effect on mammary gland development (168). However, both FGFR1 and FGR2 regulate epithelial morphogenesis in the mammary gland upon activation by FGFs (FGF2, 7, 9, 10), which are provided from the mammary gland stroma (170).

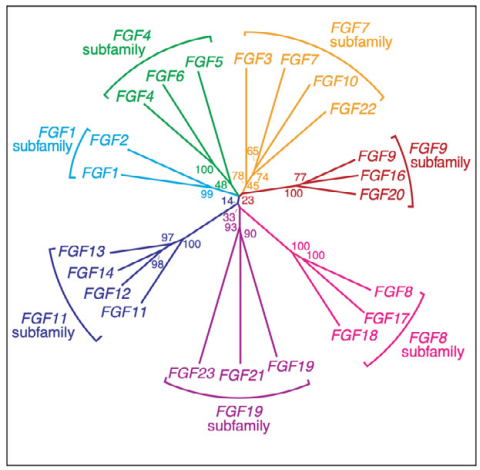

Figure 7. The phylogeny of human FGFs depicted by the branches that reflect the evolutionary distances between them (164).
Reprinted with permission from Springer Nature. Itoh N, Ornitz DM. Evolution of the Fgf and Fgfr gene families. Trends Genet. 2004;20(11):563-9.

In the breast cancer context, the carcinogenic role of FGF/FGFR system is partially attributed to the genetic aberrations of the receptors that lead to their constitutive activation. FGFR1 and FGFR2 were amplified in human breast cancer samples (171). FGFR2 was further shown as a breast cancer susceptibility gene by a genome-wise association study (172). Genetic aberrations of the receptors were also shown to participate in metastasis (173). Genetic aberrations of the ligands produced in the stroma were reported to contribute to the amplification of FGF/FGFR1 signaling (174). FGFR1 mutations are the most common FGFR aberration and are present in 15% of breast cancers: 27% of HER2+, 23% of ER+, and 7% of basal-like cancers (175). FGFR1 amplification is significantly associated with shorter survival mainly in ER/PR+ breast cancer; this is an increased risk of distant metastases (176). FGFR1 amplification was further reported to be involved in antiestrogen resistance in ER+ tumors (177).

FGFR inhibitors have been further explored in clinical trials for their potential to treat breast cancer (178). There were nine trials registered at the time when this thesis was written (https://clinicaltrials.gov/).

Aims

The overall aim of this thesis work is to bring further insight on breast cancer prevention with regards to lifestyle modifications (studies I and II) and targeted interventions (studies III and IV).

Study I

The aim of study I was to investigate the associations between the amount of pre-diagnostic physical activity and breast cancer risk in relation to time of physical activity in life and body composition in a large prospective cohort.

Study II

The aim of study II was to investigate the associations between the WHO-recommended amount and intensity of pre-diagnostic physical activity and mammographic features: mammographic breast density, mammographic appearances, and mode of breast cancer detection in breast cancer patients.

Study III

The aim of study III was to investigate the associations between FGF/FGFR1 expression, mammographic breast density, and breast tumor characteristics in paired tumor-adjacent and tumor tissues from breast cancer patients.

Study IV

The aim of study IV was to investigate the effects of tamoxifen on healthy breast epithelial cells in terms of proliferation, cell cycle regulation, and ECM adhesion.

Methods

Epidemiology

Study populations

Malmö Diet and Cancer Study (MDCS)

The study population of studies I and II was based on MDCS—a prospective population-based cohort study initiated in 1991 (179). MDCS is a part of the European Prospective Investigation into Diet and Cancer (EPIC) study which investigates the relationship between diet, nutrition, lifestyle and environmental factors and the incidence of cancer and other chronic diseases (180). For MDCS, 17,035 women aged 44 to 74 were recruited between 1991 and 1996 with a participation rate of 42.6%. Eligibility criteria for recruitment were to be a Malmö resident and fluent in writing and reading Swedish. At study baseline, participants completed self-reported questionnaires including questions about their diet, socioeconomical status, reproductive factors, lifestyle factors (including physical activity), and medical history. Anthropometric measurements and blood samples were also taken (178, 179, 181, 182).

Karolinska Mammography Project for Risk Prediction of Breast Cancer (KARMA)

In study III, the study population was based on the KARMA cohort, which is a prospective screening cohort initiated in 2010. The long-term goal of KARMA is to reduce breast cancer incidence and mortality by focusing on individualized breast cancer prevention and screening (183). KARMA recruited 70,877 women aged 40–74 years from 2011 to 2013 and comprises about 35% of the women in the southern Stockholm area and southern Sweden who were invited for mammography screening during the recruitment period. At baseline, participants donated blood and answered questionnaires. Genotyping was performed by extracting DNA from the blood samples. All mammograms were collected regardless of whether the reason for the examination was screening or clinical. This information is continuously updated (184).

Sweden Cancerome Analysis Network – Breast (SCAN-B)

The population in study III also included SCAN-B, which is a prospective population-based study introduced in 2010. SCAN-B was initiated to analyze breast cancer by using next-generation genomic technologies to identify and validate breast cancer biomarkers with the aim of developing more individualized treatment strategies. As of March 2023, the study comprised 19,718 patients whose tumor tissues, blood samples, as well as clinical and pathological data from the National Quality Registry for Cancer Patients (NKBC) were collected (185).

Data

Physical activity assessment

For studies I and II, physical activity for 17 different physical activities data were gathered at baseline of the MDCS study inclusion. Participants were asked to record the time spent on each activity retrospectively, separately for each season of the previous year (Figure 8). The minutes of physical activity per week were then calculated in the metabolic equivalent of task (MET) hours per week in order to standardize different physical activities with regard to duration and intensity. METs are determined by the Adult Compendium of Physical Activities (186) and represent ratios of the work metabolic rate to the resting metabolic rate while performing a specific activity.

In study II, moderate and vigorous intensity physical activities, which were defined as physical activities that had a MET value of 3–6 and above 6, respectively, were then analyzed in relation to mammographic features. These activities were analyzed in the study and are included in paper II along with their MET values. Additionally, WHO-recommended minutes of moderate (150-300 min/week) and vigorous (75-150 min/week) intensity physical activity per week that need to be undertaken to keep healthy and prevent cancer were used as another set of physical activity exposure variables as described in paper II.

Mammographic information

Mammograms closest to the date of diagnoses were used for study II and assessed by radiologists at the Department of Breast Radiology, Malmö. The following data were retrieved from the radiology reports: mammographic breast density, mammographic appearance, and mode of detection.

Fysisk aktivitet – på fritiden

32. Motion på fritiden och förflyttning till och från arbetet

*Frågorna gäller dels aktiviteter på fritiden och dels hur Du tar Dig till och från arbetet, men **inte** aktiviteter i arbetet.*

*Ange i tabellen nedan hur många **minuter** Du i genomsnitt **per vecka** ägnar Dig åt de uppräknade aktiviteterna **under de olika årstiderna**. Du ska försöka uppskatta den **aktiva tiden** förutom omklädning, duschning och liknande. Om Du saknar något kan Du själv lägga till det i slutet av tabellen.*

EXEMPEL	*Vår*	*Sommar*	*Höst*	*Vinter*
	Antal minuter per vecka			
Promenad (minuter/vecka) (även till och från arbetet)	*195*	*315*	*20*	*225*

Kryssa här för de aktiviteter som Du sällan eller aldrig deltar i ⬇		Vår	Sommar	Höst	Vinter
Ange antalet minuter per vecka ⬇		Antal minuter per vecka			
Badminton (minuter/vecka)	☐	.....	.....	.....	.....
Bordtennis (minuter/vecka)	☐	.....	.....	.....	.....
Fotboll/handboll (minuter/vecka)	☐	.....	.....	.....	.....
Golf (minuter/vecka)	☐	.....	.....	.....	.....
Jogging/löpning (minuter/vecka)	☐	.....	.....	.....	.....
Motionsgymnastik (minuter/vecka)	☐	.....	.....	.....	.....
Orientering (minuter/vecka)	☐	.....	.....	.....	.....
Simning (minuter/vecka)	☐	.....	.....	.....	.....
Tennis (minuter/vecka)	☐	.....	.....	.....	.....
Cykel (minuter/vecka) (även till och från arbetet)	☐	.....	.....	.....	.....
Promenad (minuter/vecka) (även till och från arbetet)	☐	.....	.....	.....	.....
Gång i trappa (minuter/vecka)	☐	.....	.....	.....	.....
Gammaldans (minuter/vecka)	☐	.....	.....	.....	.....
Sällskapsdans (minuter/vecka)	☐	.....	.....	.....	.....
Gräsklippning *(handgräsklippning)* (minuter/vecka)	☐	.....	.....	.....	.....
Grävning *(för hand)* (minuter/vecka)	☐	.....	.....	.....	.....
Trädgårdsarbete (minuter/vecka)	☐	.....	.....	.....	.....
..... (minuter/vecka)		.....	.....	.....	.....

Figure 8 Physical activity part of the MDCS questionnaire

Mammographic breast density was evaluated qualitatively according to the Swedish clinical practice and categorized into three categories: fat-involuted, which corresponds to BI-RADS A; moderately dense, which corresponds to BI-RADS B+C; and dense, which corresponds to which BI-RADS D. MBD was later dichotomized into fat-involuted + moderately dense and dense. For a subset of the study population, BI-RADS density values were also available.

The most dominant mammographic appearances were mass (well-defined, partly ill-defined, ill-defined/diffuse), spiculated mass, architectural distortion, and asymmetrical density. There are also microcalcifications that are categorized as comedo-type or non-specific. Mammographic appearances were categorized and described in paper II.

The mode of detection can be either clinical detection, which includes interval cancers, or detection through screening.

In study III, MBD was quantitatively assessed by using the density measurement tool iCAD (iReveal®,Nashua, NH, USA) and the fully automated STRATUS method.

Immunohistochemical evaluation

Immunohistochemistry (IHC) was conducted for study III, the details of which are described in the respective paper. Briefly, tissue microarrays (TMAs) were first constructed by transferring duplicate 1 mm tissue cores of representative areas from the formalin-fixed paraffin embedded (FFPE) tissue samples to a recipient microarray block. Sectioned TMAs were then deparaffinized and pretreated for antigen retrieval and stained with a monoclonal anti-FGFR1 antibody by using the fully automated DISCOVERY ULTRA IHC research platform (Ventana). We had the unique opportunity to evaluate the FGFR1 expression in both tumor-adjacent and tumor samples from the same patient with linked prediagnostic patient characteristics and clinicopathology information.

RNA-sequencing

In study III, differentially expressed genes in breast tumor samples were analyzed through RNA-sequencing by using the Illumina platform at the Canceromics Branch, Division of Oncology and Pathology, Lund University Cancer Center. The generated data were then pre-processed and $\log_2$-transformed. These steps ensured the clean-up and normalization of the raw data. RNA-sequencing data from SCAN-B were also used to create Single Sample Predictor models to further identify molecular intrinsic subtypes (187).

Statistical analyses

In study I, the Kaplan-Meier method and the LogRank test were used together to study the associations between the dichotomized physical activity levels and breast cancer incidence. In the Kaplan-Meier method, the observations of incidence are cumulatively put together with increasing time and the incidence rates are calculated as a function of time. The LogRank test is used to compare the differences between the two Kaplan-Meier curves. The null hypothesis in a LogRank test is that there is no difference between the curves. In study I, Cox regression analysis was also performed, which provided hazard ratios with 95% confidence intervals in age- and multivariable-adjusted models. Cox regression gives an estimate of the association between multiple independent variables and a time-to-event outcome. Hazard ratio provides the ratio of incidences. A 95% confidence interval means that the true measure of an association lies within that range. Adjustment is conducted to ensure that the effect is associated with the exposure and not influenced by other factors that may contribute to the outcome. While studying breast cancer incidence, breast cancer risk factors are entered as covariates in the Cox regression analysis.

In studies II and III, logistic regression analyses were conducted, which provided odds ratios with 95% confidence intervals in crude and multivariable-adjusted models to study the associations of physical activity and FGFR1 expression with mammographic features and breast tumor characteristics, respectively. All were categorical variables, and the outcome variables were binary. Binary logistic regression gives an estimate of the association between the exposure and the probability of a binary outcome to occur. Odds ratio provides the ratio of proportions. In study II, covariates for each outcome were selected individually after performing univariable logistic regression. In study III, age at diagnosis and tissue storage time were selected as covariates.

In study III, chi-square and Jonckshеere-Terspstra tests were also used. The chi-square test is used to compare whether two categorical variables are independent or if the distribution of the outcome variable differs based on the exposure variable. The null hypothesis in a chi-square test is that there is no relationship between the two variables, or the exposure and outcome are not associated. The Jonckshеere-Terspstra test assessed whether there is a trend between an ordinal exposure variable and a continuous outcome variable.

All statistical analyses were conducted using SPSS (IBM Version 27–29 for Mac).

Ethics

Research on human subjects must ethically abide by the Declaration of Helsinki and the Nuremberg Code. The Declaration of Helsinki was first issued in 1975 and demands that the detailed study protocols are reviewed by independent ethical boards. The Nuremberg Code demands that voluntary consent from human subjects must be taken, and the subjects must be further informed that they have a right to refuse to participate in the study or withdraw from the study at any time.

All types of personal data are also under legal protection in the European Union based on the General Data Protection Regulation that took effect in 2016. Any type of information relating to a living person is defined as personal data, and personal data relating to a person´s health is referred to as sensitive personal data.

Our studies were approved by the regional ethics committees, and informed consent was obtained from study participants at study inclusion. As for the present studies, personal data were subjected to pseudo-anonymization, which replaces personal information with study ID pseudonyms and encrypts sensitive information. Results were also presented at a group level, reducing the possibility of identifying the individual participants.

In vitro

Study IV

Cell culture

The human mammary epithelial MCF10A cells (ATCC) were cultured in regular growth medium and seeded at three densities for all experiments: low (6,000 cell/cm^2), intermediate (9,000 cell/cm^2), and high (12,000 cell/cm^2).

Cell proliferation

The cells were first treated with 0–30 μM of (Z)-4-hydroxytamoxifen for 48 hours. Subsequently, they were fixed with trichloroacetic acid and stained with the sulforhodamine (SRB) which electrostatically binds to proteins. After washing the excess dye, the protein-bound SRB was dissolved in tris buffer and fluorescently measured on a plate reader (VersaMaxTM Tunable Microplate Reader, Molecular Devices). The doses 0.1, 1, 3 μM were determined to be used in the subsequent

experiments. MCF10A cells seeded at the three densities were treated with the three doses of tamoxifen for 48 hours.

Cell cycle analysis

The tamoxifen treated cells were collected, fixed with 70% ethanol, stained with propidium iodide (PI), which binds to nucleic acids, and then applied to the flow cytometry device AccuriTM C6 flow cytometer (BD Biosciences) 20,000 cells at a time.

Cell adhesion assay

The tamoxifen treated cells were collected, counted, and transferred to plates precoated with laminin or fibronectin in the same cellular densities. The cells were then incubated, washed, and stained with the cell-permeable crystal violet dye that binds to proteins and DNA. After washing the cells and solubilizing the dye, the absorbance was measured on the same plate reader mentioned above.

Western blot analysis

The tamoxifen treated cells were collected and lysed. Upon determining the protein concentrations, the lysates were dissolved in a loading buffer at the same protein concentrations, boiled, and loaded to the 4-12% NuPAGE® Bis-Tris Mini Gels. The proteins in the lysates were separated by electrophoresis. The gels were blotted on nitrocellulose membranes, which were later blocked, treated with the primary antibodies of interest, washed, treated with the compatible horseradish peroxidase-conjugated secondary antibodies, washed, and visualized.

Modeling breast density

Toward establishing a more comprehensive experimental model for the study of breast density, considerable efforts were undertaken to model low and high density in vitro. which unfortunately proved difficult to produce consistently reproducible models. Briefly, I first prepared gelatin-coated 24-well plates, which were later used to seed human mammary fibroblast cells (HMF3S cell line) that were either cultured in regular culture media or adipocyte-conditioned media that was previously collected from the supernatant of mature differentiated adipocyte (3T3-L1, ATCC) culture.

Culturing with regular/adipocyte-conditioned media, I thought, would mimic the microenvironment in a fatty breast. Further, I induced ECM production in HMF3S

cells by daily ascorbic acid treatment for three and seven days with the aim of mimicking low and high dense breasts, respectively (Figure 9). At the end of the culturing period, I denuded the wells to remove the fibroblasts from the generated cell-derived ECM scaffolds and proceeded with performing immunofluorescence (IF) staining against fibronectin to observe the ECM deposition and to determine whether the model was working robustly.

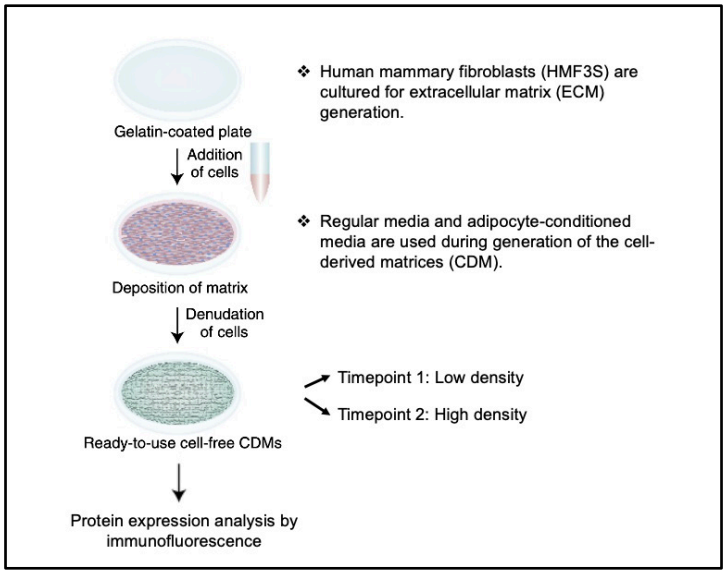

Figure 9 Workflow of the breast density modeling efforts (adapted from Kaukonen et al., 2017(188))

Results and discussion

In this section, the main results are summarized and followed by discussions involving interpretation of the results, comparison with the literature, and methodological considerations.

Study I

In study I, we investigated the associations between physical activity and breast cancer incidence in the large prospective MDCS cohort. Among 15,983 women studied, 1302 invasive breast cancer cases occurred by the end of the follow-up period with a median of 23.2 years. We found that baseline physical activity equivalent to or more than one hour of walking per day (≥28.5 MET-h/week) was associated with a 23% reduction in long term (12+ years) breast cancer risk (HR_{adj} = 0.77, 95% CI 0.66–0.90). We showed that physical activity was associated with a reduced breast cancer risk predominantly in women who participated in high levels of physical activity during or after menopause and women with lower-middle or upper-middle waist circumference, body fat percentage, and BMI values. The risk reduction effect was time-dependent with benefits beginning to show 12 years after the physical activity measurements at study inclusion. In addition to the aforementioned results, we could not observe any clear risk reduction in women who conducted high levels of physical activity before menopause or in those who had the lowest and highest body composition values at the beginning of the study. Neither could we observe any short-term risk reduction. How the changes in the physical activity levels or body compositions of the participants throughout the follow-up period would impact the results as well as the reasons behind the reduction observed only after 12 years of follow-up are worth investigating.

The results of study I further support the literature in relation to the observed overall reduction in breast cancer risk while taking the intensity and duration of 17 types of physical activities into account through the use of MET-hours and bringing further insight into breast cancer risk reduction in relation to time in life of physical activity and body composition. Physical activity has repeatedly been shown to decrease breast cancer risk by varying percentages as mentioned earlier in the introduction section of this thesis. Briefly, a meta-analysis of 31 studies including 63,786 women

showed that breast cancer risk was reduced by 12% from women in the 25th percentile to the 75th percentile of physical activity (189). A pooled analysis of ten prospective cohort studies showed that breast cancer risk was reduced by 10% from women in the 10th percentile to the 90th percentile of physical activity (118). Unlike these studies, we dichotomized our population based on the median physical activity value, which may contribute to the differences in the proportion of the reduction. As for timing in life, similar to our study, physical activity that was conducted before menopause was not shown to modify breast cancer risk (190). As for body composition, women with very high BMI appeared to benefit to less extent from physical activity alone in terms of breast cancer risk reduction relative to women having normal or overweight body compositions (191, 192). We could not observe any risk reduction among women with the lowest body compositions in contrast to the previous findings (193), although, physical activity may not be as relevant as a breast cancer preventive measure for lean postmenopausal women. Mechanistically, it is postulated that physical activity may reduce breast cancer risk via its effects on estrogen and insulin signaling (194).

In our study, a physical activity questionnaire was completed from memory for the year before study inclusion, which means that the exposure was "the average amount of physical activity that was conducted for the past year." This exposure was later dichotomized by the median physical activity level of the study population, which for the MDCS cohort, exceeded the WHO recommendations. The outcome was breast cancer incidence. We first used the Kaplan-Meier method which estimates survival and provides survival curves to visually assess whether the proportional hazard assumption in Cox regression holds for the outcome of two physical activity groups. Due to the observed divergence in the Kaplan-Meier curves emerging around 12 years, separate LogRank tests and analyses were conducted for 0–11 and ≥12 years of follow-up time. Additionally, we could have used physical activity data as a continuous variable, which would have provided information on breast cancer risk according to one unit (MET-h/week) increase in physical activity, as compared with compiled categories according to low or high physical activity levels.

The availability of data for many breast cancer risk factors is a unique strength of the MDCS. The covariates that were used in the multivariable-adjusted Cox regression analysis were all established breast cancer risk factors: age at baseline, age at menarche, parity, age at first childbirth, oral contraceptive use, current hormone replacement therapy, socioeconomic index, and alcohol consumption. Although, the results between the age-adjusted and multivariable-adjusted models were alike: the covariates had little to no influence on the effect size by physical activity in the adjusted model, demonstrating that the effect of physical activity on breast cancer risk was independent of the other established breast cancer risk factors we included in the analysis.

Overall, the well-known association between physical activity and decreased breast cancer risk was reinforced in our cohort with the use of MET hours and detailed with regard to the time of physical activity in life and body composition of study participants.

Study II

In study II, we investigated the associations between the amount and intensity of baseline physical activity and mammographic breast density, mammographic appearances, and mode of breast cancer detection in breast cancer patients in the MDCS cohort. There was no clear association for the amount or the intensity of physical activity and any of the mammographic features among the 1116 breast cancer patients that we studied. Two positive trends were observed between high mammographic breast density and WHO-exceeding levels of physical activity and high amounts of moderate intensity physical activity and high MBD. The trend in the WHO-exceeding group was driven by the high levels of moderate intensity physical activity rather than vigorous intensity. These positive trends were weakened in the multivariable-adjusted models. To the best of our knowledge, this was the first study to investigate the associations between physical activity and mammographic appearances as well as mode of breast cancer detection.

The majority of our study population engaged in physical activity levels that were adhering to (22%) or exceeding (67%) WHO guidelines. This might indicate that the participants followed an overall healthy lifestyle. The lack of a significant association between physical activity and MBD was qualitatively assessed and was somewhat consistent with the literature as discussed in paper II. The dual effects of physical activity on both fibroglandular and adipose compartments as well as the variations in the assessment of physical activity and MBD most likely contribute to the inconsistent results. For instance, high physical activity (≥50 MET-h/day) and lower absolute dense volume was quantitatively assessed by Volpara and was found to be associated (195), but there was no association between high physical activity (>2 h/week) and lower absolute dense area as quantitatively assessed by Cumulus (196). There was also no association between high physical activity (4 h/week) and MBD as qualitatively assessed in a prospective cohort with a similar design as MDCS (197).

MBD can be assessed qualitatively based on visual evaluation or quantitatively via the use of relevant software programs such as Cumulus, Volpara, STRATUS, etc. Visual assessment by radiologists is generally performed based on BI-RADS classification of density and may suffer from intra- and inter-observer variability (198, 199). Among the quantitative assessment tools, Cumulus has a high rate of

reproducibility and been the gold standard tool, however, it is a semi-automated program that has a human interactive aspect to define the breast area and train the program. Instead, fully automated programs such as Volpara may be preferred to ensure more reliability and less subjectivity. Cumulus and Volpara compute area and volume, respectively. Both measurements can be used to calculate percent MBD. A study showed strong associations between MBD (%) measurements obtained through visual assessment or Volpara and those obtained via Cumulus. However, MBD (%) values by Cumulus had a weak relationship with absolute dense volume (200). A cross-sectional study showed that MBD (%) assessed visually was strongly associated with the quantitative assessment by Volpara or Quantra—another fully automated program that measures volume. The results from Volpara and Quantra were clearly different, however (201). Taken together, more research is needed to compare and improve different methods of density assessment on mammograms. In our study, qualitative assessment of the mammograms was a limitation that prevented comparison with absolute or percent dense area or volume. Regardless, it is worth emphasizing that the relationship between MBD and increased breast cancer risk was first observed by Wolfe on visually assessed mammograms back in the 1970s (202, 203). Until the development of the software programs that quantify mammograms in the early 2000s, qualitatively assessed MBD was extensively shown to have strong associations with breast cancer risk (83-86). MRI was shown to have much higher sensitivity and specificity as a breast imaging modality than mammography irrespective of breast density (204). MRI density was also shown to be strongly correlated to MBD as quantitatively assessed by Volpara (205), thus indicating that MRI density may be closer to true density. However, MRI is not as readily available as mammography in the clinic.

Factors known to influence the mammographic features were first examined in directed acyclic graphs (DAGs) to select the covariates. Each factor was then applied to univariable logistic regression. As a result, a different set of covariates was chosen to include in the multivariable-adjusted logistic regression models to study each outcome.

In studies I and II, the unavailability of repeated measures of physical activity data until the endpoint was a limitation that prevented from analyzing how continuous physical activity over time is associated with breast cancer risk and mammographic features, respectively. Furthermore, the MET value system may still be prone to bias for activities that can be exercised at different intensities or paces.

Overall, this study is the first to explore the mammographic outcome of physical activity with regards to tumor appearance and mode of cancer detection.

Study III

In study III, we investigated the associations between FGF/FGFR1 expression, mammographic breast density, and breast tumor characteristics in paired tumor-adjacent and tumor tissues from breast cancer patients in the KARMA cohort. To the best of our knowledge, this is the first study to compare FGFR1 protein expression in between tumor-adjacent and tumor tissue samples from the same individual and the first to investigate its associations with mammographic breast density. We showed that FGFR1 expression was increased in 58% of the tumors versus their adjacent breast epithelial counterparts among the 426 breast cancer patients we studied. This novel finding may further support FGFR1 as a clinically relevant target that functions towards breast carcinogenesis. We also showed that high FGFR1 expression in tumors was associated with less favorable tumor characteristics: high histological grade (OR=1.86, 95% CI 1.00–3.44), high Ki67 proliferative index (OR=2.18, 95% CI 1.18–4.02), and luminal B-like subtype (OR=2.56, 95% CI 1.29–5.06). We further observed a positive correlation between FGF ligand expression (*FGF1*, *FGF11*, *FGF18*) in tumors and mammographic breast density. To the best of our knowledge, *FGF1*, *FGF11* and *FGF18* in tumors have not been previously studied in relation to MBD.

In this study, FGFR1 was proposed as an MBD–related breast cancer biomarker. As mentioned earlier in the introduction, FGFR1 is a widely studied protein in the context of breast development and cancer. Importantly, FGFR1 amplification was previously implicated as an early breast cancer event (206); FGFR1 amplification plays a role in paracrine signaling between epithelial cells and stromal fibroblasts (166, 207). However, no significant association between FGFR1 and MBD was observed in our study, while FGFR1 ligands were positively associated with MBD. The found associations with less favorable characteristics are consistent with the literature. For instance, a high rate of *FGFR1* amplification was associated with high Ki67 (208), luminal B-like tumors (209), ER+, and HER2- (176) breast cancers. As for the ligands, no association was found between plasma *FGF1* and MBD (210).

In biomarker studies, TMAs are generally preferred over whole section staining for reductions in time and tissue material. In contrast, tumors are heterogeneous, and therefore, bigger tissue specimens imply more representative results. This limitation of the TMAs can be slightly circumvented by sampling the tumors multiple times.

In our study, I double read and scored the staining, which I consider to be a limitation versus having two scorers. Regardless, the use of internal reference tissue cores for intensity assessments and receiving consultancy from experienced colleagues in uncertain cases reduced variability.

Tissue storage time and antibody specificity are other important parameters that may influence the protein expression data. Formalin-fixed paraffin-embedded samples (FFPE) stored up to 10 years provide consistent protein structures (211). In our study, FFPE samples were stored for 10–12 years, and the storage time did not modify the results obtained. The monoclonal FGFR1 antibody used here was previously validated in house on kidney and tonsil tissues as well as breast cancer tissues. The antibody had been validated in MDA-MB-231 and SUM159 breast cancer cells with CRISPR/Cas9 knockout of FGFR1 (212).

On a different note, breasts with different densities are distinct biological entities and most probably follow distinct routes of carcinogenesis. Therefore, I think the expression levels of an MBD-related breast cancer biomarker candidate should also be investigated in between healthy and cancerous breast tissue samples that have the same densities.

Overall, this study contributed to the field with novel findings about the FGFR1/FGF system in the context of breast cancer: the increased FGFR1 expression in tumor tissues compared to tumor-adjacent tissues as well as the correlations between *FGF1*, *FGF11*, *FGF18* and MBD.

Study IV

In study IV, we investigated the effects of tamoxifen on healthy human breast epithelial cells (MCF10A) at different cellular densities (low, intermediate, high) in terms of proliferation, cell cycle regulation, and ECM adhesion. Human mammary epithelial MCF10A cells were reduced in proliferation at all densities upon treatment with 0.1–10 μM tamoxifen for 48 hours. Tamoxifen treatment also resulted in the accumulation of MCF10A cells at G_0/G_1 phase at the low density, but only at the 3 μM dose. Upon tamoxifen treatment, the breast epithelial cells overall downregulated the expression of cell cycle regulators (total Akt, cyclin D1, and cyclin E as well as CDK2, CDK4, and CDK6), but upregulated the expression of cell cycle inhibitors p21 and p27 in a somewhat dose-dependent manner. MCF10A cells at low and intermediate densities were further reduced in their adhesion capacity to laminin and fibronectin—especially at 1 and 3 μM doses of tamoxifen. The expression levels of integrin subunits β1, β4, and αv were also decreased in a somewhat dose-dependent manner for all densities of MCF10A cells. Finally, the cells appeared overall to be more sensitive to tamoxifen exposure at low density compared to high.

Prior research on the effects of tamoxifen on healthy breast epithelial cells is limited. Tamoxifen is an ER antagonist and the gold standard treatment targeting ER+ breast

cancer cells. Tamoxifen can also exert ER-independent effects and was shown to influence the proliferation and apoptosis of ER- cell lines and ER- breast tumors (213). MCF10A cells also express ER albeit at nondetectable levels (214). which is presumably why these cells are still somewhat sensitive to tamoxifen. Previously, it was shown that tamoxifen caused breast cancer cells to be arrested at G_0/G_1 phase (215), thus decreasing cyclin D1 and increasing CKIs (216). Similar to the present findings in mammary epithelia cells, tamoxifen was also shown to impact the adhesion capacity of ER+ breast cancer cell lines (217). A randomized controlled trial, the KARISMA study, showed that low doses of tamoxifen (2.5–5 mg) reduced MBD, healthy breast epithelial area, as well as epithelial Ki67 expression in premenopausal women, thus indicating that the reduction in the epithelial area may be related to the changes in cell cycle (162). Overall, our study may provide mechanistic insight to these findings of the KARISMA trial as Ki67 is a proliferation marker that is upregulated during mitosis (218), and MCF10A cells are derived from a premenopausal woman (219).

Modelling breast density

The generation of mammary fibroblast cell-derived matrices was initially planned to be the basis for further studies in which healthy and cancerous breast epithelial cells would be observed for their proliferation and function in low and high dense breast microenvironments. The modelling efforts to recapitulate the complex features of breast density resulted in an increased and a more aligned/linearized fibronectin expression in the high versus the low density model. Mammary fibroblast exposure to adipocyte-secretome further seemed to enhance the fibronectin deposition while disrupting the aligned/linearized structure of fibronectin, especially in the high density model (Figure 10). The results were only partially reproducible, however.

The setup proved to be challenging in terms of reproducibility. One plausible explanation might be through the natural circadian rhythms of cells that regulate certain biological events. Fibronectin expression in HMF3S cells may differ due to the asynchronized circadian rhythm of the cells because fibroblasts are considered to be a well-established model to study circadian rhythms when synchronized (220). The circadian rhythm of the differentiated adipocytes may have been synchronized due to the use of dexamethasone—a circadian rhythm modulating drug—in the differentiation process (221) as previously shown with circadian adiponectin expression (222). Finally, an extraction buffer containing 30% ammonia solution was used to remove the cells and was possibly harmful to the generated ECM. This step was critical to ensure that the cells were all removed from the scaffolds so that

the denuded low/high dense matrices would be ready for further use in the investigation of healthy breast epithelial cell proliferation and function.

In retrospect, the use of a chemically defined lipid mixture could have been an alternative to the use of adipocyte-conditioned media in which the entire adipocyte secretome would be traded for consistency and reproducibility.

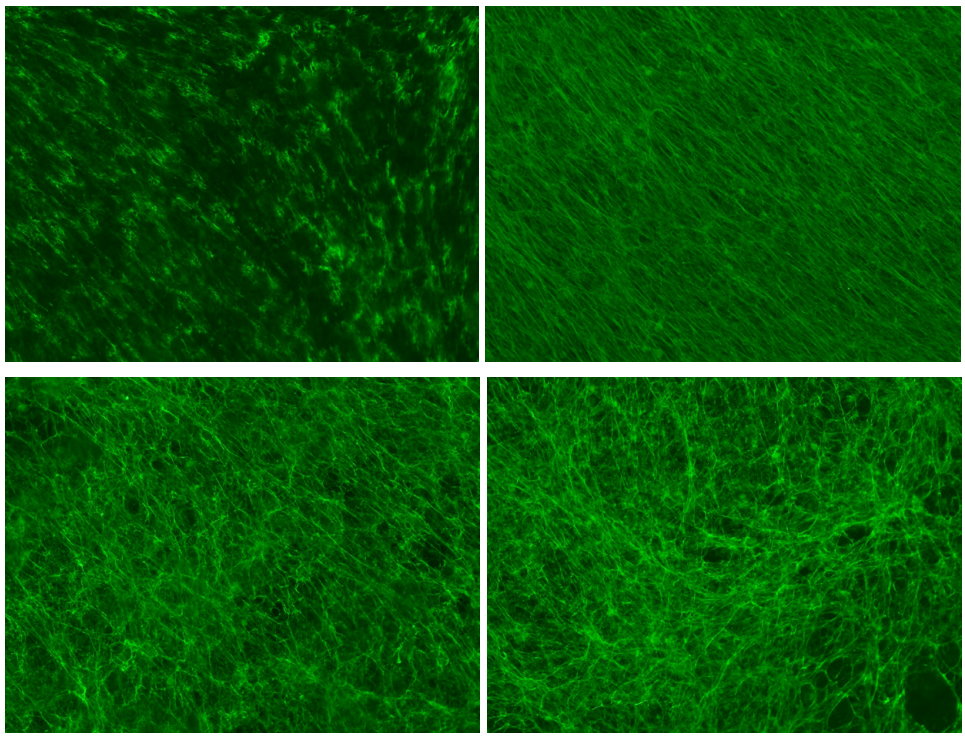

Figure 10. Immunofluorescence images of low- (left) or high- (right) density matrices generated from human mammary HMF3S fibroblasts grown in control medium (upper panel) and adipocyte-conditioned medium (lower panel) with fibronectin expression visualized in green (10X).

In addition to the observed of fibronectin protein deposition, reverse transcriptase-polymerase chain reaction (RT-PCR) results further showed that adipocyte secretome increased the mRNA expression of fibronectin in HMF3S cells, while a non-significant increase was observed for collagen I (Figure 11).

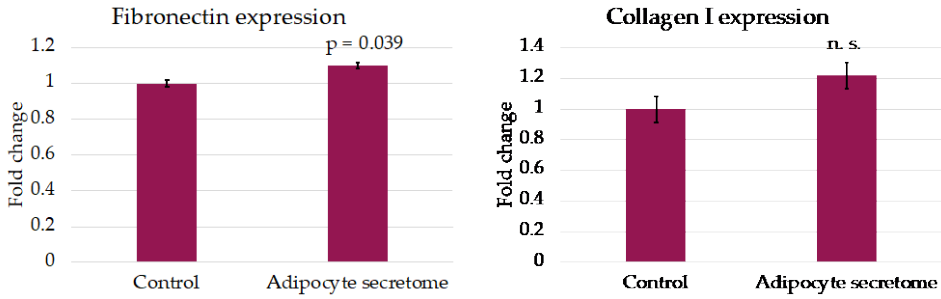

Figure 11. Adipocyte secretome increases the mRNA expression of fibronectin and collagen I in HMF3S cells.

I further examined the proliferation of the human mammary fibroblast HMF3S cells in response to adipocyte-conditioned medium with an SRB assay and observed that adipocyte secretome upregulated HMF3S proliferation relative to control medium (Figure 12).

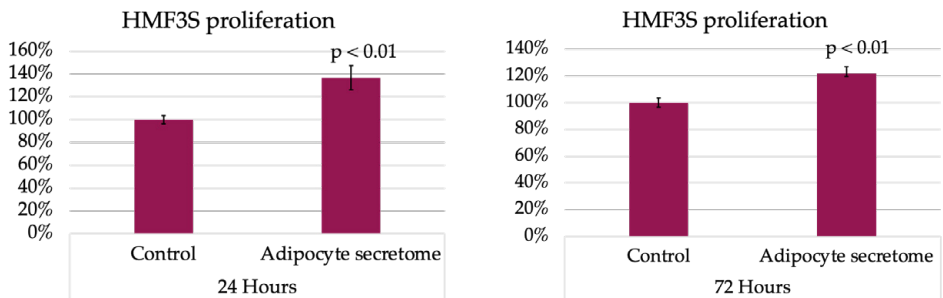

Figure 12. Adipocyte secretome stimulates the proliferation of HMF3S cells.

Taken together, these findings show that the secreted factors from differentiated adipocytes influence the proliferation as well as ECM production and organization of cell-derived matrices via human mammary fibroblasts.

Conclusions

This doctoral thesis investigated physical activity as a lifestyle modification (studies I and II), the FGF/FGFR1 system, and tamoxifen responses as potential targets (studies III and IV) in the context of breast cancer prevention with a focus on mammographic breast density.

Study I

Physical activity equivalent to ≥1 hour of walking a day was associated with a 23% breast cancer long-term risk reduction. The risk reduction was most prominent with high physical activity during or after menopause or in those with lower-middle and upper-middle values of waist circumference, BMI, and body fat. No risk modification was observed in women who exercised before menopause or had the lowest/highest values of body composition.

Study II

No clear association was observed between the amount or intensity of physical activity (according to the WHO recommendations on moderate and vigorous activities) and mammographic breast density, mammographic tumor appearance, or mode of breast cancer detection in a mostly postmenopausal group of breast cancer patients.

Study III

FGFR1 was upregulated in nearly 60% of tumors compared to their adjacent breast epithelial counterparts. High FGFR1 levels were associated with less favorable tumor characteristics in breast cancer: high histological grade, high Ki67, and luminal B subtype. *FGF1*, *FGF11*, and *FGF18* were positively associated with MBD. There results attribute the FGF/FGFR1 system a contributing role in postmenopausal breast carcinogenesis with implications on MBD.

Study IV

Tamoxifen disrupted cell cycle progression, inhibited proliferation, and decreased ECM adhesion capacity of healthy breast epithelial cells of premenopausal origin providing potential mechanistic explanations to its clinically demonstrated effects in reducing mammographic density in breast cancer preventive settings.

Future perspectives

In the Western world, 1 in every 8 women is burdened with breast cancer in her lifetime. The increased breast cancer incidence over the past few decades, despite being investigated extensively in relation each established risk factor, remains a mystery to be resolved. Preventing a disease is objectively better than curing one. The scope of this thesis was thus breast cancer prevention.

In the context of this thesis work, further research is needed to understand how additional breast cancer preventive measures, in combination with physical activity, could be applied for optimized preventive benefit among women with larger body compositions at increased risk of breast cancer. Physical activity assessment needs to be detailed further than the use of metabolic equivalents as is the standard practice currently. Heart rates measured by smart watches while performing an activity could be incorporated to the physical activity data in future studies to ensure better standardization of the data in relation to intensity.

Further research is needed to understand how breast density is associated with increased breast cancer risk and contributes to breast carcinogenesis. First, breast imaging modalities including mammography as well as MBD assessment methods need to be advanced so that breast density can be computed in the most objective way possible. The development of preclinical models that mimic breast density is an urgent need so that the molecular mechanisms behind the MBD-breast carcinogenesis association can be identified. Tangentially, risk assessment tools that integrate risk factors such as breast density should be developed to be further implemented in personalized breast cancer screening.

The understanding of breast carcinogenesis, and carcinogenesis in general, is limited despite all the research and accumulated knowledge. The disease should perhaps be tackled from new perspectives to ensure that we understand the origin of the disease first. Then we will know how to best prevent it.

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