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Mammographic breast density and hormonal, proliferative and stromal histopathologic markers in benign and malignant breast tissue of different ethnicities

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1. Introduction

1.1 Definition of breast density

Breast density, or mammographic parenchymal pattern, refers to the radiographic appearance of the female breast. The individual mammographic picture arises from variations in x-ray attenuation of different types of tissue. Fat, which is radiolucent, appears dark on a mammogram. Epithelial and stromal tissues on the other hand, appear white or radiodense and are collectively referred to as “mammographic density”. As stroma is present in much larger quantities than epithelium, it accounts for most of the radiological density (1).

The higher a woman’s breast density, the greater is her associated risk to develop breast cancer. Breast cancer is the most common cancer in women and a leading cause of death in nearly all developed countries. Several breast cancer risk factors such as increasing age, late menopause, early menarche or family history of breast cancer (among others) have been known for many years, whereas mammographic breast density is a new, recently established as independent, risk factor. There is now a substantial body of evidence describing an association between variations in mammographic parenchymal patterns and differences in breast cancer risk. Studies report risk estimates up to 6.0, making breast density one of the strongest risk factors for breast cancer (2-4). However, there is no general agreement on what causes some women’s breast to be denser than others and a reason or biological mechanism for the positive association to breast cancer risk has yet to be found.

1.1.1 Breast density and breast cancer risk

The first article about the relationship between breast density and breast cancer risk was published by Dr. John Wolfe in 1976 (5). He applied a method of classification of parenchymal patterns using qualitative criteria. Based on four patterns designated N1 (primarily fatty), P1 ($\leq 25\%$ prominent ducts), P2 ($> 25\%$ prominent ducts) and DY (dense fibroglandular tissue), he was the first to describe an increased breast cancer risk for women with high breast density. For years his hypothesis was debated and distrusted in literature until many publications confirmed his findings in the late 1990s. Thereafter high-quality epidemiological studies explored the risk-density relationship. As a minimum, 15 cohort studies, or nested case-control studies confirmed that Wolfe’s classification of mammographic patterns is associated with a difference in breast cancer risk. One group of opponents of Wolfe’s theory proposed what later became known as the “masking hypothesis” (6). They suggested that the apparent excess of cancer cases in the high density group during follow up is simply due to a decrease in mammographic sensitivity. However, in subjects reexamined over a long period of time, these effects of masking will be short-lived.

Among others, Brisson et al. showed in one of their studies that the increased risk associated with greater mammographic density persist for up to 9 years after screening (7). This argues against detection bias as the sole cause of the observed increase of cancer.

In a systemic meta-analysis of data for over 14.000 breast cancer cases and 226.000 noncases from 42 different studies, McCormack et al. found a consistent association of breast density with breast cancer risk (8). The strength of the association of breast density with cancer risk is greater than for most other established risk factors, except of age and genetic factors. The total dense area on a mammogram and percent density are both associated to higher breast cancer risk, however, percent density shows a stronger association (9). Several quantitative studies of breast density and cancer risk have been published applying visual estimation of the dense area, planimetry, or computer-assisted methods in digitized images. Although category definitions might vary, the odds ratio for developing breast cancer for very dense breasts compared with fatty breasts ranges from 1.8 to 6.0, with nearly all studies reporting risk estimates of 4.0 or higher (3, 9, 10). This risk is larger than is found with most other risk factors for breast cancer. Additionally, breast density accounts for a large number of cancer cases. Attributable risk has been estimated in a report of McCormack et al.; for the 20% and 10% of premenopausal women, who had densities over 50%-74% and $\geq 75\%$ correspondingly, the population attributable fraction was 42.8% and 26.7% respectively. For postmenopausal women the respective equivalent fractions were 9.8% and 23.2% (for 10% and 3% of women with densities of 50-74% and $\geq 75\%$) (8). Breast density is unique in its potential of being modified by hormonal (11) and dietary interventions (12).

Nevertheless, the reasons or biological mechanisms for this positive association have yet to be identified in detail. Besides, there is no general agreement on the causes of dense breasts and we do not know if a reduction in breast density would mean a reduction in risk, too.

1.1.2 Breast density measurement technique

To assess mammographic breast density, four principal methods have been used to date. Examples of breast density measurements can be seen in figure 1. As the mammographic measurement technique changed from xeromammography to screen-film mammography (which emphasized density differences in breast parenchyma rather than the visibility of prominent ducts) four categories of overall breast density replaced the four Wolfe parenchymal patterns as an index of parenchymal content. Because of difficulties in consistently applying a qualitative classification and variations in reproducibility (13, 14) researchers subsequently moved towards quantitative measures of mammographic density. Since the early 1980s, different quantitative mammographic methods of measuring breast density were developed and performed. To date, nine studies have used quantitative methods to find a risk of four or greater comparing extreme categories of the

density classifications used (15). In clinical practice, the most common method of classification is the American College of Radiology's four category *Breast Imaging Reporting and Data System* (BI-RADS) rating (extremely dense, heterogeneously dense, scattered fibroglandular tissue and almost entirely fat) (16). However, this classification, mainly used to inform referring physicians of the amount of decline in mammographic sensitivity with increasing breast density, was not intended to serve as a method of measuring breast density.

The only moderate interobserver agreement with an overall kappa value of 0.4-0.6 (17, 18) might be due to the lack of well-defined categories. Nevertheless, the great advantage of this classification is its wide and consistent use, which allows for an analysis of large populations. Concerning the interobserver agreement, more consistent quantitative computer-assisted density measurements have been developed. The earliest computer-assisted planimetry was used in 1987 (19). Agreement with this method was 70% to 94% (19, 20). Following this technique, film mammograms have been digitized and measurements are now being taken with more consistent computer systems. With either a mouse, or a digital segmentation of the mammogram the area of breast parenchyma and the total area of the breast are outlined and percent density values are computed. Interreader reliability for quantitative estimation by a radiologist its intermediate (intraclass correlation coefficient (ICC)~0.7) (21) and good for the computer-assisted measurement (ICC~0.9) (22).

Although quantitative methods are more time consuming than qualitative estimations, they are more reliable and can additionally provide information about the separate components of the ratio of percent density. In this study, computer-assisted density measurements were used, as described in detail later.

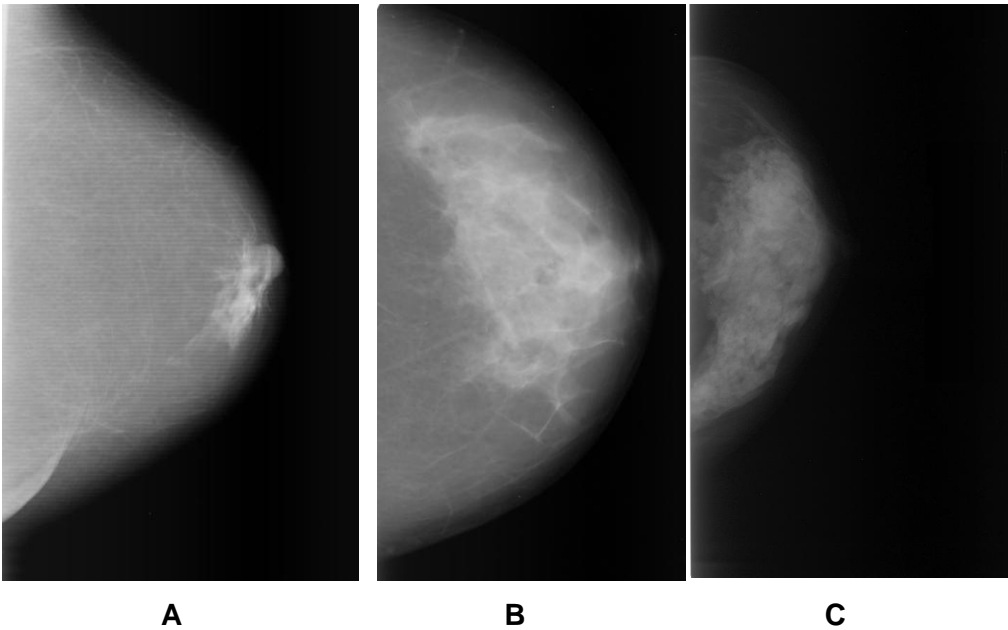


Fig. 1 Examples of mammographic breast density. Very low density (A), Medium dense breast (B), Extremely dense breast (C).

1.1.3 Histological basis for mammographic density

In order to identify biological plausible mechanisms through which breast density might be associated to breast cancer, several studies have examined the association between the mammographic and histopathological appearance of the female breast. The principal features of these studies are summarized in table 9.1 and table 9.2.

The two types of tissue that give rise to mammographically dense breasts are stroma and epithelium, where the majority of the dense part is comprised of stromal tissue. Numerous reports describe a positive association between density and proliferative lesions of stroma and epithelium (23, 24). Especially hyperplasia, atypical hyperplasia, carcinoma in situ (CIS) and fibrocystic changes have been shown to be positively associated to denser breasts (25-27). In the study published by Bartow et al., material derived from a forensic autopsy series was utilized preparing radiographs from sectioned specimens (s.c. mastectomy), reporting a positive correlation between high density and intraductal epithelial hyperplasia and lobular microcalcification (28). Three other cross-sectional studies compared the histological findings of breast biopsies and radiological qualitative classification of the whole breast and also found density to be positively associated with epithelial proliferation. They also report an association with high risk patterns such as atypia of the ductal type (24, 29, 30). Lee et al. found a direct relationship between the degree of mammographic density and cytological abnormality in nipple aspirate fluid (NAF) (31). Boyd et al. described a strong association between mammographic densities and the risk of developing

proliferative lesions in the breast epithelium. Compared with women showing no mammographic densities, women with the most extensive densities (i.e., occupying > 75% of the breast volume) had a 9.7 times greater risk of developing carcinoma in situ or atypical hyperplasia, a 12.2 times greater risk of developing hyperplasia without atypia and a 3.1 times greater risk of developing nonproliferative disease (10). On the other hand, Moskowitz et al. as well as Bland et al. did not find a significant positive association of high mammographic densities and hyperplasia in biopsies from women with benign breast disease (32, 33). In accordance with these findings, Fisher et al. who analyzed samples from breast cancer and fibrocystic breast disease patients did not report a significant correlation (34). Arthur et al. did not find an association of epithelial hyperplasia or CIS with Wolfe's patterns, but reported an association of fibrosis in the interlobular stroma with high density patterns (35).

Although not fully consistent, these findings agree with the hypothesis that breast density might be associated to breast cancer risk through mechanisms contributing to proliferative activities of breast tissue. Some researchers hypothesize that the breast cancer risk is dependent of the amount of mammary gland cells, or mammary tissue-specific stemm cells. They argue that dense breasts harvest more glandular tissue and ,therefore, more cells with the potential to transform into malignant cells (36). Breast density might therefore just reflect the number of glandular cells and therefore an increased breast cancer risk.

Further evidence that the breast tissue responsible for mammographic densities is responsive to hormonal influences is derived from reports about associations of breast density to other risk factors of breast cancer. Some of these are being described in the following section.

1.1.4 Determinants of breast density

Mammographic densisites have consistently been found to be associated with other breast cancer risk factors, including endogenous hormonal factors such as menopause, parity and number of live births, as well as exogenous hormonal factors such as hormone replacement therapy (HRT). Among others, density is inversely associated with increasing age and body mass index (BMI). Family history of breast cancer, on the other hand, is associated with more extensive densities. The evidence for some of these associations, relevant for our study, is being described in the following sections.

1.1.4.1 Age

On of the most important risk factors for breast cancer, a woman's age, is closely associated with breast density. The amount of dense tissue has been shown to decline with age, contradictory to an increase of breast cancer incidence over time (15). Few longitudinal studies of mammographic

densities have been conducted. One of the few longitudinal studies, estimated the average annual reduction in percent density as 1% (37). In a study of Maskarinec et al., it has been demonstrated that the age related decline of breast density was highest between age 45 and 55 and changed very little after age 65 (38). Others also found that women with a higher initial percent density had a faster rate of decrease in breast density over time (39).

The decline in density with age may seem paradoxical, as breast cancer incidence in contrast, increases with age. However, the apparent paradox might be resolved by reference to a model of breast cancer incidence as proposed by Pike and colleagues (40), who showed that cumulative breast tissue exposure to hormones and growth factors rather than the chronological age describes the age-incidence curve for breast cancer. Exposure is greatest at the age of menarche and consistently decreases with pregnancy, perimenopausal period and finally in menopause. Corresponding to this model, as stated by McCormack et al., cumulative exposure to breast density would be the pertinent risk factor for which density at a given age is a good proxy. It might be density at a given age rather than density per se that is related to breast cancer.

1.1.4.2 Menopause

Mammographic density has also been shown to be significantly higher in premenopausal than postmenopausal women. Density decreases during menopause. 34% of women age 75 to 79 have breasts mainly comprised of fatty tissue, compared with only 11% of women age 25 to 29 years. Equally, only 30% of women age 75 to 79 have a breast density of 50% or greater (41). On the other hand, it has been argued that these lesser values in postmenopausal density are simply a result of the decrease in density with age since most evidence is derived from density measures of different women (rather than on the change in the mammograms of one individual). To address this problem, Boyd et al. conducted a longitudinal study of the effects of menopause within subjects of the NBSS, directly comparing changes occurring in women who experienced menopause with an age-matched group who remained premenopausal. Their findings demonstrate that menopause has effects on density that, over the same period of time, are greater than the effects of age alone (37). Compared with women who remained premenopausal, percentage of breast density decreased to a significantly greater extent in women who underwent menopause (difference in change = -3.26 percentage points, $p=0.004$) during the same time period. On the contrary, Guthrie et al. did not find a significant effect of menopausal change on the amount of dense tissue after controlling for age (42). In their longitudinal study, they report a lower percent mammographic density in postmenopausal women, but no significant increase in dense area. Therefore, they argue, like other researchers, that the decrease in percent density might simply be explained by an increase in the amount of fatty, nondense tissue. Nevertheless, in results of the NBSS study and

others, the decrease in percent density is due to both, an increase in the area of nondense tissue as well as a reduction in the area of radiologically dense tissue (37). In conclusion, breast density does decline significantly with menopause. This could be explained by the biological process of menopause - the consequence of decrease in the levels of endogenous hormones in the perimenopausal period results in regression of glandular and stromal elements and in replacement by radiolucent fatty tissue.

1.1.4.3 Hormone replacement therapy

In agreement with the hypothesis that breast density is influenced by hormonal changes, it has been shown in various studies that combined HRT (with a progesterone and estrogen regimen) increases breast density (43, 44). Estrogen only, on the other hand, has just a weak influence on mammographic breast density (44-46). Exogenous hormone treatment seems to stop or delay the progress of involution during menopause, resulting in an increase in mammographic density. In a study of Laya et al. it has been demonstrated that densities increased about 6.7% (mean increase; $p=0.03$) in 30 (73%) of the 41 subjects undergoing continuous combined HRT for 1 year compared with baseline density values (43). They also showed an inverse relationship between baseline density and change in percentage density; the lower the baseline density percentage, the greater the change. Greendale et al. assessed the effects of conjugated equine estrogens (CEE) alone and CEE combined with three progestin regimens on breast density in a randomized, long-term, placebo-controlled and double-blinded trial. They report an increase in density in approximately 8% of estrogen users and 19% to 24% of estrogen-progestin users. Compared to CEE-only patients, combined HRT users had a 7-fold to 13-fold increased risk for developing denser breasts (47).

1.1.4.4 Estrogen exposure

Together with the knowledge of estrogen being a central player in the etiology of breast cancer, these findings suggest that breast density might be a marker for estrogen exposure.

Nevertheless, there are ambiguous results from studies addressing this association. Two studies did not find an association between estrogen serum levels and breast density (48-50). Similarly, Frankenfeld et al. detected an association with sex hormone binding globulin, but no association with free estradiol levels.

Given the role of estrogen receptors in estrogen response of breast tissue, expressions of these receptors in normal breast tissue could be related to breast cancer risk and incidence. However, few studies address these receptors in their relation to breast density. Results of one previous study show a positive association of breast density to both ER-positive and ER-negative breast cancer cases (51). Furthermore, bone mineral density (BMD), believed to be a marker of

cumulative estrogen exposure did not show an association with mammographic density (52). In accordance to the association of density with HRT, several studies agree in their findings that density is noticeably higher during the luteal phase of a woman's cycle, which is characterized by an increase in progesterone as well as estrogen levels (53-55). Examining continuous breast density measures in the same women at different phases of their menstrual cycle, Ursin et al. observed a statistically significant increase of 1.2% in breast density during the luteal phase. Endogenous progesterone was found to be related to mammographic density in one report among premenopausal women (56). Moreover, studies show a decrease in mammographic density after endocrine therapy with tamoxifen, a selective ER modulator with antiestrogenic effects (57, 58). This effect was most pronounced in premenopausal women and has been found in treatment with raloxifene, an analog of tamoxifen, too (59). In addition, nulliparous women and those with a later age at first birth have been shown to have significantly denser breasts than women who had children at a young age (60, 61).

One reason for the discrepancy in the results might be the use of different measures addressing the estrogen exposure of breast tissue. It is known that estrogen levels in the breast tissue differ from circulating hormones or plasma levels. Local estrogen concentrations in benign and malignant breast tissue were reported to be higher than plasma levels (62, 63). The exact correlation though has only inconsistently been reported. Furthermore, the inconsistent findings about the association to endogenous and exogenous estrogens and breast density could possibly be related to the expression of hormonal receptors, which are essential in the response to circulating estrogens.

1.1.4.5 Genetics and ethnicity

Genetics partially determine breast density as shown in a study by Boyd et al. (64). They report a 2-fold higher correlation between monozygotic compared to dizygotic twins, whereas Pankow et al. found significant correlations with breast density between sisters in their cohort study (65). There are known genetic factors that influence breast density variation such as variants in the CYP1A2 and catechol-O-methyltransferase genes, both being involved in estrogen metabolism (66-68). Among others, Crest et al. found that having a family history of breast cancer is strongly associated with higher breast density, especially when relatives were genetically similar (69).

Since race and ethnicity have a significant impact on breast cancer incidence and mortality, several studies have addressed the question, whether variations in breast density could explain these differences. Ursin et al., as well as Maskarinec et al. report similar effects of density on breast cancer in whites and Asian Americans (3, 70). These findings are consistent with the trends in breast cancer incidence among Asians living in the United States. Maskarinec et al. found percent density among Japanese women living in Hawaii to be significantly higher than among

Japanese women living in Japan (71). Conflicting results have been published, such as studies reporting higher breast density among Asian women (72). However, these differences disappeared after adjustment for BMI. Therefore, studies suggest that the size of the dense area is a better indicator of ethnic differences than percent density.

1.2 Hormonal receptors (ER α , ER β and PR)

The biological activities of endogenous and exogenous sex steroids on breast tissue are mediated by nuclear estrogen receptors alpha and beta (ER α and ER β) and progesterone receptor (PR).

Estrogen plays a crucial role in regulating growth and differentiation of the mammary gland and is a key player in the development and progression of breast cancer (73). Activation of estrogen receptors leads to expression of proteins associated with cellular proliferation and tumor. The dysregulation of ER is related to an improved overall survival and favorable response to endocrine therapy such as anti-estrogen tamoxifen. Data suggest that ER expression of normal breast tissue is fairly consistent (74). The main clinical application of ER α measurement in breast cancer is to determine prognosis and predict response to endocrine therapy. Tumors rich in ER α have a 60 to 70% chance of responding to first line endocrine treatment (75).

ER β also plays a role in carcinogenesis, progression and hormone dependency of breast cancers, however associations between expression of ER β and ER α have inconsistently been described (76, 77).

Only one prior study addressed the association between breast density and ER α status in breast tissue. In noncancerous tissue from mastectomies they did not find an association between ER expression and breast density. However, the sample size was only n=56 (78). This study did not find an association between density and PR expression. Progesterone, another major player in mammary gland biology, acts in conjunction with estrogen through its specific intranuclear progesterone receptors (PRA and PRB). Abnormal ratios of these isoforms may play some part in the initiation of breast carcinogenesis (79, 80). The prognostic and predictive values of total PR status are similar to ER status, but weaker in terms of prediction of endocrine therapy response (74).

1.3 Proliferation markers (Ki-67, PCNA and HER2/neu)

Proliferation of tumor cells is an important prognostic indicator. The estimation of growth fraction can be evaluated by immunocytochemical staining with antibodies against proliferation-associated antigens. One of the antibodies frequently used for this purpose is Ki-67 (81, 82). It reacts with a nuclear antigen present in G1, S and G2 phases, reaching a maximum in the early S phase and disappearing during mitosis. PCNA, proliferating cell nuclear antigen, is an accessory protein for

DNA polymerase and has a longer half-life than Ki-67. Therefore, it tends to overestimate proliferation but provides a longer-term, cumulative assessment of proliferative activity and is valuable for tissues with only low number of proliferating cells (83, 84). Together, these two markers provide two complementary measures of proliferation.

Their relation to breast density has been assessed in two prior studies. One reports a positive association of breast density to expression of Ki-67 in ducts of breast tissue (78). The other study conversely did not find a correlation between Ki-67 expression in FNA tissue samples and breast density (85). To date, PCNA has not been assessed in its relation to breast density.

Another receptor, distantly related to proliferation, is the tyrosine kinase HER-2/neu of the epidermal growth factor family. Amplification of the gene encoding ErbB2/HER2 is detected in approximately 20% of breast tumors. It is correlated with dramatic overexpression of the receptor, an aggressive phenotype of tumors as well as poor clinical prognosis (86). Trastuzumab, a HER-2 specific recombinant humanized antibody was the first biologic approved for treatment of metastatic breast cancer. This receptor had not yet been analyzed in its relation to breast density.

1.4 Stromal histopathology and matrix metalloproteinases (MMPs)

As mentioned earlier, the main component of the radiologically dense breast is comprised of stromal tissue whereas epithelial cells only constitute about 5% of breast tissue. In breast cancer patients, increased amounts of collagen, the main component of stromal tissue, have been shown through many studies to be associated with breast density (87-89). Growing evidence supports the notions that altered regulation of the stroma, or extracellular matrix (ECM), contributes to neoplastic progression and that disruptions in the ECM may precede epithelial changes (90). One group of enzymes, involved in the remodeling of extracellular matrix (ECM) are matrix metalloproteinases (MMPs). They constitute a family of over 20 zinc-dependent endopeptidases that have classically been viewed as being secreted by tumor cells to degrade the basement membrane and ECM components to facilitate tumor invasion and formation of metastasis. Today we know that MMPs contribute to multiple steps of tumor promotion, angiogenesis and the establishment of metastatic lesions. They are synthesized by tumor cells, as well as by the surrounding host stromal cells such as fibroblasts, endothelial and also infiltrating inflammatory cells (91-93). MMPs are produced as latent proenzymes, stored in inflammatory cells, anchored to the cell surface or within the ECM. Physiologic MMP levels are low, but their expression is rapidly up-regulated when tissues undergo remodeling such as inflammation, wound healing and tumorigenesis (94, 95).

Overexpression and unrestrained activity of MMPs have as well been linked to malignant conversion of tumor cells (96). MMPs are universally expressed during tumor progression and

have been documented in essentially every human tumor entity, including breast cancer (91). There is considerable evidence supporting a role of MMPs in early stages of tumor onset and primary tumor growth. In particular, MMP-3 and MMP-9 have been reported to be involved in mammary carcinogenesis (97). Although the matrix-degrading properties of MMPs are important, they alone do not account for the diversity of biologic effects. The ECM is a reservoir of growth factors and binding proteins which effect tumor cell behavior. Most likely, the activating ability of MMPs on these factors is what yields to the rapid responses required for tumor growth and progression. For instance, the activity of insulin like growth factors (IGFs), important growth factors in tumor progression, is controlled by numerous extracellular binding proteins (IGFBPs) (98). Cleavage of these binding proteins by MMPs possibly contributes to tumor growth (99). Other factors activated by MMPs are promoting survival (FAS-Ligand), angiogenesis (Collagen IV, VEGF) and invasion (Lam-5, CD-44) (among others) (100). It is important to note that different MMPs contribute to tumor progression at multiple or different stages. They play important roles in tumorigenesis, cancer progression, invasion and metastasis through several diverse mechanisms. Tissue inhibitor of metalloproteinases (TIMPs 1, 2 and 3), are natural inhibitor proteins, mostly produced by stromal host cells and by the tumor cell itself. They form complexes with both latent and activated MMPs, inhibiting their enzymatic activity.

1.5 Goals of the study

Breast density is a strong, independent risk factor for breast cancer, which is a leading cause of morbidity and mortality among women in nearly all developed countries. Yet, the biological basis for this association is not known. Given the aforementioned inconsistent findings about proliferative lesions and histopathological correlates of breast density, one aim of this study was to examine the correlation of breast density with the expression of markers of proliferation in breast tissue. We measured Ki-67 expression, a widely-used indicator of cell proliferation in normal and neoplastic tissues and Proliferating Cell Nuclear Antigen (PCNA), which gives a cumulative assessment of proliferative activity and is valuable for tissues with only low numbers of proliferating cells (84). As risk factors that induce proliferation, hormone replacement therapy (HRT) and endogenous sex steroids are associated with higher breast cancer risk as well as higher breast densities (44, 77, 78). The biological activities of endogenous and exogenous sex steroids on breast tissue are mediated by nuclear estrogen receptors alpha and beta (ER α and ER β) and progesterone receptor (PR). To understand how hormone receptors and cell proliferation are related to mammographic density, this investigation examined the expression of ER α and ER β , PR and HER2/neu. The effects of sex steroid hormones on the mammary gland are also mediated, at least in part, through interactions between stroma and epithelium (101). As stroma is present in much larger quantities

than epithelial tissue in the mammographically dense breast, differences in stromal qualities were also investigated, in particular the altered regulation of cell - extracellular matrix (ECM) interaction which contributes to neoplastic growth (88, 90). Finally, matrix metalloproteinases (MMPs), zinc-dependent endopeptidases involved in remodeling of ECM, contribute to all steps of tumor progression, including tumor promotion, angiogenesis, and the establishment of metastatic lesions (102, 103). A disruption of the balance between MMPs and their natural inhibitors (TIMPs) has been implicated in the progression of cancer (104). To understand how stromal matrix metalloproteinases are related to breast density, this study examined MMP1, 3, 9, 12 and TIMP3 in relation to mammographic density. Finally, this study evaluates the comparably new technique of TMAs as a tool in epidemiologic research which characterized the pilot form of this project. The combination of TMA technology with population based tissue repositories offers a powerful tool for research regarding tumor development and progression without depletion of pathological specimens.

2. Materials and Methods

2.1 Study population

2.1.1 The multiethnic cohort study (MEC study)

The study population is based on subjects who took part in the multi-ethnic-cohort (MEC) study in Hawaii and California and in a nested-case-control-study (NCC) for mammographic density in Hawaii. Participants entered the MEC study from 1993 to 1996 by completing a mail questionnaire that obtained information about their food frequency as well as demographic and other information. The MEC study investigates the association between diet and cancer in 215,251 adult men and women of different ethnic backgrounds (African-American, Latino, Japanese-American, Native Hawaiian and Caucasian). This population-based study recruited participants through driver's license, voter registration, and Health Care Financing Administration files and was approved by the Committee on Human Studies at the University of Hawaii. Data from the Hawaii component of the MEC study is linked annually to the Hawaii Department of Health vital records database and the statewide Hawaii Tumor Registry (HTR) to determine cancer incidence and deaths. Further details are provided elsewhere (105, 106).

2.1.2 The nested case-control study (NCC study)

The NCC study, on which our study population is based on, recruited all female members of the MEC study who resided in Hawaii and were diagnosed with primary breast cancer between cohort entry and December 2000 (potential cases: n=1,587). Controls were randomly selected from MEC Study participants who were not known to have breast cancer and frequency matched by age and ethnicity (107). Women with previous diagnosis of breast cancer, a history of breast augmentation or reduction, or without a mammogram were excluded from the study. After further exclusion of subjects due to death, no consent or no suitable mammogram, the final sample of the NCC study consisted of 607 breast cancer cases and 667 controls. Comparison of the women included in the study with ineligible cases showed only small differences. None of the demographic, reproductive, or anthropometric variables observed differed significantly at the 5% level between matched pairs of participants, indicating a representative sampling of the original MEC. The NCC study was approved by the Committee on Human Studies at the University of Hawaii. All subjects provided informed consent to participate for the NCC -as well as the MEC- study.

2.1.3 Recruitment of study subjects

The 607 cases of the NCC-study were selected as a starting point for our study. Study subjects were recruited by mail with a maximum of 2 reminders. We sent out letters including information about the study, consent forms for the release of tissue together with a Soy Questionnaire to 430 women. The other 177 (29.16%) women out of the NCC study were not contacted for different reasons. For 137 cases (22.57%), the respective hospitals were not participating in the tissue discharge repository organized by the HTR. Another 40 women were not contacted because of deaths at time of recruitment (26 women, 4.28%) and refusals (14, 2.31%). After we sent out the letters to potential participants, we learned about 6 deaths (0.99%) that had occurred (before the time of recruitment), 4 women were too ill to participate (0.66%) and some refused to partake in the study (37 women; 6.10%). For 52 women (8.57%) who signed a consent form, we could not receive any tissue for the analysis. In 30 of these cases, there was no tissue stored in the hospitals, 5 cases were not linked to the HTR and the tissue blocks from the remaining 17 subjects did not contain enough tissue to sample. We excluded all these women after obtaining this additional information. There were 12 women that deceased before the recruitment for our study, but had their tissue stored in the HTR. They were included in the study. Table 3 shows the numbers of women recruited and participating in the study. Our final number of cases was n=283, 46.62% of the original NCC study participants.

2.2 Data collection

2.2.1 Anthropometric and demographic information

Information on anthropometric, demographic and reproductive variables and family history of cancer were gathered with an extensive questionnaire at entry into the cohort. A follow-up questionnaire mailed during 1993-2003 provides updated body weight information for all cohort members. Additional information on HRT, menopausal status, previous breast surgery and mammograms were obtained with the breast health questionnaire when the women enrolled in the mammographic density (NCC) study.

2.2.2 Mammograms

We retrieved the mammographic films from clinics located throughout the State of Hawaii. The 33 different clinics and hospitals listed on the release forms were accredited according to the Mammography Quality Standards Act of 1992. Authorization forms signed by the study subjects were used to request these films.

2.2.3 Tissue from the Hawaii Tumor Registry

The tissue analyzed in this study was provided by the Hawaii Tumor Registry (HTR), one of the 17 state and regional central cancer registries of the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute. The SEER program has provided data on cancer incidence and survival in the United States since 1973. As a population based cancer registry, it also aims to monitor trends and identify risk/protective factors for cancer development and offers huge research potential. The HTR collects data on patient demographics as well as clinical information including tumor site, histology, stage at diagnosis, first course of treatment, and follow-up for vital status. To date it maintains information on nearly 130,000 cancer cases diagnosed in Hawaii (105). The foundation of one central repository that collects tissue specimens offers the advantage of a defined population basis, equal data quality standards and unbiased frequency measures of molecular markers. In addition, it greatly improves access to these specimens and important annotations, such as corresponding demographic, clinical and pathology information, concerning the patient. A Tissue advisory committee (TAC) provided scientific and ethical oversight and sets up policies for pathology specimens as well as guidelines for obtainment of tissue from the repository. The Tissue Discard Repository obtains tissue samples from pathology laboratories throughout the State since 1995. These tissues would have otherwise been discarded by medical facilities (after 5 -10 years) and therefore lost for research projects. For those women diagnosed with cancer during the last 5-10 years, some specimens for this project were not part of the HTR at the time of analysis. The investigators took the extra effort and requested the tissue blocks directly from the hospitals where these women received their treatment. Briefly, to request tissue from the HTR, letters of intent with a study summary, approvals regarding research on human subjects (full institutional review board (IRB) review) and completed patient informed consent forms are needed.

2.3 Assessment of breast tissue

2.3.1 Selection of tissue

The original histopathologic slides were evaluated with the help of Dr. Killeen, Clinical Pathologist and director of laboratories at Kapiolani Medical Center for Women and Children. He examined the achieved tissue blocks from a certain tumor (in general up to 10 blocks from a given patient) and taking the laboratory reports as guidance, identified 1-2 blocks with sufficient tissue to sample. After this prescreening, the blocks were sectioned at 5 microns and between 1 to 10 hematoxylin and eosin (H&E) slides were prepared for each case. Each slide was examined microscopically with a magnification of 40 times, to identify morphologically representative regions from cancer and non-cancer areas. On the slide, showing the most representative malignant and benign tissue,

these areas were marked using different colors to make a distinction. Areas about 3 mm² to 6 mm² were circled in order to have enough tissue to choose from for the construction of TMAs and in the same time not to deplete the tumor for further research. Cancerous tissue was selected applying a black marker directly on the slide. In cases in which invasive and carcinoma in-situ co-existed, the areas showing invasive carcinoma were circled. For benign tissue a green color was used (figure 2). No distinction was made between specific areas of the tumor, such as center versus periphery or depth of invasion. The samples for Immunohistochemistry (IHC) were chosen based on the best quality and quantity of normal and malignant epithelium. The benign areas selected were preferably at far distance from the malignant parts to represent normal tissue for the study. The malignant tissue areas included mostly invasive tumors. Some cases (62 cases, 23%) however only showed ductal carcinoma in situ (DCIS). Both, benign and malignant areas were surrounded by different amounts of stromal and fatty tissue. Due to difficulty finding benign ducts in some of the blocks, more stromal tissue was marked for benign than for malignant cases. Even though the amounts of fatty tissue were preferably kept as small as possible, some fatty tissue was included in the marked areas. The tissue microarrays only including fatty tissue were later excluded from the study. The marked H&E slides were sent back to the laboratory of the Cancer Research Center and aligned with the corresponding tissue blocks for the construction of the TMAs.

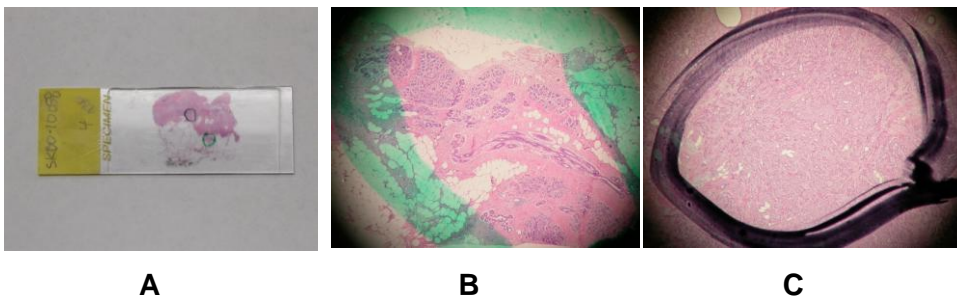


Fig. 2 Marking of benign (green) and malignant (black) areas of tissue for construction of TMAs. Slide after marking (A), magnification of marked benign (B) and malignant (C) tissue.

2.3.2 Construction of tissue micro arrays

Tissue Microarrays (TMAs) are a high-throughput and cost-effective method for the investigation of biomarkers in multiple tissue specimens simultaneously. TMAs are a collection of tissue cores arrayed into a single paraffin block which can be sectioned and mounted onto slides, producing up to 200 of nearly-identical sections. The technique of TMA preparation allows for the synchronized profiling of hundreds of specimens on a single microscopic slide, thus improving IHC efficiency. Suitable tissue can even be acquired from very small tumors. In our study, the tumor tissue blocks from the respective hospitals were used for the preparation of TMAs. From each of the formalin-fixed, paraffin-embedded (FFPE) blocks, 4 cylindrical malignant and 4 cylindrical normal core biopsies were taken, using a specialized manual arraying instrument (Model MTA1, Beecher instruments, Sun Prairie, Wis). First, the original FFPE tissue blocks were aligned with the marked histological slides to identify the corresponding area of tissue on the (donor) paraffin block. This area was then precisely marked on the block to allow for an exact sample of tissue. Out of the region identified, cores were punched out from the donor block with a 0.6 mm diameter needle. These tissue cores (approximately 0.5 mm in diameter and 3-4 mm in height) were then embedded into a preformed hole of an empty (recipient) paraffin block. Each particular hole of the recipient block was punched out (with a needle of 0.7 mm in diameter) just before the construction of the core sample in order to not deform the block. From each block, 4 benign and 4 malignant cores were sampled, if sufficient tissue was available. We documented the location of individual specimens in the recipient block using computer template files. Each array in the series had its own numerical designation (1, 2, 3, and 4). The new TMA paraffin blocks were 37 x 25 x 10 mm in dimension. Spacing between the core centers was 0.7 to 1 mm. Using this array arrangement the newly formed tissue array block can contain up to 530 specimens due to the close alignment of tissue cores. For cases with insufficient tissue to obtain 4 malignant and/or 4 benign cores, fewer cores were taken, leaving the corresponding areas in the recipient block empty. Also included were samples from liver and spleen tissue as quality controls at the top and bottom of each TMA block as well as normal breast tissue controls interspersed throughout the array. These controls assist the user to track physical considerations of the TMA block or cut sections, such as edge effects or construction artifacts that might arise during processing of the slides. The TMA blocks were subsequently cycled between 37 and 4°C to form a more rigid structure in the paraffin, binding the tissue cores to the paraffin. These blocks were sectioned at 4 microns and each cut was mounted on a single slide. In general, 100 to 200 slides can be prepared from each tissue array block. These prepared sections were used for IHC analysis.

2.3.3 Immunohistochemistry (IHC)

The TMAs were stained at the Wake Forest medical school, North Carolina in the lab of Dr. J. Mark Cline. The markers and characteristics of the antibodies are summarized in the following tables:

Antibody	Type	Company	Clone
Ki-67	(monoclonal rabbit)	Labvision NeoMarkers, Fremont, CA, USA	SP6
PCNA	(monoclonal mouse)	Novocastra Labs, Newcastle-upon-Tyne, UK	PC10
ER α	(monoclonal mouse)	Novocastra Labs, Newcastle-upon-Tyne, UK	6F11
ER β	(monoclonal mouse)	Novocastra Labs, Newcastle-upon-Tyne, UK	EMR02
PR	(monoclonal mouse)	Novocastra Labs, Newcastle-upon-Tyne, UK	1A6
HER2/neu	(monoclonal mouse)	DAKO Corporation, Carpinteria, CA, USA	

Table 1 Primary Antibodies used for staining of epithelial tissue

Antibody	Type	Company	Catalog #
MMP1	(monoclonal rabbit)	Epitomics, Inc., Burlingame, CA, USA	1906-1
MMP3	(monoclonal rabbit)	Epitomics, Inc., Burlingame, CA, USA	1908-1
MMP9	(monoclonal rabbit)	Epitomics, Inc., Burlingame, CA, USA	1939-1
MMP12	(monoclonal rabbit)	Epitomics, Inc., Burlingame, CA, USA	1906-1
TIMP3	(polyclonal rabbit)	Cell Applications, Inc., San Diego, Ca, USA	CA0577

Table 2 Primary Antibodies used for staining of stromal tissue

Staining procedures of the TMA slides were performed on the fixed, paraffin-embedded tissues. The basic staining procedure uses an avidin-biotin-alkaline phosphatase method, modified for antigen retrieval from paraffin-embedded tissue using the procedure of Shi et al. (108). Briefly, slides were deparaffinized through Xylene and alcohols to citrate buffer (pH 6.0), heat-treated for antigen recovery and exposed with the primary antibodies overnight at 4⁰C. Subsequently, tissue sections were exposed to biotin-streptavidin link and labeling antibodies (Biogenex, San Ramon, CA, USA), and finally visualized using the chromogen-substrate Vector Red (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared through p-Xylene and coverslipped. With each staining batch, positive and negative controls were prepared for each marker.

2.3.4 Image Analysis

Slides that were stained for epithelial markers (ER α and β , PR, Ki-67, PCNA and HER2/neu) were measured at the Wake Forest medical school with image analysis software for immunohistochemical stains (Image Pro Plus Software, Media Cybernetics). I evaluated the TMAs stained for stromal markers (MMPs and TIMP) at the Cancer Research Center of Hawaii. A trained pathologist (JMC) reviewed all specimens after staining to confirm their benign or malignant epithelial tissue content. If variables were discrepant, they were recoded according to the new reading.

For epithelial markers, pictures of each TMA were taken with an Infinity 3 Digital Video Camera (Lumenera Scientific) at Wake Forest medical school (figure 3). Slides were placed under the 10 X objective of a microscope which then displayed the image on the computer screen in the image analysis program. The evaluation of stains was performed on a snapshot taken from the screen. Results were recorded as continuous variables. The area of all nuclei (A) was measured by a color selection corresponding to hematoxylin, whereas the area of positively stained nuclei (B) was measured by a color selection corresponding to the Vector Red chromogen. For the nuclear stains used in this study the percentage of staining was expressed as $B/A \times 100$. For subjects with more than one measurement available, results were subsequently averaged.

In the second part of the study, the staining for stromal markers (MMPs), slides were scanned from Aperio Scanning Services (Vista, CA) and digital images for each slide were evaluated for analysis (figure 4).

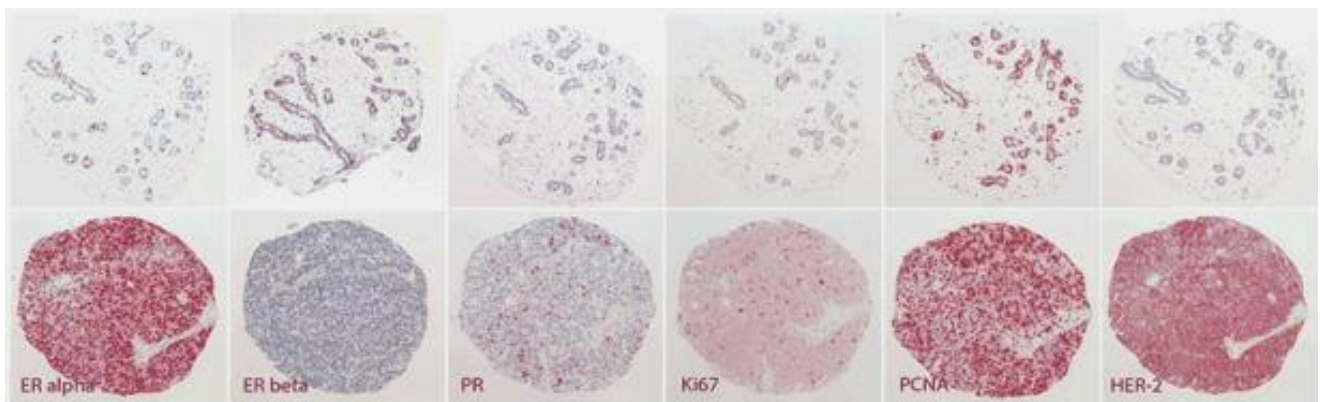


Fig. 3 Representative micrographs of the respective stains in benign (top row) and malignant (bottom row) tissue.

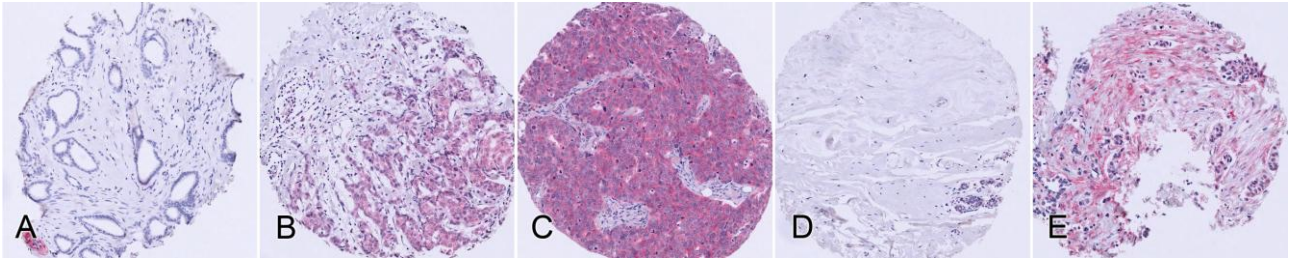


Fig. 4 Examples of staining results for stromal markers in epithelial (A-C) and stromal (D-E) tissue: no (A), medium (B) and strong stain (C) and no stain (D) versus any stain (E).

2.4. Breast density analysis

Mammograms were evaluated by a computer-assisted density assessment using the Cumulus 108 software, a program designed to operate under Windows to display digitized mammograms and allow thresholding operations. First, each film was scanned with a Kodak LS 85 Film Digitizer (absorbance range, 0.001-4.1; Eastman Kodak Company, Rochester, New York) at a resolution of 98 pixels per inch (pixel size equal to 260 μ m). For density assessment, the 8-bit images are displayed in 256 shades of gray. Originally, several mammograms were available for each patient. If possible, we only included films taken before the date of diagnosis of breast cancer. Otherwise, the mammogram of the contralateral breast was used in our study. In order to analyze histopathologic features in association with mammographic density, we included the mammogram taken closest to the diagnosis of breast cancer and, therefore, closest to collection of our tissue samples. In our analysis, only the cranio-caudal views of mammograms are studied. For participants, mammograms were taken 2.4 ± 2.6 years after entry into the cohort and 2.7 ± 2 years after completion of the baseline questionnaire. We randomly ordered all images to be read into batches of 50 mammograms each (except for the last batch, which included 30 films). The reader was blinded as to identity and year of the films and performed computer-assisted density readings. For assessment of intraobserver agreement, a separate batch with randomly chosen images of the other 9 batches was assessed in a separate session. An example for the computer-assisted density measurement is shown in figure 5. We first set a threshold for each image, to determine the outside margin of the breast. This threshold, shown by a red line, separates the image of the breast from the black background. To establish the dorsal margin of the breast and to cut off the Musculus pectoralis a line was subsequently drawn from top to bottom of the breast image. The computer calculates the number of pixels in the digitized image that constitute the total area of the breast. To measure the dense area, a second threshold is set, which surrounds the lightest areas of the breast image with a green line, indicating radiodense tissue. Percent breast density is then computed by dividing the dense area by the total area and multiplying the result with 100. To

convert the pixels into square centimeters we used a factor of 0.000676. Given the high correlation between readings from the right and the left breast, mean values were used for the analysis when both mammograms were available. 137 (48.4%) of the values are based on the readings of one side only. For the remaining 146 participants (51.6%) we calculated mean values form both readings.

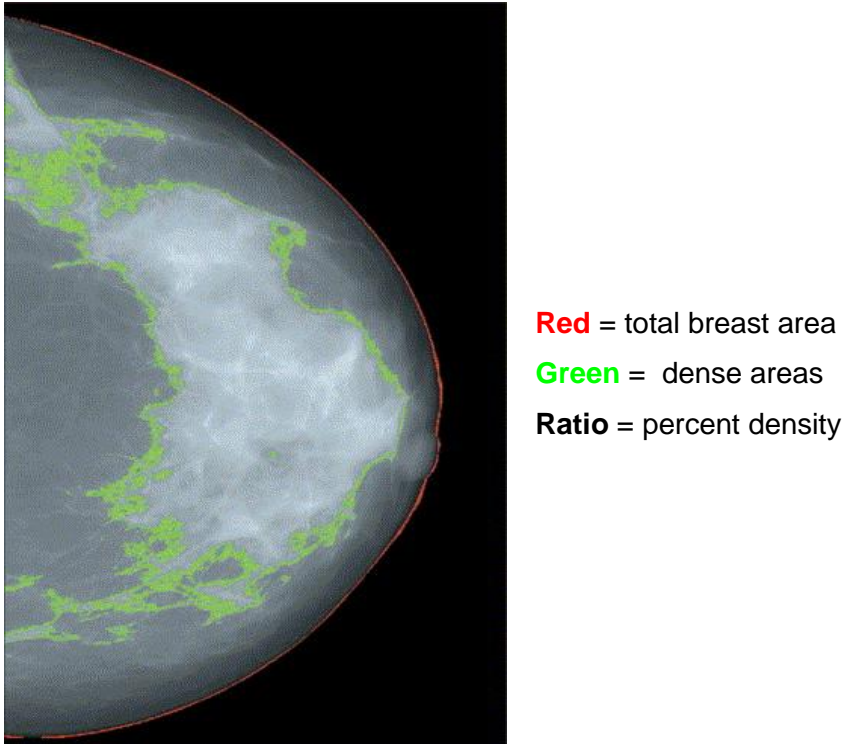


Fig. 5 Computer assisted density measurement (2)

2.5 Statistical Analysis

Data management and statistical analysis were performed using the SAS statistical software package 9.1 (SAS Institute Inc., Cary, NC). No names or other identifying information was part of the analytical database. Histopathologic results were added to the database of nutritional, demographic, and medical information obtained from the food frequency questionnaire (FFQ) using the study identification number as a link. We examined the data for completeness, outliers and possible coding errors. All continuous variables were inspected for skewness of their distribution. For those outcome variables deviating form normal distribution, we applied appropriate transformation or created categorical variables.

2.5.1 Variables

For the assessment of breast density, the variables percent density, dense area of the breast and total area of the breast were included in our analysis as continuous variables. Dense area was square root transformed for analysis to normalize the distribution. For ease of interpretation, back-transformed values are presented in the tables.

For epithelial markers, we computed means for percent staining of all available cores for each subject, separately for benign and malignant tissue. In benign tissues, distributions of all six epithelial markers were skewed with strong left tails. The interquartile ranges for benign tissue were 0.0-8.1% (ER α), 0.0-13.8% (ER β), 0.0 – 1.5% (PR), 0.0-14.5% (HER2/neu) , 0.0-0.6% Ki-67) and 0.8-16.2% (PCNA), respectively. Consequently, staining results for ER α , ER β and HER2/neu and PCNA were divided into two categories; negative staining (less than 10% of cells stained) and positive staining (at least 10% of cells stained). PR and Ki-67 were strongly left skewed; in benign tissue only 4 and 13 women respectively, had positive ($\geq 10\%$) staining. Thus we dichotomized these results into the groups no vs. any epithelial staining. In malignant tissue the individual interquartile ranges were 3.8-44.1% (ER α), 0.0-11.8% (ER β), 0.0-7.4% (PR), 3.7-40.1% (HER2/neu), 0.2-2.5% (Ki-67) and 20.9-55.0% (PCNA). Therefore, more detailed categories were created for these markers. We categorized into negative (no cells stained positive), weak staining (<10% of cells stained positive) and stronger staining ($\geq 10\%$ of cells stained positive) for ER α and ER β ; into no (0-1%), weak (1-10%) and strong staining (>10%) for HER2/neu and no (0-1%), weak (1-20%) and strong staining (>20%) for PCNA. We kept the categories no vs. any epithelial staining for PR and Ki-67 staining results.

Measurement results of the MMP stains in epithelial tissue were categorized as no, weak or strong staining, whereas staining of stromal tissue was dichotomized into no vs. any stain. A weight variable for every marker, reflecting the amount of benign and malignant samples (1 to 4) was calculated for each participant. For each subject, the benign and malignant core with the strongest level of staining was selected for the analysis. Only women with at least one measurement per marker and category (epithelial vs. stromal) were included in our analysis.

For the ethnicity variable, the ethnic background for both parents was reported on the questionnaires. We classified persons with more than one ancestry into a single ethnic category, giving first priority to Hawaiian followed by Japanese, then Caucasian, and finally Other (105). Ethnicity was categorized into Caucasian, Japanese, Hawaiian and others. Due to small numbers (Hawaiian n=31, others n=26) stratified analyses on density and markers were only performed for Japanese (n=121) and Caucasian (n=100) women separately.

In our analysis we only included the HRT variable for the mammogram, which was taken closest to the date of diagnosis of breast cancer. For women with missing HRT type information, we assigned

a type of HRT based on their hysterectomy status (109). For those who underwent hysterectomy we assigned Estrogen only therapy, otherwise combined HRT was imputed.

For menopausal status a similar approach was used. If we had the information that a woman underwent menopause, but were uncertain about the menopausal status of the specific year, we assigned a perimenopausal variable. Because of the small frequency count of women in this group (10 women, 0.35%), they were included in the premenopausal group in our analysis.

The body mass index (BMI) (weight (kg)/height in (m)²), was included as a continuous variable. However, for analysis stratified by BMI, we created four categories: <20, 20-25, 25-30 and >30 kg/m².

2.5.2 Analysis

To examine differences between the study population of our study and the original NCC study we employed χ^2 analysis for categorical variables and t-tests for continuous variables. For all epithelial markers, intraclass correlation coefficients (ICCs) were computed for benign and malignant tissue to assess marker agreement between the four samples per subject. Distributions of all staining results were analysed for the overall population and ethnic subgroups. Agreement between markers in benign and malignant tissue of each participant and correlation between the different markers were computed with χ^2 -and Spearman correlation coefficients, respectively.

We applied general linear models to determine the relation between markers and mammographic densities while adjusting for covariates that are known to be related to breast cancer risk. Potential confounders included age at mammogram, BMI as a continuous variable, ethnicity (Hawaiian, Japanese, Caucasian and Other), parity (0-1 children, 2-3 children and > 3 children), age at first live birth (< 21 years, 21-30 years, or > 30 years, or no children), age at menarche (< 13 years, 13-14 years, or > 14 years), menopausal status, HRT use at mammogram (no use, estrogen only, combined estrogen and progesterone), family history of breast cancer in a first degree relative (yes, no) and tumor stage (in-situ, localized, regional, unknown). All analyses were conducted separately for malignant and benign tissue and stratified by ethnicity.

3. Results

3.1 Baseline characteristics of study participants

For this analysis we recruited 283 breast cancer cases with primarily Caucasian (35.3%) and Japanese (42.8%) ancestry. Also included were 12.7% Hawaiians and 9.2% women of other ancestries. The mean age at the time of the analyzed mammogram was 60.2 ± 8.6 years. This relatively old mean age is partly due to the age limit of 45-75 years for entry into the MEC on which this population was based. More than two-thirds of the participants were postmenopausal (79.5%), the majority of whom did not use HRT (44.9%). The other 30.7% reported use of HRT with only estrogen, whereas 24.4% of postmenopausal women reported combined HRT treatment. Nearly all women in our study had early-stage disease consisting of either carcinoma in situ (21.9%) or localized invasive carcinoma (60.8%). The characteristics of the 283 participants included in this study compared with those of the NCC study are presented in table 3. Comparison of the women included in the analysis with the excluded cases showed only small differences. Japanese cases made up 51% of the excluded cases, Caucasians 26% and Hawaiians 14%, representing a similar distribution to the included women. The included cases were 3.2 years younger ($p < 0.0001$) and more likely to be postmenopausal at the time the mammogram was taken ($p = 0.0002$). They also showed a higher prevalence of use of oral contraceptives in the past (50.2% vs. 37.0%; $p < 0.0004$). Mean percent breast density was slightly higher in participants (38.9% vs. 35.4%; $p = 0.053$). There were no significant differences in BMI (kg/m^2), family history of breast cancer, age at menarche, age at first life birth, parity, HRT, use or class of HRT used. These findings indicate a representative sample of the population.

Table 3 Characteristics of women recruited for the NCC-study and for the TMA study

Variable	NCC-Study	Patho-Study	p-value^o
Sample Size (No.)	607	283	
Ethnicity			0.09
Caucasian	185 (30.5%)	100 (35.3%)	
Hawaiian	80 (13.2%)	36 (12.7 %)	
Japanese	287 (47.3%)	121 (42.8%)	
Other	55 (9.1%)	26 (9.2%)	
Age at mammogram*	63.7 ± 8.1	60.2 ± 8.6	< 0.0001
Body mass index (kg/m²)*	25.3 ± 5.6	24.7 ± 4.5	0.19
FH of breast cancer¹	103 (17.0%)	42 (14.8%)	0.19
Age at menarche			0.72
< 13 years	334 (55.0%)	159 (56.2%)	
13-14 years	218 (35.9%)	97 (34.3%)	
>14 years	55 (9.1%)	27 (9.5%)	
Number of children			0.35
0-1	166 (27.4%)	85 (30.0%)	
2 to 3	314 (51.7%)	143 (50.5%)	
>3	127 (20.9%)	55 (19.4%)	
Age at first live birth			0.27
<21 years	80 (13.2%)	41 (17.7%)	
21-30 years	377 (62.0%)	170 (14.5%)	
>30 years	56 (9.2%)	22 (60.1%)	
N/A	94 (15.5%)	50 (7.8%)	
Oral contraceptive (OC) use			0.0004
Never	341 (56.4%)	137 (48.8%)	
Ever	264 (43.6%)	144 (51.2%)	
HRT use at mammogram:			0.19
No use	264 (43.5%)	127 (44.9%)	
Estrogen only	174 (28.7%)	87 (30.7%)	
Combined HRT	169 (27.8%)	69 (24.4%)	
Breast density in percent*	33.5 ± 23.0	37.5 ± 23.5	0.035
Menopausal Status			0.0002
Postmenopausal	517 (85.2%)	225 (79.5%)	
Premenopausal	90 (14.8%)	58 (20.5%)	
Tumor Stage			0.30
In-situ	124 (20.4%)	62 (21.9%)	
Localized	368 (60.6%)	172 (60.8%)	
Reginal	96 (15.8%)	38 (13.4%)	
Unknown	18 (3.0%)	11 (3.9%)	

* Mean values are given

¹ Diagnosis of breast cancer in any first-degree relative

^o p-values from χ^2 - and Student's T test comparing cases included in the TMA study and those excluded

3.2 Quality of mammographic measurements

For assessment of intraobserver agreement (JSS), an individual batch with 45 randomly chosen images of the other 9 batches was assessed in a separate session. Based on these duplicate readings, the reproducibility of the density measurement was high, showing an intrareader correlation coefficient for percent density of 0.95 and 0.93 for dense area.

The mammograms from the NCC study participants have also been assessed by only one reader (G.M.); the intraclass correlation coefficients derived from duplicate readings were 0.96 for the size of the dense area and 0.97 for percent density.

Comparison of measurements from both observers showed an interreader correlation coefficient of 0.87. To evaluate agreement more precisely we created categories for percent breast density. Measurements were divided into the categories low (<10), medium (10 - 24.9), medium high (25 - 49.9) and high (≥ 50). Comparison of both readings can be seen in figures 6.1 and 6.2. Computing kappa statistics, we found a moderate interreader agreement ($\kappa=0.49$). When comparing only two categories of density (high versus low), comprised of values higher or lower than the median of the respective evaluation, we calculated a kappa value of 0.65 indicating substantial agreement between readers. To ensure consistency of results with prior published studies, we only used readings by G.M. in our analyses.

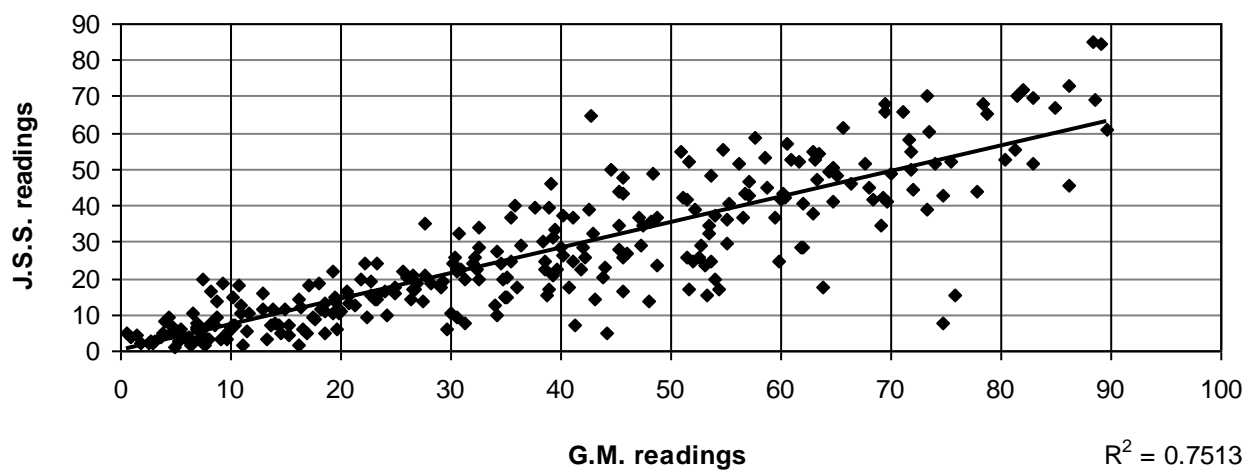


Fig. 6.1 Interreader agreement of percent density measurements

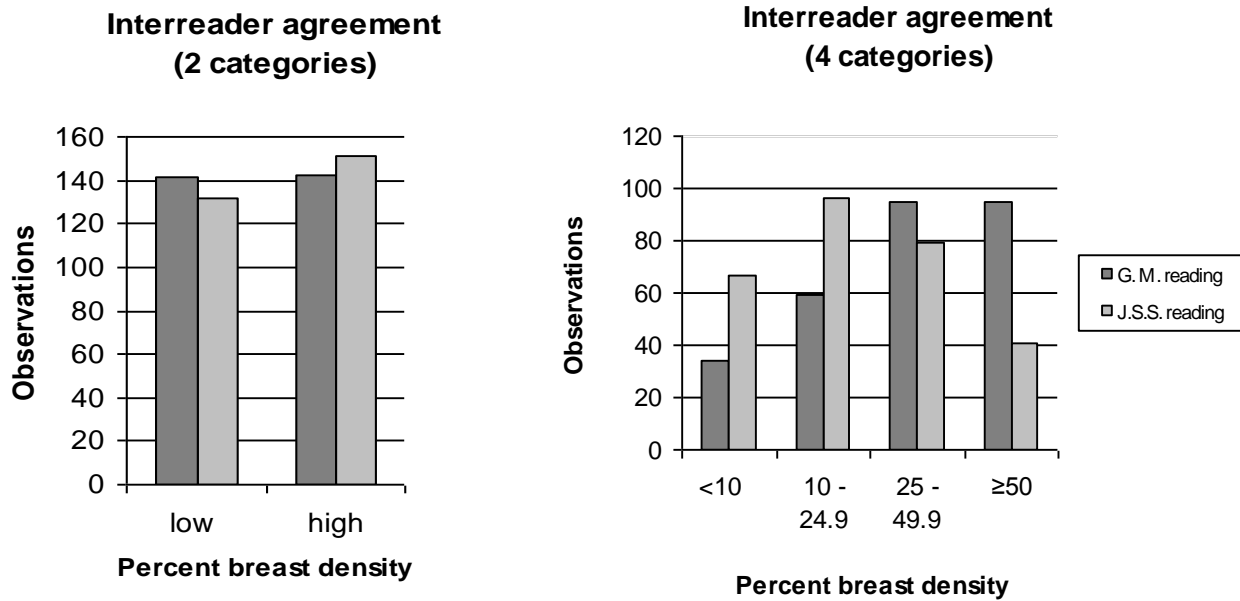


Fig 6.2 Interreader agreement of categorized percent density measurements

3.3 Association between breast density and risk factors

The percent breast density in our study population (n=283) ranged from 0.5% to 89.6% with a mean of 37.5% (± 23.5). Mean dense area was 37.8 cm² (± 25.36) with a range from 1.4 to 134.9 cm². The mean total breast area as measured on the mammograms was 116.0 cm². Conducting analysis of variance we found percent breast density to differ significantly by age ($p=0.001$), menopausal status ($p=0.001$), HRT ($p=0.002$), BMI ($p<0.0001$), parity ($p=0.002$) and age at first live birth ($p=0.007$) (table 4). Separate analyses stratified by ethnicity are shown in table 5. Mean percent density adjusted for age was lowest in women of Hawaiian and Caucasian ancestry, i.e. 30.2% and 36.0% respectively. Japanese had a mean density of 39.8%, whereas women of other ancestries had the highest density measurements with a mean of 42.7%. However, these differences were non significant ($p=0.07$). After adjustment for other known confounders only small differences in percent density were observed in the different ethnic categories. Mean percent density was lowest in Caucasian (35.4%) compared to the other subgroups, i.e. 38.9% (Hawaiian), 39.6% (Japanese) and 39.6% (other ancestries). The mean age when the mammogram was taken differed by group ($p=0.04$): Caucasian 60.0 years, Hawaiian 57.5 years, Japanese 60.0 years and for the group 'Others' 55.0 years. Also BMI differed significantly by ethnic group ($p=0.0001$). Hawaiians had the highest mean BMI with 28.03 kg/m² whereas the mean BMI for others was lowest with 23.62 kg/m².

The difference of a lesser density by age in the overall population remained significant ($p=0.0005$)

after full adjustment for all confounders. Density was also significantly higher in premenopausal (48.6% [42.7-54.4]) than in postmenopausal women (34.7% [31.7-38.5]) ($p<0.0001$). BMI was quite similar in pre- and postmenopausal participants ($p=0.79$). Premenopausal women had a mean BMI of 24.65 kg/m²; postmenopausal women had a mean BMI of 24.83 kg/m². Among postmenopausal women breast density differed significantly by type of HRT, with highest density percentages in the combined HRT group ($p=0.002$). Mean breast density was 30.7% in 127 women not taking HRT, 32.2% in 87 women taking estrogen alone and 42.9% in 69 women taking combined HRT with progestin and estrogen.

Table 4 Association of breast density with breast cancer risk factors

	n	Percent density		p-value ¹
		Mean %	(95%CI)	
Overall	283	37.5	(34.7 - 40.2)	
Menopausal status				
Premenopausal	58	48.6	(42.7 - 54.5)	0.001
Postmenopausal	225	34.6	(31.6 - 37.6)	
HRT use				
No HRT	127	30.6	(25.7 - 35.5)	0.002
Estrogen only	87	32.3	(27.3 - 37.2)	
Estrogen + Progest.	69	43.0	(37.3 - 48.6)	
BMI kg/m²				
< 20	33	52.8	(45.3 - 60.3)	< 0.0001
20-25	137	41.5	(37.8 - 45.2)	
25-30	81	30.2	(25.4 - 35.0)	
>30	32	22.9	(15.2 - 30.6)	
Number of children				
0-1	85	41.9	(37.0 - 46.9)	0.002
2 to 3	143	38.6	(34.7 - 42.3)	
>3	55	27.8	(21.6 - 33.9)	
Age at first live birth				
<21 years	50	46.3	(39.9 - 52.7)	0.007
21-30 years	41	27.4	(20.3 - 34.4)	
>30 years	170	36.4	(33.0 - 40.0)	
N/A	22	44.3	(34.7 - 54.0)	

¹ p-value for general linear models (percent density)

Table 5 Mean percent density in different ethnic groups

Ethnicity	n	Percent Density ^a	Dense Area ^a	BMI ^b	Age ^c
		(95%CI)	(95%CI)		
Caucasian	100	35.4 (31.2 - 39.5)	40.2 (35.3 - 45.2)	24.5 (23.54-25.46)	61.4 (59.7-63.1)
Hawaiian	36	38.9 (31.6 - 46.2)	42.1 (33.3 - 50.8)	28.03 (26.12-29.93)	57.8 (54.7-60.9)
Japanese	121	39.6 (35.8 - 43.4)	35.7 (31.2 - 40.3)	24.31 (23.72-24.91)	60.6 (59.1-62.1)
Other	26	39.3 (31.0 - 47.7)	38.5 (28.5 - 48.5)	23.62 (21.81-25.36)	57.2 (53.8-60.5)

^a Adjusted for age at mammogram, body mass index, menopausal status, age at menarche, HRT use at time of mammogram, age at first life birth, parity and family history of breast cancer

^b Body mass index; weight (kg)/height in (m)²

^c Age at time of mammogram

3.4 Number of cores on TMA

For 4 of the 283 recruited subjects, not enough tissue was available on the selected tissue blocks for placement on TMAs. We therefore only constructed TMA blocks for 279 women. Out of the 2232 possible TMAs (four malignant and four benign samples for 279 women), 459 cores (20.6%) could not be placed due to insufficient tissue on the respective block. Of these, 29% (134 cores) were of malignant tissue and 71% (325 cores) of benign samples. Overall 1773 (79.4%) tissue cores were successfully placed on the respective blocks. During staining for stromal markers, about 4 – 8% of samples fell off the slides. The numbers were quite similar throughout the particular stromal markers.

During staining for epithelial markers, about 7-9% TMAs fell off and numbers were also quite similar across individual markers. For all the markers collectively, about twice as many benign vs. malignant samples were missing after staining.

On average, 3.1 malignant tissue samples and 1.7 benign samples were available per women for the epithelial markers. For stromal markers on the other hand, 3.5 malignant and 2.9 benign TMAs were available on average per women. For stromal markers up to 3% of cores could not be properly evaluated due to artifacts (vascular stain, fluid in duct, stained red blood cells, among others). About 11.6-13.7% of cores had less than 25% of tissue present and were, therefore, not evaluated. For subsequent analyses, we used data sets including only those women who had at least one measureable stain for the respective markers. For epithelial markers, 268 women (159 women with only benign samples and 253 women with only malignant measurements) were

included. In our stromal marker datasets we had 277 women, 259 women with at least one benign and 274 with at least one malignant measurement (figures 7.1 and 7.2).

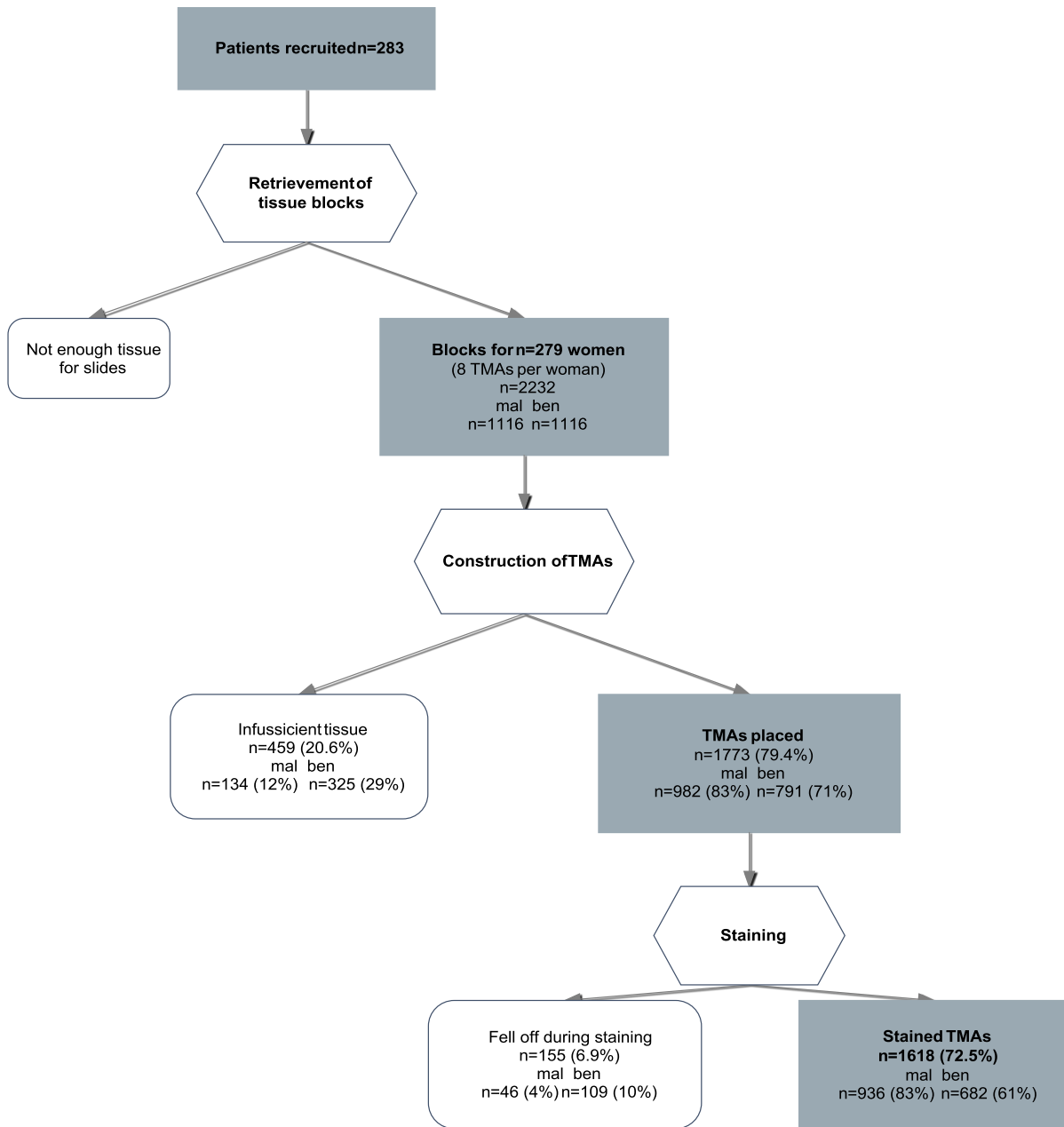


Fig. 7.1 Loss of subjects and tissue throughout recruitment and TMA construction

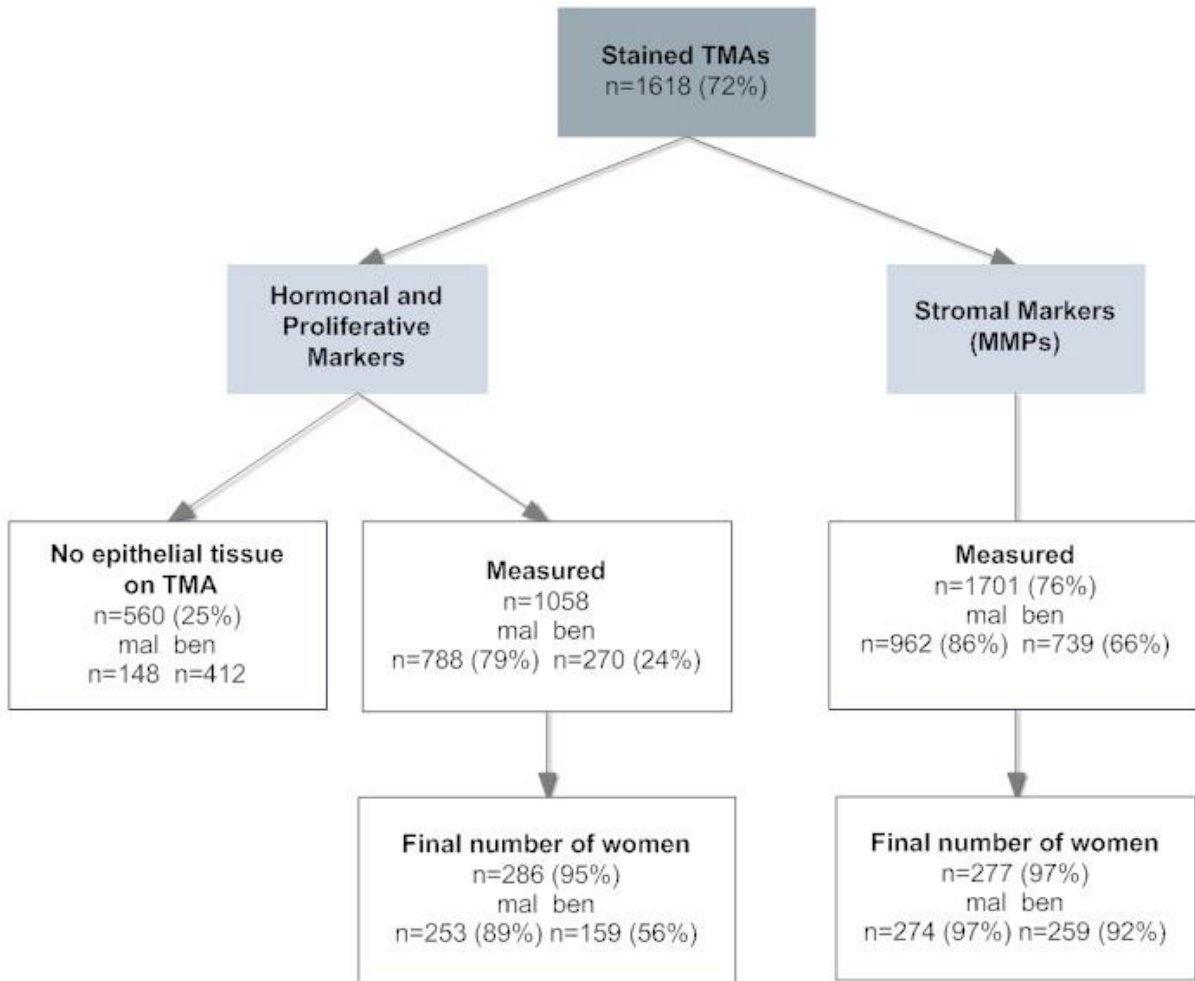


Fig. 7.2 Cores placed on TMAs

3.5 Descriptive analysis of markers

3.5.1 Hormonal and proliferation markers

To assess the agreement per subject, ICCs were computed for all epithelial markers. Across malignant samples, ICCs varied between 0.45 and 0.74 depending on the specific marker, whereas ICCs for benign tissue were 0.20-0.42 indicating fair and moderate to substantial agreement. Distributions of all six epithelial markers were skewed with strong left tails (figure 8.1). A substantial proportion of the benign samples were in the low or no staining categories. The percentages were 78% (ER α), 70% (ER β), 49% (PR), 67% (HER2/neu), 42% (PCNA) and 60% (Ki-67). In malignant specimens low or no staining was considerably less frequent for all epithelial markers except for ER β (70% of TMAs were in the no or < 10% staining categories). Percentages for other markers were 33% (ER α), 23.4% (HER2/neu) and 28% (PR). The proliferation markers were negative or low in 9% (PCNA) and 11.9% (Ki-67) of malignant specimens (see figure 8.2). A

similar proportion of Japanese and Caucasian women were in the highest staining category for all markers. None of the differences were statistically significant. The samples size was too small to examine other ethnic groups.

There was substantial, statistically significant agreement for the markers ER α ($p=0.002$), ER β ($p<0.0001$) and PR ($p=0.03$) in benign and malignant specimens of the same subjects. The proliferation markers Ki-67 and PCNA as well as HER2/neu did not correlate ($p=0.30$ and $p=0.20$, and $p=0.13$ respectively). In benign tissue, based on Spearman correlation coefficients, all epithelial histopathologic markers were significantly positively correlated with each other except for ER β and PR. There was an especially strong correlation between ER α and PCNA ($\rho=0.64; p<0.0001$) as well as Ki-67 ($\rho=0.31; p<0.0001$) and HER2/neu ($\rho=0.38; p=0.0001$). These associations were also significant in malignant tissue, but not as strongly. ER α was positively associated to PCNA ($\rho=0.47; p<0.0001$), but not significantly correlated to Ki-67 expression, whereas ER β was positively correlated with Ki-67 ($\rho=0.33; p<0.0001$).

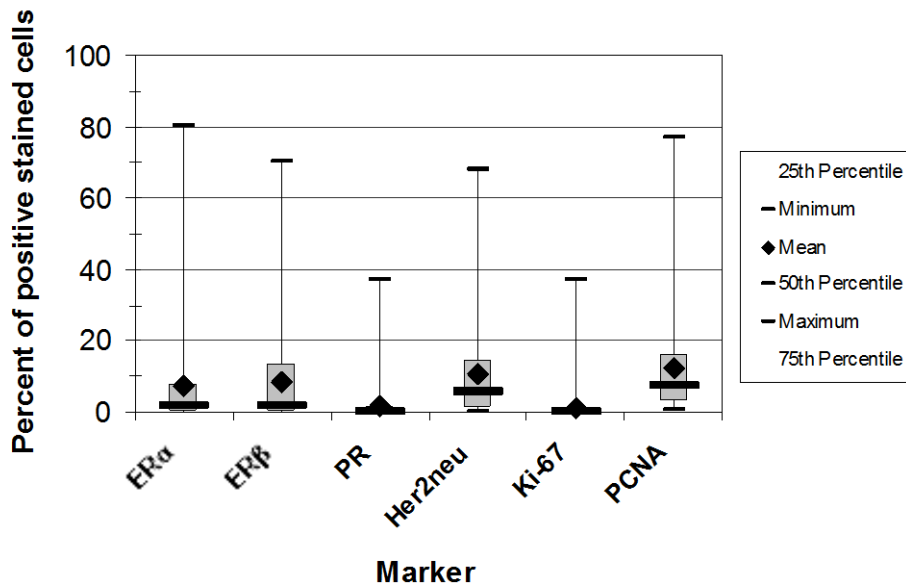


Fig. 8.1 Distribution of positive staining results in benign tissue

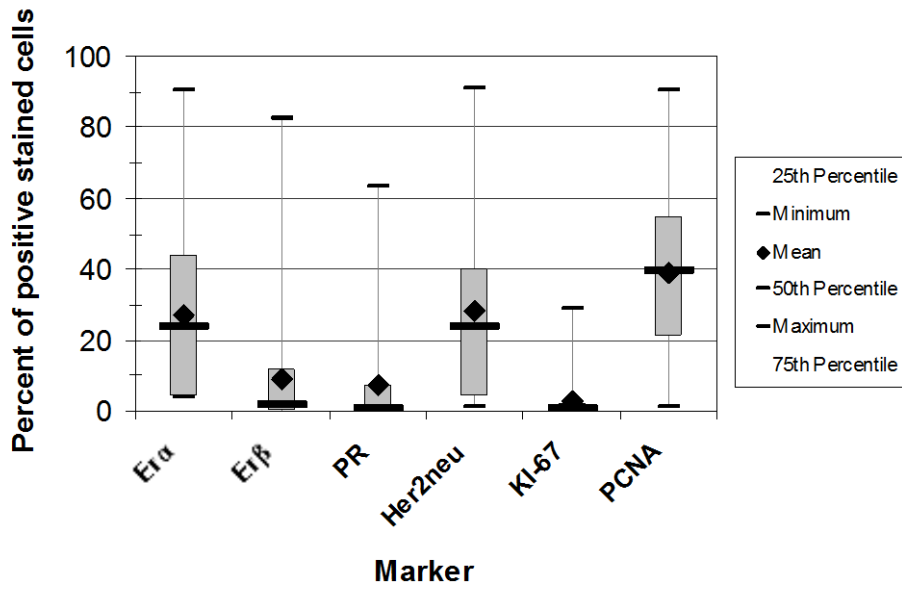


Fig. 8.2 Distribution of positive staining results in malignant tissue

3.5.2 Stromal markers

The percentages of positive stained cells for the respective stromal markers and tissue origin are shown in figures 9.1 and 9.2. Of all subjects in the stromal marker subgroup only 2% of benign and 8% of malignant epithelial specimens stained positive for MMP3; No stromal samples showed positive staining. For MMP9, only 5% stained positive in benign and 18% in malignant epithelium. In stromal tissue samples, 5% and 1% stained positive in malignant and benign tissue, respectively. Due to this lack of staining we did not include MMP3 and MMP9 in further analyses. For MMP1 and MMP12, epithelial as well as stromal marker expressions were more likely to be higher in malignant than in benign tissue. In stromal tissue, the proportion of positive stained cells was 28% (malignant) and 20% (benign) for MMP1 ($p < 0.0001$) and 38% (malignant) and 10% (benign) for MMP12 ($p = 0.001$). In epithelium, the percentages of strongly positive stained cells were 52% and 34% for MMP1 and 75% and 44% for MMP12 for malignant and benign tissue, respectively.

In epithelium, expression of TIMP3 was higher in malignant (16% strongly positive) than benign (8%) tissue ($p < 0.0001$), whereas stromal tissue expression was higher in benign (33%) than malignant (19%) samples ($p < 0.0001$). We observed no significant difference in staining between Japanese and Caucasian except for MMP12 in malignant tissue; the proportion stained was higher in Japanese than Caucasian women both in stromal ($p = 0.056$) and epithelial tissue ($p = 0.03$).

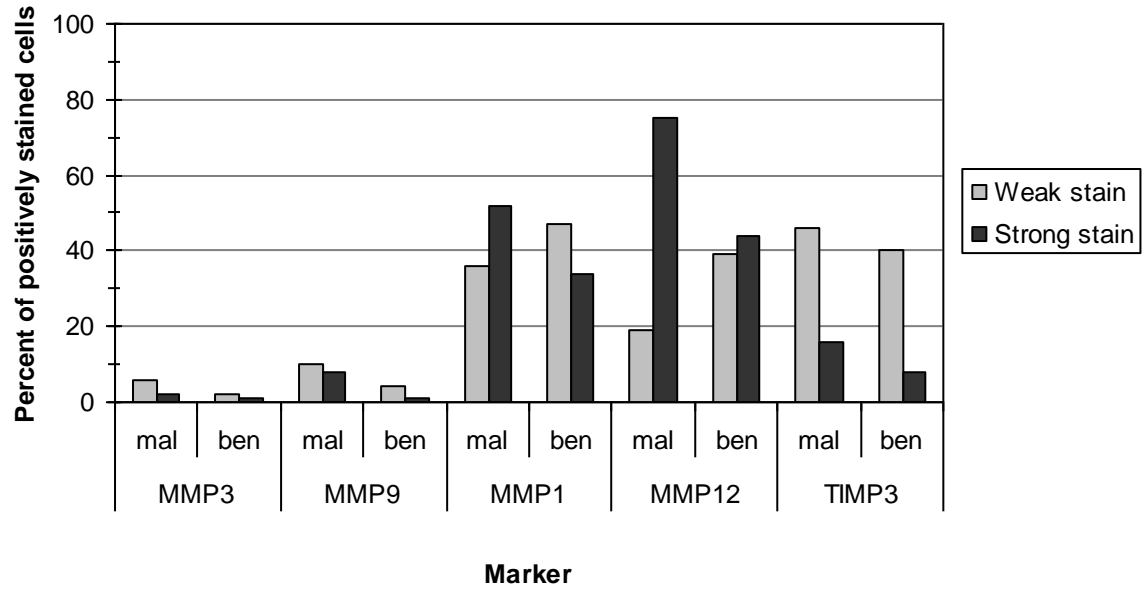
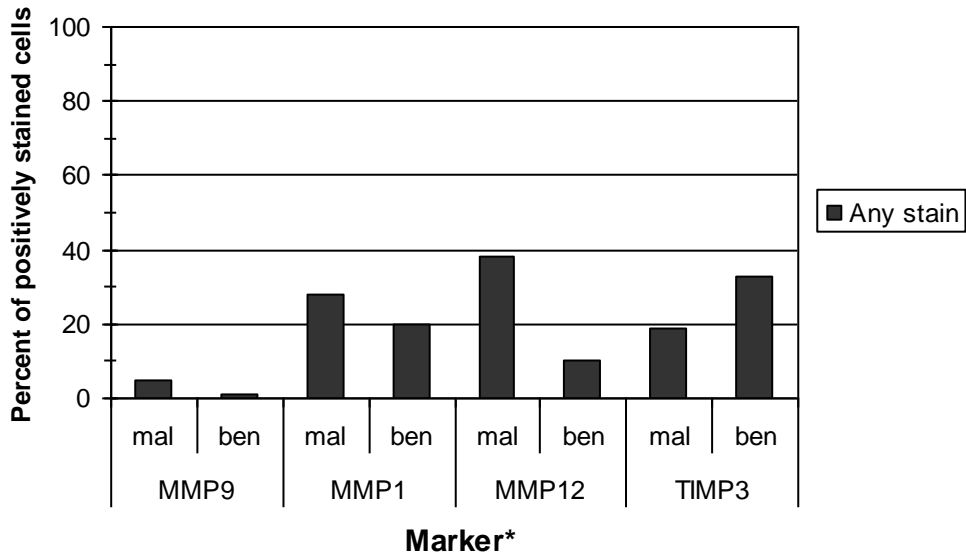


Fig. 9.1 Percent of positive staining results for MMPs in epithelial tissue



*no stroma stained positive for MMP-3

Fig. 9.2 Percent of positive staining results for MMPs in stromal tissue

3.6 Association of density with histopathologic markers

The association between breast density as a continuous measure and categories of histopathologic markers was analyzed using general linear models. We adjusted for covariates that are known, as mentioned above, to be associated with breast density.

3.6.1 Hormonal and proliferative markers

Overall, only small differences in breast density were seen between staining categories of several markers (figures 10.1 – 10.3). Most of our findings were not statistically significant (tables 6 and 7). We observed consistently higher mammographic density for women with PR expression across the two major ethnic groups (all women: 3.4%; Caucasian: 5.8%; Japanese: 3.5%). These findings were similar in malignant samples of all women. For women with stronger ER β staining, percent density was higher in the overall population and in Caucasians (2.4% and 11.6%) but not in Japanese. The difference among Caucasian women was statistically significant ($p=0.05$). There was no consistency in malignant samples. Furthermore, no consistent associations of percent density with ER α and HER2/neu were observed. Among Caucasian women, percent breast density was significantly less extensive in women with low than high staining for ER α (39.8% and 23.9% respectively, $p=0.04$). However, Japanese women showed an inverse correlation which was also observed for staining in malignant samples; Mean percent density was 31.9% (no stain), 43.5% (weak stain) and 38.0% (strong stain, $p=0.05$).

Percent density was slightly higher in women with Ki-67 staining both in the overall population (3.4%) as well as in ethnic groups (Caucasians: 3.8% and Japanese 4.4%). Only 12% of malignant samples were in the low staining group, and percent density did not differ significantly between low and high staining for Ki-67.

Women with positive staining for PCNA showed slightly higher percent density. This finding was consistent among the overall study group and Japanese but not among Caucasians. In malignant samples, breast density was lower in women with higher PCNA staining.

Restricting the analyses to postmenopausal women did not substantially change the results of the overall analyses of benign samples for ER α , HER2/neu, and the proliferative markers, whereas the associations of benign ER β and PR staining with mammographic density became somewhat stronger. The respective mean percent densities for ER β were 26.4% vs. 32.0% ($p=0.22$) and 25.3% vs. 30.8% ($p=0.18$) for PR (data not shown).

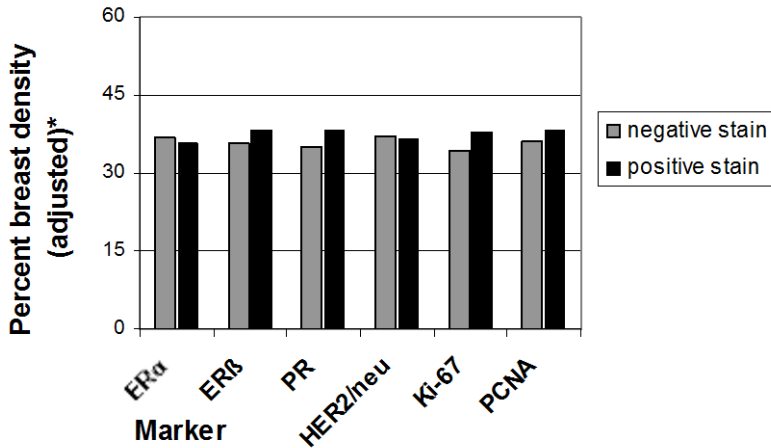


Fig. 10.1 Density and epithelial markers in benign tissue of all women^o

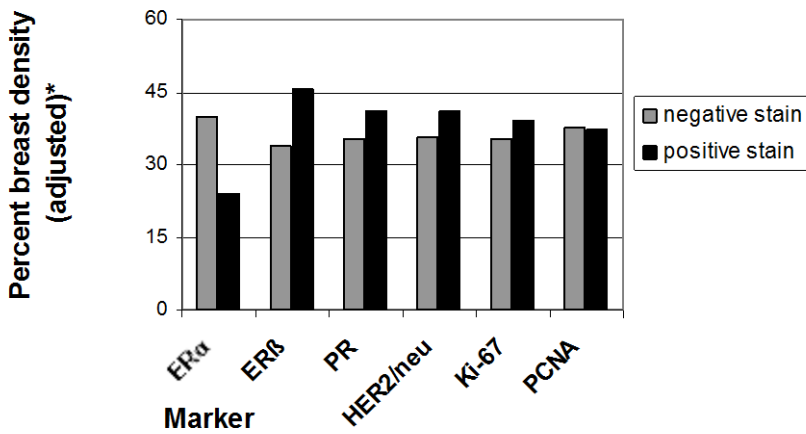


Fig. 10.2 Density and epithelial markers in benign tissue of Caucasian women^o

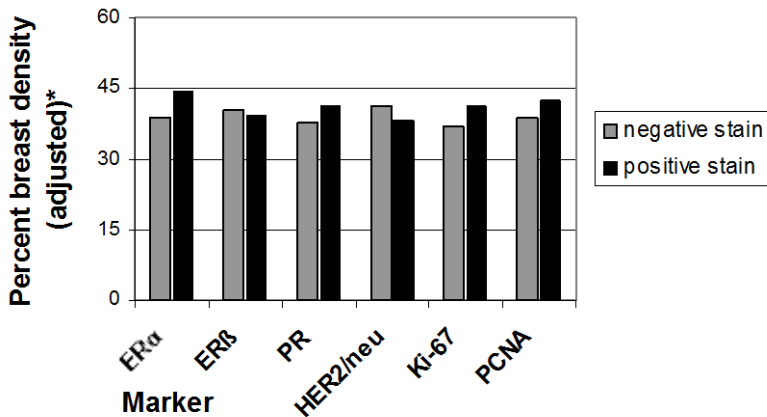


Fig. 10.3 Density and epithelial markers in benign tissue of Japanese women^o

^o Categories for staining and numbers of subjects per group are shown in table 6.

* adjusted for age at mammogram, BMI, menopausal status, age at menarche, HRT use at time of mammogram, age at first life birth, parity and family history of breast cancer

Table 6 Numbers of subjects per group for analysis of benign samples

Marker	All women (n=159)			Caucasian (n=49)			Japanese (n=70)		
	Staining		p-value ^a	Staining		p-value ^a	Staining		p-value ^a
	low	high		low	high		low	high	
ERα	122	35	0.75	38	11	0.04	53	16	0.39
ERβ	110	47	0.49	36	13	0.05	47	21	0.85
HER2/neu	104	52	0.82	34	15	0.38	45	23	0.57
PCNA	95	63	0.50	28	20	0.91	42	28	0.52
PR*	77	81	0.28	23	25	0.30	35	35	0.57
Ki-67*	65	90	0.32	16	32	0.53	31	39	0.46

^a p-values given for glm-analysis of difference in percent breast density by staining group; adjusted for known confounders as mentioned above

* Staining categories for PR, Ki-67: low (no staining); high (any staining); for ER α , ER β , HER2/neu and PCNA: low (<10% staining), high (\geq 10% staining).

Table 7 Breast density and marker expression in malignant sampels

Marker		Staining category			p-value ^a
		1	2	3	
ERα	Percent density	31.9	43.5	38.0	0.05
	Number	29	55	168	
ERβ	Percent density	38.0	41.4	35.5	0.17
	Number	68	110	73	
HER2/neu	Percent density	35.5	40.0	37.7	0.60
	Number	25	57	194	
PCNA	Percent density	53.1	39.6	35.8	0.24
	Number	20	59	196	
PR	Percent density	37.1	39.7		0.39
	Number	70	183		
Ki-67	Percent density	40.7	38.4		0.58
	Number	30	221		

Staining categories: ER α and ER β : 1 (negative), 2 (<10%); 3 (\geq 10%)

PR and Ki-67: 1 (no staining); 2 (any staining);

PCNA: 1 (0-1%); 2 (1-20%); 3 (>20%); HER2/neu: 1 (0-1%); 2 (1-10%); 3 (>10%)

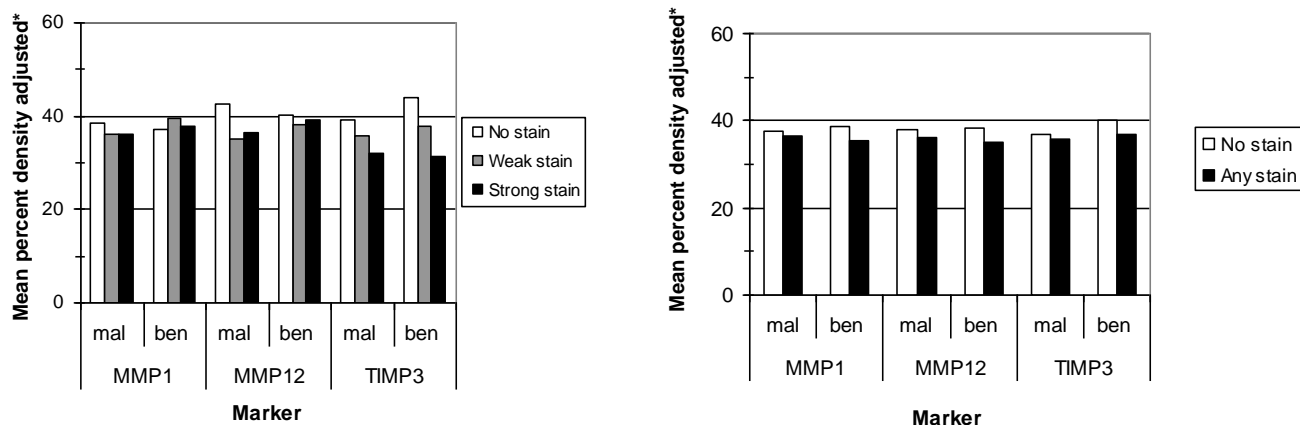
^ap-values given for glm-analysis of difference in percent breast density by staining group; adjusted for known confounders as mentioned above

3.6.2 Stromal markers

There was no statistically significant association of MMP1, MMP12, and TIMP3 with breast density in the entire study population (figure 11.1). After stratification by ethnicity, we noted several discrepant associations (figures 11.2 and 11.3). In Japanese women, density was inversely associated with MMP1 expression (31.5% for any stain vs. 41.3% for no stain in malignant stromal tissue; $p=0.04$). The same trend, though not significant, was observed in epithelium. In contrast, percent density was higher in Caucasian women with more extensive MMP1 expression; the respective values for malignant stromal tissue were 40.7% vs. 32.1% ($p=0.12$).

Although not statistically significant, mean percent density was lower with more extensive TIMP3 expression both in stromal and epithelial tissue. In stromal tissue, mean density was 40.0 vs. 36.9 ($p=0.28$) for no vs. any stain in benign tissue; the respective values for malignant tissue were 36.0 vs. 35.7 ($p=0.73$). In benign epithelial tissue, mean density was 43.9% vs. 31.5% for no vs. strong stain ($p=0.10$); the respective values for malignant epithelial tissue were 39.2% vs. 31.9% ($p=0.18$). With one exception (malignant stromal tissue of Caucasians), the inverse association was consistent across ethnic groups.

Fig. 11.1 Density and MMPs in epithelial and stromal tissue of all women^o



^o Categories for staining and numbers of subjects per group are shown in table 6.

* adjusted for age at mammogram, BMI, menopausal status, age at menarche, HRT use at time of mammogram, age at first life birth, parity and family history of breast cancer

Fig. 11.2 Density and MMPs in epithelial and stromal tissue of Caucasian women^o

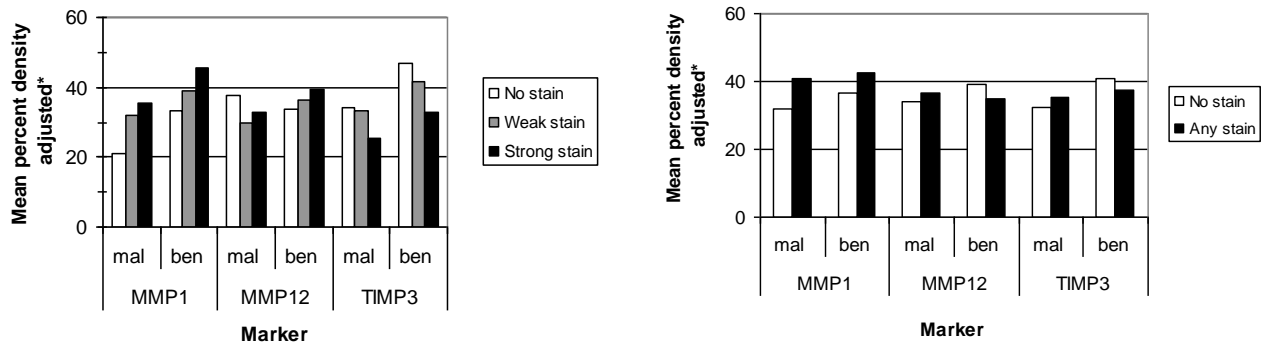
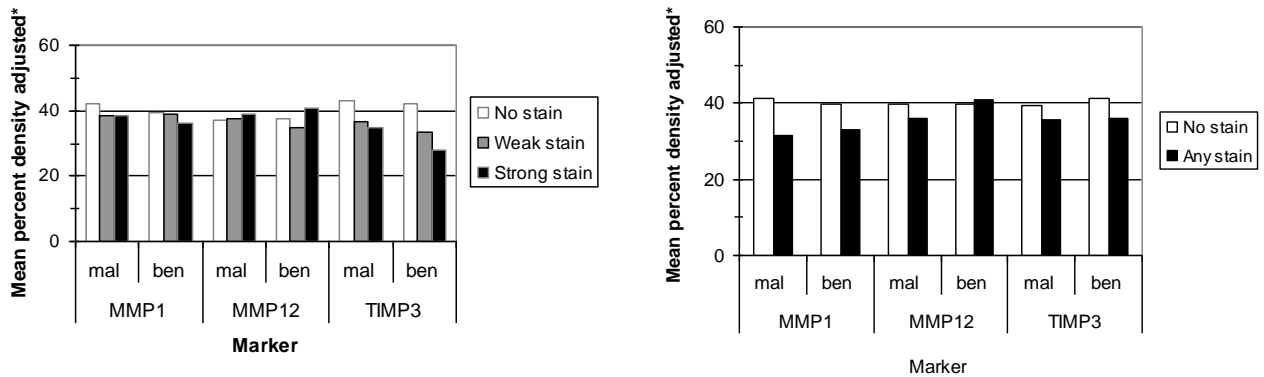


Fig. 11.3 Density and MMPs in epithelial and stromal tissue of Japanese women^o



^o Categories for staining and numbers of subjects per group are shown in table 6.

* adjusted for age at mammogram, BMI, menopausal status, age at menarche, HRT use at time of mammogram, age at first life birth, parity and family history of breast cancer

Table 8.1 Numbers of subjects per group for analysis of epithelial tissue

Marker	Tissue	All women (n=277)				Caucasian (n=100)				Japanese (n=121)			
		Staining			p-value	Staining			p-value	Staining			p-value
1	2	3	1	2		3	1	2		3			
MMP1	mal	30	89	130	0.85	8	31	43	0.19	18	39	53	0.80
	ben	28	72	53	0.82	7	22	17	0.38	16	37	20	0.87
MMP12	mal	15	49	192	0.50	10	21	55	0.70	4	20	87	0.90
	ben	28	61	69	0.90	6	17	24	0.81	17	30	26	0.60
TIMP3	mal	96	118	40	0.18	41	32	12	0.47	37	54	22	0.29
	ben	77	57	12	0.09	22	20	6	0.42	29	23	3	0.22

Staining categories: 1 (no stain); 2 (weak stain); 3 (strong stain); p-values given for glm-analysis of difference in percent breast density by staining group; adjusted for known confounders as mentioned above

Table 8.2 Numbers of subjects per group for analysis of stromal tissue

Marker	Tissue	All women (n=277)			Caucasian (n=100)			Japanese (n=121)		
		no stain	any stain	p-value	no stain	any stain	p-value	no stain	any stain	p-value
MMP1	mal	192	75	0.71	69	24	0.11	83	31	0.04
	ben	189	49	0.37	67	15	0.34	82	22	0.20
MMP12	mal	165	99	0.45	66	26	0.62	66	46	0.40
	ben	197	23	0.49	66	10	0.58	84	9	0.88
TIMP3	mal	218	51	0.73	73	20	0.60	94	23	0.48
	ben	158	78	0.27	53	29	0.50	72	30	0.26

4. Discussion

Breast density, the radiographic appearance of the female breast, is one of the strongest risk factors for breast cancer. Although a vast body of literature describes a positive association between breast density and breast cancer, reporting risk estimates up to 6.0 (3, 9), the biological mechanisms underlying this association are largely unknown. Density has inconsistently been reported to be associated to histopathologic changes, such as proliferative lesions of epithelium, stroma or both (88, 89). Several other breast cancer risk factors, especially those connected to an increase in estrogen exposure, have as well been shown to increase the extend of mammographically dense tissue.

Since stroma compromises most of the radiographic dense tissue, alterations in the extra-cellular matrix might also be connected, in a causal matter to the mechanisms underlying the associated breast cancer risk. MMPs play a crucial role in breast carcinogenesis and have not yet been analyzed in their relation to breast density.

In this study, we recruited 283 breast cancer cases of mainly Caucasian and Japanese ancestry and examined breast density and its association with hormonal (ER α , ER β , PR) proliferative (Ki-67, PCNA, HER2/neu) and stromal markers (MMP1,3, 9 and 12 and TIMP3).

In TMAs of benign and malignant breast tissue samples, we did not observed any significant association in the overall study population. The topic of this investigation is of importance because the histological correlates of mammographic density are very poorly understood, and yet, mammographic density is widely used as a biomarker for breast cancer risk and considered an important tool for breast cancer risk assessment and management.

4.1 Major findings and interpretations

4.1.1 Density and hormonal and proliferative markers

We found all hormonal (ER α , ER β and PR) and proliferative markers (Ki-67, PCNA, and HER2/neu) to be much more frequently expressed in malignant than in benign tissue. For all these markers, findings did not differ significantly by ethnicity. In benign tissue ER α was significantly correlated to proliferative markers.

These results agree with the general understanding of higher expression in malignant samples. Especially markers indicating proliferation were expected to be more strongly expressed in malignant than in benign tissue samples. Compared to our similar findings among Caucasian and Japanese subjects, other studies report lower levels of ER and PR expression in normal breast tissue of Asian women (110) and generally lower frequencies of ER-positive and PR-positive breast carcinomas in Asian than western women (111, 112). Our results disagree with these prior

reports, possibly due to the fact, that our Japanese women are 2nd to 4th generation immigrants, and in their risk pattern more similar to Caucasians.

To our knowledge, no previous results for associations between mammographic density and ER β , HER2/neu and PCNA have been reported.

As in previous studies, we found percent breast density to differ significantly by age, menopausal status, HRT, BMI, parity and age at first live birth. The higher percent density among women with Japanese ancestry despite their lower breast cancer risk was also observed (38).

This might be due to the smaller breast sizes which result in a higher percent of breast occupied by dense tissue. As shown in cross-sectional comparisons, the size of the dense area appears to be a better indicator of risk when different ethnic groups are compared (70, 71).

Breast density has also been shown to be affected by cumulative lifetime estrogen exposure. Our findings agree with prior studies consistently reporting increased density with earlier age at menarche, later menopause, and use of combined HRT (42-44).

In our analysis of breast density and hormonal and proliferative histopathologic markers in benign and malignant tissue samples, we did not observe any consistent significant association between markers and mammographic density. However, it appeared that mammographic density was slightly higher in women with PR expression (compared to no PR expression in tissue samples). Though not significant, this observation was consistent across the two major ethnic groups and tissue origins (malignant and benign tissue). The difference in benign samples was 3-4% in mammographic density which, if a true finding, may translate into a 6-8% higher breast cancer risk (113).

Although we observed a higher percent density with expression of ER β in the overall population as well as in Caucasian women, findings differed among Japanese subjects and in malignant samples. Given the inconsistent findings, and the fact that to date, no other studies have examined ER β in its relation to density, future studies are needed to interpret a possible association.

Furthermore, our findings of higher percent breast density in women with less extensive ER α staining are inconsistent with our hypothesis. The tentative findings among Japanese women and malignant samples of higher density with higher expression of ER α are not statistically significant and might be due to small numbers.

To our knowledge, only one prior study assessed the association of ER α and PR expression with mammographic breast density and reports similar results (78). In noncancerous tissue from mastectomies of a relatively small study population (n=56), no association was found between ER or PR status and breast density (78). These findings agree with prior investigations that do not report any association of high risk pathologic changes or fibrocystic breast disease with mammographic breast density (32, 33, 114).

The slightly higher PR expression in women with high breast density that we observed in our study may reflect a stronger estrogenic response in mammographically dense breasts which might possibly lead to stronger cell proliferation. This hypothesis is supported by reports describing associations of breast density with hyperplasia and other proliferative lesions in breast tissue (4, 29, 115).

In benign samples, breast density was slightly higher for expression of Ki-67 and PCNA, both, in the overall study population and ethnic groups, except for PCNA among Caucasians. Density did not differ significantly with expression in malignant samples.

Quite a few studies addressed the correlation of mammographic breast density and histopathological correlates of proliferation, using different sources for breast tissue. Most of these studies describe a positive association between breast density and proliferation in epithelial and stromal tissue components. In particular, a positive correlation between high density and intraductal epithelial hyperplasia and lobular microcalcification (28), and an association with high risk patterns such as atypia of the ductal type (24, 29, 30) and carcinoma in situ or atypical hyperplasia (115) have been described.

This agrees with the hypothesis that high breast density is associated to higher breast cancer risk through mechanisms which contribute to the proliferative activity of breast tissue. A higher index of proliferation exposes more cells to potential environmental alteration and subsequent malignant conversion. To date, two studies examined mammographic density and Ki-67 expression; one reported no significant association (85), the other found a positive association (78). Harvey et al. found breast density to be associated to an increased Ki-67 activity in the ducts of breast tissue ($p=0.031$) (78), whereas the other investigation, which assessed FNA tissue samples of 344 pre- and postmenopausal women, did not find any association between density and Ki-67 expression (85).

4.1.2 Density and stromal markers

Besides the consistently reported association of density with proliferative lesion of the epithelium (23, 24, 30), density has also been found to correlate with intra- and extralobular fibrosis (29) as well as higher collagen density (88, 89). Since the stroma comprises most of the mammographically dense area, these associations might be of importance for the associated increase in breast cancer risk.

Altered regulation of the stroma, or extracellular matrix (ECM), contributes to neoplastic progression and disruptions in the ECM, which possibly precede changes in the epithelium (90).

Matrix metalloproteinases (MMPs), involved in the remodeling of ECM, contribute to multiple steps of tumor promotion, angiogenesis, and the establishment of metastatic lesions (116, 117). A

disruption of the balance between MMPs and their natural inhibitors (TIMPs) has been implicated in the progression of cancer (118). To our knowledge, no previous studies have examined MMPs and their effect on breast density as of this date.

For MMP3 and MMP9 less than 20% of cells stained positive in different tissue origins. Therefore we did not include these markers in further analyses. MMP1 and MMP12 expression was higher in malignant than in benign samples, TIMP3 was more frequently expressed in benign stromal, but malignant epithelial tissue. Except for MMP12, which stained higher in Japanese women, expression of stromal markers was similar among different ethnicities.

This exploratory study detected no statistically significant associations between mammographic density and expression of MMP1, MMP12, and TIMP3 in breast tissue. However, we found TIMP3 to be consistently less expressed in breast tissue with high than low mammographic density. Despite its lack of statistical significance, the consistency of this trend across ethnic groups and tissue types may suggest an inverse association. Although for MMP1 and 12, no significant association with breast density was observed, the relations appeared to differ between Caucasian and Japanese women. Whereas the expression of markers differed little by ethnicity, higher MMP1 expression was related to higher breast density among Caucasian women and was inversely related to breast density in Japanese women. These results were analogous in epithelial and stromal tissue as well as malignant and benign samples.

Contrary to our findings, a previous study with a very small sample size found high breast density to be associated to an increased amount of TIMP3 (119). The authors hypothesized that TIMP3 may influence matrix deposition leading to higher breast density or that TIMP3 may act through alteration of insulin-like growth factors, which influence cell proliferation. A possible explanation for the discrepancy of the two studies is that the balance between MMPs and TIMPs is more important than just the expression of one protein.

MMPs are thought to be secreted not only by tumor cells, but mainly by the surrounding reactive microenvironment. These proteinases degrade the basement membrane and ECM components to a more “watery” consistence, which radiographically is denser than fat, which facilitates tumor invasion and formation of metastasis (90, 94). Thus, MMPs also contribute to early steps of tumor progression, including tumor promotion and angiogenesis (116, 117). As a result of the activity of MMPs, cell-cell and cell-ECM interactions are altered, new biologically active ECM molecules are generated, and the bioavailability and activity of many growth factors and cytokines is modified. We indeed found stronger expression of all MMPs in malignant than benign tissue. Our findings of higher MMP1 expression in Caucasian women with higher densities agree with the hypothesis that MMPs might contribute to the associated higher breast cancer risk in these women. MMP-1 is an interstitial collagenase that is often up regulated in breast cancer (120). Additionally, in vitro studies

show that MMP-1 facilitates the invasive properties of breast cancer cells (121). However, the opposing findings in Japanese women are difficult to explain; although breast cancer is less frequent in Japanese women, there is little difference in risk factors by ethnicity (table 5). Almost 90% of our participants are born in the United States, most of these are probably at least second generation immigrants, so lifestyles and breast cancer incidence are more similar to Caucasian women.

To thoroughly evaluate the role of MMP1, concise staining panels including its counterpart TIMP-1 are needed. Because of the high number of comparisons the statistically insignificant differences might be due to chance or they may also indicate variation in genetic or other risk factors for breast cancer across ethnic groups.

Growing evidence supports the notions that altered regulation of the extracellular matrix contributes, in a causal matter, to neoplastic progression. An imbalance between MMPs and their inhibitors has been found to be a factor in different steps of tumor growth (94, 118). Therefore, a role for MMPS and TIMPs in the association between mammographic breast density and breast cancer risk as well as survival appears likely.

Overexpression of TIMPs has been shown to inhibit primary tumor growth in mice (122, 123) as well as reduce tumor invasion and metastasis (118, 124, 125). In the present study, TIMP3 expression was lower in stromal cells of malignant than benign tissue; however, this was not the case for epithelial cell expression. One possible hypothesis would be that the lower expression of TIMP3 in women with high breast density may indeed affect the activity of different growth factors and influence matrix deposition that translates into altered breast density. If low TIMP expression leads to greater MMP activity, which in turn may lead to higher breast density and a greater carcinogenic potential of breast cells, it may be partially responsible for the increased breast cancer risk associated with high breast density or with further progression and greater mortality. However, TIMP3 also has other specific bio-cellular functions, not related to MMP-inhibition, such as direct induction of apoptosis (118, 126) or inhibition of endothelial cell motility and proliferation (127) which might also be possible mechanisms leading to low breast density and associated breast cancer risk.

4.2 Strength and limitations

The benefits and drawbacks of TMA-technology have been discussed in detail in other publications by Hernandez and colleagues (128). One disadvantage of the TMA approach is the loss of samples during immunohistostaining due to lack of adhesion to the glass slides. This might be due to the small diameter (0.6 mm) used in our study and larger cores might reduce these losses.

Another issue with TMAs is the confounding that might have taken place throughout the preparation of TMAs. When choosing a section on the slide for construction of a TMA, no effort was made to distinguish between specific areas of the tumor (center versus periphery, depth of invasion, et cetera). Bias due to intratumor heterogeneity may have been introduced when these small pieces of tissue were selected. Also, sectioning these constructed TMA blocks might lead to a depletion of the tumor, leaving only the first slides with useable tissue specimens (about 1 to 25 slides are said to contain tumor tissue). Subsequently cut slides might not contain sufficient tumor tissue and therefore decrease the accuracy of results.

Though these issues might be important concerns in studies directly assessing tumor variables, these differences are not likely to have a big impact on our analysis, because we used 4 different cores of each tumor block to assure consistency of findings. To further reduce the risk of misclassification, we also reassessed TMAs after staining as to ensure tissue entity (benign vs. malignant tissue). As shown before, benign tissue samples had to be reclassified more often than malignant cores (129, 130). There might also be differences in the staining results due to different tissue fixation and storage procedures used in the several hospitals, from which we received the tissue blocks.

Additionally, there might be some measurement inaccuracy in the analysis of slides which contained a lot of inflammatory cells. When choosing the negative stained epithelial cells (light and dark blue color), granulocytes and mastocytes would simultaneously be marked as well. However, only very few slides included tumors that showed a high amount of inflammation. All slides of one study part (i.e. histopathological marker) were read by one person, assuring consistency in our results. Even though the exact amounts of positively or negatively stained cells might differ slightly, the analyses for each marker were still correlated.

Our investigation illustrates the problems of obtaining benign breast tissue specimens for a representative sample of participants in epidemiologic research. We acknowledge that breast tissue considered normal or benign in this study represents specimens from women diagnosed with breast cancer. Therefore, these samples may not be equivalent to breast tissue from women with no clinical or mammographic abnormalities. Breast cancer is considered a systemic disease, and even in the absence of cancer, breast tissue obtained from biopsies may be different from tissue of women not requiring a biopsy. It is rather unfortunate, that past research addressing density and histopathologic markers in healthy women is limited to forensic studies (23) and investigations of breast reduction samples (131). Our study would have benefited from tissue samples from healthy women, but constraints for obtaining human breast tissue samples for research projects limit this approach. The amount of material available from stereotactic biopsies is often too small for TMA preparation, and women with benign biopsies cannot be identified through

tumor registries. Although breast reduction surgery would yield large amounts of tissue, women undergoing that procedure represent a selected subgroup with predominantly fatty breast tissue and different mammographic patterns (132).

As to the pilot nature of this project, we chose our array of MMPs based on review of the literature regarding relevance to breast cancer, availability of antibodies and prior staining experience at our institution. Our choice of markers represents only part of the markers that are needed for a concise evaluation of MMP and TIMP activity. Therefore one major disadvantage of our study is the incompleteness of MMP panels. In particular the low staining results of MMP9 and MMP3 might be due to high levels of TIMP-1, their natural inhibitor, which needs to be assessed in future studies for a thorough understanding.

Although mammographic density assessment is a well established technique, some limitations need to be mentioned. While assessing mammographic density, differences in the quality of films in respective clinics and between subjects may have introduced some measurement error. The level of compression of the breast, the amount of radiation as well as the processor chemistry used during mammography introduce possible confounding. Through differences in assessing density, breast cancer risk might be underestimated. Densities also differ on digital and film mammograms. However, these differences are minimal. We chose a density measurement technique that is currently the highest standard with consistently reported interreader reliability (ICCs) of 0.9 and higher.

For development of future measurement techniques, it has been criticized that the two-dimensional projection used in mammography is limited in terms of measuring a three-dimensional tissue feature such as density. A technique that determines the total glandular content from a three-dimensional construction of the breast might give a better estimation of breast density. Pixels are only used in a binary fashion, either representing fat or parenchyma. Therefore pixels with large amounts of parenchyma are seen equal to pixels with small quantities of parenchyma. Several three dimensional techniques are under investigation (133, 134).

Given a sample size of 78 women in our smallest subgroup, $\alpha = 0.05$ (two-sided) and power = 0.80, a standard deviation of 22.2 for percent breast density, the minimum detectable difference in means is 11%. Since this was a pilot-project in this particular field of research, we are aware that the power is low and some results may only be suggestive.

On the other hand, this study had several strengths. Our distinctive study population included a large sample of breast cancer cases with ethnic diversity and pathologic specimens from a population-based tissue repository. The availability of demographic and other risk factor variables in a study of tissue characteristics is rather unique and gives us the opportunity to apply suitable

adjustments to our analyses. The women in this investigation also span a wide age range which includes menopause and important feature when assessing breast density.

The questionnaires collecting data from our participants were designed and tested by highly trained staff at the cancer center. Furthermore, mammograms were obtained before the participants were diagnosed with breast cancer, which gives us the opportunity to assess density on mammograms without confounding by tumor masses.

This study benefited from the use of a high throughput TMA approach which enabled us to assess marker expression in a large number of samples under comparative conditions. Additionally, multiple markers could cost-effectively be analyzed using sections of the TMA block without depletion of the original tissue blocks for future research. Previous studies report a nearly 100% concordance between TMA data and data from staining of corresponding whole-section tumor slides (135-137). The investigation adds to the limited literature of TMAs in epidemiologic studies and to the variety of markers measured in TMAs (128, 129). The access of tumor blocks through tumor registries will allow future population-based research with pathologic specimens.

4.3 Implications and future research

To benefit from the advantages of the TMA technique in future research, it is recommended that a trained pathologist carefully selects the areas for subsequent TMA preparation and to minimize the loss of specimens during immunostaining. Especially the loss of benign tissue samples during staining seriously limited our power to detect associations with breast density. Using cores with a larger diameter could possibly alleviate this problem and the pathologist needs to be very careful in marking benign areas. Alternatively, immunohistochemistry could be performed on full sections, if available, but may be unacceptable because blocks will be used up. Constructing TMAs from large cohorts of patients in future clinical studies offers a useful tool for the association between biomarkers and clinically important end-points, as well as screening of potentially promising new biomarkers (138, 139).

To further assess multiple pathways such as inflammation, proliferation, apoptosis and hormonal pathways possibly associated with breast density, gene expression studies may be a more efficient technique. Many different signaling pathways controlling cell proliferation, differentiation and death are involved in cancer development. A powerful tool for analyzing multiple pathways simultaneously is the analysis of gene expression with microarrays. In recent years, gene expression profiling has successfully been used in breast cancer research and has, for instance, identified distinct subtypes of breast cancer with expression profiles, representing biologic entities with differences in clinical outcome (140, 141). The use of this technique particularly in cancer research has clearly been shown in numerous studies (142-144). Analyzing gene expression at

different stages of cancer development and in women with different risk factors, such as the amount of dense tissue in their breasts, could give valuable insight in disease processes.

Based on our few suggestive findings, future mammographic density investigations may pursue estrogenic effects as assessed by PR and cell proliferation using Ki-67 and other such markers to examine the question of local estrogen activity in relation to breast density.

Given the weak associations in the current investigations and the discrepant findings across studies, the roles of hormonal receptors and growth factors in shaping breast density deserve further attention (87, 89).

No prior investigations have examined the association of mammographic density with MMPs. This novel approach showed trends which might suggest a possible association of breast density with MMPs and TIMPs. However, to fully understand the role of MMPs and their inhibitors, it is of utmost interest to study complete panels of these markers. In particular MMP2, MT1-MMP, TIMP-1 and TIMP-2 would be desirable stains to study. Future investigations into activities and ratios of these proteases as well as other pathways such as inflammation, may lead to identification of patients at high risk for developing breast cancer or developing remarkably aggressive tumors (145).

Due to small sample sizes, we could only stratify our analyses by Japanese and Caucasian ethnicity. The issue of difference in ethnic groups needs to be addressed by future studies with larger numbers.

Although breast density has consistently been shown to be strongly associated with breast cancer risk, the reasons or biological mechanisms for this positive association are largely unknown. In addition, there is no general agreement on what causes some women's breasts to be mammographically more dense than others. More importantly we do not know if a reduction in breast density would mean a reduction in risk, too. Cuzick et al. report a reduction in percent density and a subsequent reduction in breast cancer incidence with use of tamoxifen, a drug with antiestrogenic effects on breast tissue (146). In comparison, as described in detail above, HRT with combined regimens leads to an increase in percent density. Density could be an indicator of the cumulative breast cancer risk after exposure to different risk factors such as past estrogen exposure. Through its distinctive capability of being modifiable, it might also be a potential response biomarker.

If the underlying pathology for mammographic density were better understood, prediction of breast cancer risk in individuals could allow for improved design of preventive strategies, planning of

interventions and improved clinical decision making. For instance in cardiovascular medicine, modification of risk factors based on risk prediction has been estimated to account for approximately half of the 40% reduction in age-specific mortality from cardiovascular disease (147). For breast cancer risk prediction, the Gail model is currently most widely used. This model includes age, age at menarche, age at first live birth, number of benign breast biopsies and number of first-degree relatives with breast cancer (148). The addition of percent mammographic density, as one of the strongest risk factors, to the Gail model might lead to more precise risk estimation. Addition of percent density as measured by BI-RADS, however has only shown modest levels of individual prediction, increasing the concordance statistics from ranges between 0.58 and 0.63 to ranges between 0.62 and 0.66 (149). However, this increase is higher than an addition of the seven SNPs (single nucleotide polymorphisms) found reproducibly to be associated with breast cancer (concordance statistics: 0.63) (150).

Further research in this area might be productive in providing a better understanding which is needed if density is to be used to estimate individual risk and to monitor changes in a meaningful way.

The vast clinical use of screening mammography offers unique research potential for preventive studies. Mammograms are available for large numbers of women and acquired with comparable techniques. With the availability of prediagnostic mammograms, it would be reasonable to reanalyze breast cancer clinical trials for differences in outcomes according to breast density. Few studies have addressed recurrence rates after breast-conserving surgery and report positive associations of higher density with local recurrences (151, 152). Furthermore it would be of interest to study the possible effects of breast density on breast cancer survival.

If exact mechanisms were known about the underlying pathology for increased breast density and increased breast cancer risk, medications acting on these specific mechanisms could be developed to decrease breast density and possibly also decrease the associated risk for breast cancer.

5. Summary

Background. Mammographic breast density is a strong, independent risk factor for breast cancer, yet the underlying biologic mechanisms are not clear. Studies inconsistently report associations of breast density with histopathologic correlates including proliferative lesions of the epithelium, stroma or both. Matrix metalloproteinases (MMPs), endopeptidases regulating degradation of extracellular matrix play an important role in tumorigenesis and cancer progression. Imbalance of MMPs and their inhibitors might be a mechanism contributing to the elevated breast cancer risk among women with high densities. We explored the association of mammographic density, with hormonal, proliferative and stromal histopathologic markers in benign and malignant tissue from tumor blocks of breast cancer patients and the possible use of pathology blocks and TMA-technology in epidemiological research.

Methods. We recruited 283 cases from a case-control study on breast density. Mammographic density was assessed on digitized prediagnostic mammograms using a computer-assisted method. For 279 participants of the original study with primarily Caucasian (36%) and Japanese (41%) ancestry, we obtained tumor blocks and prepared tissue microarrays (TMA). Up to 4 malignant and 4 benign cores per woman were placed in the TMA block; slides were immunohistochemically stained for ER α and ER β , PR, HER2/neu, Ki-67, and PCNA. At least one benign core could be assessed for 159 women, one malignant core for 253 women. In a second part of this study, we investigated the association of breast density with stromal and epithelial expression of MMPs 1, 3, 9, and 12 and a tissue inhibitor of MMP3 (TIMP3). In the stromal marker datasets, there were 259 women with at least one benign and 274 with at least one malignant measurement. The microscopic pathological evaluation categorized each core as no, weak, and strong staining (depending on the specific marker). We applied general linear models to compute breast density according to marker expression while adjusting for known confounders.

Results: For staining of hormonal and proliferative markers a substantial proportion of the samples were in the low or no staining categories (range of 0-10%). None of the results were statistically significant, but women with PR and ER β staining had 3.4% and 2.4% higher percent density. The respective values for Caucasians were 5.7% and 11.6% but less in Japanese women (3.5% and -1.1%). Percent density was 3.4% higher in women with any Ki-67 staining and 2.2% in those with positive PCNA staining.

Strong expression of all MMPs was about twice as high in malignant as compared to benign tissue, while TIMP3 expression was higher in benign cores. For MMP3 and 9, less than 10% of cores

stained positive; thus, they were not further analyzed. No statistically significant association of TIMP3, MMP 1 or 12 with breast density was found. However, mean density was consistently lower with more extensive TIMP3 expression both in epithelial and stromal tissue of Japanese and Caucasian women. In particular, in benign epithelial tissue, mean density was 43.9% vs. 31.4% for no vs. strong stain ($p=0.09$); the respective values for malignant epithelial tissue were 39.2% vs. 31.9% ($p=0.18$). Although overall results were non-significant for MMP-1, we noted a discrepant association by ethnicity. In Japanese women, density was inversely associated with MMP-1 expression (31.5% for any stain vs. 41.3% for no stain in malignant stromal tissue; $p=0.04$). The same trend, though non-significant, was observed in all other tissue categories. In contrast, density was higher in Caucasian women with more extensive MMP1 expression; the respective values for malignant stromal tissue were 35.4% vs. 20.9% ($p=0.11$).

Conclusions. This study detected little evidence for an association between mammographic density and expression of hormone receptors and proliferation markers in breast tissue, but it illustrated the problems of locating tumor blocks and benign breast tissue samples for epidemiologic research. Given the suggestive findings, future studies examining tissue estrogen levels, steroid receptors, and density in the breast may be informative. Our findings indicate that breast density may be related to higher proliferative activity through lower TIMP3. For MMP 1 and 12, no consistent association with breast density was observed. These data suggest possible mechanisms that may mediate the higher breast cancer risk in women with dense breasts and may, in the future, lead to preventive medications that lower mammographic breast density. Further studies of concise MMP and TIMPs panels and gene expression studies might lead to a better understanding of the underlying mechanisms.

6. List of abbreviations

BI-RADS	Breast Imaging Reporting and Data System
BMD	Bone mineral density
BMI	Body mass index
CEE	Conjugated equine estrogens
CIS	Carcinoma in situ
DCIS	Ductal carcinoma in situ
ECM	Extra cellular matrix
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin embedded
FFQ	Food frequency questionnaire
Fig.	Figure
H&E	Hematoxylin and Eosin
HER-2/neu	Human epidermal growth factor receptor 2
HRT	Hormone replacement therapy
HTR	Hawaii Tumor Registry
ICC	Intraclass correlation coefficient
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IHC	Immunohistochemistry
IRB	Institutional review board
MEC	Multi ethnic cohort
MMP	Matrix metallo proteinase
NAF	Nipple aspirate fluid
NCC	Nested case control
PCNA	Proliferating cell nuclear antigen
PR	Progesterone receptor
s.c.	Sub cutaneous
SEER Program	Surveillance, Epidemiology and End Results Program
SNP	Single nucleotide polymorphism
TAC	Tissue advisory committee
TIMP	Tissue inhibitor of metalloproteinases
TMA	Tissue micro array
VEGF	Vascular endothelial growth factor

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9. Appendix

9.1 Overview of previous studies published

9.1.1 Mammographic density and histopathologic markers

Author (Ref.)	Population ^a			Markers	Material	Radiology Classification	Results ^b	
	Design	Source	Size				Menopausal Status	Ethnicity
Byrne (2001)	NCC	BCDDP	347 B, 410 C	Pre. + Post.	N/A	Biop. + Mamm.	Quant.	N/A
Guo (2001)	CS	HS	92	Unknown	Unknown	Fine-Needle Biop.	High vs. Low Dens.	High density correlated with larger total collagen area, TIMP3, Assoc. with IGF-1 and nuclei only for age <50
Alowami (2003)	CH	MSP	62	Post.	N/A	Biop. + Mamm.	Semi-Quant.	No association with mammographic density and density of epithelial components
Ziv (2004)	CH	Mamm. Registry	44,811	Pre. + Post.	Multi.	Biop. + Mamm.	BIRADS	Density was strongly associated with ER+ and ER- breast cancer
Li (2005)	Series	Office of Medical Investigator	519	Pre. + Post.	White, Hispanic, Am. Indian	Mast. at Autopsy + Mamm.	Wolfe	Percent density pos. associated with proportion of area of epithelial and nonepithelial nuclei and glandular structures
Khan (2007)	CS	BSP	344	Pre. + Post.	N/A	RPFNA	Quant.	No association between percent density and Ki-67 or cytomorphology
Harvey (2008)	CS	BCP	56	Post.	Unknown	Mast. +Mamm.	Quant.	No association between ER, PR, Ki-67 and density

9.1.2 Mammographic and histological appearance of the female breast

Author (Pub.Date)	Population			Material	Radiology		Results		
	Design	Source	Size		Age	Classification		Subclassification	Epithelium
Fisher (1978)	CS	HS	50 CA, 50 FCD	N/A	Mast.	Wolfe	N, P, DY	No association of proliferative FCD and mammographic densities	Greater incidence of fibrous mazoplasia in DY patterns
Wellings and Wolfe (1978)	CS	HS	143	31-78	Biop.	Wolfe	N ₁ , P ₁ , P ₂ , DY	Atypical lobules more frequent in DY pattern	Parenchymal fibrosis greater in DY than NI pattern
Moskowitz (1980)	CH	BCDDP	8,033	35-74	Biop.	Mod. Wolfe	F ₁ + NOS ₁ , P ₁ , P ₂₋₄ , NOS ₂₋₄	No association of any mammographic pattern and high-risk pathologic markers	N/A
Bland (1982)	CH	BCDDP	863	35-74	Biop.	Wolfe	N ₁ , P ₁ , P ₂ , P _{2b} , P _{2c} , DY	Nonproliferative and proliferative FCD associated with P2 and DY patterns	N/A
Bright (1988)	CS	HS	320	N/A	Biop.	Wolfe	N ₁ , P ₁ , P ₂ , DY + Nodular vs. Homogeneous	Epithelial hyperplasia is associated to nodular densities in postmenopausal women	Extralobular fibrosis associated with homogeneous density
Urbanski (1988)	CS	HS	160	<50	Biop.	Quant.	Extent of dysplasia and ductal prominence in %	Epithelial hyperplasia with atypia associated with extensive densities	N/A
Arthur (1990)	CH	Screening trial	162	45-64	Biop. + Mast.	Wolfe	N ₁ , P ₁ , P ₂ , DY	No association of epithelial hyperplasia or CIS and Wolfe pattern	Fibrosis associated with P2 and DY patterns
Bartow (1990)	CS	FAS	486	15-98	Mast.	Wolfe	N ₁ /P ₁ versus P ₂ /DY	Epithelial hyperplasia and intralobular calcification more frequent in P2/DY patterns	N/A
Boyd (1992)	CH	NBSS	353 B and 501 C	40-49	Biop.	Quant.	0-10%, <25%, <50%, <75%, ≥75%	Extensive density (>75% area), 9.7 greater risk of CIS or AH, 12.2 times greater risk of hyperplasia than no density	Increasing collagen and decreasing proportion of fat in biopsies with increasing radiological density
Lee (1994)	CS	Referral clinic	588	25-65	NAF	Quant.	N1, P1, P2, DY and <25%, -49%, -74% ≥75%	Highly dense mammograms are associated with cytological atypia (4.4 (p=0.8)) in women with BMI<25	N/A
Boyd (2000)	CH	NBSS	353 B, 465 C	40-49	Biop.	Quant.	0-10%, <25%, <50%, <75%, ≥75%	Density >75% associated with increased hyperplasia without atypia, AH and CIS	N/A
Byrne (2001)	NCC	BCDDP	347 B, 410 C	59.5*	Biop.	Quant.	0-10%, 10-49%, 50-74%, ≥75%	Positive association between degree of proliferation and amount of dense tissue	N/A
Helvie (1991)	CS	HS	58	32-77	Biop.	Quali.	Microcalcification, nodular/spiculated masses, asymmetric opacity	Clustered microcalcifications are directly correlated with AH	N/A

** mean age **Abbreviations:** BCDDP breast cancer detection demonstration project, BCSP breast cancer screening program, BSP breast screening program, CAM computer assisted method, CH cohort, CS cross-sectional, E1 estrone, E2 estradiol, ER estrogen receptor, FAS Forensic autopsy series, FCD fibrocystic disease, HS hospital series, I incidence, MSP mammographic screening program, NCC nested case-control study, NHS nurses' health study, P prevalence, PR progesterone receptor, RPFNA random periareolar fine needle aspiration, SHBG sex hormone binding globulin, F1, NOS1, P1, P2-4, NOS2-4 as well as P2f, P2n, P2c- see text

9.3 Curriculum Vitae

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Publikationen

1. Mammographic Density and Matrix Metalloproteinases in Breast Tissue.
Steude JS, Maskarinec G, Erber E, Verheus M, Hernandez BY, Killeen J, Cline JM.
Cancer Microenviron.2010. [Epub ahead of print] PMID 20012240
2. The relation of leptin and adiponectin with breast density among premenopausal women.
Maskarinec G, Woolcott C, Steude JS, Franke AA, Cooney RV.
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3. Mammographic density and epithelial histopathologic markers.
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BMC Cancer. 2009 Jun 13;9:182
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Maskarinec G, Steude JS, Franke AA, Cooney RV.
J Inflamm (Lond). 2009 Apr 7;6:9.

Präsentationen

1. Mammographic density and histopathologic markers: An example of using tissue microarrays in breast cancer research. Maskarinec G, Erber E, Steude JS, Verheus M, Killeen J, Hernandez BY, Cline JM., Jahrestagung der Deutschen Gesellschaft für Epidemiologie, Berlin, Germany, 9/2010.
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3. Mammographic Density and Matrix Metalloproteinases in Breast Tissue (Poster).
Steude JS, Maskarinec G, Erber E, Verheus M, Hernandez BY, Killeen J, Cline JM. 100th Annual Meeting of the American Association for Cancer Research, Denver, USA, 4/2009.
4. Inflammatory Markers in a 2-Year Soy Intervention among Premenopausal Women (Poster).
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5. Mammographic density and hormone receptors in TMAs of breast cancer cases (Poster).
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