ASSOCIATION BETWEEN PROLACTIN AND MAMMOGRAPHIC BREAST DENSITY

by

Claudia Costa Leiras

BS, College of Mount Saint Vincent, 1997

MS, University at Buffalo, 2000

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This dissertation was presented

by

Claudia Costa Leiras

It was defended on

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and approved by

M. Michael Barmada, PhD, Director, Center for Computational Genetics and Associate Professor, Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh

Joel L. Weissfeld, MD, MPH, Associate Professor, Department of Epidemiology, Graduate School of Public Health and Assistant Professor of Medicine, School of Medicine, University of Pittsburgh

John W. Wilson, PhD, Assistant Professor, Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh

Anthony J. Zeleznik, PhD, Profesor, Department of Cell Biology and Physiology, School of Medicine, University of Pittsburgh

Jane A. Cauley, DrPH, Vice Chair for Research and Professor, Department of Epidemiology, Graduate School of Public Health and Associate Professor, School of Nursing, University of Pittsburgh
Dissertation Advisor

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Claudia Costa Leiras, PhD

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Breast density affects mammographic sensitivity and is predictive of breast cancer risk. Factors that increase breast density may compromise the reduction in mortality gained by mammographic screening. Understanding these factors is crucial as it may help us improve mammographic screening and reduce breast cancer risk. Prolactin, an endogenous hormone that acts as a mitogen and differentiating agent in the breast, may be one such factor. To our knowledge, this is the first study to examine the association between prolactin and mammographic breast density in a cross-sectional study of healthy, cancer-free postmenopausal women.

A weak, but statistically significant correlation was observed between prolactin and percent breast density (spearman correlation coefficient of 0.1197; p-value 0.013) after adjusting for every being pregnant and ever breast feeding. Prolactin is most likely one of several factors that contribute to increased mammographic breast density, and further analyses are needed to determine its' full contribution. No statistically significant associations were observed for the prolactin gene single nucleotide polymorphisms (SNPs) examined in relation to prolactin, percent breast density, or proportion of dense breast area. However, two SNPs in the prolactin receptor gene (rs7734558 and rs7705216) were significantly associated with serum prolactin level at the 0.10 significance level. Women with the G allele (AG and GG) at SNP rs7734558 have a slightly elevated level of prolactin when compared with women homozygous for the A allele (AG 10.76 ± 6.40 ng/mL, GG 10.77 ± 4.60 ng/mL vs. AA 9.86 ± 6.32 ng/mL); and those

with the GG allele at SNP rs7705216 have a slightly elevated prolactin level when compared with individuals with the C allele (GG 11.71 ± 2.78 ng/mL vs. CG 11.15 ± 6.22 ng/mL, CC 10.27 ± 5.99 ng/mL). These SNPs need to be further investigated to determine their full contribution in relation to serum prolactin levels.

Having an understanding of factors that affect breast density is an important public health issue has it may lead to improvements in breast cancer screening and help identify not only women at an increased risk for breast cancer, but women who may benefit from prevention strategies.

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1.0 Introduction

1.1 Breast Cancer Epidemiology

Among women, breast cancer is the most common cancer and is second only to lung cancer in the number of deaths per year. In 2006, approximately 212,920 new cases of invasive and 61,980 cases of in situ breast cancer will be diagnosed in the United States, accounting for 31% of all female cancers.[1] Approximately, 40,970 women will die from breast cancer, accounting for approximately 15% of cancer mortality in women.[1] During the past twenty years, while the annual breast cancer incident rate has been steadily increasing, the mortality rate has declined by approximately 2.3% per year.[2] This discrepancy between incident and mortality rates in most likely due to cancer screening improvements, leading to the early detection of breast cancer, as well as more effective treatment regimens. Data from the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) program show that breast cancer mortality rate has declined 2.3% each year between 1990 and 2003.[2] The percentage of women surviving at least five years after diagnosis has risen to 88% and the 5-year survival is 98% among women diagnosed with early stage, localized disease.[2]

1.1.1 Age and Breast Cancer Risk

With the exception of gender, age is the most scientifically proven breast cancer risk factor. Breast cancer seldom occurs in women under the age of 25 years.[3] The overall incidence rate for women ages 20-24 years is 1.4 per 100,000 women. However, after the age of 25 years, the incidence rate rises linearly until the age of menopause, at which point the rates begin to plateau.[3] Data from SEER indicate that between 1999 and 2003, the incidence rate for breast

cancer was 119.3 per 100,000 for women ages 40-44; 249.0 per 100,000 for women ages 50-54; and 388.3 for women ages 60-64. The highest rate of breast cancer (490.4 per 100,000) was observed in women aged 75-79.[3] More than 80% of breast cancer cases occur in women over the age of 50 years.[4]

1.1.2 Race/Ethnicity and Breast Cancer Risk

Breast cancer rates vary by race and/or ethnicity. African-American women have a lower overall incidence of breast cancer when compared to Caucasian women (118.9 per 100,000 versus 137.6 per 100,000, respectively, for 1999-2003, SEER Data),[3] but have a higher incidence of breast cancer before 35 years of age.[2] In addition, African-American women have an overall higher rate of breast cancer mortality at all ages than Caucasian women (34.4 deaths per 100,000 versus 25.4 deaths per 100,000, respectively, for 1999-2003, SEER Data).[3] These breast cancer disparities are difficult to explain, but several theories have been put forth in an attempt to explain them, including: differential utilization of mammographic screening and stage at diagnosis; differential effect and/or distribution of breast cancer risk factors; differences in inherent genetic susceptibility; differences in tumor characteristics; differential access to treatment; and differences in the prevalence of co-morbidities.[5] All of these proposed factors have been examined, and it appears that the most likely factor responsible for the observed disparities is the differential distribution of risk factors, most notably obesity.[6] Tumor characteristics[7,8] and differences in co-morbid conditions[9] also appear to contribute, and most likely genetic susceptibility[10,11] will also play a role (not well understood at this time). As can be seen from the lack of evidence, still much research is needed to fully explain the breast cancer disparities between African-Americans and Caucasians.

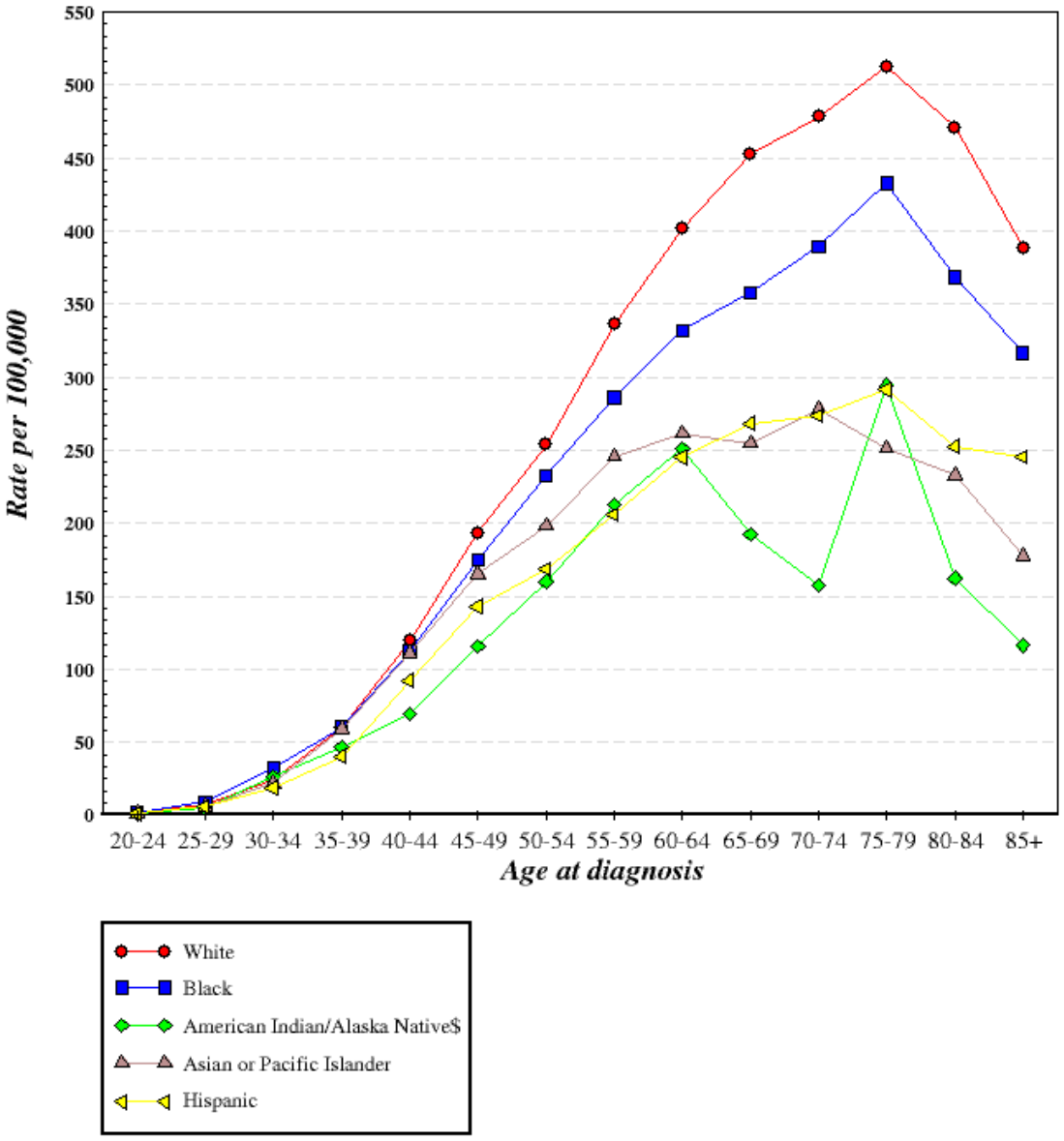


Figure 1: Age-specific breast cancer incidence rates among U.S. women by race, 1999-2003.

Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence-SEER 9 Regs Public-Use, Nov 2005 Sub (1973-2003), National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch, released April 2006, based on the November 2005 submission.[3]

As noted above, between 1999-2003, SEER reported female breast cancer incidence rates for Caucasians as 137.6 per 100,000 and for African Americans as 118.9 per 100,000.[3] These rates are much higher than those reported for other racial groups. Among Asians and Pacific Islanders, the breast cancer incidence rate was (per 100,000) 93.5; 87.1 for Hispanics; and 74.4 for Native Americans and Alaskan Natives.[3] Mortality rates during the same period were also lower than for both Caucasians and African-Americans (25.4 deaths per 100,000 versus 34.4 deaths per 100,000, respectively). The mortality rates (per 100,000) for Asians and Pacific Islanders was 12.6; 16.3 for Hispanics; and 13.8 for Native Americans and Alaskan Natives.[3] These differences may be attributed to varying genetic, environmental, and behavioral factors.

Interestingly, age-adjusted breast cancer incidence rates also vary greatly around the world. There exists more than a 3-fold difference between high- and low-risk countries.[12] High-risk countries are those considered to be industrialized, like North American and Western Europe. The low-risk countries are those considered to be developing nations, most commonly found in Asian and Africa. As women migrate from low-risk countries to high-risk countries, their breast cancer risk increases. For example, Asian women who live in the East have one of the lowest breast cancer rates of any population in the world. When Asian women migrate to North America, however, they acquire breast cancer incidence rates similar to the women in the host country.[13,14] These trends may be partially explained by acculturation and adoption of a Western diet.[15]

1.1.3 Family History and Breast Cancer Risk

Aside from increasing age and geographical region, family history of breast cancer is one of the strongest breast cancer risk factors. Women who have a first-degree relative with a history of

breast cancer are themselves at an increased risk (OR 1.80, 95% CI 1.69-1.91) as are women with two first-degree relatives with a history of breast cancer (OR 2.93, 95% CI 2.36-3.64) when compared with women without an affective relative.[16] This risk is further increased if the breast cancer in the relative is diagnosed at an early age. A woman with a first-degree relative with a history of breast cancer diagnosed before the age of 40 years has a 5.7 times greater risk (95% CI 2.7-11.8) of being diagnosed with breast cancer herself before she is 40 years of age compared to a woman of the same age without a family history of breast cancer. [16]

The BRCA1 and BRCA2 genes have been implicated in familial breast cancer, which accounts for less than 10% of all breast cancers.[17] These mutations are more strongly related to premenopausal breast cancer, which occurs at a younger age. Among women diagnosed with breast cancer before 40 years of age, 9% have a BRCA mutation compared to 2% of women of any age diagnosed with breast cancer.[18] For BRCA1 mutations, the lifetime risk is 50-73% of developing breast cancer by the age of 50 years and 65-87% by 70 years of age, and for BRCA2 mutations, the risk is 59% by the age of 50 years and 82% by 70 years of age. [18]

1.1.4 Breast Cancer Risk Factors

How cancer of the breast arises is still not completely understood, but is most likely due to a combination of reproductive, hormonal, environmental and genetic factors. The difficulty in fully understanding the etiology of breast cancer is partly due to the long duration of sub-clinical disease as well as the heterogeneity of the cancer once it is diagnosed. Compounding these issues is the observation that risk factors may differ between pre- and post-menopausal breast cancer. However, epidemiological studies have been able to identify both risk factors as well as protective factors for breast cancer development (**Table 1**; reviewed by Dumitrescu et al.[19]).

These factors can be classified into two categories – modifiable factors (example: diet and physical activity) and non-modifiable factors (example: family history of breast cancer). Research has yet to identify a major risk factors that if modified can be used for primary breast cancer prevention.

Table 1: Summary of Breast Cancer Risk Factors.

Factors that increase breast cancer risk:	
Well-confirmed factors:	**Magnitude of risk:**
Increasing age	++
Geographical region (USA and Western countries)	++
Family history of breast cancer	++
Mutations in BRCA1 and BRCA2 genes	++
Mutations in other high-penetrance genes (p53, ATM, NBS1, LKB1)	++
Ionizing radiation exposure (in childhood)	++
History of benign breast disease	++
Late age at menopause (>54)	++
Early age at menarche (<12)	++
Nulliparity and older age at first birth	++
High mammographic breast density	++
Hormone replacement therapy	+
Obesity in postmenopausal women	+
Tall stature	+
Alcohol consumption (~1 drink/day)	+
Probable factors:	
High insulin-like growth factor I (IGF-I) levels	++
High prolactin levels	+
High saturated fat and well-done meat intake	+
Polymorphisms in low-penetrance genes	+
High socioeconomic status	+
Factors that decrease breast cancer risk:	
Well-confirmed factors:	
Geographical region (Asia and Africa)	--
Early age at first full-term pregnancy	--
Higher parity	--
Breast feeding (longer duration)	--
Obesity in premenopausal women	-
Fruit and vegetable consumption	-
Physical activity	-
Probable factors:	
Non-steroidal anti-inflammatory drugs (NSAIDs)	-
Polymorphisms in low-penetrance genes	-

++ (moderate to high increase in risk) -- (moderate to high decrease in risk)
+ (low to moderate increase in risk) - (low to moderate decrease in risk)

Reproductive factors have long been implicated in the etiology of breast cancer. Research has shown that early age at menarche (<12 years), late age at menopause (>54 years), late age at first full-term birth are breast cancer risk factors. Women who started menstruating at 12 years of age or younger have a 30% greater risk of developing breast cancer when compared with women who started menstruating on or after the age of 15.[20] Women who become menopausal at ≥55 years have a 71% greater risk (95% CI 1.37-2.12) of breast cancer than women who go through menopause at <40 years of age.[21] Additionally, women who had a first-full term pregnancy after the age of 31 years also have an increased breast cancer risk when compared to women whose first full-term pregnancy occurred before 18 years of age (RR 1.65, 95% CI 1.40-1.93).[22] For every five year increase in age for first full-term birth, there is a 20% greater risk (95% CI 1.16-1.24) for breast cancer development. [22]

1.1.5 Estrogens and Breast Cancer Risk

Based on the association between hormonally related risk factors (early age at menarche, late age at menopause, and late age at first full-term pregnancy) with increased breast cancer risk, differences in endogenous estrogen levels have been theorized to affect breast cancer risk.[23-26] Epidemiological studies have consistently shown that prolonged exposure to high levels of estrogen that occur in the menstrual cycle are related to an increased risk of breast cancer among postmenopausal women.[23-26] In a pooled analysis of nine prospective studies of endogenous hormone concentrations and breast cancer risk, serum estradiol concentrations predicted the risk of postmenopausal breast cancer. The relative risk for women with the highest quintile of free estradiol concentration was 2.58 (95% CI 1.76-3.78) relative to women in the lowest quintile.[26]

In postmenopausal women, body mass index (BMI) is a critical determinant of estrogen production. BMI is calculated as weight in kilograms divided by height in meters squared. The World Health Organization classifies BMI into four categories: underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2), and obsese (≥30.0 kg/m^2).[27] Results from the Women's Health Initiative (WHI) Observational Study confirmed the effect of increasing BMI on breast cancer risk among postmenopausal women who have never taken HT. Heavier women (baseline BMI >31.1) had an increased risk of postmenopausal breast cancer (RR 2.52, 95% CI 1.62-3.93) compared with slimmer women (baseline BMI <22.6).[28] Also, bone mineral density (BMD), which is considered a surrogate measure of lifetime estrogen exposure, is positively associated with breast cancer risk.[29]

Extensive data also link the use of HT after menopause, a major source of exogenous estrogen exposure in postmenopausal women, to the risk of developing breast cancer. Estimates vary, but it is estimated that breast cancer risk increases 10-80% depending on duration of use.[30-32] Results from the WHI indicate that this increased risk may occur among users of combined estrogen and progestin formulations[30,32], but not among users of unopposed estrogen[33,34]. Women randomized to take a combination formulation had a 24% increased risk of developing invasive breast cancer when compared to those randomized to placebo (HR 1.24, 95% CI 1.01-1.54).[30] In a separate arm of the WHI, women randomized to an unopposed pill had a risk of invasive breast cancer similar to those randomized to placebo (HR 0.80, 95% CI 0.62-1.04).[34]

When there is an abrupt arrest of estrogen by the ovaries, such as in surgical menopause, the risk of breast cancer decreases. In the Multiple Outcomes of Raloxifene Evaluation (MORE) trial, women in the highest tertile of estradiol levels had a 2.1 fold increased breast cancer risk when compared to women in the lowest tertile of estradiol.[35] Women with circulating levels of

estradiol >10pmol/L in the Raloxifene group had a breast cancer rate 76% lower (95% CI 53%-88%) than women with similar levels of estradiol in the placebo group.[36] Thus, inhibiting the action of estrogen reduces the risk of breast cancer.

Taken together, these studies all suggest that increased lifetime endogenous and exogenous estrogen exposure appears to increase breast cancer risk. Despite the evidence implicating estrogens in breast cancer, the underlying mechanism by which estrogens exert their effects remains unclear.

1.2 Mammographic Breast Density

Mammography represents the most accepted method for the early detection of breast cancer. A radiologist examining a mammogram is able to detect a tumor(s) or other breast abnormalities that may lead to breast cancer. Excluding high-risk women with a strong family history of breast cancer, the American Cancer Society currently recommends that women over the age of 40 years receive annual mammograms to screen for early breast cancer.[1] This recommendation is based on the observation that the earlier the cancer is detected, the greater the chances for survival. Although a mammogram is able to detect tumors (breast cancer), research has indicated that it is in fact the composition of the breast that may provide information on breast cancer risk itself.

The histological composition of the breast is composed of different types of tissue which are reflected mammographically by density and parenchymal patterns.[37] The dark areas on a mammogram represent fatty breast tissue. Because fatty tissue is radiolucent, it allows the x-ray beams to pass through it resulting in a dark appearance. The light areas on a mammogram represent fibroglandular tissue, which is composed of stroma, ductal, and glandular tissue. Because it is more dense than fatty tissue, the fibroglandular tissue absorbs the x-rays, thus

resulting in a light appearance.[37] The higher the fat content of the breast, the lower the radiologic density. Conversely, a high proportion of stroma or ductal and glandular tissue increases density.[38,39] Thus, mammographic breast density is an estimate of the proportion of dense tissues in the breast as opposed to fatty tissue. At menopause, glandular and ductal tissue decreases and fibrous connective tissue is usually replaced by fat, explaining the decrease in breast density that occurs with age.[40-46]

Research suggests that breast tissue aging rather than chronological age has the greatest influence on breast cancer risk.[47] The theory of breast tissue aging relates to the aging of breast tissue – most rapid at menarche, slows with each pregnancy, slows further in the peri-menopausal period, and is the lowest after menopause. This pattern of breast tissue aging corresponds to the pattern observed for breast density. A higher density is observed for pre-menopausal women, a lower density for women during the peri-menopausal period, and the lowest density observed for postmenopausal women.[47]

1.2.1 Methods of Measuring Breast Density

Since there is no standardized method to assess breast density, a variety of methods, both qualitative and quantitative have been developed.

1.2.1.1 Qualitative Breast Density Measurements

Dr. John Wolfe, in 1976, described the first method to associate breast parenchymal patterns and breast cancer risk [48]. Breasts were classified by one of four parenchymal patterns – "Wolfe's patterns":

1. N1 – primarily fat tissue; no duct pattern is visible; low risk

2. P1 – mostly fat tissue but with some dense areas (ductal prominence) of less than 25% of the total breast; intermediate risk
3. P2 – more than 25% of the breast composed of dense tissue along with a noticeable ductal pattern; intermediate risk
4. DY – primarily homogeneous dense tissue and no conspicuous ductal pattern; radiographically dense; highest risk

Several studies have used Wolfe's classification to measure breast cancer risk associated with breast density.[42,49-54] In three cohort studies,[52-54] the DY pattern was associated with an increased risk of breast cancer when compared to the N1 pattern. In both case-control and cohort studies, breast density determined by Wolfe's method has been associated with increased breast cancer risk (ORs ranging from 1.4-6.2).[42,49-54]

Another qualitative method is the Tabar classification.[55] This classification is based on the anatomic-mammographic correlation and utilizes a three-dimensional, sub-gross (thick-slice) technique to describe 5 patterns of breast density:

1. Pattern I – scalloped contours with some areas of fatty replacement; 1mm evenly distributed nodular densities (typical appearance of the premenopausal breast); low risk
2. Pattern II – complete fatty replacement of tissue; 1 mm evenly distributed nodular densities; low risk
3. Pattern III – prominent ductal pattern in the retroareolar area; low risk
4. Pattern IV – extensive nodular and linear densities throughout the breast, with nodular size larger than normal lobules; high risk
5. Pattern V – homogenous, extensive fibrosis with an appearance similar to ground glass; high risk

Gram et al. reported a poor agreement between the Tabar classification and Wolfe's patterns when comparing high-risk versus low-risk mammograms.[55] This mostly liked was because a large proportion (45.6%) of the evaluated mammograms were classified as Wolfe pattern DY (high risk), but as Tabar Pattern I (low risk).[55] This discrepancy may be due to assessment bias, since the author performed both measurements.

A third qualitative method that is commonly used to assess density by radiologists and is often reported on mammograms is the Breast Imaging Reporting and Data System (BI-

RADS).[56,57] In this method, four classes of density are reported: (1) breast is composed almost entirely of fat; (2) breast contains scattered fibroglandular densities; (3) breast is heterogeneously dense; and (4) breast is extremely dense.[56]

The above mentioned methods are all qualitative as hence subject to a high-degree of subjectivity and low to moderate reproducibility. In one study, the intra-observer intraclass correlation coefficient (ICC) for the Wolfe patterns was reported as 0.68 and the inter-observer ICC as 0.65.[58] The intra-rater reliability of the Tabar patterns was reported in one study as κ=0.65.[59] These indicate good to moderate reliability. A study of inter-observer agreement of the BI-RADS method reported an overall reliability of κ=0.43, with extremely poor agreement for the "extremely dense" category (κ=0.17) and highest agreement for the "fatty" category (κ=0.76).[60]

1.2.1.2 Quantitative Breast Density Measurements

In order to reduce intra- and interobserved variability associated with Wolfe patterns and Tabar patterns, various methods have been developed to quantitatively assess mammographic parenchymal patterns.

One of the first methods developed was the visual estimation of the proportion of the breast area occupied by dense tissue.[61] Since then, planimetry, both manual and computerized, has been used to assess breast density. Planimetry involves tracing both the total breast area as well as areas of dense tissue (excluding biopsy scars, Cooper's ligaments, and breast masses). The percent density is then calculated by dividing the area of the dense breast tissue by the total breast area. In manual planimetry, a wax pencil is used to trace the total area of the breast and all dense tissue areas onto a clear acetate sheet placed over the mammogram. A compensating polar

planimeter is then used to measure the total area of the breast and the area of breast density.[62-64] In computerized planimetry, digital mammograms or film mammograms that have been dititized are utilized. On a computer, a mouse is used to outline the total area of the breast as well as the dense areas and then the respective areas are calculated by the computer.[65,66] The intra-reader reliability is high, with intra-class correlation coefficients reported as 0.97 for non-dense area, 0.82 for absolute dense area, and 0.93 for percent density.[66]

Another quantitative method is interactive thresholding. Using digitized images of the breast, the reader selects a "threshold brightness" to distinguish the breast tissue from the background of the mammogram. Then another "threshold brightness" is chosen that differentiates the dense and non-dense tissue. The computer uses these thresholds to identify both the total are of the breast as well as the areas of density. The number of pixels within these areas are summed to give a measure of the total breast area, the dense area, and the percent density.[61,67-70] The intra-reader and inter-reader reliability using this computerized interactive thresholding method have been reported to be extremely high, with ICCs>0.90.[70]

Similarly to Wolfe's method, quantitative methods have shown an increased association (ORs ranging from 2.0-3.8).[49,61,71] Finally, studies using both methods have verified these findings and indicate that quantitative methods are more strongly associated with breast cancer risk than Wolfe's method.[45,49,72] As can be seen, regardless of the method used breast density has been shown to be a strong risk factor for breast cancer in the general population.

1.2.2 Breast Density and Mammographic Screening Sensitivity

Breast density is one factor shown to affect mammographic sensitivity and specificity,[73,74] and it is predictive of breast cancer risk.[75-77] Higher density is associated with lower sensitivity and

specificity.[78] Agents that affect breast density, therefore, also affect the mammographic screening success. Breast density decreases with greater parity, greater body weight, and increased age,[75] and increases with use of hormonal therapy (HT).[79] For example, current use of HT, which increases breast density, is associated with a lower specificity (false-positive readings) and lower sensitivity (false-negative readings) of mammograms when compared with women not currently using HT.[80] The estrogens and progestins present in the various HT combinations reversibly increase mammary cell proliferation. This increase in mammary cell proliferation is observed on a mammogram as an increase in breast density and occurs in 17-37% of women on HT.[81] If HT use is discontinued, the mammographic sensitivity is increased, decreasing the number of false-positives, and hence reducing the need to have a breast biopsy performed.[80] Given this evidence, the current recommendation is that women on HT discontinued taking the medication 2 weeks prior to a mammogram.[81]

1.2.3 Breast Density and Breast Cancer Risk

The higher the breast density, the stronger the association with an increased risk of breast cancer. Comparing the highest category of breast density to the lowest, there is a 4- to 6-fold increased risk of breast cancer development. A meta-analysis[57] published in 2006 examined both qualitative and quantitative measures of breast density with breast cancer risk. The authors reported combined point estimates from general population prevalence studies using Wolfe patterns to be 1.25 (95% CI 1.02-1.54) for P1 versus N1, 1.97 (95% CI 1.29-3.00) for P2 versus N1, and 2.92 (95% CI 1.98-2.97) for DY versus N1. These point estimates were slightly higher for general population incidence studies (combined relative risks): 1.76 (95% CI 1.41-2.19) for P1 versus N1, 3.05 (95% CI 2.54-3.66) for P2 versus N1, and 3.98 (95% CI 2.53-6.27) for DY

versus N1.[57] This meta-analysis did not include the one study that used the Tabar classification in its combined estimates. This study reported an increased risk of breast cancer among women with Tabar pattern IV (adjusted OR 2.42, 95% CI 0.98-5.97) when compared with Tabar pattern I.[82]

In regards to studies that utilized quantitative measures of breast density, similar combined estimates of relative risk were observed in this meta-analysis. For general population prevalence studies, the authors reported combined relative risk estimates of 1.39 (95% CI 1.10-1.76) for 5-24% density, 2.22 (95% CI 1.75-2.81) for 25-49% density, 2.93 (95% CI 2.27-3.79) for 50-74% density, and 3.67 (95% CI 2.72-4.96) for ≥75% density when compared with <5% density.[57] Similarly, to the qualitative studies, the point estimates were slightly higher for general population incidence studies. The authors reported combined relative risks of 1.79 (95% CI 1.48-2.16) for 5-14% density, 2.11 (95% CI 1.70-2.63) for 25-49% density, 2.92 (95% CI 2.49-3.42) for 50-74% density, and 4.64 (95% CI 3.64-5.91) for ≥75% density when compared to <5% density.[57]

The meta-analysis referred to above by McCormack and dos Santos Silva[57] illustrates the strong evidence for an association between breast density and breast cancer risk. Regardless of the methods used in the individual studies, whether qualitative or quantitative, or the type of study conducted, whether using incident or prevalent cases, similar and consistent results were obtained.

Additionally, this meta-analysis[57] illustrates the importance of masking bias when examining the association between breast density and breast cancer. Masking bias refers to the "masking" of breast cancer by high breast density. Tumors in dense tissue may be missed at an initial mammogram, but manifest themselves at subsequent mammographic examinations, thus

giving the appearance that women with higher density have an increased risk of breast cancer.[83] If masking bias is present, one would observe an underestimated relative risk in prevalence studies (cancers were detected at the time of screening) and an overestimated relative risk in incidence studies. This is exactly what the authors observed. The estimates for prelavence studies were lower than those of incidence studies. The authors also observed combined relative risks of 4.64 (95% CI 3.64-5.91) for all cancers, 4.52 (95% CI 3.54-5.78) when cancers diagnosed in the first year were excluded, and 13.38 (95% CI 2.73-66.6) when including cancers diagnosed in the first year when comparing women with ≥75% density to those with <5% density.[57] However, one could agree that since similar risk estimates where observed for both prevalence and incidence studies, that masking bias is not a major factor.[75] From this meta-analysis the authors also concluded that the association between breast density and breast cancer risk remains strong regardless of age, menopausal status, or race.[57]

Since the publication of the meta-analysis indicated above, only one additional article has been published that has examined the association between breast density and breast cancer risk. In this manuscript, Mitchell et al.[84] reported that higher percent breast density remains a strong risk factor for breast cancer among women with known BRCA1/2 mutations. The odds of breast cancer among mutation carriers with density ≥50% were twice that of mutation carriers with <50% density (OR 2.29, 95% CI 1.23-4.26).[84]

Changes in breast density have also been reported to be associated with subsequent changes in breast cancer risk. For each 1% increment in percent breast density an estimated 1.5-2% increase in breast cancer risk occurs.[75,85] This indicates that breast density is a stronger predictor of breast cancer risk than most traditional breast cancer risk factors.

1.2.4 Age, Menopausal Status and Breast Density

Typically, breast density decreases with postmenopausal status and increasing age [71]. However, Byrne et al. [86] found a greater effect of breast density on breast cancer risk in postmenopausal women. Among premenopausal women, a woman with 1-24% breast density had an OR of 1.37 (95% CI 0.95-2.3) and a woman with ≥75% breast density had an OR of 3.79 (95% CI 2.3-6.2) when compared to a premenopausal woman with 0% density. Similar increases in breast cancer risk with increasing breast density were observed for postmenopausal women, but the magnitude of the association was higher. A postmenopausal woman with 1-24% breast density had an OR of 1.79 (95% CI 1.3-2.5) and a woman with ≥75% breast density had an OR of 5.82 (95% CI 3.0-11.3) when compared with a postmenopausal woman with 0% breast density.[86] Similarly, Boyd et al.[47] found a higher risk in women ages 50-59 with a RR 7.1 (95% CI 2.0-25.5) compared to women ages 40-49 RR 6.1 (95% CI 1.5-24.2).

It appears that the menopausal transition has a stronger influence on breast density than age. Boyd et al.[87] examined longitudinally the effects of menopause on breast density. The authors compared women who were premenopausal at a baseline mammogram and then postmenopausal at a subsequent mammogram to women who remained premenopausal at both mammograms, matched on age. The percent density decreased more among the women who transitioned through menopause as opposed to those who remained premenopausal.[87] However, age may not be a factor in women over the age of 70 years. Modungo et al.[88] examined 239 participants from the Study of Osteoporotic Fractures (SOF) reported that only BMI, parity, surgical menopause, and current smoking status were significantly associated with mammographic breast density in multivariate analyses. The mean age of the women used for the study was 78.6 (SD 3.8). This indicates that factors associated with breast density may be

different in older women when compared with either premenopausal or younger postmenopausal women.[88]

1.2.5 Breast Density and Breast Cancer Risk Factors

In general, breast cancer risk factors are also associated with an increase in breast density. Early age at menarche, late age of menopause, nulliparity and later age at first full-term birth have been associated with increased density.[89-95] Hormone therapy (HT) in the form of combined formulations of estrogen and progesterone increase density,[79,96-102] while SERMS decrease density.[76,103-105] Because the effects of both HT and SERMS on mammary cell proliferation are reversible, their respective effects on breast density cease with discontinuation of use. Changes in breast density are also observed among oral contraceptive users and by phase of the menstrual cycle. Breast density increases in premenopausal women during the luteal phase, which is characterized by elevated levels of estradiol.[106-108] Because of the above-mentioned associations between reproductive and hormonal exposures and breast density, it has been hypothesized that mammographic breast density may be a marker of estrogen and other hormonal effects on the breast tissue.

There is limited information on the relationship between circulating estradiol and breast density. Among postmenopausal women, not using exogenous hormones, one study found a positive association between estradiol levels and percent density; two studies found an inverse relationship; two found null relationahsips, one only after adjustment for sex hormone binding globulin (SHBG), age, and waist circumference.[109-115] It has been suggested that localized estrogen production in the breast tissue may be more relevant to breast density than circulating estrogen levels. As with endogenous estrogens and breast density, the relationship between

breast density and bone mineral density, an established breast cancer risk factor and surrogate marker of estrogen exposure, remains unclear.[114,115]

Increased BMI is associated with an increased risk of breast cancer among postmenopausal women. However, studies of mammographic breast density have consistently reported that increased weight or BMI is associated with lower percent breast density.[59,66,94,116-119] Vachon et al.[94] observed a difference in percent density when the 3rd and 1st quartiles of BMI were compared – a 5.2 unit decrease among premenopausal women and a 4.7 unit decrease among postmenopausal women.

The majority of studies examining the interaction between weight or BMI and breast density on the risk of breast cancer have noted significant effect modification. Ursin et al.[85] reported a U-shaped relationship, with women having the lowest and the highest BMI demonstrating the strongest association between breast density and risk of breast cancer. Duffy et al.[120] reported that when evaluating the relationship between high-risk Tabar patterns and breast cancer risk, only those women who were both overweight and had dense breasts showed an increased risk of breast cancer (OR 2.30, 95% CI 0.98-5.40 for women with BMI >25 kg/m^2 and dense breasts compared to those with BMI <25 kg/m^2 and non-dense breasts). Boyd et al.[121] also noted that BMI was not significantly associated with breast cancer in either pre- or postmenopausal women prior to adjustment for breast density. When percent breast density was controlled for, there was an increase and statistically significant association with breast cancer risk overall and for postmenopausal women. Although the association between BMI and breast cancer risk among premenopausal women increased, it was not statistically significant.[121] The authors concluded that anthropometry and breast density are confounders of one another in relation to breast cancer risk and failure to adjust for breast density in previous studies may

explain the negative associations between BMI and breast cancer that are commonly reported among premenopausal women.[121]

There may also be a genetic component to mammographic breast density. Boyd et al.[122] reported the results of two twin studies in women ages 40 to 70 years. The correlation coefficients for percent breast density were 0.61 for Australian monozygotic twins and 0.67 for North American monozygotic twins. For dizygotic twins, the correlation coefficients for percent density were 0.25 for Australian twins and 0.27 for North American twins. In these studies genetic factors explained 60-75% of the variability in percent breast density.[122]

1.3 Prolactin

Prolactin (PRL) is a 23kDa polypeptide protein with 199 amino acids arranged in four antiparallel α helices with three disulfide loops (Figure 2)[123] and is located on chromosome 6. Its amino acid sequence and tertiary structure is similar to both growth hormone and placental lactogen.[124] Genes encoding all three growth factors evolved from a common ancestral gene by gene duplication. It is believed that the PRL and growth hormone lineages diverged approximately 400 million years ago.[125] Along with growth hormone and placental lactogen, PRL has been classified as belonging to an extended family of proteins, known as the hematopoietic cytokines.[126] Alternative splicing, proteolytic cleavage, phosphorylation, glycosylation, deamination of asparagines or glutamate residues, sulfonation of tyrosine residues, and polymerization and formation of complexes with other molecules result in a number of PRL variants.[127] Aside from some limited research on proteolytic cleavage and phosphorylation of PRL, the physiological functions of the majority of these modifications are not yet known. What is known is that posttranslational modifications are often more harmful than helpful for the

activity of PRL – glycosylation lowers biological activity; phosphorylation generates PRL antagonisits; and proteolytic cleavage of PRL into 16KDa PRL abolishes receptor binding and has antiangiogenic properties.[127,128] In addition, polymerization and conjugation to IgG can form large molecular species of PRL, such as "big" PRL (50-60 kDa) and macro-PRL (150-170 kDa) which are present in the serum of patients with hyperprolactinemia.[123]

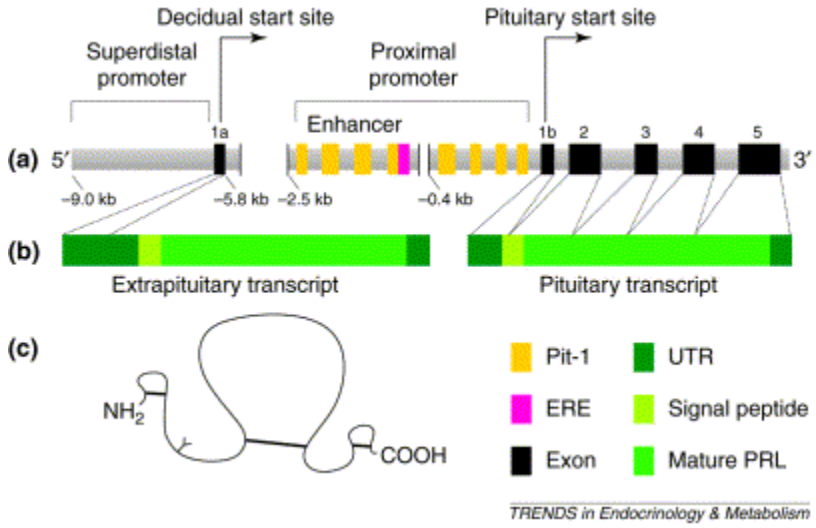


Figure 2: Comparison of pituitary and extrapituitary prolactin (PRL)

Notes: (a) Diagram of the gene encoding human PRL illustrating the proximal and superdistal promoter regions; (b) pituitary and extrapituitary transcripts; (c) PRL protein and the location of the three disulfide bridges. Note the use of exon 1a as the transcription start site for extrapituitary PRL and the longer 5′-untranslated region. Adapted from Ben-Jonathan et al.[123]

Prolactin is synthesized in and secreted by the lactotrophs – specialized cells of the anterior pituitary gland.[124] Within the secretory granules of the lactotrophs, p21-activated protein kinase 2 (PAK2) mediates the phosphorylation of PRL.[129] Phosphorylated PRL (pPRL) has both antagonistic and agonistic effects, as measured thru Nb2 lymphoma cells. These cells proliferate after treatment with lactogenic hormones and are often used to measure PRL activity.[130,131] pPRL

inhibits the proliferation of Nb2 lymphoma cells in a dose-dependent, antagonistic manner.[132] However, a molecular mimic of pPRL (S179D) antagonizes PRL activity in Nb2 lymphoma cells.[133] This mimic of pPRL also inhibits rat mammary gland growth[134] and can antagonize PRL induced proliferation in breast cancer cell lines.[135] Given the contrary roles of pPRL, much research is still needed in this area, as well as in the physiological functions of the various PRL variants.

Although, pituitary PRL secretion can be either positively or negatively regulated, it is mainly controlled by inhibitory factors, such as dopamine, from the hypothalamus. The lactotrophs contains the D2 subclass of dopamine receptors which bind dopamine, thus inhibiting pituitary PRL secretion.[124] The synthesis of pituitary PRL is driven by a proximal promoter, which is divided into a proximal region and a distal enhancer (**Figure 2**). Both of these are necessary for optimal pituitary-specific expression. The proximal promoter requires the Pit-1 transcriptional factor for trans-activation and is regulated by dopamine, estrogens, neuropeptides and other growth factors.[124]

Secondary secretion of PRL occurs at extrapituitary sites, such as the mammary epithelium, placenta, uterus, brain and the immune system.[124] Because PRL can be synthesized in extrapituitary sites, it is considered a growth factor, as opposed to being classified as a hormone. Hormones are exclusively produced by endocrine glands and not by other cell types. The synthesis of extrapituitary PRL is driven by a superdistal promoter, located 5.8kb upstream of the pituitary start site (**Figure 2**). The superdistal promoter is silenced in the pituitary, does not bind the Pit-1 transcriptional factor and is not regulated by dopamine, estrogen or neuropeptides, unlike the pituitary PRL transcript.[123] The alternative transcriptional start site, exon 1a, is spliced into exon 1b, thus yielding a coding region identical to the pituitary transcript.

However, since extrapituitary PRL is not stored in secretory granules it does not depend on Ca^{+2} dependent exocytosis. Although, the superdistal promoter contains several binding sites for various transcription factors, its regulation is poorly understood. It is possible that these transcriptional factors are needed for the secretion of PRL at the various extrapituitary sites. For example, progesterone decreases PRL synthesis in the myometrium, while increasing PRL synthesis in the endometrium.[123]

1.3.1 Prolactin Receptor

The prolactin receptor (PRLR) is a member of the class I cytokine receptor superfamily and is located on chromosome 5.[136] It is a transmembrane receptor, consisting of an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and an intercellular domain. Alternative splicing of the PRLR gene leads to multiple isoforms which differ in length and composition of their cytoplasmic tail, but have identical extracellular domains. Three different forms of the PRLR have been described – long form (90 kDa), intermediate form (50 kDa), and short form (40 kDa). The intermediate form is the result of a deletion mutant in the long form, resulting in the lack of 198 amino acids in the cytoplasmic tail. It is believed to be more sensitive to PRL than either the long or short forms of the receptor and is the predominant form found in Nb2 rat lymphoma cells.[125] Both the long and intermediate forms are able to induce differentiation (measured by induction of milk protein gene expression), while the short form is a negative regulator of this differentiation. In the rat, the expression of the short and long PRLR isoforms have been shown to vary as a function of the stage of the estrous cycle, pregnancy, and lactation. The PRLR is not only capable of binding PRL, but PL (placental lactogen) and primate GH (growth hormone) as well.[124]

This receptor is virtually expressed in all organs and/or tissues, with its expression varies from very low (cells of the immune system) to ~200 to ~30,000 receptors per cell.[124] Interestingly, PRLR have been found in breast cancer cells. Mertani et al.[137] found PRLR mRNA in normal breast, inflammatory lesions (mastitis), benign proliferative breast disease (fibroadenoma, papilloma, adenosis, epitheliosis), intraductal carcinoma or lobular carcinoma in situ, and invasive ductal, lobular, or medullary carcinoma. There was no correlation between the level of PRLR mRNA and the histological type of lesion. In another study, more than 90% of breast cancer surgical samples were also positive for PRLR mRNA.[138] The cancerous tissue had significantly more mRNA than the adjacent, noninvolved tissue from the same patient.

1.3.2 Activation of the Prolactin Receptor

The extracellular domain of the PRLR consists of a NH_2-terminal D1 sub-domain and a membrane-proximal D2 sub-domain, consisting of a total of 210 amino acids. The D1 sub-domain is composed of two pairs of disulfide bonds between cysteine residues (Cys^{12}-Cys^{22} and Cys^{51}-Cys^{62}), while the D2 sub-domain has a "WS motif" (Tryptophan-Serine-x-Tryptophan-Serine), both of which are essential for proper folding and trafficking of the receptor to the cell membrane, but not ligand biding.[125]

Prolactin (**Figure 3**) itself has two binding sites – binding site 1 (α helix 1 and 4) and binding site 2 (α helix 1 and 3). PRL binding site 1 binds with the D1 sub-domain of the PRLR. This initial interaction/binding then allows the same prolactin molecular to bind to another PRLR via binding site 2. This receptor dimerization is essential for the activation of a number of signaling cascades. It induces the phosphorylation of a tyrosine kinase, Janus kinase 2 (Jak2) that binds to the proline-rich box 1 motif of the intracellular domain of the PRLR. The activation of

Jak2 occurs approximately one minute after prolactin binding thru the transphosphorylation of two Jak2 molecules. However, this transphosphorylation can only occur if two conditions are met: (1) presence of a proline-rich box 1 motif; and (2) homodimeric stoichiometry of the ligand-induced PRLR dimmers. Once the Jak2 kinases transphosphorylate one another, they then phosphorylate tyrosine residues (Y) in the PRLR itself.[125]

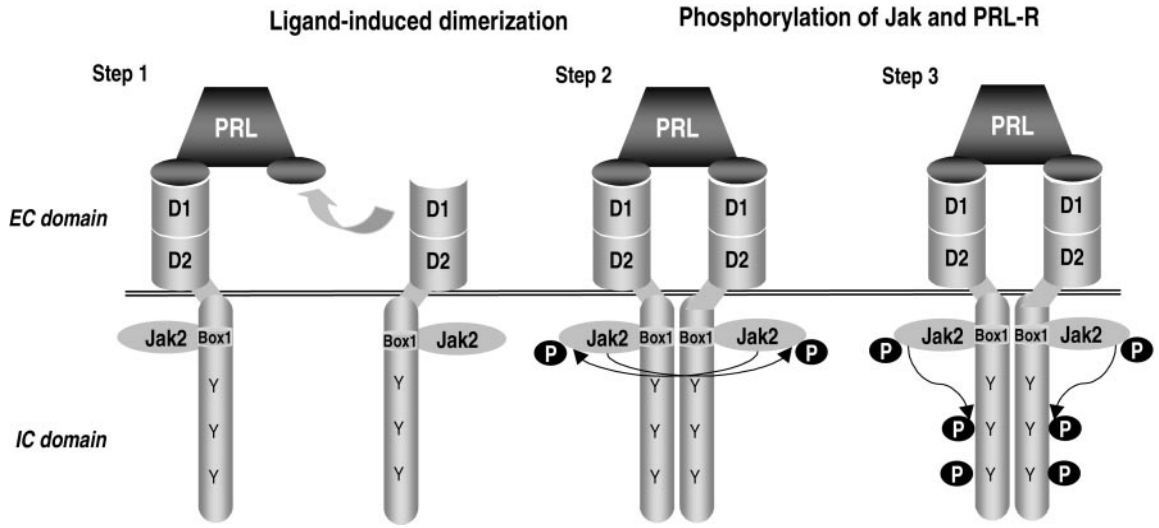


Figure 3: Prolactin Receptor Activation

Mammary glands containing Jak2-/- epithelium in virgin animals develop normally, but the mammary glands fail to develop lobuloalveoli at parturition. The terminal end buds are present at the ends of ducts indicating an effect in the earlier stage of differentiation to alveolar buds. This phenotype is accompanied by a reduction in proliferation, maintenance of ductal cell markers and lack of secretory cell markers in Jak2-/- mammary epithelium.[136,139]

The phosphorylation of the tyrosine residues of the PRLR by Jak2 allow signal transducer and activator of transcription (Stat) protein family members (Stat1, Stat3, Stat5) to bind to the prolactin receptor (**Figure 4**).[125] The Stat family proteins contain five conserved regions: a DNA-binding domain, an SH3-like domain, an SH2-like domain, and a NH_2- and

COOH-terminal transactivating domain. A phosphorylated tyrosine residue (Y) of the PRLR interacts with the SH2-like domain of Stat. This interacts causes the Stat to dock at the PRLR, leading to its activation by Jak2. Once the Stat is phosphyrated, it then dissociates from the PRLR and either hetero- or homodimerizes through its phosphorylated tyrosine residues with the SH2-like domain of another phosphorylated Stat molecule. The Stat dimmer is then able to translocate to the nucleus where it activates a Stat DNA-binding motif (GAS – γ-interferon activated sequence with a palindromic sequence of TTCxxxGAA) in the promoter of the prolactin gene to initiate gene transcription.[140] Aside of prolactin, Stat is also able to interact with other signal transducers to initiate a cell- and/or cytokine-specific response. The above activation occurs in the long-form of the prolactin receptor. The tyrosine residues of the short PRLR are not phosphorylated by Jak2, but the phosphotyrosine of Jak2 serves as a docking site for Stat1.[125]

Lack of Stat5 in mice lead to impaired mammary gland development due to a reduced formation of lobuloalveoli.[141] Stat5-/- mammary epithelium develop normally in virgin animals but lack lobuloalveolar development at parturition, demonstrating a role for Stat5 in mammary gland development.[141] This phenotype may be more extreme than the PRLR-/- mammary phenotype due to presence of a closed epithelial lumen and aberrant cell-cell contacts. Stat5 -/- epithelium retains markers of ductal epithelium and fails to express markers of secretory epithelium.[142] The PRLR -/- mammary epithelium has a greater defect in proliferation in response to estrogen and progesterone than the Stat5-/- mammary epithelium, indicating that Stat5 mediates the effect of PRL on differentiation, but not proliferation.[142] However, other hormones and growth factors are able to stimulate Stata5 activation. GH and epidermal growth factor are able to stimulate Stata5 activation in PRLR -/- mammary epithelium.[142]

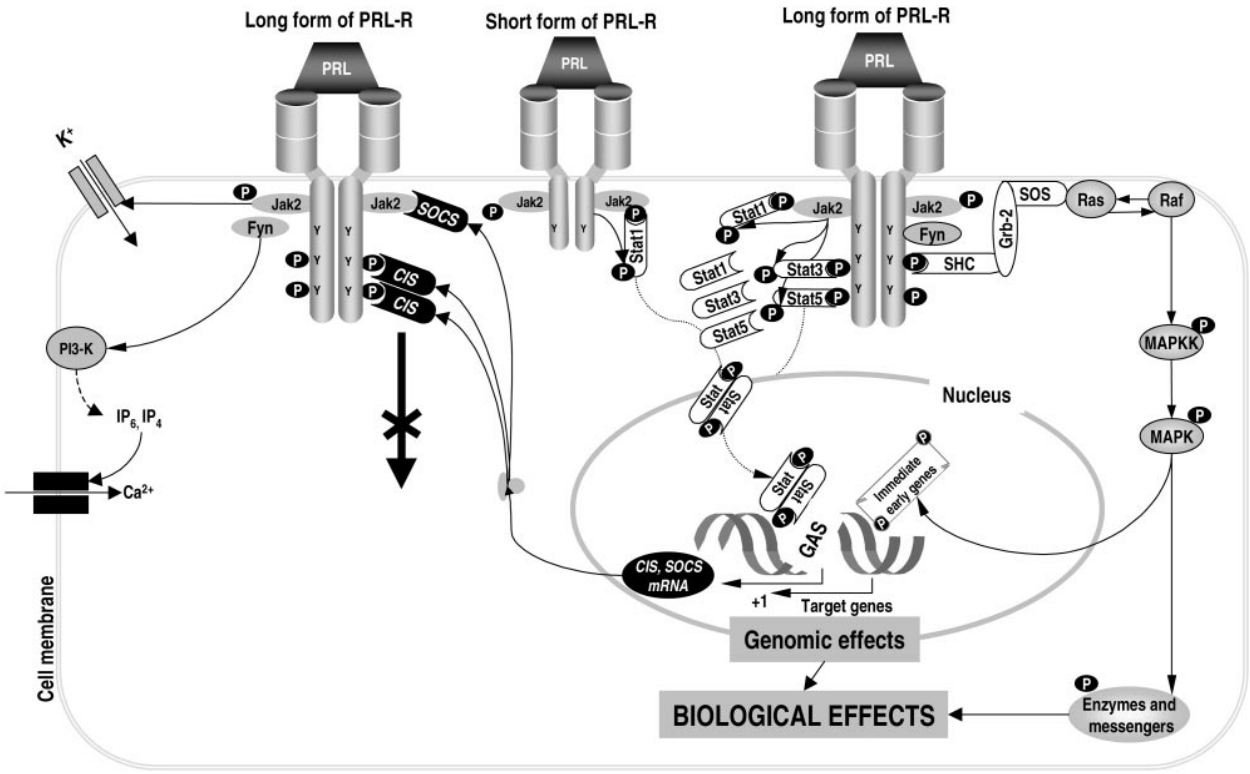


Figure 4: Signal transduction pathways initiated by activation of the prolactin receptor (PRLR)

The tyrosine phosphorylation sites in the PRLR also serve as docking sites (**Figure 4**) for the adapter proteins Src homoly 2 domain containing transforming protein (Shc), growth factor receptor bound protein 2 (Grb2) and son of sevenless (SOS).[125] Binding of these proteins result in the activation of Ras and Raf, which in turn activate the mitogen activated protein (MAP) kinase pathway.[125,136] Activation of the MAP kinase pathway is believed to mediate the effect of prolactin on the proliferation of mammary epithelial cells.[143] In addition, the PRLR also facilitates docking of Src family kinases, c-src and Fyn. These kinases can then activate protein kinase B and the phosphatidylinositol 3'-kinase (PI3 kinase) pathway.[144] Activation of the PI3 kinase pathway is believe to regulate prolactin's anti-apoptotic and cell proliferation effects.[145]

Additionally, box 1 of the intracellular domain of the prolactin receptor is involved in the activation of a tyrosine kinase-dependent, calcium-sensitive K^+ channels through Jak2. The COOH terminal of the PRLR's intracellular domain is involved in the production of the intracellular messengers inositol 1,3,4,5-tetrakisphosphate (IP_4) and inositol hexakisphosphosphate (IP_6), which open voltage-independent Ca^{+2} channels in the cell membrane (**Figure 4**).[125] These Ca^{+2} channels are regulated by dopamine released from the hypothalamus, acting as a prolactin release-inhibiting factor.[146] Dopamine can activate several interacting intracellular signaling pathways to suppress PRL gene expression and lactrotroph proliferation.[147]

1.3.3 Downregulation of the Prolactin Receptor

Reduction of PRLR signaling is mediated by members of the suppressor of cytokine signaling (Socs) gene family. For example, Socs1 and Socs3 are activated thru the inhibition of the Jak/Stat pathways.[148] Socs1-/- mice exhibit accelerated lobuloalveolar development characterized by an increase in lobuloalveoli, enlargement of the lumen, an increase in milk protein expression, and Stat5 phosphorylation during pregnancy and lactation. Deletion of a single Socs1 allele is able to restore lobuloalveolar development in PRL +/- mammary epithelium, demonstration that Socs1 is a key regulator of PRL signaling in the mammary gland.[149]

1.3.4 Pathologies Associated with Prolactin

Prolactinoma is a tumor of the pituitary gland, accounting for 25-30% of functioning pituitary adenomas[150] and is the most frequent cause of persistent hyperprolactinaemia – defined as the

presence of an abnormally high level of prolactin in the blood; normal levels for non-pregnant women are typically 20-25 ng/mL and for pregnant women, prolactin levels can reach 500 ng/mL.[151] In premenopausal women, hyperprolactinaemia may manifest itself as galactorrhea, oligomenorrhea, loss of libido, and sexual dysfunction. Prolactinomas in premenopausal women tend to be microprolactinomas (<10 mm in diameter).[152] In contrast, prolactinomas in postmenopausal women tend to be macroprolactinomas (>10 mm in diameter).[152] In addition to having hyperprolactinaemia, postmenopausal women may also experience headache, visual disturbances, cranial nerve palsies, hypopituitarism, or rarely spontaneous cerebrospinal fluid leakage, epilepsy due to temporal lobe compression and obstructive hydrocephalus.[152]

Aside from prolactinomas, certain medications can also cause hyperprolactinaemia. These include anti-psychotics (phenothiazines, haloperidol, butyrophenones, risperidone, monoamine oxidase inhibitors, fluoxetine, sulpiride), anti-emetics (metoclorpramide, domperidone), anti-hypertensives (methyldopa, calcium channel blockers, reserpine), and tricyclic depressants that inhibit the release of dopamine.[150] In addition, certain opiates can stimulate hypothalamic opioid receptors that promote prolactin secretion and protease inhibitors increase prolactin levels via unknown mechanisms.[150]

Treatment of prolactinomas usually involves dopamine agonists. Dopamine is an inhibitor of PRL, regulating prolactin gene expression and lactrotroph proliferation. Dopamine receptors are classified into D1 and D2 sub-types. Aside from both receptor sub-types being found in the brain (susbstantia nigra and striatum, and limbic cortex and associated structures), the D1 sub-type is also found in blood vessels and the proximal tubule cells, while the D2 sub-type is found on the cell surface of lactotroph cells and in the sympathetic nerve terminals.[147] The dopamine agonists, bromocriptine, cabergoline (Dostinex®, Pfizer, Inc., New York, USA),

and quinagolide (Norprolac®, Ferring Pharmaceuticals, Lausanne, Switzeralnd) have a high-affinity for the D2 receptor sub-types on the lactotroph cells of the anterior pituitary and act to reduce prolactin secretion[147,150].

1.3.5 Circadian Rhythm of Prolactin Secretion

Sassin et al.[153] in 1972 measured plasma prolactin levels at 20-minute intervals for a 24-hour period in normal adults. They were also to demonstrate that prolactin levels "undergo episodic changes and there is a diurnal variation in release, with highest concentrations during the nocturnal sleep period and the lowest during the waking hours." Since this initial report, other researchers have demonstrated that prolactin levels begin to rise as one falls asleep and reach a maximum 1-2 hours before waking, with peaks of prolactin secretion occurring during slow-wave sleep. This rhythm changes if the sleep pattern is disturbed or rearranged.[146,154]

1.3.6 Polymorphisms in the Prolactin Gene and Prolactin Receptor Gene

Recent studies have established an immuno-regulatory function for prolactin.[155-157] Prolactin is not only produced by the anterior pituitary gland, but at various extrapituitary sites, including T lymphocytes. The prolactin receptor is a member of the class I cytokine receptor superfamily, suggesting that PRL may have a multifunctional cytokine role in addition to its endocrine role. Prolactin, acting through its receptor, stimulates cell proliferation and survival,[158] hence modulating the function of the immune system. Additional proof for the role of PRL in the immune system comes from animal models, in which ablation of the pituitary gland or treatment with bromocriptine (a dopamine agonist that reduces pituitary PRL release) induced anemia, leucopenia and thrombocytopenia, as well as an impaired humoral and cellular immune

response.[159,160] In all of these studies, the lympho-hemopoietic function was restored when either PRL was injected or a syngenic pituitary gland was implanted.[159,160] Studies in patients with a deficient immune system, including individuals with systemic lupus eythematosus,[161,162] multiple sclerosis,[163] rheumatoid arthritis,[164] Reiter's disease,[165] Sjogren's syndrome,[166] Hashimoto's thyroiditis,[167] and uveitis,[168] have found moderately increased levels of PRL. In addition, genetic studies have confirmed that PRL maps to chromosome 6p, and is telomeric to the HLA region.[155] The HLA region has been linked to autoimmune diseases, most notably type 2 diabetes mellitus.

Several SNPs have been identified in the PRL gene. These include -1149 extrapituitary promoter G/T (rs1341239);[156,157,169] -733 extrapituitary promoter C/T;[156] -328 pituitary promoter G/A;[156] +78 exon 5 G/A;[156] and +214 intron C T/C (rs7739889).[156] However, the study populations used to identify these SNPs have had a deficient immune system.[156,157,161-169]

Only one study to date has examined the association between SNPs in the PRL and PRLR gene with breast cancer risk. Vaclavicek et al.[170] conducted a case control study of 441 German familial unrelated breast cancer cases and 552 hospital-based controls matched by age, ethnicity, and geographical region. Although women carrying BRCA1/2 mutations were excluded, all cases had a strong family history of breast cancer – 131 cases with two or more breast cancer cases in the family, with at least two cases with onset before 50 years of age; 68 cases with at least one breast cancer and one ovarian cancer in the family; 201 cases with at least two breast cancer cases in the family, not meeting the above two criteria; 16 cases with a single breast cancer case in the family diagnosed before the age of 15 years; 3 cases with both female and male breast cancer in the family; 13 cases with bilateral breast cancer diagnosed before the age of 50 years; and 9 cases with missing family history. A total of 7 SNPs were selected for genotyping. Within the PRL gene, the SNPs chosen were rs1341239, rs12210179, rs2244502,

and rs1205960. Two SNPs (TT for rs1341239 and GG for rs12210179) were statistically significantly associated with a higher breast cancer risk (OR 1.67, 95% CI 1.11-2.50, and OR 2.09, 95% CI 1.23-3.52, respectively). When haplotypes where constructed from the four SNPs listed above, in the order listed, the TGTG haplotype was also statistically significantly associated with a higher breast cancer risk (OR 1.42, 95% CI 1.07-1.90). For the PRLR gene, three SNPs were chosen - rs13354826, rs9292573, and rs37389. None were statistically associated with an increased breast cancer risk, but interestingly the TCC haplotype conferred a statistically significant reduction in breast cancer risk (OR 0.69, 95% CI 0.54-0.89).[170]

No study to date has identified PRL and PRLR SNPs in a healthy population without a comprised immune system. Also, the functional significance of PRL and PRLR SNPs has not yet been established nor have the various SNPs been correlated with serum prolactin levels.

1.3.7 Effect of Prolactin and Prolactin Receptor on the Mammary Gland: Animal Studies

In wild-type animals ductal elongation results from the formation of terminal end buds (TEBs) – specialized structures with high rates of cell division at their invasive leading edge and a central zone of apoptotic cells that produce the canalized duct. This structure differentiates into a terminal alvelolar bud once ductal elongation has terminated. In mammary glands from prolactin receptor gene deficient (PRLR-/-) mice, ductal elongation occurs normally as the animals enter puberty, however secondary side branching does not occur as the animals age and the ducts become more distended.[171,172] In mature PRLR-/- animals, the TEB-like structure lacks the cap cell layer and the multiple layers of highly mitotic and apoptotic cells. This suggests a defect in the final stage of differentiation.[171]

Transplantation of PRLR-/- mammary epithelium to the mammary fat pad of an immuno-compromised but otherwise normal animal allows examination of PRLR-/- mammary gland development in a normal endocrine environment, thus allowing development during pregnancy to be examined. The host fat pad is cleared of endogenous epithelium and the transplanted epithelium is allowed to penetrate and fill the fat pad.[136] Transplanted PRLR-/- epithelium develop side branches in virgin animals, as the epithelium experiences normal levels of sex steroid hormones. During pregnancy, a complete failure of lobuloalvelolar development is observed and only alveolar-buds are formed. This implicated PRLR in mediating the formation of milk producing structures during pregnancy.[171]

In order to form functional lobuloaveoli during pregnancy, two processes must occur within the alveolar bud. The first is proliferation to increase cell number to provide greater epithelial surface area for milk production and secretion (lobuloalveolar development). The second process is final stage differentiation to allow the cells to produce and secrete milk (lactogenesis). Both processes require the activation of the PRLR.[136] PRLR-/- epithelium do not proliferate to form lobuloalveoli in transplants[171] or after treatment with estrogen and progesterone.[173] However, in PRLR+/- mice lack of proliferation is only observed at the later lactogenesis stage, indicating a defect in differentiation. In addition, PRLR +/- mice mated at 8 weeks of age have less than a 50% chance of successfully nursing their young, although these mothers have a greater chance of successful lactation following their second pregnancy. The mammary glands of these mothers who fail to lactate show the formation of lobules, but they are unable to form an open lumen or secrete milk – their development is stopped at mid-pregnancy.[174] The PRLR +/- phenotype suggests that the level of signaling flux initiated by PRL can modulate mammary gland development, with the later developmental stage of lactogenesis

requiring higher levels of PRLR signaling that cannot be met from a single functional PRLR allele.[174]

PRL produced by the mammary gland may also play a role in proliferation of mammary epithelial cells during lactogenesis. PRL -/- mammary epithelium transplanted to a wild-type cleared mammary fat pad showed a decrease in proliferation. This indicates that mammary-produced PRL has a role in sustaining milk production, rather than development of the gland during pregnancy.[136]

1.3.8 The Role of Prolactin in Breast Development

Unlike most organs in women, the mammary gland completes its development postnatally at three distinct points in a woman's life: during puberty, throughout pregnancy and during lactation.[175,176] The exact mechanisms linking these events to breast development remain unknown, but estrogen is believed to play a major role. There is, however, much evidence linking prolactin to breast development as both a mitogen and differentiating agent,[138,177] especially during pregnancy and lactation. During pregnancy, breast epithelial cells rapidly proliferate to create additional ductal branches and promote lobuloalveolar growth.[178] Prolactin has been shown to act directly on the mammary epithelium to produce lobuloalveolar development.[171] Although, estrogen and progesterone are also involved in ductal growth and branching, PRL is essential for full lobuloalveolar development. Progesterone appears to increase the level of PRLR, thus acting synergistically with prolactin to exert its effects on ductal growth and lobuloalveolar development.[179] If PRL is absent during puberty, the mammary gland is underdeveloped. Additional during lactation, the alveoli serve as the unit of milk production and are directly under the control of circulating PRL. Upon ceasing lactation, PRL levels drop

causing the mammary gland to undergo extensive restructuring and apoptosis leading to involution and a return to the primary ductal structure.[180]

1.3.9 Prolactin as a Risk Factor for Breast Cancer: Epidemiologic Studies

There have been several case/control studies[181-186] examining the breast cancer – prolactin association with inconsistent findings (**Table 2**). Small studies of postmenopausal women, ranging in size from 12 to 48 cases, have reported a significant positive association[184,186] or a significant negative association.[183] Six small studies of pre-menopausal women, ranging in size from 6 to 66 cases, have reported positive[183,184,187] and no association.[186,188,189] Three studies in which menopausal status was not evaluated reported no significant associations.[181,182,185] Small sample size and the use of post-diagnostic PRL levels, which may be affected by the presence of a tumor, likely explain these contradictory findings.

Several small (21-71 cases) prospective studies (**Table 3**) have failed to find any positive association between PRL levels and subsequent breast cancer.[144,190-192] Similarly, two small prospective studies[190,191] failed to find a significant association with post-menopausal disease, although in both studies, the relative risk was elevated, suggesting a positive association. The Nurses Health Study[193] has reported a significant positive association for post-menopausal disease (RR 2.03, 95% CI 1.24-3.31, highest versus lowest quartile). The association was stronger when cases were limited to invasive disease (RR 2.64, 95% CI 1.54-4.51), and it was not altered when adjusted for circulating estrogens, androgens and IGFs. Using some of the same cases and controls from the Nurses Health Study, Tworoger et al.[194] examined the association between plasma prolactin concentrations and breast cancer risk. A significant positive association was also found in this study of postmenopausal women (RR=1.34, 95% CI

1.02-1.76, highest versus lowest quartile of prolactin concentrations). The association was stronger when limited to invasive breast cancer (RR=1.41, 95% CI 1.08-1.86), for tumors >2 cm (RR=1.66, 95% CI 1.04-2.64), and for ER+/PR+ tumors (RR=1.78, 95% CI 1.28-2.50).[194] The estrogen receptor (ER) and PRLR appear to be co-expressed (through an unknown mechanism),[195] but previous studies have not observed any association between the two receptors.[26,183,196] This positive relationship between PRL and post-menopausal breast cancer is similar in magnitude to that recently observed in a pooled analysis of nine prospective studies for estrogen and breast cancer (RR 2.0, 95% CI 1.47-2.71, highest versus lowest quartile).[26] These findings need to be validated in other populations.

Tworoger et al.[197] in a follow-up study of the Nurses' Health Study II, observed a positive association between prolactin and breast cancer risk (RR 1.5, 95% CI 1.0-2.5, highest versus lowest quartile) among premenopausal women. The observed association was stronger when the sample was restricted to women diagnosed with ER+/PR+ breast cancer (RR=1.9, 95% CI 1.0-3.7). The estrogen receptor (ER) and PRLR appear to be co-expressed (through an unknown mechanism),[195] but previous studies have not observed any association between the two receptors.[26,183,196] These initial data suggest that prolactin may be involved in the development of ER+ tumors, although further studies are needed to confirm this observation.

Table 2: Case-Control Studies of Prolactin and Breast Cancer Risk

Study	Description	Blood Collection	Menopausal Status	Cases/Controls	Comparison	Odds Ratio (95% CI)
Wilson et al. 1974 [181]	Hospital-based	Collected in the late afternoon	Not Evaluated	14 primary breast cancer 18 localized advanced BC 13 metastatic breast cancer 39 controls	Mean prolactin level (mamp/ml)	10.86 ± 2.20 8.44 ± 0.99 7.69 ± 1.64 8.31 ± 1.16
Sheth et al. 1975 [182]	Tata Medical Hospital	Collected at intervals of 48 hours during the 7th to 23rd day of menstrual cycle	Premenopausal; Postmenopausal	Breast cancer cases 42 – ages 31-40 30 – ages 42-50 26 – ages 51-60 12 Cystic mastitis 10 gynaecomastia Premenopausal 10 – follicular phase 8 – luteal phase 12 Postmenopausal	Mean serum prolactin level (ng/ml)	 22 ± 3 19 ± 2 17 ± 2 14 ± 2 16 ± 2 21 ± 4 43 ± 5 20 ± 3
Malarkey, et al. 1977 [183]		Hourly blood collection	Premenopausal; Postmenopausal	Pre follicular phase 1 breast cancer case 5 benign breast disease 7 controls Pre luteal phase 5 breast cancer cases 7 benign breast disease 9 controls Postmenopausal women 12 breast cancer cases 4 benign breast disease 9 controls Matched on age and weight	Mean nocturnal (12pm-7am) serum PRL levels (ng/ml)	**Pre follicular phase** 19.8 ± 1.9 19.8 ± 3.5 15.9 ± 1.7 **Pre luteal phase** 23.5 ± 2.6 22.5 ± 2.8 19.1 ± 0.9 **Postmenopausal** 9.3 ± 0.9 11.4 ± 2.1 13.4 ± 1.3

Table 2 (Continued)

Study	Description	Blood Collection	Menopausal Status	Cases/Controls	Comparison	Odds Ratio (95% CI)
Rose et al. 1981 [184]		Collected between 7-9am with subjects in a resting state	Premenopausal; Postmenopausal	Premenopausal 14 premastectomy BC cases 21 postmastectomy BC cases 9 advanced BC cases 34 controls Postmenopausal 20 premastectomy BC cases 17 postmastectomy BC cases 29 advanced BC cases 39 controls Overall 18 premastectomy BC cases 20 postmastectomy BC cases 23 advanced BC cases 18 controls	Plasma prolactin levels (ng/ml)	**Premenopausal** 22.9 ± 32.4 (p<0.001) 32.6 ± 25.3 (p<0.001) 11.4 ± 2.8 9.1 ± 3.6 **Postmenopausal** 13.5 ± 15.9 (p<0.001) 27.2 ± 31.2 (p<0.001) 15.6 ± 15.6 (p<0.001) 4.9 ± 2.2 **Overall** 21.9 ± 31.4 (p<0.001) 30.2 ± 27.8 (p<0.001) 16.5 ± 15.2 (p<0.001) 6.8 ± 3.6
Meyer et al. 1986 [187]	Caucasian; 1978-1981; 8 institutions in Boston SMSA	Luteal phase blood collection	Premenopausal	<140 mU/liter – Reference 6 breast cancer cases 25 controls 140-240 mU/liter 11 beast cancer cases 53 controls >240 mU/liter 19 breast cancer cases 25 controls >500 mU/liter (above upper limit of normal range) 6 breast cancer cases 2 controls	Plasma levels	<140 mU/liter RR = 1.0 140-240 mU/liter RR = 0.8 (95% CI 0.2-2.5) >240 mU/liter RR = 2.7 (95% CI 0.9-8.5) >500 mU/liter RR = 11.9 (95% CI 1.6-90.1)

Table 2 (Continued)

Study	Description	Blood Collection	Menopausal Status	Cases/Controls	Comparison	Odds Ratio (95% CI)
Anderson et al 1989 [185]	Family History Breast Cancer Clinic	Collected between 10am and 3pm	Premenopausal; Postmenopausal	Familial breast cancer 67 cases 55 controls Premenopausal familial 39 cases 43 controls Matched breast cancer cases and controls 20 cases 20 controls Matched by age, parity, weight, menopausal status	Median PRL levels (ng/ml)	**Familial breast cancer** Cases = 7.8 Controls = 7.9 **Premenopausal familial** Cases = 8.1 Controls = 7.8 **Matched sample** Cases = 9.9 Controls = 6.9 All associations were statistically non-significant
Ingram et al. 1990 [186]	Western Australia; 1985 to 1987	Fasting blood sample collected between 8am and 12 noon; collected on day 21 or 22 of menstrual cycle for premenopausal women	Premenopausal; Postmenopausal	Benign fibrocystic dx 86 cases 102 controls Benign epithelial hyperplasia of the breast 82 cases 101 controls Breast cancer 78 cases 122 controls Matched by age & residence	Mean prolactin levels	**Benign fibrocystic dx** OR=1.03 (95% CI 0.59-1.80) **Benign epithelial hyperplasia of the breast** OR =1.70 (95% CI 0.89-3.27) **Breast cancer** OR=2.12 (95% CI 1.00-4.51) **Premenopausal BC** RR=1.56 (95% CI 0.56-4.29) **Postmenopausal BC** RR=3.57 (95% CI 1.16-11.02)
Love, et al. 1991 [188]	Caucasian; from upper Midwest US	Between 8-9am on day 22±2 of their cycle; 1-2 hours after awakening	premenopausal	18 cases w/ mastectomy (BC); 23 controls with a strong family hx of breast cancer (FH); 39 controls with no family hx (Controls)	Mean serum PRL RIA levels (log transformed levels)	**Parous women** (p=0.94) BC mean = 2.18 FH mean = 2.24 Controls mean = 2.24 **Nulliparous women:** (p=0.41) BC mean = 2.59 FH mean = 2.89 Controls mean = 2.61

Table 3: Prospective Studies of Prolactin and Breast Cancer Risk

Study	Description	Blood Collection	Menopausal Status	Cases/Controls	Comparison	Relative Risk (95% CI)
Wang et al 1992 [190]	Island of Guernsey; 1968 to 1976	Collected at time of enrollment	Premenopausal; Postmenopausal	Premenopausal women I – 14 cases; 9903 person-yrs II – 11 cases; 10314 person-yrs III – 10 cases; 10062 person-yrs IV – 19 cases; 9764 person-yrs V – 17 cases; 9898 person-yrs Postmenopausal women I – 6 cases; 4526 person-yrs II – 6 cases; 4521 person-yrs III – 11 cases; 4510 person-yrs IV – 8 cases; 4412 person-yrs V – 9 cases; 4391 person-yrs	Serum prolactin levels (ng/ml)	Quintiles of Prolactin **Premenopausal women** I – RR=1.00 II – RR=0.70 (95% CI 0.31-1.56) III – RR=0.67 (95% CI 0.29-1.53) IV – RR=1.25 (95% CI 0.62-2.55) V – RR=1.07 (95%CI 0.51-2.23) **Postmenopausal women** I – RR=1.00 II – RR=1.05 (95% CI 0.33-3.34) III – RR=1.83 (95% CI 0.66-5.06) IV – RR=1.32 (95% CI 0.45-3.92) V – RR=1.63 (95% CI 0.57-4.71) Adjusted for age, parity, height, and hx of benign dx
Helzlsouer et al. 1994 [192]	Follow-up Washington County Cohort; 1974 to 1991		Premenopausal	21 incident breast cancer cases not on oral contraceptives or HRT 42 controls	Serum prolactin levels (ng/ml)	Matched OR by tertiles Low OR=1.0 Med OR=0.6 (95% CI 0.1-2.5) High OR=1.1 (95% CI 0.3-4.1) Matched for age, parity, height, benign breast dx

Table 3 (Continued)

Study	Description	Blood Collection	Menopausal Status	Cases/Controls	Comparison	Relative Risk (95% CI)
Hankinson et al. 1999 [193]	Follow-up of the Nurses' Health Study; 1989 to 1994	Collected at time of enrollment	Postmenopausal	All women 306 breast cancer cases 448 controls	Quartiles of plasma prolactin level (ng/ml)	**All women:** I – RR=1.0 II – RR=1.05 (95% CI 0.65-1.71) III – RR=1.45 (95% CI 0.91-2.31) IV – RR=2.03 (95% CI 1.24-3.31) **Invasive breast cancer:** I – RR=1.0 II – RR=1.26 (95% CI 0.75-2.13) III – RR=1.61 (95% CI 0.98-2.64) IV – RR=2.64 (95% CI 1.54-4.51) **Excluding first 2 yrs of follow-up:** I – RR=1.0 II – RR=0.69 (95% CI 0.37-1.32) III – RR=1.34 (95% CI 0.72-2.51) IV – RR=2.39 (95% CI 1.24-4.61) Adjusted for BMI at age 18 yrs, family history of breast cancer, age at menarche, age at first birth/parity, age at menopause, duration of postmenopausal hormone use, month & time of blood draw, fasting
Kabuto et al 2000 [191]	Life Span Study Pop of the Radiation Effects Research Foundation in Hiroshima & Nagasaki, Japan; follow-up from 1970 to 1983	Collected between 1968 and 1970	Premenopausal; Postmenopausal	Premenopausal women 46 cases 94 controls Postmenopausal women 26 cases 56 controls	Unit increase in log10 of serum prolactin levels	**All** OR=1.76 (95% CI 0.02-43.9) **Premenopausal** OR=1.01 (95% CI 0.02-47.4) **Postmenopausal** OR=6.45 (95% CI 0.01-43.9) Matched on age, date of blood collection, exposure, radiation dose, and city

Table 3 (Continued)

Study	Description	Blood Collection	Menopausal Status	Cases/Controls	Comparison	Relative Risk (95% CI)
Tworoger et al. 2004[194]	Nurses' Health Study; 1989-2000	Collected between 1989 and 1990	Postmenopausal	**All postmenopausal women:** 851 cases; 1,275 controls **Invasive breast cancer:** 722 cases **Ductal breast cancer:** 588 cases **Lobular breast cancer:** 93 cases **Tumor size ≤2 cm:** 531 cases **Tumor size >2 cm:** 162 cases **ER+/PR+ breast cancer:** 397 cases **ER-/PR- breast cancer:** 96 cases **ER+/PR- breast cancer:** 91 cases Matched on age, postmenopausal hormone use, fasting status & time of day and month of blood collection	Quartiles of plasma prolactin level (ng/ml)	**RR comparing quartile IV to I** **All postmenopausal women:** RR=1.34 (95% CI 1.02-1.76) **Invasive breast cancer:** RR=1.41 (95% CI 1.08-1.86) **Ductal breast cancer:** RR=1.38 (95% CI 1.04-1.85) **Lobular breast cancer:** RR=1.76 (95% CI 0.95-3.26) **Tumor size ≤2 cm:** RR=1.35 (95% CI 1.00-1.83) **Tumor size >2 cm:** RR=1.66 (95% CI 1.04-2.64) **ER+/PR+ breast cancer:** RR=1.78 (95% CI 1.28-2.50) **ER-/PR- breast cancer:** RR=0.76 (95% CI 0.43-1.32) **ER+/PR- breast cancer:** RR=1.94 (95% CI 0.99-3.78) Adjusted for BMI at age 18 yrs, weight change from age 18 to blood draw, family history of breast cancer, age at menarche, age at first birth/parity, age at menopause & matching factors

Table 3 (Continued)

Study	Description	Blood Collection	Menopausal Status	Cases/Controls	Comparison	Relative Risk (95% CI)
Tworoger et al. 2006[197]	Nurses' Health Study II; 1996 to 1999	Collected between 1996 and 1999	Premenopausal	**All premenopausal women:** All – 235 cases; 470 controls Invasive – 159 cases; 318 controls ER+/PR+ – 109 cases; 218 controls T1 – 86 cases; 172 controls T2 – 79 cases; 158 controls T3 – 70 cases; 140 controls **Follicular phase prolactin:** All – 193 cases; 386 controls Invasive – 130 cases; 260 controls ER+/PR+ – 89 cases; 178 controls T1 – 63 cases; 126 controls T2 – 64 cases; 128 controls T3 – 66 cases; 132 controls **Luteal phase prolactin:** All – 197 cases; 394 controls Invasive – 131 cases; 262 controls ER+/PR+ – 89 cases; 178 controls T1 – 63 cases; 126 controls T2 – 66 cases; 132 controls T3 – 68 cases; 136 controls Matched on age, fasting status, time of day & month of blood collection, race/ethnicity, and timing of blood draw within the menstrual cycle	Quartiles of plasma prolactin level (ng/ml) & time between blood draw and breast cancer diagnosis T1 - <2 yrs; T2 – 2 yrs to <3.875 yrs; T3 – ≥3.875 yrs	**RR comparing quartile IV to I** **All premenopausal women:** All cases – RR=1.5 (95% CI 1.0-2.5) Invasive – RR=1.6 (95% CI 0.9-2.7) ER+/PR+ – RR=1.9 (95% CI 1.0-3.7) T1 - RR=1.6 (95% CI 0.8-3.0) T2 – RR=1.8 (95% CI 0.9-3.5) T3 – RR=1.1 (95% CI 0.5-2.3) **Follicular phase prolactin:** All cases – RR=1.3 (95% CI 0.8-2.1) Invasive – RR=1.3 (95% CI 0.7-2.3) ER+/PR+ – RR= 1.5 (95% CI 0.7-3.1) T1 - RR=1.5 (95% CI 0.7-3.3) T2 – RR=1.5 (95% CI 0.7-3.4) T3 – RR=0.8 (95% CI 0.3-1.9) **Luteal phase prolactin:** All cases – RR=1.0 (95% CI 0.6-1.7) Invasive – RR=0.9 (95% CI 0.5-1.7) ER+/PR+ – RR= 0.8 (95% CI 0.4-1.6) T1 - RR=1.7 (95% CI 0.8-3.7) T2 – RR=1.1 (95% CI 0.5-2.4) T3 – RR= 0.6 (95% CI 0.2-1.2) Adjusted for BMI at age 18 yrs, weight change from age 18 to blood draw, family history of breast cancer, age at menarche & matching factors

1.4 Summary of Background

Breast density, a reflection of the histologic composition of the breast, is one factor that affects mammographic sensitivity and is predictive of breast cancer risk. Factors that increase breast density may compromise the reduction in mortality gained by mammographic screening. Understanding these factors may help us improve mammographic screening and reduce breast cancer risk. Prolactin, an endogenous hormone that acts as a mitogen and differentiating agent in the breast, may be one such factor. The majority of both case-controls and prospective studies examining the association between prolactin and breast cancer risk have yielded inconsistent results. However, two large prospective study reported a significant positive association between prolactin and post-menopausal breast cancer[193,194] similar in magnitude to that observed for estrogen.[26] Prolactin and estrogen act synergistically to exert their mitogenic effects on the normal breast. Whereas increased levels of estrogen (for example from HT use) have been linked to increased breast density, whether increases in prolactin levels also increase breast density remains unknown.

2.0 Methods

2.1 Specific Aims

The goal of this study was to correlate serum prolactin levels and breast density in postmenopausal women using a cross-sectional study design. In addition, genetic variability in both the prolactin gene and prolactin gene receptor were examined to understand how these genes may influence mammographic density. To date, no study has examined the association of genetic polymorphisms in the prolactin gene nor the prolactin receptor gene and breast density among healthy postmenopausal women. The specific aims of this proposal were:

Specific Aim 1: to examine the correlation of serum prolactin levels to breast density.

> **Hypothesis 1.1**: among postmenopausal women, higher levels of prolactin are associated with higher breast density.

Specific Aim 2: to determine the association between single nucleotide polymorphisms (SNPs) in both the prolactin gene and prolactin gene receptor with serum levels of prolactin and breast density in postmenopausal women.

> **Hypotheses 2.1**: postmenopausal women with polymorphisms in the prolactin gene that lower the level of prolactin will lower breast density; and conversely women with polymorphisms in the prolactin gene that increase the level of prolactin will have higher breast density.

2.2 Parent Study Overview

This is an ancillary study to The Mammograms and Masses Study (MAMS), a case-control study of estrogen metabolites, mammographic density, and breast cancer risk. 869 cancer-free women and 264 recently diagnosed breast cancer cases were recruited into MAMS through the Magee Women's Hospital Mammographic Screening and Diagnostic Imaging Program in the greater Pittsburgh, PA area between September 2001 and May 2005. Women who were 18 years of age or older, could provide written informed consent and reported no previous personal history of cancer, with the exception of non-melanoma skin cancer, were eligible for study enrollment. Participants in the MAMS can be classified into three separate groups: (1) recruited from the Magee-Women's Surgical Clinic for an initial evaluation after newly diagnosed primary breast cancer; (2) women who were undergoing outpatient needle breast biopsy through the Breast Biopsy Service, but who were not subsequently diagnosed with breast cancer (control group 1 – benign breast masses); and (3) women receiving screening mammography through Magee-Women's Hospital or through Pittsburgh Magee-Womancare Centers (control group 2 – negative mammograms). In order to increase recruitment of control group 2, study flyers were attached to screened negative mammogram reports mailed to Magee-Womancare Center patients between November 2003 and April 2005.

2.3 Ancillary Study Population

Participants were selected for this ancillary study if they met the following eligibility criteria: (1) screen negative women (control groups 1 and 2) recruited via study flyers through Magee-Women's Hospital or through Pittsburgh Magee-Womancare Centers; (2) 40 years of age or older; (3) postmenopausal defined according to the methods described by the Women's Health

Initiative (CITE)[198], where age at menopause corresponds to the age at which the participant last had any natural menstrual bleeding, had a bilateral oophorectomy, or began using HT. For a hysterctomized woman without a bilateral oophorectomy, age at menopause was the earliest age at which she began using HT or first had menopausal symptoms. If neither occurred and her age at hysterectomy was 50 years or older, then age at menopause as her age at hysterectomy; (4) cancer-free; and (5) had a mammogram within three months of blood draw.

A total of 1,133 women enrolled in the MAMS Study between September 2001 and May 2005. Out of these, 264 were women with newly diagnosed breast cancer (case groups), and thus excluded from the ancillary study population. From the remaining 869 women (control group), 222 women were premenopausal and thus excluded from the ancillary study population. From the remaining 647 postmenopausal women, 202 women were excluded due a variety of reasons, including: 28 non-Caucasian women, 40 women without a blood sample, 47 women without a mammogram, 2 women with a previous breast cancer diagnosis, and 85 women had missing information on key study variables. Due to the above mentioned exclusions, the resulting sample size for this ancillary study consisted of 445 white, cancer-free, healthy, postmenopausal women – 104 control group 1 women (benign breast masses) and 341 control group 2 women (negative mammograms).

2.4 Protection of Human Subjects

The MAMS was approved by the Magee-Women's Hospital and the University of Pittsburgh's Institutional Review Boards. All participants provided written informed consent for participation and permission to release medical records and mammograms at the time of study entry. All participants were provided copies of the signed study consent forms. No study procedures were

performed until after written informed consent was given. All data was stored and coded to ensure confidentiality. The study coordinator examined all study forms for completeness before data entry and removed all identifiers, i.e. name, address, before data entry. Each study participant was assigned a study identification number, which was used to label all study documents, copies of mammograms, and biological specimens. Identifying information was stored separately from the data with the participants study identification number. All hard copies were kept in locket file cabinets in a locked office. Information linking the study identifier with personal identifiable information was kept under strict security, and only authorized study personnel had access to this information. Linking the study ID number to personal identification will occur only when strictly necessary. Participants' records and biological specimens will be stored for at least five years subsequent to the completion of the study per University of Pittsburgh policy. If a participant withdraws from the study, all study related forms, including biological specimens, will be destroyed.

2.5 Questionnaire Data Collection

A standardized self-administered questionnaire was used to obtain exposure information at the time of enrollment. Data collected include personal demographic factors (age, race/ethnicity, education); medical history; lifestyle factors (exercise, smoking, and alcohol intake); reproductive history (age of menarche, parity, age at first full-term birth, duration of breastfeeding, age at last natural menstrual bleeding, surgical versus natural menopause, history of benign breast disease, and history of breast biopsy); family history of cancer in first-degree relatives (mother(s), father(s), sister(s), brother(s)); past HT use and years since HT use. Participants were asked to list the type of compounds and dosages for each agent they regularly

used. During the baseline clinic visit, the questionnaire was returned to the study coordinator, who reviewed it for completion and removed all personal identification information.

2.6 Anthropometric Measurements

The study coordinator obtained anthropometric measurements at the time of study enrollment and recorded the information on a standardized form. After the participant removed her shoes and heavy clothing, weight was measured at a standing position to the nearest 0.1 kilogram (kg) using a standard balance beam. Standing height was measured at full inspiration to the nearest 0.1 centimeter (cm). All anthropometric measurements were taken twice and were repeated if the first two measurements differed by more than 0.5 kg or 0.5 cm. The mean of the measurements will be used for all analysis. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared.

2.7 Mammographic Density Measurements

Mammographic density and parenchymal patterns were determined from mammographic film copies and recorded on a standardized form. The readings were performed by Ms. Martine Salane, an expert reader trained by Dr. Wolfe in planimetry. The reader was blinded to the identity and other personal characteristics of the study participants. Mammographic measurements were made using a randomly selected craniocaudal view (CC) of one breast from each subject. To calculate percent breast density, areas of radiographically dense tissue (excluding isolated calcifications, Cooper's ligaments, biopsy scars, and breast masses) were outlined with a china marker. Total breast area and outlined regions were measured using a

compensating polar planimeter (LASICO, Los Angeles, CA). Percent breast density was calculated by dividing the outlined regions of density by the total breast area. Nondense breast area was determined by dividing the dense breast area by the total area of the breast. All measurements are taken twice to ensure accuracy. The reader also recorded the quality of the image (poor ++, poor +, poor, fair, good, excellent).

In order to assess the reliability of Ms. Salane's readings, twenty-eight randomly selected mammograms (eight from the lowest tertile of percent breast density, and ten each from remaining two tertiles of percent breast density) were sent for re-review. The intraclass correlation coefficient (ICC) was calculated from an F value that was derived by dividing the mean square error terms for the between-patient variance by that of the within-patient variance. The intraclass correlation coefficient (ICC)for intra-observer agreement was ρ=0.86 for area of density, ρ=0.99 for total area of the breast, and ρ=0.89 for percent breast density. Our ICC for percent density is consistent with reports from computer-assisted measurements in the Canadian National Breast Screening Study (ICC ρ=0.897 for 150 sets of films),[61] the Nurses' Health Study (ICC ρ=0.93),[199] and for Ms. Salane's reproducibility in the Breast Cancer Detection Demonstration Project (BCDDP) (ICC ρ=0.915 for 193 sets of films).[64] Ms. Salane's validity was evaluated against computer-assisted density measurements showing excellent correlation (ρ=0.90).[62]

2.8 Biological Specimen Collection

At the time of study enrollment, the study coordinator drew 40mL of peripheral non-fasting blood from each participant using standardized phlebotomy procedures. Before blood draw, information on current medication use (example, aspirin) and time from last meal was recorded

for all participants on a standardized form. The 40mL of blood draw consisted of 20mL collected without anticoagulant (2 red top tubes), which provides serum as well as clot, and 20mL collected into EDTA (purple top tube), which provides plasma and buffy coat. All samples were processed on site at the Magee Women's Hospital Satellite Clinical Research Center (MWH-S-CRC) according to well-established protocols. After processing, the samples were aliquotted into 1mL cryovials in which red blood cell, serum, plasma, and buffy coat were separated. Samples are stored in –70°C freezers in the Epidemiology Department at the University of Pittsburgh.

2.9 Prolactin Measurements

Serum prolactin levels were assayed at the laboratory of Dr. Patrick Sluss in the Reproductive Endocrine Unit Reference Laboratory at Massachusetts General Hospital. This is the same laboratory that is currently used by the Nurses' Health Study (NHS)[193,194,200] Prolactin was measured using a fully automated system [AxSYM, Abbott Diagnostics]. The method is a microparticle enzyme immunoassay. All reagents for this FDA approved *in vitro* diagnostic method are manufactured and quality controlled by the manufacturer. This method has no "hook" effect up to PRL levels of 10,000 ng/mL. The reportable range of the method is 1-200 ng/mL. Specimens containing higher than 200 ng/mL are diluted and retested. The assay's detection limit (sensitivity) is 0.6 ng/mL. Variability within assay duplicates for positive control sera are typically less than 5%; between assay variability is less than 8%. The reported intra-assay coefficient of variation was 7.6%.

Plasma samples (250 microliters each) were sent to Dr. Sluss' laboratory for analysis. Technicians were blinded to the source of each sample. Blinded, duplicate samples were sent for 5% of subjects to evaluate assay reproducibility. There was a high level of agreement between

the duplicate measures of prolactin. All samples were run in duplicate. Each set of duplicate samples were evaluated according to Dr. Sluss' protocol and the average concentrations for each set was reported (ng/mL). The coefficient of variation for duplicate samples must be less than 10% or the sample was re-assayed. Research personnel were blinded as to the breast density results in order to avoid potential bias in the conducting of the laboratory assays.

2.10 Single Nucleotide Polymorphisms (SNP) Selection

Population genetics research has shown that approximately 90% of the sequence variation among individuals is due to common variants.[201] In addition, most of those variants arose from single historical mutation events and are therefore associated with nearby variants that are present on the ancestral chromosome on which the mutation occurred. These two concepts are important for the discussion that follows.

Any two copies of the human genome vary from one another by approximately 0.1% of nucleotide sites or one variant per 1,000 bases on average.[202,203] The most common type of variant, observed at a frequency of ≥1%, is called a single nucleotide polymorphism (SNP). It represents a difference between chromosomes in a base present at a particular site in the DNA sequence – one individual might have a G (the "G allele") at the site of interest, whereas another individual might have an A (the "A allele") at that same site. It is estimated that there are approximately 10 million sites (1 variant per 300 bases on average) with different alleles. These 10 million sites are believed to constitute 90% of the variation that is observed in the DNA sequence.[201] The other 10% of the variation can be attributed to a variety of different variants that are rare (occurring at a rate <1%).[201]

Each SNP is the result is a single historical mutational event. The mutation rate is very low, in the order of 10^{-8} per site per generation, when compared to the number of generations since the most recent common ancestor of any two humans (in the order of 10^4 generations). Because of this low mutation rate, each new allele is associated with other alleles that were present on the particular chromosomal background from which that new allele arose.[204] This combination of alleles that are observed on either a single chromosome or part of a chromosome is referred to as a haplotype. In addition to the above scenario, new haplotypes can also rise as a result of additional mutations or due to recombination. Recombination occurs when the maternal and paternal chromosomes exchange corresponding segments of DNA, thus resulting in an off-spring chromosome that is a mosaic of both parental haplotypes.[205]

In a haplotype, one observes the coinheritance of different SNP alleles. This coinheritance leads to associations between the SNPs, which is a concept known as linkage disequilibrium (LD). Because the likelihood of recombination between two alleles increases with the distance between them, the associations between alleles decreases with distance. Research has indicated that in the human genome there exists highly significant levels of LD, often with strong associations to nearby SNPs.[206-208] What this means is that in many chromosomal regions, there are only a few haplotypes which can account for most of the variation across individuals.

In order to account for all of the individual variation within either a chromosome or part of a chromosome, one would need to genotype all of the SNPs within that region. This can not only be very labor intensive, but expensive as well. By utilizing the concept of LD one is able to genotype a few, carefully chosen SNPs in the region of interest. These carefully chosen SNPs – termed "tagging or tag SNPs" can provide enough information to predict much of the information about the remainder of the SNPs found in the region of interest.[208,209] It is estimated

that approximately 200,000 to 1,000,000 tagging SNPs can account for most of the genetic variation represented by the 10 million common SNPs in the human population.

The importance of using tagging SNPs in research is that it does not require the researcher to have prior knowledge of putative functional variants. By using this so-called "indirect approach," a set of sequence variants can serve as genetic markers to detect an association between a particular region and the disease in question, whether or not the markers themselves have any functional effect. Regions that show an association with the disease can then be further tested to find the causative variant(s).[204,208,209]

The above observations lead to the development of International HapMap Project in 2002. The aim of this project is "to determine the common patterns of DNA sequence variation in the human genome, by characterizing sequence variants, their frequencies, and correlations between them, in DNA samples from populations with ancestry from parts of Africa, Asia, and Europe."[204] The International HapMap Project will genotype a total of 270 DNA samples – 90 samples from a US Utah population with Northern and Western European ancestry (30 trios of two parents and an adult child); 90 samples from the Yoruba people in Ibadan, Nigeria (30 trios of two parents and an adult child; samples collected in 1980 by the Centre d'Etude du Polymorphisme Humain (CEPH)); 45 unrelated samples from Tokyo, Japan; and 45 unrelated samples from the Han Chinese in Beijing, China. All of the information collected will be available free of charge on the world wide web at http://www.hapmap.org.[204]

HapMap and its accompanying program, Haploview, were used to identify tagging SNPs for both the prolactin gene and the prolactin receptor gene.

2.10.1 Prolactin SNP Selection

Using the HapMap Data Rel 19/phase II Oct05, on NCBI 34 assembly, dbSNP b124 dataset, the code for prolactin (PRL) was inputted into the landmark or region section. The SNP genotype data for the CEU (US Utah population with Northern and Western European ancestry) population extending to 40 kbp was exported into Haploview. For PRL, a total of 16 SNPs account for the total variability within the PRL gene. Using a minor allele frequency (MAF) of 10%, the Tagger program was run using the 2-3-marker haplotype selection feature. The 2-3-marker haplotype selection allows the Tagger algorithm to test all possible two- and three-way SNP combinations, to see if they give you additional information that each of the individual SNPs by themselves. The program selected seven tagging SNPs – rs1205955, rs2744119, rs1205961, rs12210179, rs849877, rs1341239, and rs6456483. These seven tagging SNPs result in the formation of one LD block (**Figure 5 and Figure 6**), capture 100% of alleles with an r^2 >0.8 and capture 7 of 7 alleles (SNPs) with a mean r^2 of 1.0.

2.10.2 Prolactin Receptor SNP Selection

Using the HapMap Data Rel 19/phase II Oct05, on NCBI 34 assembly, dbSNP b124 dataset, the code for the prolactin receptor (PRLR) was inputted into the landmark or region section. The SNP genotype data for the CEU (US Utah population with Northern and Western European ancestry) population extending to 200 kbp was exported into Haploview. For the PRLR, a total of 86 SNPs account for the total variability within the PRLR gene. Using a minor allele frequency (MAF) of 10%, the Tagger program was run using the 2-3-marker haplotype selection feature. The program selected 15 tagging SNPs – rs1609500, rs4703505, rs401694, rs7734558, rs7727306, rs6866465, rs6897600, rs1587608, rs1587059, rs7720677, rs685193, rs1587607,

rs249522, rs4235652, rs7705216. These fifteen tagging SNPs result in the formation of six LD blocks (**Figure 7 and Figure 8**), capture 100% of alleles with an r^2 >0.8 and capture 31 of 31 alleles (SNPs) with a MAF ≥10% with a mean r^2 of 0.956.

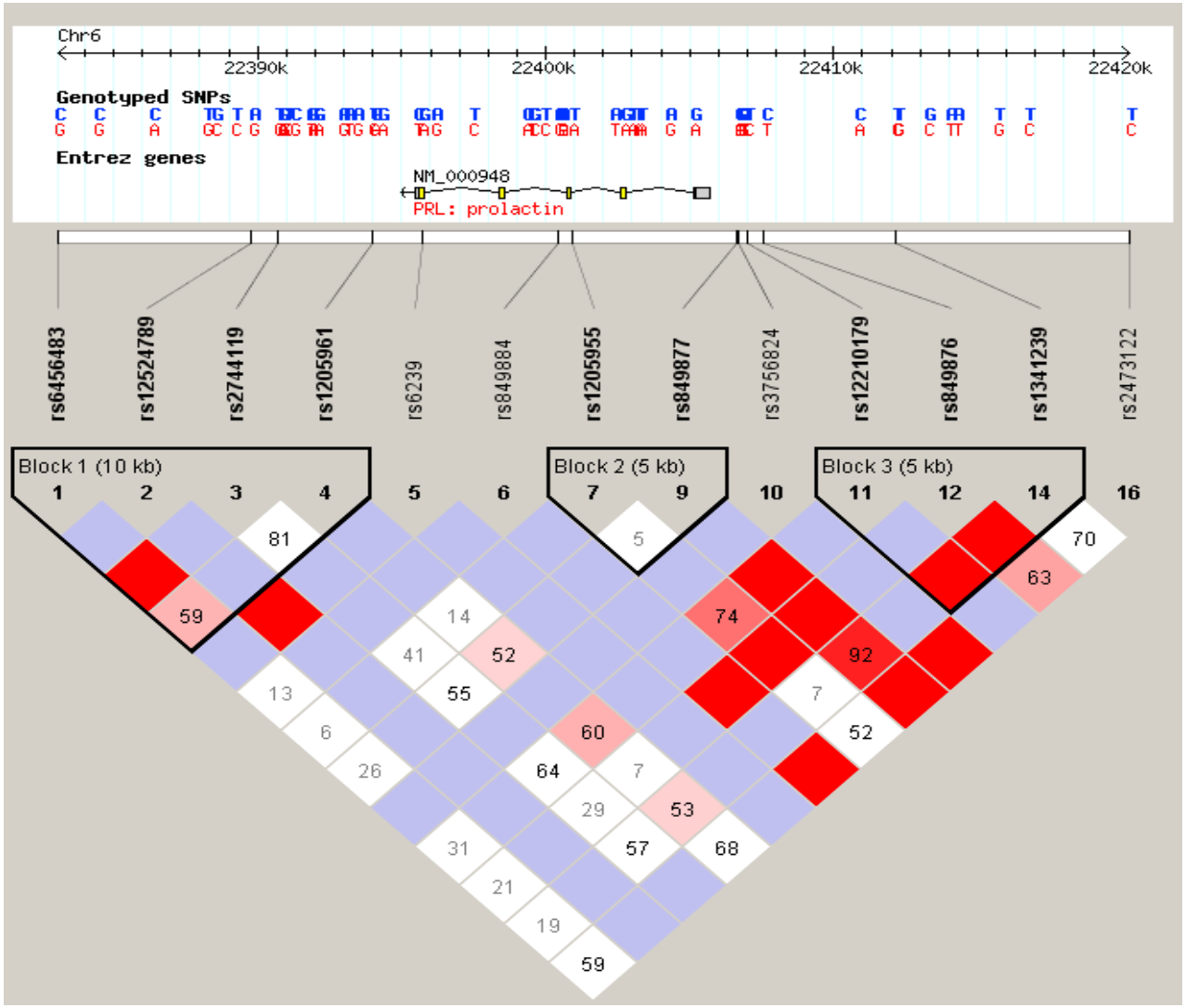


Figure 5: Linkage disequilibrium block structure of the prolactin gene

Notes: Linkage disequilibrium (LD) block structure of the prolactin (PRL) gene based on a minor allele frequency of 10%.

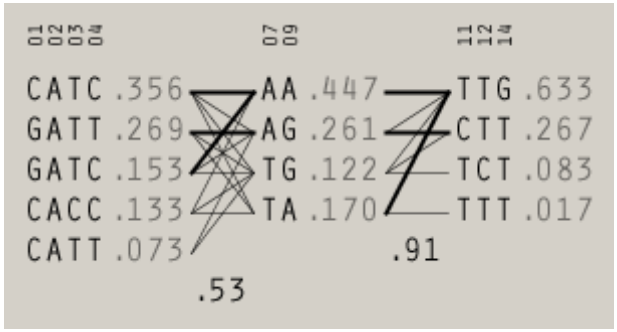


Figure 6: Haplotype of the prolactin gene based on a minor allele frequency of 10%.

Notes: The numbers on top of each block correspond to each individual SNP number:

#1 – rs6456483	#9 – rs849877
#3 – rs2744119	#11 – rs12210179
#4 – rs1205961	#14 – rs1341239
#7 – rs1205955	

The pattern of, for example, CATC, represents a haplotype. As can be seen in block 1, approximately 63% of genotypes will have either the CATC or GATT haplotype – here the difference being which allele is present in SNP #1 and #4. The numbers between each block can be thought of as a correlation between blocks. For example, block 2 captures 53% of the allelic variability in block 1.

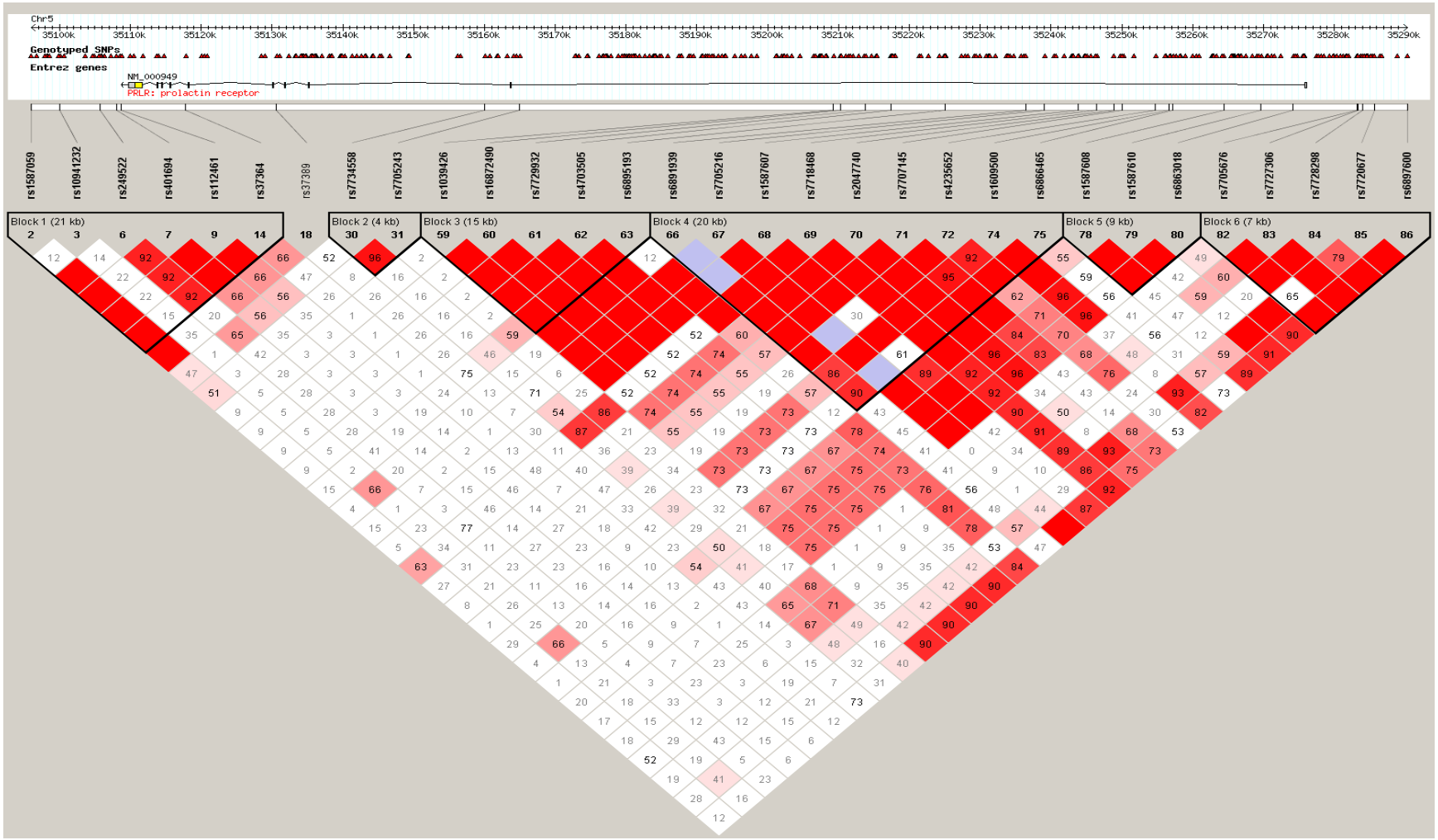


Figure 7: Linkage disequilibrium block structure of the prolactin receptor gene

Notes: Linkage disequilibrium (LD) block structure of the prolactin receptor (PRLR) gene based on a minor allele frequency of 10%.

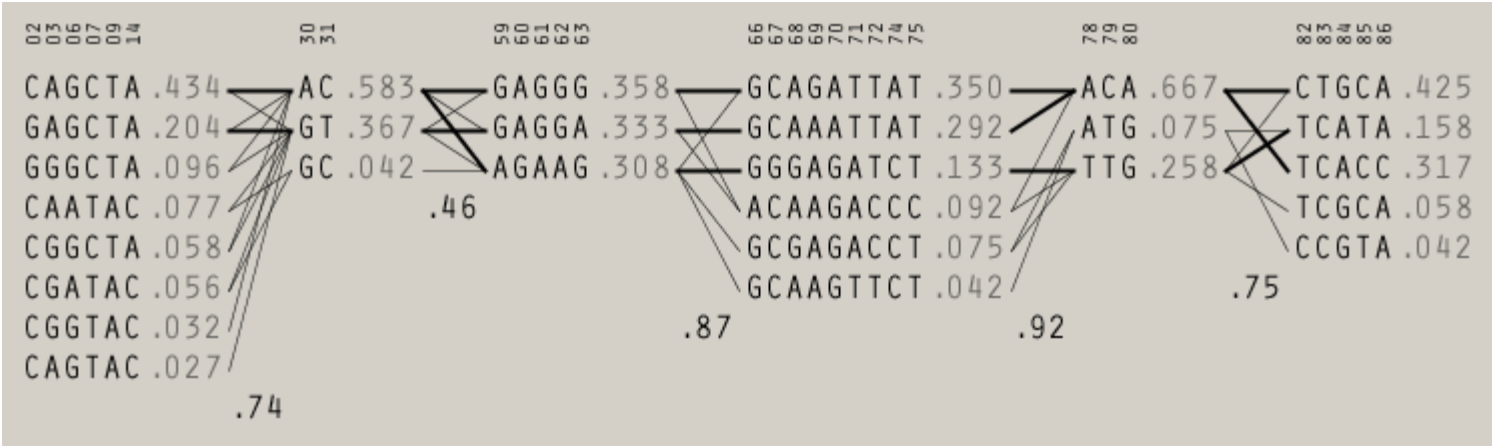


Figure 8: Haplotype of the prolactin receptor (PRLR) gene based on a minor allele frequency of 10%.

Notes: The numbers on top of each block correspond to each individual SNP number:

#2 - rs1587059	#63 – rs4235652
#6 – rs249522	#65 – rs1609500 (not shown)
#7 – rs401694	#66 – rs6866465
#30 – rs7734558	#78 – rs1587608
#53 – rs4703505 (not shown)	#83 – rs7727306
#54 – rs6895193 (not shown)	#85 – rs7720677
#58 – rs7705216 (not shown)	#86 – rs6897600
#59 – rs1587607	

The pattern of, for example, CAGCTA, represents a haplotype. As can be seen in block 1, approximately 63% of genotypes will have either the CAGCTA or the GAGCTA haplotype – here the difference being which allele is present in SNP #2. The numbers between each block can be thought of as a correlation between blocks. For example, block 2 captures 74% of the allelic variability in block 1.

2.11 SNP Genotyping

The SNPs were genotyped in the Molecular Genetics Laboratory in the Department of Human Genetics at the Graduate School of Public Health, University of Pittsburgh ran by Dr. Robert Ferrell. High molecular weight DNA was isolated from banked buffy coat specimens using a commercial protocol (PureGene DNA Isolation Kit, Gentra Systems, Inc. Minneapolis, MN). SNPs were genotyped by the 5′-nuclease (TaqMan) assay[210] using the ABI 7900HT sequence detector. Assay development used the Assay-by-Design and Assay-on-Demand services of Applied Biosystems, Inc., and assays were carried out in a 384 well format. Each genotyping run included multiple controls of known genotype, established by direct sequencing, and no template controls. Genotype assignments were reviewed by Dr. Ferrell, and any disagreements resolved by consensus or retyping. Samples were only labeled with an ID number and study investigators were blind to serum prolactin levels and breast density results to avoid potential bias in the conducting of the laboratory assays.

2.12 Data Quality Management

To prevent data errors a variety of procedures were implemented. All anthropometric measurements were taken twice and standardized data collection instruments were used. Completed questionnaires were checked and edited by both the study coordinator and a research assistant before data entry. If any missing information was found, several attempts were made to contact the participant. In addition, double entry verification was used to enter all data and to ensure data reliability. The data manager routinely checked for ID validation, unusual codes and extreme values to further reduce the likelihood of data-entry errors. Each data file was thoroughly cleaned before being merged with the master data set.

2.13 Statistical Analysis

2.13.1 Specific Aim 1: Correlation of Prolactin with Breast Density

Because prolactin (PRL) levels were not normally distributed, non-parametric methods were used to test the correlation between breast density and serum PRL levels. Breast density and serum PRL levels were analyzed using Spearman's correlation. To test for possible confounders that have been shown to affect breast density, serum prolactin levels and breast density were regressed on each potential confounder. For each potential confounder, two linear regression models were run – the first regressed the potential confounder on prolactin levels, and the second regressed the potential confounder on breast density levels. Spearman's correlation was then utilized to examine the correlation between the residuals obtained from each of the two regression models. This same procedure was utilized to examine all potential confounders – age, weight, BMI, age at menarche, ever pregnant, age at first full-term pregnancy, number of live births, ever breast fed, number of children breast fed, age at menopause, surgical menopause, current hormone therapy user, history of breast cysts, family history of breast cancer, current smoker, drank alcohol in the past twelve months, and walks for exercise, individually. Those confounders whose residuals were significant (p-value 0.05) based on the results from the Spearman's correlation coefficient (ever pregnant and ever breast fed) were then combined and analyzed in the same manner as described above.

2.13.2 Specific Aim 2: Association of Prolactin and Prolactin Receptor Polymorphisms with Prolactin Levels and Breast Density

For each SNP, allele frequencies were analyzed and each genotypic distribution was tested for Hardy-Weinberg equilibrium using a chi-square goodness of fit statistic.

Using freely available software found online and specifically created for genetic analysis, such as R, the linkage disequilibrium of each SNP within each gene will be tested to determine the efficacy of haplotype-based analyses. If the results are positive, haplotype analyses will be conducted using existing techniques. Multivariable linear models for each SNP/haplotype with either breast density or serum PRL level as the outcome variable will be utilized, controlling for potential confounders. If either breast density or PRL levels are not normally distributed, they will be appropriately transformed. In order to deal with the problem of multiple corrections, permutation testing, in addition to other methods, may be utilized.

2.14 Power/Sample Size Calculations

All power calculations were done using PASS 6.0.

2.14.1 Specific Aim 1: Correlation of Prolactin with Breast Density

As can be seen from Table 4, in order to detect a significant correlation of 0.15 between serum prolactin levels and mammographic density, 462 subjects will be needed to obtain 90% power. If the observed correlation increases, the sample size would then decrease. Anticipating that up to 10% of the samples will be unsuable due to factors unrelated to either breast density or prolactin levels, increasing the sample size to 500 will be more than adequate to detect a significant correlation between PRL levels and breast density of 15% or higher.

Table 4: Sample Size and Power Calculations for Specific Aim 1.

Power	N	Alpha	Ho	Ha
0.90002	462	0.05	0.00	0.15
0.80048	346	0.05	0.00	0.15
0.95045	319	0.05	0.00	0.20
0.95040	138	0.05	0.00	0.30
0.95132	75	0.05	0.00	0.40
0.95351	46	0.05	0.00	0.50
0.95548	30	0.05	0.00	0.60

Ho: The is no correlation between PRL levels and breast density
Ha: There is a correlation between PRL levels and breast density

2.14.2 Specific Aim 2: Association of Prolactin and Prolactin Receptor Polymorphisms with Prolactin Levels and Breast Density

To determine the sample size for the prolactin gene SNP analysis, a Bonferroni correction for multiple comparisons will be utilized. For the PRL gene, seven SNPs will be analyzed, corresponding to a comparison-wise type I error of 0.007 (0.05/7). To detect an effect size of 0.20 with a type I error of 0.007 and 80% power, a sample size of 308 participants is required (**Table 5**).

Table 5: Sample Size and Power Calculations for Specific Aim 2 – PRL Gene.

N	Alpha	Ho	Ha
308	0.007	0.00	0.20
134	0.007	0.00	0.30
73	0.007	0.00	0.40

Ho: The is no correlation between PRL SNPs and PRL levels and breast density
Ha: There is a correlation between PRL SNPs and PRL levels and breast density

For the PRLR gene, 15 SNPs will be analyzed, corresponding to a comparison-wise type I error of 0.0033 (0.05/15). To detect an effect size of 0.20 with a type I error of 0.0033 and 80% power, a sample size of 351 participants is required (**Table 6**).

Table 6: Sample Size and Power Calculations for Specific Aim 2 – PRLR Gene.

N	Alpha	Ho	Ha
351	0.0033	0.00	0.20
152	0.0033	0.00	0.30
83	0.0033	0.00	0.40

Ho: The is no correlation between PRLR SNPs and PRL levels and breast density
Ha: There is a correlation between PRLR SNPs and PRL levels and breast density

3.0 Results

3.1 Descriptive Analysis

In the overall sample of white, postmenopausal, cancer-free healthy women (**Table 7**), we observed a mean serum prolactin level of 10.62 ± 6.89 ng/mL (standard deviation), a mean breast dense area of 43.53 ± 28.30 cm^2, and a mean percent breast density of 31.41 ± 19.87. The majority of women were ever pregnant (84.04%), with most giving birth to their first child in their 20s (44.13% between 20-24 years of age and 27.93% between 25-29 years of age), having at least 2 children (36.03% had 2 live births and 46.93% had 3 live births), and breast feeding at some point (41.94% of those who gave birth to a live child). The women in our sample experienced menopause before the age of 50 years (mean age at menopause 48.19 ± 5.18 years), 30% had a hysterectomy, and most (43.24%) were former hormone therapy users. Approximately 14% had a family history of breast cancer in first-degree relatives and 7% had a history of benign breast cysts.

Because our sample population consisted of two groups of women – control group 1 (benign breast masses) and control group 2 (negative mammograms) – comparison analysis were run on the descriptive variables. Women in control group 2 started their menses at a younger age (31.67% vs. 19.23% at 12 years of age), and were more likely to be former hormone therapy users (48.68% vs. 25.24%) when compared with control group 1 women. In contrast, group 1 women were more likely to be younger (mean age of 57.44 ± 7.41 years vs. 62.07 ± 8.27 years), more likely to be current smokers (32.65% vs. 11.11%), had a greater proportion of breast dense area (50.57 ± 31.57 cm^2 vs. 41.38 ± 26.91 cm^2), and had greater breast density (36.44 ± 19.86% vs. 29.87 ± 19.65%) when compared with group 1 women.

Table 7: Descriptive characteristics of white, postmenopausal, cancer-free healthy women in the Mammograms and Masses Study (MAMS)

Descriptive Variable	Overall		Group 1	Group 2	p-value*
	n = 445		n = 104	n=341	
Prolactin ± SD, ng/mL	10.62 ± 6.89		10.94 ± 5.87	10.52 ± 7.18	0.585
Proportion of dense breast area, cm^2	43.53 ± 28.30		50.57 ± 31.57	41.38 ± 26.91	**0.004**
Percent breast density, %	31.41 ± 19.87		36.44 ± 19.86	29.87 ± 19.65	**0.003**
Age ± SD, years	60.99 ± 8.30		57.44 ± 7.41	62.07 ± 8.27	**<0.001**
Weight ± SD, kg	73.70 ± 16.09		71.94 ± 15.30	74.23 ± 16.31	0.207
BMI ± SD, kg/m2	27.99 ± 5.89		27.36 ± 5.38	28.18 ± 6.03	0.218
Age at Menarche, %					
<12 years	17.30		16.35	17.60	**0.034**
12 years	28.76		19.23	31.67	
13 years	33.03		43.27	29.91	
≥14 years	20.90		21.25	20.82	
Ever Pregnant, %	84.04		86.54	83.28	0.428
Age at 1st full-term pregnancy, %					
<20 years	11.17		14.29	10.22	0.874
20-24 years	44.13		44.05	44.16	
25-29 years	27.93		26.19	28.47	
30-34 years	12.01		10.71	12.41	
≥35 years	4.75		4.76	4.74	
Number of live births§, %					
1	17.04		20.00	16.12	0.647
2	36.03		32.94	37.00	
≥3	46.93		47.06	46.89	
Ever breast fed§, %	41.94		35.58	43.94	0.132
Number of children breast fed‡, %					
1	40.66		48.65	38.62	0.513
2	30.22		24.32	31.72	
≥3	29.12		27.03	29.66	
Age at menopause ± SD, years	48.19 ± 5.18		47.26 ± 5.19	48.48 ± 5.15	**0.039**
Surgical menopause, %	30.56		36.54	28.74	0.131
Hormone therapy (HT), %					
Never HT user	32.88		16.50	37.83	**<0.001**
Former HT user	43.24		25.24	48.68	
Current HT user	23.87		58.25	13.49	
History of breast cysts, %	6.97		54.84	36.84	0.135
Family history of breast cancer, %	14.38		11.65	15.34	0.351
Current smoker, %	7.19		32.65	11.11	**<0.001**
Drank alcohol in past 12 months, %	25.39		66.67	60.96	0.527
Walks for exercise, %	60.22		56.44	61.88	0.326

§ Among parous women; ‡ Among parous women who breast fed

* Level of significance (p-value) determined from either the Chi-square test for categorical variables or the t-test for continuous variables

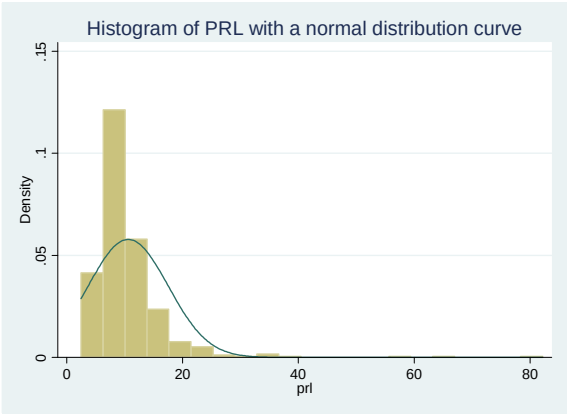
Abbreviations used: Group 1 – women with benign breast masses; Group 2 – women with negative mammograms; SD – standard deviation

Although a significant difference was observed for current smokers, there was no difference in mean prolactin levels between women in control group 1 and 2 (10.81 ± 6.19 ng/mL vs. 10.91 ± 7.66 ng/mL). Additionally, no differences in mean prolactin levels were observed for either former hormone therapy users (10.15 ± 5.97 ng/mL for control group 1 women vs. 10.87 ± 5.18 ng/mL for control group 2 women) or current (within the past three months prior to study enrollment and blood draw) hormone therapy users (10.00 ± 4.70 ng/mL for control group 1 women ng/mL vs. 11.34 ± 6.75 ng/mL for control group 2 women).

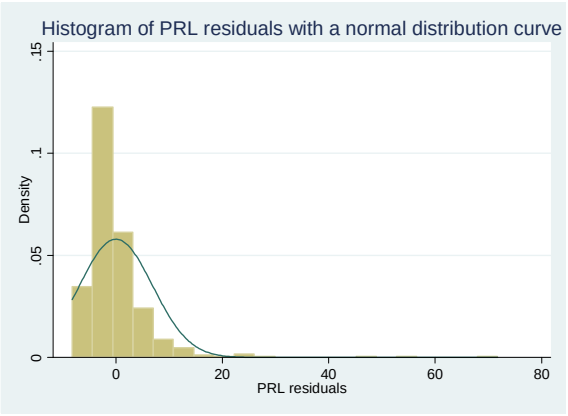
3.2 Analysis of Serum Prolactin Level Predictors

Neither serum prolactin levels nor prolactin residuals were normally distributed (**Figure 9**). After an analysis of various transformations, it was determined that the log transformation would be the most appropriate to bring prolactin values closer to normality (**Figure 9**).

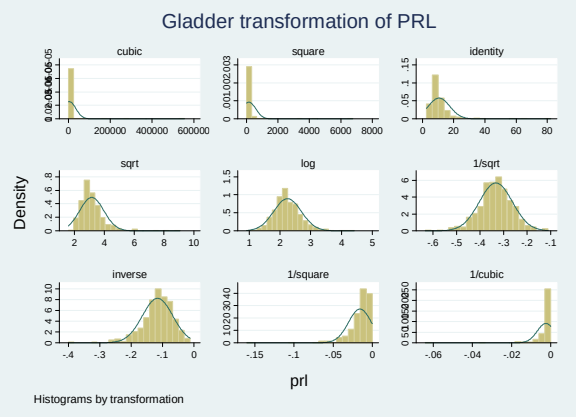
In order to determine potential predictors for prolactin serum levels, linear regression analysis was performed and the residuals were analyzed. Two separate models were constructed. The first utilized the variables found to be significant at the 0.10 level in the bi-variate analysis (ever pregnant, number of live births, ever breast feeding, and number of children breast fed) as potential predictors of serum prolactin levels. The second model utilized the variables listed in the literature (age, age at first full-term pregnancy, age at menarche, age at menopause, number of live births, ever breast feeding, family history of breast cancer in first-degree relatives, history of benign breast cysts, hormone therapy use and body mass index) as potential predictors of serum prolactin levels.



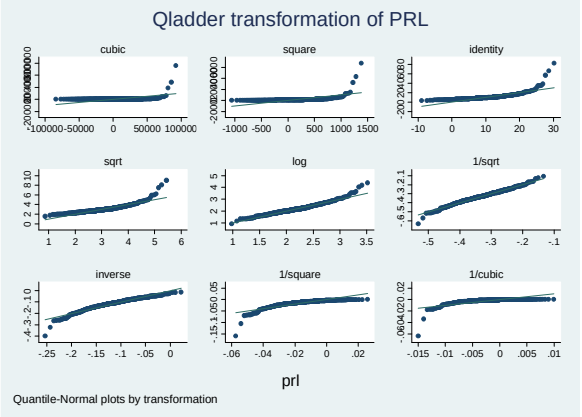
(a) Serum prolactin levels



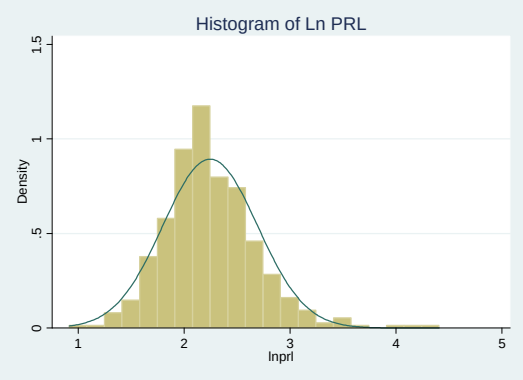
(b) Prolactin residuals



(c) Potential prolactin transformations



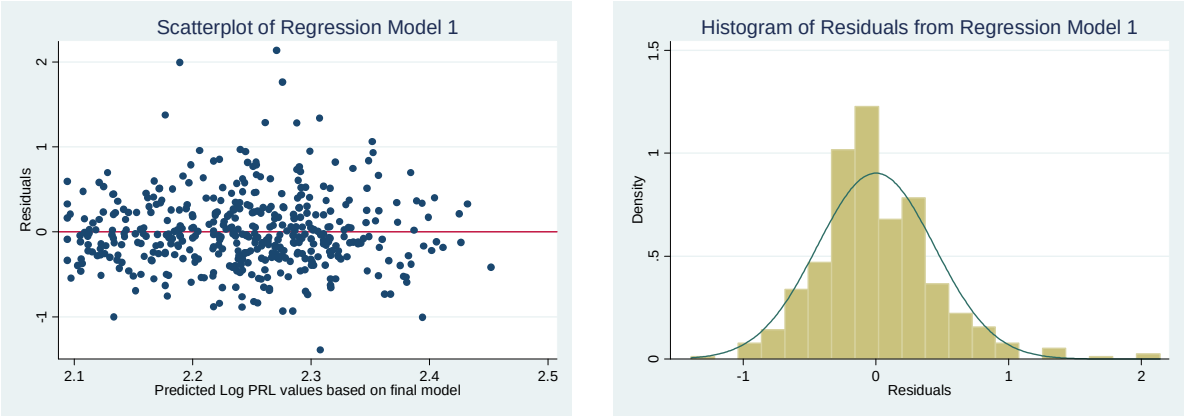
(d) Potential prolactin transformations



(e) Log transformation of prolactin

Figure 9: Normality analysis of serum prolactin levels

Both models yielded the same exact results – the only variable found to be a statistically significant predictor of serum prolactin levels (log transformed) was ever breast feeding. Women who breast fed were more likely to have a decreased level of log transformed prolactin (0.884, 95% CI 0.812-0.962, p-value 0.004). A histogram and scatterplot of the residuals from this model can be found in **Figure 10**.



(a) Scatterplot of residuals (b) Histogram of residuals

Figure 10: Analysis of the residuals from the final linear regression model of predictors of serum prolactin levels.

3.3 Correlation Analysis of Prolactin (PRL) and Percent Breast Density

A spearman correlation coefficient of 0.1167 (p-value 0.018) was obtained for the correlation between prolactin and percent breast density. In order to determine if any other variables could impact this correlation, several analyses were run between prolactin and each of the descriptive variables (**Table 8**). The only significant correlations observed were between prolactin and ever being pregnant (p-value 0.005) and between prolactin and ever breast feeding (p-value 0.007).

Table 8: Correlation between prolactin and descriptive variables among white, postmenopausal, cancer-free healthy women in the Mammograms and Masses Study (MAMS).

Variable	Correlation	p-value
% density	0.12	**0.018**
Age	-0.09	0.073
Weight	-0.07	0.141
BMI	-0.06	0.249
Age at Menarche		
12 years	0.00	0.934
13 years	0.03	0.516
≥14 years	-0.05	0.309
Ever Pregnant	-0.16	**0.005**
Age at 1st full-term pregnancy		
20-24 years	0.04	0.507
25-29 years	-0.00	0.969
30-34 years	0.01	0.825
≥35 years	-0.00	0.948
Number of live births		
1	0.05	0.341
2	-0.09	0.064
≥3	-0.06	0.184
Ever breast fed	-0.13	**0.007**
Number of children breast fed		
1	-0.07	0.123
2	-0.09	0.059
≥3	-0.03	0.530
Age at menopause	0.03	0.543
Surgical menopause	0.06	0.217
Current hormone therapy user	0.03	0.587
History of breast cysts	-0.06	0.215
Family history of breast cancer	-0.03	0.575
Current smoker	-0.09	0.222
Drank alcohol in past 12 mths	0.09	0.251
Walks for exercise	0.08	0.109
Aspirin in last 48 hrs before blood draw	-0.09	0.077

In order to determine if ever pregnant and/or ever breast feeding could confound the observed correlation between prolactin and percent breast density, separate spearman correlation analyses were run to examine the impact of the above mentioned variables. After adjusting for ever being pregnant and ever breast feeding (**Table 9**), the observed correlation between prolactin and percent breast density increased slightly (spearman correlation coefficient of 0.1197; p-value 0.013) and remained statistically significant.

Table 9: Spearman correlation matrix between prolactin, percent breast density, ever pregnant, and ever having breast fed in white, postmenopausal, cancer-free, healthy women in the Mammograms and Masses Study (MAMS).

	Prolactin	**% breast density**	**Ever pregnant**	**Ever having breast fed**
Prolactin	1.0000			
% breast density	0.1197	1.0000		
	p-value 0.013			
Ever pregnant	-0.1515	-0.1401	1.0000	
	p-value <0.001	p-value 0.004		
Ever having breast fed	-0.1295	0.0823	0.3404	1.0000
	p-value 0.007	p-value 0.087	p-value <0.001	

Separate spearman correlation analyses were performed to examine the potential correlation between percent breast density and each of the descriptive variables. Aside from ever being pregnant (spearman correlation coefficient of -0.1259; p-value 0.000), none of the other descriptive variables were found to be statistically significant (data not shown). Additionally, the correlation between prolactin and dense breast area (spearman correlation coefficient of 0.0749; p-value 0.115) was not statistically significant.

3.4 Analysis of Prolactin (PRL) Single Nucleotide Polymorphisms

Seven single nucleotide polymorphisms (SNPs) were identified as tagging SNPs. Out of these, rs1205955 could not be genotyped and was dropped from the analysis. All of the remaining six SNPs (rs6456483, rs2744119, rs1205961, rs849877, rs12210179, and rs1341239) were in Hardy-Weinberg equilibrium (**Table 10**). No differences in genotype frequency were observed between control group 1 (benign breast masses) women and control group 2 (negative mammograms) women (**Table 10**). No statistically significant differences were found in regards to either serum prolactin levels, percent breast density, or proportion of dense breast area among the various SNPs (**Table 11**).

Table 10: Prolactin single nucleotide polymorphism (PRL SNP) genotype frequency among white, postmenopausal, cancer-free healthy women in the Mammograms and Masses Study (MAMS).

PRL SNP	Genotype	Overall n (%)	HapMap Distribution§ (%)	HWE p-value‡	Group 1 n (%)	Group 2 n (%)	p-value*
rs6456483	CC	157 (35.9)	32.8	0.536	45 (44.6)	112 (33.3)	0.072
	CG	215 (49.2)	50.0		40 (39.6)	175 (52.1)	
	GG	65 (14.9)	17.2		16 (15.8)	49 (14.6)	
rs2744119	AA	347 (80.5)	75.0	0.503	80 (81.6)	267 (80.2)	0.912
	AG	78 (18.1)	21.7		17 (17.3)	61 (18.3)	
	GG	6 (1.4)	3.3		1 (1.1)	5 (1.5)	
rs1205961	GG	159 (36.6)	45.0	0.963	34 (34.3)	125 (37.3)	0.704
	AG	207 (47.7)	40.0		47 (47.5)	160 (47.8)	
	AA	68 (15.7)	15.0		18 (18.2)	50 (14.9)	
rs849877	CC	58 (13.6)	15.5	0.330	9 (9.3)	49 (14.9)	0.246
	TC	211 (49.5)	43.1		54 (55.7)	157 (47.7)	
	TT	157 (36.9)	41.4		34 (35.1)	123 (37.4)	
rs12210179	TT	220 (50.5)	52.5	0.145	49 (49.0)	171 (50.9)	0.208
	TC	188 (43.1)	42.4		48 (48.0)	140 (41.7)	
	CC	28 (6.4)	5.1		3 (3.0)	25 (7.4)	
rs1341239	CC	153 (35.7)	38.3	0.527	35 (36.1)	118 (35.6)	0.319
	AC	211 (49.3)	50.0		52 (53.6)	159 (48.0)	
	AA	64 (15.0)	11.7		10 (10.3)	54 (16.3)	

§ Single nucleotide polymorphism (SNP) distribution for whites with Northern and European ancestry

‡ HWE – Hardy Weinberg equilibrium chi-square goodness of fit statistic

* p-value determined by a chi-square

Abbreviations used: Group 1 – women with benign breast masses; Group 2 – women with negative mammograms

Table 11: Association between prolactin single nucleotide polymorphisms (PRL SNP) and serum prolactin levels, percent breast density, and proportion of dense breast area among white, postmenopausal, cancer-free healthy women in the Mammograms and Masses Study (MAMS).

PRL SNP	Genotype	PRL, ng/mL (mean ± SD)	p-value§	Percent Breast Density, % (mean ± SD)	p-value§	Proportion of Dense Breast Area, cm^2 (mean ± SD)	p-value§
rs6456483	CC	10.31 ± 4.97	0.684	31.19 ± 20.50	0.176	45.15 ± 30.22	0.660
	CG	10.87 ± 7.15		32.50 ± 19.41		41.48 ± 24.67	
	GG	9.57 ± 3.96		27.43 ± 18.74		41.90 ± 27.00	
rs2744119	AA	10.46 ± 6.28	0.556	31.58 ± 19.51	0.120	42.46 ± 26.17	0.196
	AG	10.55 ± 5.23		29.59 ± 20.98		45.57 ± 34.79	
	GG	10.66 ± 2.11		43.24 ± 14.09		58.03 ± 18.67	
rs1205961	GG	9.83 ± 4.24	0.256	30.74 ± 18.70	0.321	42.70 ± 27.87	0.468
	AG	10.49 ± 6.20		32.82 ± 21.29		44.67 ± 28.67	
	AA	11.80 ± 8.54		27.68 ± 17.73		40.36 ±27.03	
rs849877	CC	10.19 ± 4.14	0.898	29.50 ± 19.30	0.346	40.46 ± 24.35	0.790
	TC	10.73 ± 6.57		30.98 ± 20.11		43.51 ± 27.81	
	TT	10.41 ± 6.01		32.92 ± 19.53		44.27 ± 28.98	
rs12210179	TT	10.19 ± 5.77	0.270	27.42 ± 18.09	0.352	44.39 ± 28.06	0.525
	TC	10.78 ± 6.59		30.66 ± 20.51		42.13 ± 27.98	
	CC	10.64 ± 3.80		27.42 ± 18.09		38.83 ± 24.14	
rs1341239	CC	10.36 ± 6.09	0.659	32.84 ± 19.41	0.389	43.98 ± 27.20	0.869
	AC	10.65 ± 6.47		31.18 ± 20.12		43.42 ± 28.84	
	AA	10.53 ± 6.08		29.67 ± 19.54		42.24 ± 26.14	

§ Krustal-Wallis test for siginificance

Abbreviations used: SD – standard deviation

3.5 Analysis of Prolactin Receptor (PRLR) Single Nucleotide Polymorphisms

Fifteen single nucleotide polymorphisms (SNPs) were identified as tagging SNPs. Out of these, rs1587607 could not be genotyped and was dropped from the analysis. All of the remaining thirteen SNPs (rs1587059, rs249522, rs7737558, rs4703505, rs6895913, rs7705216, rs4235652, rs1609500, rs6866465, rs1587608, rs7727306, rs7720677, and rs6897600) were in Hardy-Weinberg equilibrium (**Table 12**). SNP rs401694 was not in Hardy-Weinberg equilibrium, mainly because the heterozygous genotype was not observed for this SNP, thus representing a genotyping error and resulting in it being dropped from the analysis. Additionally, SNP rs6866465 was also not in Hardy-Weinberg equilibrium, mainly because the homozygous minor allele genotype was not observed for this SNP, thus representing an additional genotyping error and resulting in it being dropped from the analysis. In regards to SNP rs4703505, women with negative mammograms (control group 2) were more likely to have the GG homozygous genotype (54.6% vs. 49.5%) than the benign women (control group 1), who were more likely to have the AA homozygous genotype (14.1% vs. 4.5%). No other differences in genotype frequency were observed between control groups (**Table 12**). No statistically significant differences were found in regards to either serum prolactin levels, percent breast density, or proportion of dense breast area among the various prolactin receptor SNPs (**Table 13**).

Table 12: Prolactin Receptor (PRLR) SNPs genotype frequency among white, postmenopausal, cancer-free healthy women in the Mammograms and Masses Study (MAMS).

PRLR SNP	Genotype	Overall n (%)	HapMap Distribution§ (%)	HWE p-value‡	Group 1 n (%)	Group 2 n (%)	p-value*
rs1587059	CC	179 (41.4)	48.3	0.803	39 (39.8)	140 (41.9)	0.432
	CG	200 (46.3)	43.3		50 (51.0)	150 (44.9)	
	GG	53 (12.3)	8.3		9 (9.2)	44 (13.2)	
rs249522	CC	331 (76.8)	73.3	0.358	76 (76.0)	255 (77.0)	0.767
	TC	91 (21.1)	25.0		21 (21.0)	70 (21.1)	
	TT	9 (2.1)	1.7		3 (3.0)	6 (1.8)	
rs401694	CC	395 (97.1)	63.3	0.000	94 (96.9)	301 (97.1)	0.923
	CT		35.0				
	TT	12 (2.9)	1.7		3 (3.1)	9 (2.9)	
rs7734558	AA	130 (29.7)	31.7	0.516	34 (34.0)	96 (28.4)	0.559
	AG	211 (48.2)	55.0		45 (45.0)	166 (49.1)	
	GG	97 (22.1)	13.3		21 (21.0)	76 (22.5)	
rs4703505	GG	233 (53.4)	43.3	0.648	49 (49.5)	184 (54.6)	0.003
	AG	174 (39.9)	51.7		36 (36.4)	138 (40.9)	
	AA	29 (6.7)	0.05		14 (14.1)	15 (4.5)	
rs6895913	GG	190 (43.8)	Not reported	0.598	51 (51.0)	139 (41.6)	0.251
	AG	198 (45.6)	Not reported		40 (40.0)	158 (47.3)	
	AA	46 (10.6)	Not reported		9 (9.0)	37 (11.1)	
rs7705216	CC	332 (75.6)	73.3	0.588	72 (72.0)	260 (76.7)	0.574
	CG	101 (23.0)	0.0		26 (26.0)	75 (22.1)	
	GG	6 (1.4)	26.7		2 (2.0)	4 (1.2)	
rs4235652	TT	311 (73.0)	67.8	0.199	71 (72.4)	240 (73.2)	0.795
	TC	102 (23.9)	28.8		23 (23.5)	79 (24.1)	
	CC	13 (3.1)	3.4		4 (4.1)	9 (2.7)	
rs1609500	AA	196 (47.6)	41.7	0.497	45 (45.9)	151 (48.1)	0.045
	AC	172 (41.7)	46.7		36 (36.7)	136 (43.3)	
	CC	44 (10.7)	11.7		17 (17.3)	27 (8.6)	
rs6866465	TT	350 (83.9)	84.7	0.074	85 (87.6)	265 (82.8)	0.258
	CT	67 (16.1)	13.6		12 (12.4)	55 (17.2)	
	CC	0	1.7				
rs1587608	AA	241 (55.4)	51.7	0.634	52 (52.0)	189 (56.4)	0.222
	AT	163 (37.5)	45.0		37 (37.0)	126 (37.6)	
	TT	31 (7.1)	3.3		11 (11.0)	20 (6.0)	

Table 12 (Continued)

PRLR SNP	Genotype	Overall n (%)	HapMap Distribution§ (%)	HWE p-value‡	Group 1 n (%)	Group 2 n (%)	p-value*
rs7727306	CC	144 (33.0)	36.7	0.848	32 (32.0)	112 (33.2)	0.944
	CT	212 (48.5)	41.7		50 (50.0)	162 (48.1)	
	TT	81 (18.5)	21.7		18 (18.0)	63 (18.7)	
rs7720677	CC	283 (64.6)	63.3	0.480	60 (59.4)	223 (66.2)	0.242
	CT	141 (32.2)	33.3		39 (38.6)	102 (30.3)	
	TT	14 (3.2)	3.3		2 (2.0)	12 (3.6)	
rs6897600	AA	195 (44.8)	45.0	0.608	49 (48.5)	146 (43.7)	0.422
	AC	196 (45.1)	46.7		45 (44.6)	151 (45.2)	
	CC	44 (10.1)	8.3		7 (6.9)	37 (11.1)	

§ Single nucleotide polymorphism (SNP) distribution for whites with Northern and European ancestry

‡ HWE – Hardy Weinberg equilibrium chi-square goodness of fit statistic

* p-value determined by a chi-square

Abbreviations used: SD – standard deviation

Table 13: Association between prolactin receptor (PRLR) SNPs and serum prolactin levels, percent breast density, and proportion of dense breast area among white, postmenopausal, cancer-free healthy women in the Mammograms and Masses Study (MAMS).

PRLR SNP	Genotype	PRL, ng/mL (mean ± SD)	p-value§	Percent Breast Density, % (mean ± SD)	p-value§	Proportion of Dense Breast Area, cm^2 (mean ± SD)	p-value§
rs1587059	CC	9.54 ± 3.65	0.354	32.07 ± 20.66	0.601	43.16 ± 28.17	0.623
	CG	11.36 ± 7.68		30.98 ± 18.99		43.94 ± 27.41	
	GG	10.40 ± 5.09		29.32 ± 20.95		41.38 ± 31.03	
rs249522	CC	10.48 ± 6.49	0.494	31.49 ± 20.36	0.819	43.65 ± 28.36	0.963
	TC	10.40 ± 4.50		30.95 ± 18.21		42.53 ± 28.04	
	TT	10.34 ± 2.94		28.17 ± 22.04		43.20 ± 26.36	
rs7734558	AA	9.86 ± 6.32	0.070	32.98 ± 20.49	0.208	46.54 ± 28.16	0.182
	AG	10.76 ± 6.40		31.49 ± 19.78		41.95 ± 27.96	
	GG	10.77 ± 4.60		28.25 ± 18.87		41.36 ± 28.11	
rs4703505	GG	10.46 ± 6.67	0.148	30.53 ± 19.95	0.487	42.92 ± 28.07	0.482
	AG	10.24 ± 4.89		31.75 ± 19.53		42.34 ± 27.05	
	AA	12.18 ± 6.66		34.73 ± 20.62		48.96 ± 30.20	
rs6895913	GG	10.53 ± 6.45	0.902	32.26 ± 19.65	0.461	44.77 ± 28.15	0.402
	AG	10.41 ± 5.87		30.90 ± 19.69		42.57 ± 26.54	
	AA	10.59 ± 5.01		28.02 ± 17.77		37.78 ± 22.84	
rs7705216	CC	10.27 ± 5.99	0.080	31.21 ± 19.85	0.575	42.91 ± 26.93	0.757
	CG	11.15 ± 6.22		31.91 ± 19.14		43.86 ± 29.73	
	GG	11.71 ± 2.78		25.22 ± 26.73		38.60 ± 39.98	
rs4235652	TT	10.69 ± 6.68	0.183	30.97 ± 19.49	0.200	41.97 ± 26.57	0.083
	TC	10.10 ± 4.10		35.08 ± 21.11		49.20 ± 31.96	
	CC	7.76 ± 1.85		27.13 ±16.74		39.61 ± 28.33	

Table 13 (Continued)

PRLR SNP	Genotype	PRL, ng/mL (mean ± SD)	p-value§	Percent Breast Density, % (mean ± SD)	p-value§	Proportion of Dense Breast Area, cm^2 (mean ± SD)	p-value§
rs1609500	AA	10.68 ± 7.05	0.555	30.78 ± 20.08	0.540	48.65 ± 35.02	0.558
	AC	10.31 ± 5.01		32.83 ± 19.25		42.43 ± 25.34	
	CC	11.10 ± 5.74		31.47 ± 20.26		48.65 ± 35.02	
rs1587608	AA	10.53 ± 6.65	0.655	30.60 ± 19.96	0.448	41.75 ± 25.56	0.618
	AT	10.35 ± 5.31		32.79 ± 19.53		44.92 ± 29.56	
	TT	10.29 ± 3.79		31.92 ± 21.35		49.04 ± 38.39	
rs7727306	CC	10.35 ± 4.34	0.662	30.88 ± 20.12	0.654	40.36 ± 23.87	0.569
	CT	10.45 ± 6.34		32.40 ± 20.64		45.17 ± 30.31	
	TT	10.93 ± 7.62		29.50 ± 17.31		42.70 ± 28.71	
rs7720677	CC	10.59 ± 6.52	0.668	30.50 ± 19.15	0.410	43.05 ± 27.54	0.689
	CT	10.33 ± 5.11		33.12 ± 21.21		44.03 ± 29.08	
	TT	9.39 ± 3.63		25.98 ± 18.99		36.76 ± 28.80	
rs6897600	AA	10.43 ± 6.35	0.741	31.20 ± 19.56	0.300	44.05 ± 29.96	0.305
	AC	10.47 ± 5.82		32.23 ± 20.17		43.73 ± 26.92	
	CC	10.63 ± 5.35		26.97 ± 19.40		35.93 ± 21.59	

§ Krustal-Wallis test for siginificance

Abbreviations used: SD – standard deviation

4.0 Discussion

Both case-control[181-189] and prospective studies[144,190-194] have yielded inconsistent results regarding the association between prolactin and breast cancer risk. Recently, two large prospective studies[193,194] reported a significant positive association between prolactin and post-menopausal breast cancer. The exact mechanism is still unknown, but it has been hypothesized that prolactin and estrogen may act synergistically to exert their mitogenic effects on the normal breast[180]. Whereas increased levels of estrogen have been linked to increased breast density, whether elevated prolactin levels also lead to increases in breast density remains unknown. To our knowledge, this is the first study to examine the association between prolactin and breast density and variations in the prolactin gene and its' receptor in a cohort of postmenopausal, cancer-free healthy women.

Women with benign breast masses observed on a routine screening mammogram had a greater proportion of breast dense area (50.57 ± 31.57 cm^2 vs. 41.38 ± 26.91 cm^2), and had greater percent breast density (36.44 ± 19.86 vs. 29.87 ± 19.65) when compared with women who had negative screening mammograms. This result is not surprising given that higher breast density is associated with lower sensitivity and specificity of mammograms, thus resulting in false-positive readings (due to lower specificity).

No statistically significant differences were observed for mean serum prolactin levels between both groups of women. This may be the result of the composition of our sample population – healthy, cancer-free postmenopausal women.

A weak, but statistically significant correlation was observed between prolactin and percent breast density (spearman correlation coefficient of 0.1197; p-value 0.013) after adjusting for ever being pregnant and ever breast feeding, thus confirming our hypothesis that women with

elevated prolactin levels will have elevated percent breast density. Ever being pregnant and ever breast feeding were the only two variables for which a positive bi-variate correlation with prolactin was observed. This result is not surprising, given that prolactin acts like both a mitogen and differentiating agent during pregnancy and lactation[138,177]. During pregnancy, breast epithelial cells rapidly proliferate to create additional ductal branches and to promote lobuloalveolar growth[178]. Prolactin acts directly on the mammary epithelium to produce lobuloalveolar development[171]. During lactation, the alveoli serve as the unit of milk production and are directly under the control of circulating prolactin[180].

No statistically significant differences were observed for the prolactin gene single nucleotide polymorphisms in relation to serum prolactin level, percent breast density, or proportion of dense breast area. Prolactin levels, regardless of the SNP tested, fluctuated around the mean value of 10.62 ± 6.89 ng/mL, as did percent breast density (31.41 ± 19.87) and proportion of dense breast area (43.53 ± 28.30 cm^2).

Two SNPs (rs7734558 and rs7705216) within the prolactin receptor gene failed to reach statistical significance at the 0.05 level, they were significant at the 0.10 level (p-value of 0.070 for rs7734558 and p-value of 0.080 for rs7705216). Although further analyses are needed, it appears that healthy, cancer-free, postmenopausal women with the G allele (AG and GG) had a slightly elevated level of prolactin when compared with women homozygous for the A allele (AG 10.76 ± 6.40 ng/mL, GG 10.77 ± 4.60 ng/mL vs. AA 9.86 ± 6.32 ng/mL) for SNP rs7734558. In regards to SNP rs7705216, healthy, cancer-free, postmenopausal women who were homozygous for the GG allele have a slightly elevated prolactin level when compared with women with the C allele (GG 11.71 ± 2.78 ng/mL vs. CG 11.15 ± 6.22 ng/mL, CC 10.27 ± 5.99 ng/mL).

Our results are somewhat consistent with a recently published study by Lee et al.[211] which examined the common genetic variation in both the prolactin and prolactin receptor genes in relation to plasma prolactin levels and breast cancer risk in the Multiethnic Cohort. No statistically significant associations were observed between any of the tagging SNPs chosen (covered 59 kb of the prolactin locus and 210 kb of the prolactin receptor locus) and either plasma prolactin levels or breast cancer risk. The authors did not specifically examine the two SNPs that were significant at the 0.10 level, thus not allowing for comparison.

In contrast to our results, Vaclavicek et al.[170] using a case-control study design observed that two SNPs (rs1341239 and rs12210179) in the prolactin gene were both statistically significantly associated with a higher breast cancer risk (OR 1.67, 95% CI 1.11-2.50, and OR 2.09, 95% CI 1.23-3.52, respectively). We did not observe any association between these two SNPs and elevated levels of breast density in our sample of healthy, cancer-free postmenopausal women. One would hypothesize that if both of these SNPs were in fact associated with breast cancer risk, that they may also be associated with higher breast density, given the consistently observed strong association between breast density and breast cancer risk. However, this discrepancy could be related to the study population. Whereas Vaclavicek et al.[170] utilized a case-control study design in which the cases had a very strong family history of breast cancer, we utilized a cross-sectional study design of healthy, cancer-free postmenopausal women, of which only 14% had a family history of breast cancer in a first-degree relative. In addition, both the Lee et al.[211] and Vaclavicek et al.[170] studies examined the association between prolactin and prolactin receptor genes in relation to breast cancer risk, not breast density.

The main limitation of this study is that the women were all Caucasian and postmenopausal, thus limiting the generalizability of the findings to Caucasian post-menopausal

women only. We are not able to draw any conclusions about the association of breast density and prolactin in pre-menopausal women or women of other ethnic groups.

Another limitation is the use of a one-time serum specimen for the evaluation of prolactin levels which may not accurately reflect more etiologically-relevant long-term levels. Additionally, prolactin has a notable circadian variation, and levels can also be affected by food. Specimens were not fasting and were drawn at various times of day, which could impact the findings. Because we were unable to adjust for time of day of blood draw and/or month of blood draw, it is possible that the results obtained could be an underestimation.

Additionally, a common concern in candidate-gene association studies is population stratification – the variation in allele frequencies across subgroups of a population that also differ in risk factors profiles for the disease.[212,213] Population stratification is a form of confounding that could introduce false associations.[214] However, data suggest that in studies of U.S. non-Hispanics of European descent, it is unlikely that the population contains a make-up that would lead to appreciably biased estimates of association. Since most of the Caucasian women in this study are of European descent, population stratification should not be a problem. We were unable to utilize "genomic control" methods to address this issue. The basic idea of genomic control would be to genotype extra markers that are unlinked to the genes under investigation and that are unlikely to be associated with breast density. A standard panel of genomic control markers can then be used to estimate any stratification and calculate an adjustment parameter. The adjustment is applied to the test statistics in order to make them more conservative and account for the estimated level of stratification (if any). This method is well-supported by both statistical and population-genetic theory, and is in common use for this type of study[215-218], although it was not conducted by the laboratory used for genotype analyses. It is

also possible that any observed associations found between a SNP and breast density/prolactin may be due to linkage disequilibirum between the assessed polymorphism and the true functional polymorphism at a nearby locus.

Despite these limitations, this proposal has several strengths, including the novel hypotheses and large sample size (n=445), which will provide ample power to assess the association of prolactin with breast density. In addition, this is the first study of prolactin gene variation and breast density in a healthy postmenopausal population.

5.0 Conclusion

Although, elevated levels of mammographic breast density are a risk factor for breast cancer development, little is correctly known regarding what factors lead to such levels. A factor that may contribute to elevated levels of breast density is prolactin. Prolactin acts like both a mitogen and differentiating agent during pregnancy and lactation[175,176]. Although the majority of studies have yielded inconsistent results regarding the association between prolactin and breast cancer risk, the Nurses Health Study[194] reported a significant positive association among postmenopausal women (RR=1.34, 95% CI 1.02-1.76, highest versus lowest quartile of prolactin concentration). However, to date and our knowledge, this is the first study to examine the association between prolactin and breast density and variations in the prolactin gene and the prolactin receptor gene in a sample of healthy, cancer-free, postmenopausal women.

A weak, but statistically significant correlation was observed between prolactin and percent breast density (spearman correlation coefficient of 0.1197; p-value 0.013) after adjusting for ever being pregnant and ever breast feeding, thus confirming our hypothesis that women with elevated prolactin levels will have elevated mammographic breast density.

No statistically significant differences were observed for single nucleotide polymorphisms (SNPs) in the prolactin gene. However, two SNPs in the prolactin receptor gene (rs7734558 and rs7705216) were significantly associated with serum prolactin level at the 0.10 significance level. Healthy, cancer-free, postmenopausal women with the G allele (AG and GG) at SNP rs7734558 have a slightly elevated level of prolactin when compared with women homozygous for the A allele (AG 10.76 ± 6.40 ng/mL, GG 10.77 ± 4.60 ng/mL vs. AA 9.86 ± 6.32 ng/mL). In regards to SNP rs7705216, healthy, cancer-free, postmenopausal women who were homozygous for the GG allele have a slightly elevated prolactin level when compared with

individuals with the C allele (GG 11.71 ± 2.78 ng/mL vs. CG 11.15 ± 6.22 ng/mL, CC 10.27 ± 5.99 ng/mL). Although further analyses are needed, such as examining the potential association between the SNPs and serum levels via a dominant, co-dominant, and recessive model, the results appear to be promising and warrant further investigation.

6.0 Public Health Significance

The current study adds to the ever growing body of public health literature on the determinants of breast cancer risk, specifically mammographic breast density. As mentioned previously, breast density, outside of age and BRCA1/2 mutations, is the strongest risk factor for breast cancer. Although it is well known that screening mammography is the best way to reduce morbidity and mortality from breast cancer, elevated levels of breast density affect the sensitivity and specificity of mammograms, thus reducing the benefits of screening. It has been proposed that if breast density is taken into account in the standard breast cancer risk assessment, that up to 20% of postmenopausal women would be eligible for chemoprevention. It is therefore imperative that we understand not only the underlying factors that contribute to breast density, but their underlying mechanisms as well, in order to improve breast cancer screening and identify women who are at an increased risk for breast cancer and for whom prevention strategies may be useful.

Although we only observed a weak correlation between prolactin and percent breast density (spearman correlation coefficient of 0.1197), it was highly significant (p-value 0.013) and confirmed our hypothesis that women with elevated prolactin levels will have an elevated percent breast density. Breast cancer is a multi-factorial disease and there is no reason to believe that one of it's most predictive risk factors – percent breast density – would not also be multi-factorial. It is very likely that prolactin is only one of several factors that contribute to increased breast density. Further studies are needed to not only confirm our findings, but to further explore the relationship between prolactin and other breast cancer predictive factors, such as estrogen levels in a cohort of healthy, cancer-free postmenopausal women.

Because increased breast density reduces the sensitivity of mammograms, it is essential that we understand the determinants of breast density, which may in turn help improve mammographic screening. In addition, the current study was limited in that our study population only consisted of Caucasian women. It is possible that prolactin may have a stronger correlation with breast density in other population groups, such as African-American women. If a stronger correlation/association between prolactin and percent breast density is observed in other populations, it could provide the impetus to begin investigating prolactin antagonists as potential chemopreventive agents for breast cancer.

In addition, two SNPs in the prolactin receptor gene (rs7734558 and rs7705216) were significantly associated with serum prolactin levels at the 0.10 significance level. These SNPs need to be further investigated not only in our study population, but in other populations as well. If polymorphisms in the prolactin gene and/or the prolactin receptor gene are found to be statistically associated with increased percent breast cancer, it may be possible to use these genetic markers to identify women at an increased risk for breast cancer.

Our results, along with those of future studies, may lead to improved prevention and early detection of breast cancer, thus having an important contribution to public health.

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