ESTROGEN METABOLISM, BREAST DENSITY, AND BREAST CANCER

by

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BACKGROUND: Estrogen metabolites, sex-steroid hormones, and breast density are associated with breast carcinogenesis.

OBJECTIVE: Complete a systematic study of the contribution of two biological measures (breast density and hormone metabolism) to an endocrine-based model of breast cancer risk.

METHODS: The study groups included breast cancer-free participants (N=282) in the Study of Osteoporotic Fractures (SOF), and participants in the Mammogram and Masses Study (MAMS), inclusive of 176 cases (55 pre-menopausal, 121 post-menopausal) and 380 controls (124 pre-menopausal, 256 postmenopausal). Sex-steroid hormones, percent breast density, serum concentrations of 2-hydroxyestrone (2-OH) and 16 alpha-hydroxyestrone (16α-OH), and breast cancer risk factors were evaluated to determine associations.

RESULTS: In SOF, 16α-OH was positively associated with body mass index (BMI) (r=0.162); however, this association was not significant in multivariate analyses that controlled for the serum sex-steroid hormone concentrations (total estradiol, total testosterone, SHBG). Women who reported a surgical menopause were significantly more likely to have higher levels of 16α-OH (OR=(tertile 3 vs tertile 1) 7.37, 95% Confidence Interval (CI) 2.20-24.70), but there was no type of menopause difference with respect to 2-OH tertile. In all MAMS control subjects (N=380), breast density correlated weakly with log-transformed serum concentrations of 16α-OH (Pearson correlation coefficient (ρ) = 0.10, p-value < 0.1). Stratification according to

menopausal status substantially reduced or eliminated associations between breast density and the estrogen metabolite concentrations. Logistic regression analyses showed a 3-4 fold increased risk of breast cancer among pre-menopausal women in the highest tertile of breast density compared with those in the lowest tertile of density, even with adjustment for the estrogen metabolites. A statistically non-significant 1.5-fold increased risk of breast cancer in high vs. low tertile of density was observed among post-menopausal women taking hormone therapy (HT) after adjusting for estrogen metabolites, BMI, and age. Breast density did not appear to substantially increase breast cancer risk among post-menopausal women not taking HT.

CONCLUSION: In SOF, results did not show consistent associations between risk factors and estrogen metabolites except for a positive association between BMI and 16α-OH and surgical menopause and 16α-OH. With respect to MAMS, menopausal status may influence substrate estrogen hormone levels primarily, and, estrogen hormone levels may influence breast density secondarily, through pathways not involving the estrogen metabolites. The breast density-breast cancer association remains significant even with adjustment for the estrogen metabolites, at least in pre-menopausal women, suggesting that breast density may relate to breast cancer risk through pathways not involving estrogen metabolism.

PUBLIC HEALTH SIGNIFICANCE: Understanding factors that affect breast density and their underlying mechanism is an important public health issue. Such an understanding will help us improve breast cancer screening and may help us identify women who are at an increased risk of breast cancer and for whom prevention strategies may be useful.

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Jennifer K. Simpson

1. INTRODUCTION

In the following review of literature, a brief description of the epidemiology of breast cancer, mammographic density, and estrogen metabolism will be followed by a review of the potential relationship among these factors.

Among women, breast cancer is the most common cancer and is second only to lung cancer in the number of deaths per year. In 2005, approximately 211,240 new cases of invasive breast cancer and 58,490 in situ cases will be diagnosed in the United States and 40,410 women will die from breast cancer(1). The five-year relative survival rate is 98% if the cancer is limited to the breast at diagnosis, 81% if the regional lymph nodes are involved, and 26% if the cancer has spread to a distant site(1).

Thus, breast cancer represents a major burden to women, and methods to impact risk identification and modification are paramount. An improved understanding of risk factors has led to recommendations for risk reduction, as well as raised additional questions as to the underlying mechanisms of this disease.

1.1. Age

The greatest risk of breast cancer occurs with increasing age, with incidence doubling every 10 years until menopause(67, 85). When evaluating the distribution of breast cancer diagnosis by age, there is a steady incline (Figure 1). While the slopes are different between pre- and postmenopausal women, it is evident that the rise is steady without evidence of a plateau. This increasing risk may be indicative of the lifetime accumulation of exposures to those risk factors, known and unknown, as well as genetic events throughout the lifetime.

TABLE 10 Probability of Developing Invasive Cancers Within Selected Age Intervals, by Sex, US, 1998 to 2000*

		Birth to 39 (%)	40 to 59 (%)	60 to 79 (%)	Birth to Death (%)
All Sites †	Male	1.36 (1 in 73)	8.03 (1 in 12)	33.92 (1 in 3)	44.77 (1 in 2)
	Female	1.92 (1 in 52)	9.01 (1 in 11)	22.61 (1 in 4)	38.03 (1 in 3)
Bladder ‡	Male	.02 (1 in 4603)	.40 (1 in 250)	2.36 (1 in 42)	3.46 (1 in 29)
	Female	.01 (1 in 9557)	.12 (1 in 831)	.64 (1 in 157)	1.10 (1 in 91)
Breast	Female	.44 (1 in 229)	4.14 (1 in 24)	7.53 (1 in 13)	13.36 (1 in 7)
Colon &	Male	.06 (1 in 1678)	.86 (1 in 116)	3.94 (1 in 25)	5.88 (1 in 17)
Rectum	Female	.06 (1 in 1651)	.67(1 in 150)	3.05 (1 in 33)	5.49 (1 in 18)
Leukemia	Male	.15 (1 in 649)	.20 (1 in 495)	.82 (1 in 122)	1.45 (1 in 70)
	Female	.13 (1 in 789)	.14 (1 in 706)	.46 (1 in 219)	1.00 (1 in 100)
Lung &	Male	.03 (1 in 3439)	1.02 (1 in 98)	5.80 (1 in 17)	7.69 (1 in 13)
Bronchus	Female	.03 (1 in 3046)	.79 (1 in 126)	3.93 (1 in 25)	5.73 (1 in 17)
Melanoma	Male	.12 (1 in 809)	.49 (1 in 205)	.97 (1 in 103)	1.81 (1 in 55)
of the Skin	Female	.19 (1 in 532)	.39 (1 in 255)	.51 (1 in 197)	1.22 (1 in 82)
Non-Hodgkin	Male	.14 (1 in 739)	.45 (1 in 224)	1.27 (1 in 79)	2.10 (1 in 48)
Lymphoma	Female	.08 (1 in 1258)	.30 (1 in 332)	.98 (1 in 102)	1.76 (1 in 57)
Prostate	Male	.01 (1 in 12833)	2.28 (1 in 44)	14.20 (1 in 7)	17.15 (1 in 6)
Uterine Cervix	Female	.16 (1 in 632)	.31 (1 in 322)	.27 (1 in 368)	.78 (1 in 128)
Uterine Corpus	Female	.05 (1 in 1832)	.69 (1 in 144)	1.57 (1 in 64)	2.60 (1 in 38)

* For those free of cancer at beginning of age interval. Based on cancer cases diagnosed during 1998 to 2000.

†The "1 in" statistic and the inverse of the percentage may not be equivalent due to rounding.

‡ All Sites exclude basal and squamous cell skin cancers and in situ cancers except urinary bladder.

Source: DEVCAN Software, Probability of Developing or Dying of Cancer Software, Version 5.1. Statistical Research and Applications Branch, National Cancer Institute, 2003. http://srab.cancer.gov/devacan.

Figure 1-1: ACS Facts and Figures 2004

1.2. Geographic Variation

While age is a significant risk factor, age alone cannot explain the great variation noted among different countries. The influence of environment and lifestyle factors is probable in explaining the difference among countries. In studies evaluating migrants, the incidence of breast cancer assumes that of the host country within one or two generations(85). Currently, there is approximately a five fold difference in age-adjusted incidence and mortality between Far Eastern and Western countries.

1.3. Race

In figure 2, it is apparent that while Caucasian women have a higher incidence, the mortality in Black women remains higher. Potential reasons include disparities in access to care which may in turn lead to prognostic features such as later stage at diagnosis and increased mortality. Additionally, the incidence of comorbid conditions may impact the mortality rate. However, different biologic features may be the etiology in explaining the differences among race. When looking at estrogen receptor (ER) and progesterone receptor (PR) status across Caucasians, Blacks, and Hispanics, it is evident that Caucasians are more likely to present with ER/PR positive tumors which is a good prognostic feature (figure 3)(46). This supports the idea that although access to care is a real issue, biologic differences exist and warrant further investigation.

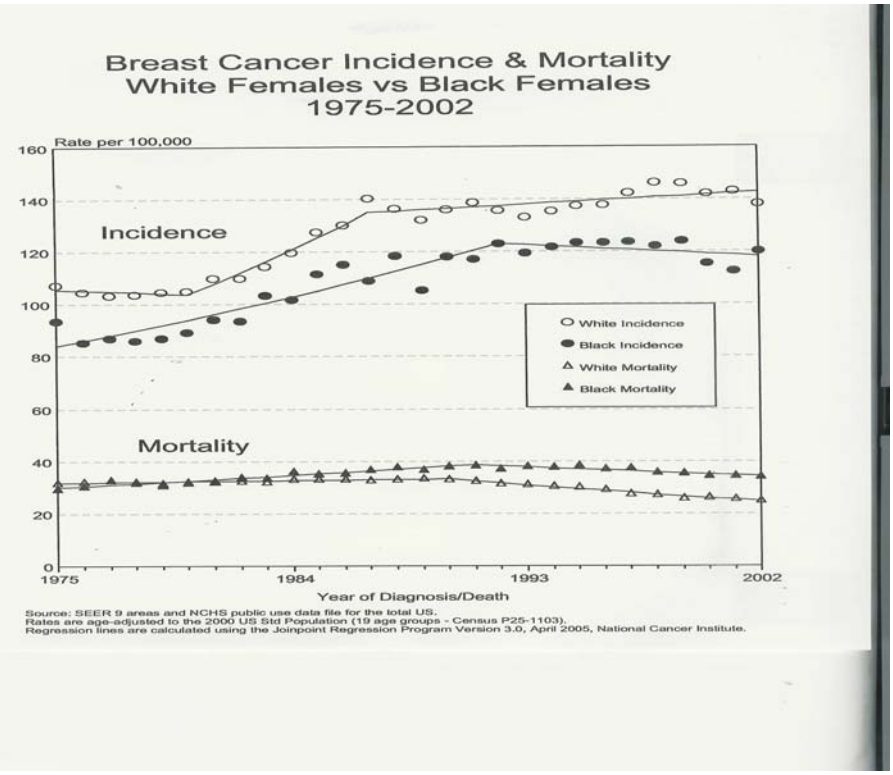
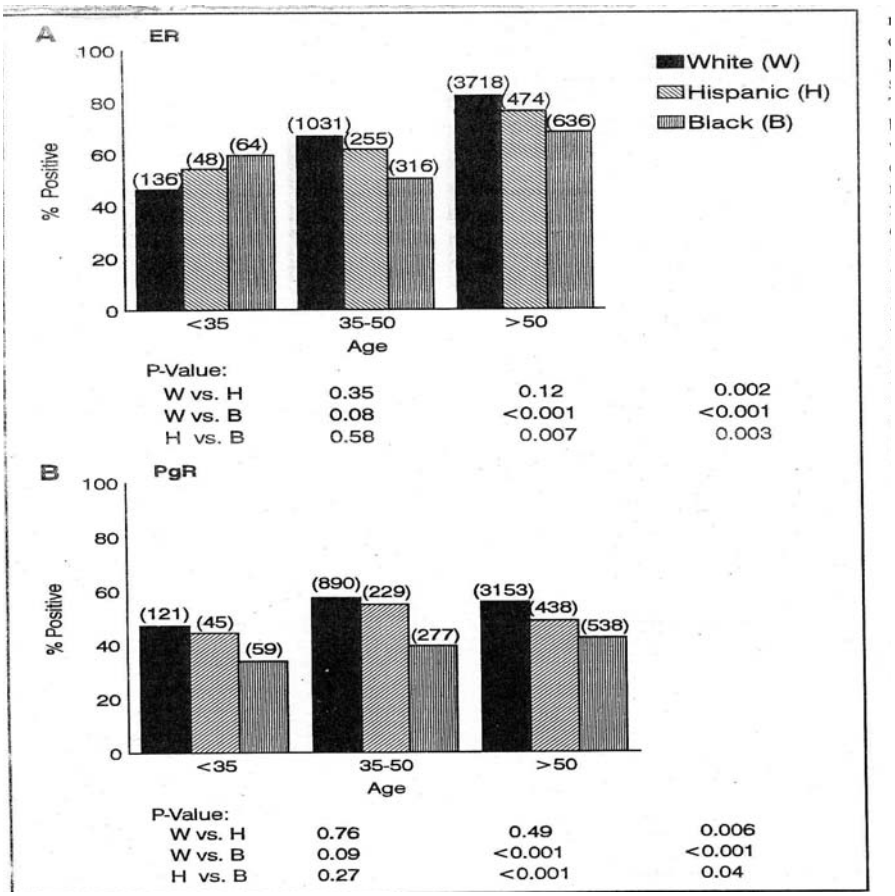


Figure 1-2: SEER Data Incidence & Mortality Rates by Race 1975-2002



Steroid receptor status by age in years and ethnic group. A) Estrogen receptor (ER). B) Progesterone receptor (PgR). Numbers in parentheses = total number of patients

Figure 1-3: ER/PR Status Across Race, Elledge, JNCI 1994

1.4. Family History/Genetics

The notion that breast cancer "runs in families" is not a new one. However, the etiology is questionable. Certainly these women generally share the same environmental exposures and lifestyle factors which may alone contribute to the increased risk. If a woman has a first degree relative with bilateral breast cancer or ovarian cancer, or a first degree relative diagnosed with breast cancer under the age of 40, her risk is three times that of the population(85). However, the

role of genetics has also been questioned. While there are probably many unidentified breast cancer genes, to date we are aware of two: BRCA1 and BRCA2. These genes are located on chromosomes 17 and 13 respectively and account for 5%-10% of all breast cancer cases. Mutations in these two independent, highly penetrant autosomal dominant loci are thought to account for the majority of inherited cancer cases(27, 92). In 100 families with at least one case of breast and ovarian cancer evaluated at a high-risk breast evaluation clinic, deleterious germline mutations in BRCA1 and BRCA2 were found in 55%(82). Methods to measure BRCA1 and BRCA2 as well as methods to reduce risk continue to be studied(20).

1.5. Benign Breast Disease

In clinical follow-up studies evidence has indicated that there is a relationship between the presence of histologically proven benign breast disease and breast cancer risk, and the level of risk varies according to the histologic category of benign breast disease. Particularly, proliferative lesions without atypia are associated with a 1.5- to 2-fold increase in risk, whereas atypical hyperplasias are associated with a fourfold to fivefold increase in breast cancer risk. There are many clinical factors which appear to modify the risk associated with these lesions, including the time since biopsy, menopausal status, and family history of breast cancer. Recent studies have begun to evaluate the potential role of biologic, molecular, and genetic markers in assessing breast cancer risk in patients with benign breast disease. New insights into benign breast disease and breast cancer risk will be derived from clinicopathologic follow-up studies, epidemiologic studies, and molecular and genetic studies(107)

1.6. Mammographic Density

It is clear in studies that the risk of breast cancer is higher in those women with dense breasts, which will be discussed in greater detail below. Factors which increase breast density include hormone therapy (HT), smoking, and family/genetic trends. As women age or pass through menopause, fatty tissue replaces glandular tissue therefore decreasing breast density. Additionally, greater body mass index (BMI) generally equates with less breast density. Potential etiologies of breast density include epithelial and stromal proliferation, sex hormone induced response by growth factors, and DNA damage(3, 5, 6, 11, 14, 21, 52, 117, 121). Lastly, in the PEPI study, mammographic density and bone mineral density (BMD) were reported to be positively associated in women who had not recently used exogenous hormones(42).

1.7. Diet/Alcohol

Generally, it has been difficult to explain the relationship between diet and breast cancer risk. In a meta-analysis of papers published up until July 2003 including case-control and cohort studies, the association of dietary fat and breast cancer was evaluated. A total of 45 risk estimates for total fat intake was obtained. The summary relative risk, comparing the highest and lowest levels of intake of total fat, was 1.13 (95% CI**:** 1.03-1.25). Cohort studies (N=14) had a summary relative risk of 1.11 (95% CI**:** 0.99-1.25) and case-control studies (N=31) had a relative risk of 1.14 (95% CI 0.99-1.32). Significant summary relative risks were also found for saturated fat (RR, 1.19; 95% CI**:** 1.06-1.35) and meat intake (RR, 1.17; 95% CI 1.06-1.29). Combined estimates of risk for total and saturated fat intake, and for meat intake, all indicate an association between higher intakes and an increased risk of breast cancer. Case-control and cohort studies gave similar results(17).

With respect to alcohol intake, the picture is clearer with a strong linear relationship indicating an increased risk with alcohol intake. In a pooled analysis of 6 prospective studies conducted in Canada, the Netherlands, Sweden, and the United States the risk of invasive breast cancer associated with total and beverage-specific alcohol consumption was evaluated. In a total of 322,647 women followed for up to 11 years, including 4,335 participants with a diagnosis of incident invasive breast cancer, the risk increased linearly with intake, and the pooled multivariate relative risk for an increment of 10 g/d of alcohol (about 0.75-1 drink) was 1.09 (95% CI, 1.04-1.13). This association did not appear to be modified by other factors(110). Similarly, Ellison et al(47) reported results from a meta-analysis of over 40 epidemiologic studies which suggested a 21% increase in breast cancer risk with an intake of 24 g of alcohol per day. Potential mechanisms include effect on steroid hormone production, insulin growth factor-1(IGF-1), generation of reactive oxygen radicals, lipid peroxides, acetaldehyde or perhaps interaction with low folate levels. Additionally, it is postulated that that increased risk of breast cancer is related to increased estrogen and androgen levels(109). Among women who consume alcohol regularly, reducing alcohol consumption is a potential means to reduce breast cancer risk(110).

1.8. Anthropometry

Weight, height, and BMI associations with breast cancer risk have been noted. HT has been shown to modify the association between body weight and breast cancer risk, with the exception of the Cancer Prevention Study-II, few studies are sufficiently large to examine the risk of breast cancer associated with BMI and weight gain separately among current HT users and nonusers(51). Additionally, variations are noted among pre- and postmenopausal women.

Among premenopausal women, an inverse relationship between baseline weight and BMI and breast cancer risk was evident in a pooled analysis from seven prospective studies, while a positive relationship was noted among postmenopausal women(118). In a study of 62,756 postmenopausal women in the Cancer Prevention Study-II Nutrition Cohort with 1,934 incident breast cancer cases, the association of BMI and adult weight gain (since age 18 years) with breast cancer risk was stratified by HT use. Total adult weight gain strongly predicted breast cancer risk among former and never HT users (P for trend < 0.0001). Weight gain of 21–30 pounds was associated with a rate ratio of 1.4 (95% confidence interval 1.1–1.8); rates doubled among women gaining >70 pounds compared with women who maintained their weight within 5 pounds of their weight at age 18. Among current HT users, no association was seen between breast cancer and either BMI or weight gain(51).

1.9. Endogenous Hormone Factors

Exposure to endogenous estrogen and its role as a risk factor for breast cancer has been described by many via the natural female processes of menarche and menopause. Menarche before age 12 yields a RR of 1.0 compared to menarche at age greater than 15 where the RR is 0.77. Menopause occurring after age 55 yields a two-fold increase in breast cancer compared with those who undergo menopause before age 45(85). Additionally, menarche before age 12 in combination with later menopause leads to a presumed increase in estrogen exposure, thereby increasing breast cancer risk(67). Furthermore, as early as 1956, it was observed that women who underwent bilateral oophorectomy before their natural menopause hadreduced breast cancer risk(79).

Similarly, the protective effect of pregnancy is apparent in those women who give birth to their first child before age 20 where the risk is reduced two-fold compared to women who give birth to their first child after age 30. Further risk reduction is noted if a second birth occurs at an early age as noted in a large population-based cohort study(126)and case-control study(33). However, it is felt that the mechanism of protection cannot solely be explained by exposure to endogenous hormones and may be related to the direct effect on breast tissue. In mouse models where early pregnancy was induced, it was noted that the breast glands became fully differentiated which may be protective against carcinogens (Presentation by Mary Daly).

1.10. Exogenous Hormones

1.10.1 Oral Contraceptives (OC)

A meta-analysis in 1996 evaluated 54 epidemiologic studies consisting of 53,297 breast cancer cases and 100,239 controls. In current users of oral contraceptives, there was a RR of 1.24 of developing breast cancer compared to never users. This increased risk was seen for up to 10 years after use, and family history of breast cancer did not appear to impact risk(40). In a retrospective cohort study(54) of sisters and daughters of women with breast cancer [ever users of (compared with never users)] had a threefold increase in breast cancer risk. However this increased risk was only noted in those who took OC before 1975 when hormone concentrations were higher(20). Thus, the association between women with a family history of breast cancer and OC use remains unclear.

1.10.2 Hormone Replacement Therapy (HT)

It is widely accepted that estrogen plays a role in the development of breast cancer(37). Before menopause, estrogen is produced primarily by the ovaries. After menopause, estrogen is

produced endogenously by the aromatization of androgens in fat tissue. The primary source of exogenous estrogens in postmenopausal women is HT. In the past, HT was generally prescribed to reduce symptoms associated with menopause, such as hot flashes and vaginal dryness(76). Subsequently, HT was used to reduce the risk of osteoporosis(76), although with the availability of agents specific for the prevention of osteoporosis, HT is generally used for symptoms related to the deficit in estrogen and progesterone . In a national survey, with a representative cohort that was followed from the mid 1970s until 1992, approximately 45% of women had used HT, and about 43% of ever-users had been on HT for at least 5 years(19).

Compelling data linking HT to postmenopausal breast cancer comes from a recent collaborative re-analysis of 51 epidemiologic studies, consisting of 53,865 postmenopausal women(8). In that study, 33% of the women had used HT at sometime, and 34% of ever-users had used HT for 5 years or more. Among current or recent (within 4 years) users, the relative risk of breast cancer increased by a factor of 1.02 (95%CI 1.01-1.04) for each year of use. The relative risk for women who had used HT for at least 5 years was 1.35 (95%CI 1.21-1.49), an increase comparable to delaying menopause for an equivalent period. Interestingly, the relative risks of breast cancer associated with HT use decreased with increasing body weight, which is consistent with data from the Nurses Health Study(19, 65) and the Breast Cancer Demonstration Project(106). This is the opposite of the normal association between BMI and breast cancer risk, namely an increase in BMI is associated with an increase in breast cancer risk. Therefore, it appears that HT modifies the association between BMI and risk of breast cancer. Finally, combination estrogen-progestin regimens may increase breast cancer risk beyond that associated with estrogen only(106).

In the Women's Health Initiative, a randomized controlled primary prevention trial (planned duration, 8.5 years) in which 16,608 postmenopausal women aged 50-79 years with an intact uterus at baseline were recruited by 40 US clinical centers in 1993-1998, the data and safety monitoring board recommended stopping the trial of estrogen plus progestin vs placebo after a mean of 5.2 years of follow-up because the test statistic for invasive breast cancer exceeded the stopping boundary for this adverse effect and the global index statistic supported risks exceeding benefits. The estimated hazard ratio was 1.26 (95% CI 1.00-1.59) for breast cancer, with a total of 290 cases. Absolute excess risks per 10000 person-years attributable to estrogen plus progestin were 8 more invasive breast cancers(129). Additionally, while mammographic density was not routinely measured, Chlebowski et al reported that in the Women's Health Initiative mammographic abnormalities occurred in 9.4% of the estrogen plus progestin group versus 5.4% in the placebo group, $p < .001$(34).

Another study investigated the effects of specific types of HT on incident and fatal breast cancer. In the Million Women Study, 1,084,110 UK women aged 50-64 years were recruited between 1996 and 2001. Of those recruited 50% had used HT; 9364 incident invasive breast cancers and 637 breast cancer deaths were registered after an average of 2.6 and 4.1 years of follow-up, respectively. Current users of HT at recruitment were more likely than never users to develop breast cancer (adjusted relative risk=1.66, 95% CI 1.58-1.75, $p<0.0001$) and die from it (RR=1.22, 95% CI 1.00-1.48, $p=0.05$). Past users of HT were, however, not at an increased risk of incident or fatal disease (RR=1.01, 95% CI 0.94-1.09 and RR=1.05, 95% CI 0.82-1.34, respectively). Incidence was significantly increased for current users of preparations containing estrogen only 1.30 (95% CI 1.21-1.40, $p<0.0001$), estrogen-progestin 2.00 (95% CI 1.88-2.12, $p<0.0001$), and tibolone 1.45 (95% CI 1.25-1.68, $p<0.0001$), but the magnitude of the associated

risk was substantially greater for estrogen-progestin than for other types of HT ($p<0.0001$). In current users of each type of HT, the risk of breast cancer increased with increasing total duration of use(89).

Current use of HT and duration of use for former HT users, appears to be associated with an increased risk of breast cancer and the effect is substantially greater for estrogen-progesterone combinations than for other types of HT.

1.11. Breast Density, Mammographic Screening Sensitivity and Breast Cancer Risk

The histologic composition of the breast is reflected mammographically by density and parenchymal pattern. 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 (21, 52, 94, 121). At menopause, glandular and ductal tissue decreases and fibrous connective tissue is usually replaced by fat, explaining the decrease in mammographic density that occurs with age(2, 26, 57, 59, 74, 104, 119, 122, 123). Breast density is one factor shown to affect mammographic sensitivity(72, 81) and specificity(10, 50, 72, 81, 124, 127), and it is predictive of breast cancer risk(4, 13, 28, 29, 44, 53, 55, 58, 61, 71, 93, 104, 114, 122, 124, 127).

In 1976, the first method to associate breast parenchymal patterns and breast cancer risk was proposed by Dr. John Wolfe. His classification consisted of four patterns: N1-radiolucent breast, low risk, P1-linear radiographic densities or ductal prominence of lesser extent than P2, intermediate risk, P2-ductal prominence to a greater extent, intermediate risk, and DY-radiographically dense, risk highest(14, 128). Several studies have used Wolfe's classification to measure breast cancer risk associated with mammographic density (Table 1). In 3 cohort studies, the DY pattern was associated with an increased risk of breast cancer when compared with the

N1 pattern. To date there have been several studies which have utilized Wolfe's method to assess mammographic density. A statistically significant increase in breast cancer risk was associated with increased density in thirteen of the fifteen cohort or case-control studies nested within cohort studies and fifteen of the nineteen case-control studies (OR 1.4-6.2)(14, 29, 36, 43-45, 55, 71, 74, 75, 90, 102, 113, 115, 116, 125). No association between breast cancer risk and density was found among six cross-sectional studies. However, this may reflect a difference in the cancer detection time among the different parenchymal patterns(14-16, 18, 23-26, 30, 32, 44, 49, 58, 64, 66, 73, 75, 88, 98, 100, 113, 115, 120, 124).

Table 1-1: Wolfe's studies

Reference	Type	Subjects	Results
Brisson 1982	Case-control	408 Cancer 1021 Controls	RR DY vs N1 1.9 (1.1-3.3)
Chaudry 1983	Case-Control	104 Cancer 937 Controls	OR DY vs N1 1.4
Carlile 1985	Case-Control	706 Cases 1412 Controls	OR DY vs N1 3.1
Saftlas 1989	Case-Control	266 Cases 301 Controls	OR DY vs N1 2.5
Tabar & Dean 1982	Prospective	1857/21,157 screen 31 incident cases	RR DY vs N1 Prevalent 2.9 Incident 6.2 Age 60+ 0.97
Gravelle 1986	Prospective	4,044 women 31 cancer	RR DY vs N1 4.4 (0.54-36.7)
De Stavola 1990	Prospective	4,044 women 69 cancer	RR P2/DY vs P1/N1 1.7 (0.72-4.0)

Despite the reported association between mammographic density and risk of breast cancer, there is great disparity among the studies with respect to the risk estimates when Wolfe's classification is utilized. It is believed that is largely due to the variation in observer assessments

of mammographic density. Additionally, variations in study design have led to varying risk estimates.

In an effort to reduce observer variability, various methods have been developed to quantitatively assess mammographic parenchymal patterns. These methods encompass visual estimation of dense tissue, digitized images utilizing computer-assisted methods, and planimetry to measure the area of density within the total breast area. To date a total of seventeen studies consisting of 7,410 cases and 14,421 controls (OR 1.2-6.0) have been published utilizing quantitative measurement (Table 2).

Table 1-2: Quantitative Studies

REFERENCE	Type	Method	Results
Boyd 1982	Case-control	Estimation	OR 6.0 (2.5-14.1)
Brisson 1982	Case-control	Estimation	OR 5.4 (2.5-11.4)
Brisson 1984	Case-control	Estimation	OR 4.4 (2.5-7.9)
Brisson 1989	Case-control	Estimation	OR 4.6 (2.4-8.5)
Wolfe 1987	Case-control	Planimetry	OR 4.3 (1.8-10.4)
Saftlas 1991	N. Case-control	Planimetry	OR 4.3 (2.1-8.8)
Boyd 1995	N. Case-control	Estimation/computer	OR 6.0 (2.8-13.0)
Kato 1995	N. Case-control	Planimetry	OR 3.6 (1.7-7.9)
Byrne 1995	N. Case-control	Planimetry	OR 4.3 (3.1-6.1)
Lam 2000	N. Case-control	BIRADS	OR 4.5 (1.9-10.6)
Van Gils 1999	N. Case-control	Computerized (automated)	OR 3.3 (1.5-7.2)
Maskarinec/Meng 2000	Case-control	Computerized (thresholding)	OR 1.8 (1.1-3.0)
Ursin 2003	Case-Control	Computer-assisted	OR 5.2 (1.7-16.1)
Torres-Mejia 2005	Cohort	Computer-assisted	OR 3.5 (1.4-5.2)
Thomas 2002	Case-Control	Planimetry	OR 4.4 (3.0-6.7)
Maskarinec 2005	N. Case-Control	Computer-assisted	OR 1.2 (1.11-1.24)

In both case-control(23, 30, 32, 49, 103) and cohort studies(55, 90, 113, 115), increased breast density as determined by Wolfe's method has been associated with increased breast cancer risk (ORs ranging from 1.4-6.2). Similarly, quantitative methods have shown an increased

association (ORs ranging from 2.0-3.8)(26) (12, 23, 25, 29, 29, 71, 103). 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(23, 25, 26, 26).

In a recent analysis, Brisson and colleagues sought to compare Wolfe's method with percent density to determine if there was any added benefit when using both to determine density and subsequent breast cancer risk. They looked at 3 case-control studies(23) (25, 26)for a total of 1060 newly diagnosed unilateral cases and 2352 controls who had undergone a routine screening mammogram. The percent density was scored in categories and the original four categories were utilized for Wolfe's method. When comparing percent density of 85% or greater to women with zero density, there was a 5-6 fold increase risk of breast cancer. However, with Wolfe's method, only a 2-3 fold increase was noted in women with P2 or DY compared to N1 patterns. Additionally, among those women with P2 or DY, the RR varied greatly with percent density. Conversely, there was little variation in the RR with the corresponding Wolfe pattern when given a percent density value(22).

1.12. Factors that affect breast density

1.12.1 Age and Menopausal Status

Typically, breast density decreases with postmenopausal status and increasing age(71). However, Byrne et al(29) found a greater effect of breast density on breast cancer risk in postmenopausal women OR 5.8 (95% CI 3.0-11.3) than in premenopausal women OR 3.8 (95% CI 2.3-6.2). Similarly, Boyd et al(12) found a higher risk in women ages 50-59 with a RR 7.1 (95% CI 2.0-25.5) than in to women ages 40-49 RR 6.1 (95% CI 1.5-24.2). The overlap in the CIs indicates that the association between breast density and cancer risk may not depend on

menopausal status or age(61). Therefore, the association between breast density and age is most likely attenuated by other breast cancer risk factors(60).

1.12.2 Breast Density, and Breast cancer Risk

HT has been shown to increase breast density in some, but not all women(9, 38, 48, 77, 78, 83, 84, 86, 97, 99, 112), although this association is attenuated when HT is discontinued(9, 62, 95, 105). Exactly which women will experience an increase in risk with HT use remains unknown. Age may be one determining factor. In one study of HT and breast density(111), there were no differences in breast density between HT users and non-users younger than age 55; in women over 55, the density was significantly greater in HT users. HT regimen also affects breast density. In the PEPI Trial(56), a double-blinded placebo controlled trial of HT, estrogen-progestin users had a greater increase in breast density than estrogen only users (24% verses 8%). Similarly, McTiernan et al(86)reported an increase in mammographic density most notably from baseline to year one. At year two the median increase in breast density percent persisted with a slight attenuation yielding an overall absolute increase in percent density of 4.9%. Other studies(80, 83, 96)confirm this finding. Additionally, the use of low dose HT(35) and transdermal HT(60) is associated with a smaller increases in mammographic density.

1.13. Estrogen Metabolism

Although the evidence linking estrogen and breast cancer is compelling(37), there is substantial evidence that the way estrogen is metabolized is associated with the risk of breast cancer. Estradiol metabolism is predominantly oxidative. Estradiol is first (reversibly) converted to estrone, which is irreversibly converted to either 2- or 16α-hydroxy estrone in order to eliminate it from the body. Both 2- and 16α-OH estrone have estrogenic properties.

In both case-control studies(39, 63, 69, 70, 130) and a prospective study(87), higher levels of 16α-OH, the more active metabolite, are associated with increased risk. Conversely, higher levels of 2-OH, the less active and non-genotoxic metabolite, are associated with reduced risk(39, 63, 69, 130). Because the 2-OH and 16α-OH metabolites compete for a limited substrate pool, a rise in one pathway will reduce the amount of product in the competing pathway. Thus, the relative activity of these two metabolic pathways (2:16α-OH) may be an endocrine biomarker for breast cancer risk.

In a study of 513 nulliparous women(68), aged 17-35, lifestyle factors, such as ethnicity, body size, age at menarche, oral contraceptive use, smoking, vegetarian diet, coffee and alcohol consumption were evaluated with respect to the 2-OHE/16alpha-OHE ratio in plasma. Among oral contraceptive users, there was a significantly lower 2-OHE/16alpha-OHE ratio than OC non-users, and among non-OC users, Asian women had significantly lower 2-OHE/16alpha-OHE ratios than white women which remained after adjustment for age and day of menstrual cycle. Among women not using oral contraceptives, the median 2-OHE/16alpha-OHE ratio in plasma was similar across all ethnic groups even after adjusting for age and menstrual cycle phase. Daily coffee consumption was significantly positively correlated with 2-OHE/16alpha-OHE ratios ($r(s) = 0.18$, $P = 0.002$) only among OC non-users. The study findings suggest that the plasma 2-OHE/16alpha-OHE ratio is associated with constitutional factors and with modifiable lifestyle factors. Additionally, modulation of estrogen metabolism to favor the less genotoxic metabolite 2-OH through physical activity was reported by Bentz et al(7).

In a nested case-control study among10,786 women ages 35-69 years enrolled in the Hormones and Diet in the Etiology of Breast Cancer (ORDET) Study, 67 pre-menopausal cases, 264 matched controls and 71 post-menopausal cases, 274 matched controls were evaluated for

the association of breast cancer risk with estrogen metabolism, specifically the ratio of 2-OH to 16α-OH. Among premenopausal women, a higher ratio of 2-OH to 16α-OH at baseline was associated with a reduced risk of breast cancer: women in the highest quintile of the ratio had an adjusted odds ratio (OR) for breast cancer of 0.58 (95% CI 0.25-1.34). The corresponding adjusted OR in postmenopausal women was 1.29 (95% CI 0.53-3.10). These results support the hypothesis that the estrogen metabolism pathway favoring 2-hydroxylation over 16alpha-hydroxylation is associated with a reduced risk of invasive breast cancer risk in premenopausal women(91).

In the Guernsey Study(87), a prospective study, postmenopausal women not using HT who went on to develop breast cancer over a 19-year period had about a 15% lower 2:16α-OH urinary metabolite ratio compared to matched control subjects, although the results were not statistically significant. However, not all studies have found a relationship between estrogen metabolite levels and breast cancer risk. In a case-cohort study conducted by Cauley et al the 2-OHE/16alpha-OHE ratio did not predict breast cancer risk(31).

1.13.1 Estrogen Metabolism and Breast Density

To date there has been one study that has evaluated the relationship between estrogen metabolism and breast density. Riza et al evaluated the role of estrogen metabolites with respect to their relationship with high-density Wolfe mammographic parenchymal patterns (P2/DY). The study was nested within a large cross-sectional survey on determinants of mammographic patterns carried out in a population-based breast screening program in Northern Greece. Urinary levels of 2-OHE1 and 16(alpha)-OHE1 were measured in a random sample of 70 postmenopausal women with P2/DY mammographic patterns and in a random sample of 70 women with N1 mammographic patterns, individually matched to the P2/DY women on year of

birth, years since menopause and date of urine collection. Women with a P2/DY pattern had 58% higher levels of 2-OHE1 (P = 0.002) and 15% higher levels of 16α-OHE1 (P = 0.37) than those with an N1 pattern. The ratio of 2-OHE1:16α-OHE1 was 35% higher (P = 0.005) in women with a P2/DY pattern. Women in the highest one-third of this ratio were six times more likely to have a P2/DY pattern than those in the lowest one-third after adjusting for potential confounders (prevalence odds ratio, 6.2; 95% CI, 1.7-22.9; test for linear trend, P = 0.002). These findings seem to suggest that a high, rather than a low, 2-OHE1:16α-OHE1 ratio may be associated with an increase in breast cancer risk in postmenopausal women(101).

1.13.2 Estrogen Metabolism, HT and the Risk of Breast Cancer

The effect of secreted or administered estrogen depends on the balance between these metabolic pathways(132), and exogenous estrogens may alter this balance. In particular, combined estrogen-progestin (E+P) regimens may cause a greater shift to the 16-OH pathway compared to estrogen-alone (E)(108). This observation may explain in part the observed greater breast cancer risk observed with combined HT regimens beyond that observed with estrogens alone.

1.14. Estrogen/Progesterone Receptors

In a study by Cotterchio et al(41), the relationship between hormonal factors and estrogen receptor (ER) and progesterone receptor (PR) status was evaluated in two recent population-based case-control studies. Breast cancer cases, ages 25-74 years diagnosed 1995-1998, were sampled from the Ontario Cancer Registry. Controls were frequency-matched to cases within 5-year age groups. ER/PR data was available for 87% of the breast cancer cases. Significant differences were observed in the risk factor profiles for ER+PR+ and ER-PR- breast cancer. Among premenopausal women, late age at menarche was only associated with a reduction in

ER+PR+ breast cancer risk; obesity was associated with an increased ER-PR- and decreased ER+PR+ cancer risk; and the association between alcohol intake and breast cancer risk was heterogeneous across ER/PR subgroups, although the direction varied across the levels of alcohol intake. Among postmenopausal women, there were no statistically significant differences observed in the risk factor profiles for ER+PR+ and ER-PR- breast cancer. In a report by Ziv et al(131), high mammographic density was associated with an increased risk of ER-positive and ER-negative breast cancers.

Evidence suggests hormonal factors may be more strongly associated with ER+PR+ than ER-PR- breast cancer risk. Measures of estrogen metabolism have not been studied in relation to type of breast cancer, that is, breast cancer according to receptor status.

1.15. Research Questions

First: What are the determinants of serum sex hormones and estrogen metabolite levels in postmenopausal women in the Study of Osteoporosis and Fractures (SOF)? In the first article, we evaluated the relationship between estrogen metabolite levels and breast cancer risk factors, independent of sex-steroid hormones, in an attempt to provide insight into the underlying biologic mechanisms.

Second: Is there a relationship between breast density and estrogen metabolism? Breast density and measures of estrogen metabolism (blood and urinary levels of 2-hydroxyestrone (2-OH) and 16alpha-hydroxyestrone (16α-OH)) have been studied in relation to breast cancer risk. In the second article, we endeavored to characterize the relationship between breast density and the serum concentrations of two major estrogen metabolites (2-OH and 16α-OH) in pre- and post-menopausal women without breast cancer.

Third: What is the relationship between breast density, estrogen metabolism and the risk of breast cancer? The objective of the third paper was to compare the joint distribution of density and metabolites between cases and controls and calculated associated measures of risk association, unadjusted and adjusted for other traditional or conventional breast risk factors.

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2. FIRST PAPER: Determinants of Estrogen Metabolite Levels in Postmenopausal Women

(To be submitted for publication)

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2.1. Abstract

BACKGROUND: Estrogen metabolites and sex-steroid hormones have been shown to be related to the risk of breast cancer. In the current report, we evaluated the relationship between estrogen metabolite levels and breast cancer risk factors, independent of sex-steroid hormones, in an attempt to provide insight into the underlying biologic mechanisms.

METHODS: We analyzed data from 282 randomly selected women without breast cancer, enrolled in the Study of Osteoporotic Fractures (SOF), a longitudinal cohort study.

RESULTS: 16 alpha-hydroxyestrone (16α-OH) was positively associated with body mass index (BMI) (r=0.162), estradiol (r=0.171), and testosterone (r=0.158) and inversely associated with sex hormone binding globulin (SHBG) (r=-0.165), all (p=0.01). However, these associations were not significant in multivariate analyses that controlled for the serum sex-steroid hormone concentrations (total estradiol, total testosterone, SHBG). Neither 2-hydroxyestrone (2-OH) nor the 2:16 ratio was related to the sex-steroid hormones. Women who reported a surgical menopause were significantly more likely to have higher levels of 16α-OH (OR=(tertile 3 vs tertile 1) 7.37, 95% Confidence Interval (CI) 2.20-24.70) but there was no difference in 2-OH levels.

CONCLUSION: Except for BMI, 16α-OH, and surgical menopause, we did not see associations between the estrogen metabolites and the traditional risk factors. Control for substrate hormones eliminated the BMI association, but not the association with type of menopause. While it appears that the substrate hormone levels appear to mediate the association between BMI and 16α-OH, the apparent lack of residual association between BMI (and other risk factors) with 16α-OH or 2-OH suggests that inter-individual differences in hormone metabolism, per se, are insensitive to external factors.

Key Words: Breast Cancer, Sex-Steroid Hormones, Estrogen Metabolism, Postmenopausal women

2.2. Introduction

Although the evidence linking estrogen and breast cancer is compelling(9), there is substantial evidence that estrogen metabolites may be associated with the risk of breast cancer. Estradiol metabolism is predominantly oxidative. In order to eliminate estradiol from the body it is first (reversibly) converted to estrone, then is irreversibly converted to either 2-hydroxyestrone (2-OH) or 16alpha-hydroxyestrone (16α-OH). Both 2-OH and 16α-OH have estrogenic properties.

In case-control studies(10, 15, 18, 31) and a prospective study(22), higher levels of 16α-OH, believed to be the more active and genotoxic metabolite, are associated with increased breast cancer risk. Conversely, higher levels of 2-OH, the less active and non-genotoxic metabolite, are associated with reduced risk(10, 15, 18, 31). Because enzymes responsible for the conversion of 2-OH and 16α-OH metabolites compete for a limited substrate pool, increased activity in one pathway will reduce the amount of product in the competing pathway. However, not all studies have found a positive association. We measured serum estrogen metabolites in a case-cohort study of 272 women with confirmed incident breast cancer and 291 random controls. The risk of breast cancer in women with the highest quartile of the 2:16α-OH ratio compared with those in the lowest quartile was 1.17 (95% confidence interval (CI)= 0.73-1.87) and therefore did not support the hypothesis that the ratio of 2-OH to 16α-OH predicts breast cancer risk. Thus, the relative activity of these two metabolic pathways (2:16α-OH) as an endocrine biomarker for breast cancer risk deserves further study.

This metabolic pathway appears to vary by ethnicity and various lifestyle factors. In a study of 513 nulliparous women, aged 17-35, lifestyle factors (ethnicity, body size, age at menarche, oral contraceptive (OC) use, smoking, vegetarian diet, coffee, alcohol consumption) were evaluated with respect to the 2-OH:16α-OH ratio in plasma. There was a significantly

lower 2-OH:16α-OH ratio in OC users versus non-users, and Asian OC users had significantly lower 2-OH:16α-OH ratios than white OC users which remained after adjustment for age and day of menstrual cycle. No significant difference was noted across ethnic groups in non-users. Daily coffee consumption was significantly positively correlated with 2-OH:16α-OH ratios (r(s) = 0.18, P = 0.002), but only among OC non-users. The study findings suggest that the plasma 2-OH:16α-OH ratio is associated with constitutional factors and with modifiable lifestyle factors(17).

In the current manuscript, we examined the relationship of breast cancer risk factors (age, weight, body mass index, age at menarche, age at first birth, age at menopause, family history of breast cancer, history of fibrocystic disease, alcohol consumption), additional lifestyle factors (smoking, exercise), sex steroid hormones, and estrogen metabolites in breast cancer-free postmenopausal women at risk from the the Study of Osteoporotic Fractures (SOF)cohort(7). To our knowledge, the association of sex-steroid hormones, estrogen metabolites, and breast cancer risk factors has not been reported. The long range goal of the analysis is to gain a better understanding of the underlying biologic mechanisms guiding the relationship between the active hormones, metabolites, and breast cancer risk factors.

2.3. Materials and Methods

2.3.1 Study Population

All women in this study were participants in SOF, a longitudinal study that evaluated risk factors for osteoporosis and falls in 9,704 White women age 65 and older. They were recruited from 1986-1988 using population-based lists in Baltimore, MD, Pittsburgh, PA, Minneapolis, MN, and Portland, OR. The complete study design and methodology have been described in previous

publications(11). Previous case-cohort studies within SOF have examined estrogen metabolites and breast cancer risk(7) and the role of sex hormones and breast cancer(6, 8). Breast cancer free controls (over 8.7 years) were randomly chosen using a case-cohort approach. Women with prevalent breast cancer or reported use of hormone therapy at baseline were excluded (7). We included only those cancer free controls who participated in the estrogen metabolite study and who also had available sex serum hormones. Analyses included nine women missing estradiol, one missing testosterone, and one missing testosterone and SHBG for a total sample N=282. The institutional review boards at each institution approved the study and informed consent was obtained from all women.

2.3.2 Data Collection

All women underwent an interview and were given a self-administered questionnaire at each bi-annual clinic visit. Reproductive history and anthropometric measurements were obtained at baseline via the interview and questionnaire process and were utilized in this analysis. Breast cancer risk factors included age at menopause, age at menarche, age at first full term birth, parity, weight, body mass index (BMI) and alcohol use(drinks per week). Categorical variables included type of menopause (surgical/natural), ever pregnant (yes/no), family history of breast cancer (yes/no), history of benign breast disease (yes/no), walks for exercise (yes/no), past estrogen use (yes/no), and ever smoke (ever/never).

2.3.3 Laboratory Measurements

Serum was collected at baseline and was used to assess total estradiol, total testosterone, sex hormone binding globulin (SHBG), 2-OH, and 16α-OH. All women were instructed to adhere to a fat free diet the evening before and the morning of the blood draw to minimize lipemic sera. The blood was drawn between 8:00 AM and 2:00 PM and was frozen to –20^{o}C. All samples

were shipped to a central repository within 2 weeks where they were stored at –190°C until assay.

The 2-OH and 16α-OH estrogen metabolite levels were measured by Immuna Care Corporation (Bethlehem, PA) with the ESTRAMET 2/16 enzyme immunoassay kits (ELISAs) using blinded serum samples(7, 20). The ESTRAMET 2/16 ELISA was previously validated against gas-chromatography-mass spectroscopy (GC-MS) in pooled serum with known amounts of 2-OH and 16α-OH(7). The sensitivity of the 2-OH and 16α-OH assays is approximately 20 pg/mL and 10 pg/mL, respectively. Variability of within-assay duplicates for positive control sera for these serum samples were less than 5% and the between-assay variability was less than 15% for both estrogen metabolite assays. To assess reproducibility serum levels of 2-OH and 16α-OH from 25 postmenopausal women measured in blind duplicate in different batches were measured yielding a correlation of r=.98(7).

The Endocrine Sciences (Calabassas, CA) and Corning Nichols Institute (San Juan Capistrano, CA) were utilized for analysis of the sex-steroid hormones. Total estradiol was measured using liquid-liquid organic extraction, column chromatography and radioimmunoassay (RIA), (intra- and inter- assay variability, 4-12% and 6-12%, respectively; sensitivity of 2 pg/ml)(8). Total testosterone was measured by using radioimmunoassay with chromatographic purification (coefficient of variation for intra-assay and total assay, 6% to 14% and 5% to 13%, respectively; sensitivity, 0.03 nmol/L). Sex hormone-binding globulin was measured by using radioimmunoassay (coefficient of variation for intra-assay and total assay, 7% and 7.8% respectively; sensitivity, 5.0 nmol/L) (8).

2.4. Statistical Analysis

Demographic characteristics of the study sample were compared to the remainder of the SOF population (Table 1) using t-tests for independent samples (continuous variables) and chi-square test (categorical variables). Estrogen metabolite levels and sex-steroid hormones were logarithm-transformed to normalize the values for all analyses. All hormone levels were within the assay range. Log transformed values were back-transformed to present the geometric means in their initial units of measure. Pearson correlation coefficients were calculated to determine significant relationships between sex steroid hormones, estrogen metabolite levels, anthropometric measurements, and reproductive factors. Variables with a significant (p-value <0.10) univariate association with target hormone levels were further evaluated with logistic regression. We used logistic regression to examine the independent association between the level of a particular estrogen metabolite and various factors including hormones, binding protein, and behavioral/reproductive breast cancer risk factors. Analyses were conducted with a base model consisting of standardized log transformed estradiol, testosterone, and SHBG. Age, BMI, and other statistically significant variables ($p <0.10$), identified in univariate analyses were added to the base model singly, to evaluate the metabolite levels in mid versus low tertile as well as high versus low tertile. The Hosmer-Lemeshow goodness of fit was assessed for all models. All tests of statistical significance were two-tailed. Probability values of ≤ 0.05 were considered statistically significant.

All analyses were conducted using SPSS for windows, version 11.0.

2.5. Results

Descriptive characteristics of the study population compared to the remainder of the SOF cohort are shown in Table 1. Women included in this study ranged in age from 65-86 (mean 71.49) and 18.6% were nulligravid. Among parous women, the average age at first birth was 25 years (SD=4.8). The study sample was essentially identical to the remaining SOF participants.

Correlations between the hormones and estrogen metabolites are shown in Table 2. All were statistically significant, with the exception of the correlations involving the primary hormones and 2-OH and the 2:16 ratio. Among the estrogens, SHBG was negatively correlated with estradiol (r=-0.287, p=0.01) and 16α-OH (r=-0.165, p=0.01). The 16α-OH metabolite levels positively correlated with estradiol and testosterone whereas the 2-OH was not correlated with estradiol or SHBG. Additionally, the 2:16 ratio correlated with its components 2-OH and 16α-OH, but not estradiol (r=-0.076, p=0.213), SHBG (r=0.058, p=0.337), or testosterone (r=-0.092, p=0.126).

In univariate analyses, weight, BMI, and surgical menopause were found to be significantly associated with 16α-OH levels (Tables 3 and 4). However, the associations between weight and 16α-OH and BMI and 16α-OH disappear in multivariate analyses controlling for estradiol, testosterone, and SHBG. With the exception of surgical menopause, no variables were significantly associated with either the 2-OH levels (Tables 3 and 4) or 2:16 ratio (Tables 3 and 4).

In logistic regression models, a one standard deviation unit increase in log transformed testosterone increased the likelihood of being in the high tertile vs low tertile 16α-OH by 1.62 fold while a one standard deviation unit increase in SHBG resulted in a decreased likelihood (OR=0.64) of having a 16α-OH in the high vs the low tertile (Table 5). Surgical menopause

increased the likelihood of high tertile vs low tertile 16α-OH by 7-fold (OR= 7.37, p<0.001). The geometric mean 16α-OH level in women with surgical menopause was 14% greater than women with natural menopause (254 pg/mL versus 222 pg/mL, $p < 0.05$). After adjusting for estradiol, testosterone, and SHBG, the geometric mean 16α-OH is 20% higher in women with surgical menopause compared to those with natural menopause (263 pg/mL vs 220 pg/mL) (95% CI ratio of 9-33% ($p < 0.05$)).

In multivariate models, no factors were found to be associated with 2-OH levels (Table 5).

2.6. Discussion

We observed significant associations between the sex-steroid hormones and estrogen metabolite levels. The relationship between weight and 16α-OH and BMI and 16α-OH were statistically significant in univariate analyses. However, in multivariate analyses, these relationships disappeared once we controlled for the sex-steroid hormones. In general, there were no significant relationships between the putative breast cancer risk factors and the 2-OH levels and the 2:16 ratio.

Previous studies have focused primarily on differences in estrogen metabolism across case-control status rather than the relationship between the estrogen metabolites and lifestyle and reproductive factors(15, 18, 25, 30, 31). Additionally, there is a paucity of data incorporating serum estrogen metabolite levels along with sex-steroid hormones as potential mediators of breast cancer risk.

Prior studies have focused on breast cancer risk relationships involving either reproductive and lifestyle factors, sex-steroid hormones, or lastly estrogen metabolites. While

each of these factors (risk factors, sex-steroid hormones, estrogen metabolites) singly have been implicated in increased breast cancer risk, the interaction between the circulating hormones, estrogen metabolites, and breast cancer risk factors remains unclear. This dataset allowed the opportunity to examine the relationship among these factors in an attempt to better understand the biologic model at work.

A significant positive relationship was seen between 16α-OH and testosterone and 16α-OH and estradiol, while a significant inverse relationship was noted between 16α-OH and SHBG. Estradiol, testosterone and SHBG have been associated with breast cancer risk. In a reanalysis of nine prospective studies, testosterone has been associated with a 2-3 fold increase in relative risk of breast cancer for women with levels in the highest versus lowest quintile(27), with estrogen receptor (ER) positive breast cancer(3), and most recently with breast cancer recurrence(4). Evidence increasingly supports the relationship between estrogen and breast cancer risk(10, 29) with a 3.6-fold increased risk of breast cancer in those with a high serum concentration versus low concentration of bioavailable estradiol in a previous SOF analysis(8). In a follow-up report with a larger sample, the association between estradiol and breast cancer was not significant after controlling for testosterone(3). Prospective studies have identified an inverse relationship between SHBG and breast cancer risk(12, 14, 28).

In univariate analyses, we observed significant relationships between 16α-OH weight, and BMI. Aromitazation of androstenedione to estrone in fatty tissue has been implicated as the source of estrogen and contributes to the increased breast cancer risk in women who are obese or experience weight gain throughout life(8, 21, 26). In our analyses, the association between weight and BMI disappeared after controlling for estradiol, SHBG, and testosterone suggesting that obesity is influencing 16α-OH through mechanisms independent of aromitazation. In a

study of 62,756 postmenopausal women in the Cancer Prevention Study-II Nutrition Cohort with 1,934 incident breast cancer cases, the association of BMI and adult weight gain (since age 18 years) with breast cancer risk was reported. Additionally, the analyses were stratified by hormone therapy (HT) use. Total adult weight gain strongly predicted breast cancer risk among former and never HT users (*P* for trend < 0.0001). Weight gain of 21–30 pounds was associated with a rate ratio of 1.4 (95% confidence interval 1.1–1.8); rates doubled among women gaining >70 pounds compared with women who maintained their weight within 5 pounds of their weight at age 18. Among current HT users, no association was seen between breast cancer and either BMI or weight gain(13). Additionally, we know that obesity has been associated with testosterone(6)and body weight(19) in women. In our analyses, we observed a borderline relationship between testosterone and BMI (p=0.069).

In our analyses, we found no relationships between traditional risk factors (age, age at menarche, age at first birth, age at menopause) and the estrogen metabolite levels. This may have reflected the small sample size but it is possible that these factors influence breast cancer through mechanisms that do not involve estrogen metabolites.

We found a significant positive relationship between surgical menopause and the 16α-OH metabolite, independent of estradiol, testosterone, and SHBG. Women who had a surgical menopause had seven times the odds of having a 16α-OH level in the high versus low tertile (OR=7.37, p<0.001). Indications for surgical menopause often include endometriosis, premenstrual syndrome, and fibroids. Many of these processes may result from abnormal hormone regulations, including estrogen(2, 5, 23). In a study of 15,844 women, a non-significant increase in breast cancer risk was noted in women who underwent gynecologic surgery for endometriosis(24). It has also been postulated that the exposure to estrogen between

menarche and first live birth is the critical period as breast tissue is most actively copying DNA and cells dividing during that life phase(1, 16). Hence, the higher 16α-OH associated with surgical menopause may reflect the underlying hormone dysregulation associated with the indication for the hysterectomy.

This study has several strengths. The study population consists of well-characterized women who were cancer free over an 8.5-year period. Standardized data were collected independent of breast cancer outcomes and laboratory results. Additionally, with an age range of 65-86 (mean 71), this allows insight into an aged population that few studies can report.

Limitations include the use of all white women limiting the ability to apply these results across diverse populations. Self-administered questionnaires have the potential for recall bias. Lastly, issues with data include colinearity of hormones and a relatively small sample size.

These same factors (weight, BMI, estradiol, testosterone, SHBG, and surgical menopause) were not related to 2-OH nor to the 2:16 ratio. It is believed that the 2-OH levels can be increased by changes in dietary habits, thereby increasing the 2:16 ratio and decreasing the risk of breast cancer. What remains unclear is whether or not these lifestyle changes can alter the 16α-OH and subsequently result in decreased 16α-OH levels, increased 2:16 ratio and decreased breast cancer risk.

Except for BMI, 16α-OH, and surgical menopause, we did not see associations between the estrogen metabolites and the traditional risk factors. Control for substrate hormones eliminated the BMI association, but not the association with type of menopause. While it appears that the substrate hormone levels appear to mediate the association between BMI and 16α-OH, the apparent lack of residual association between BMI (and other risk factors) with 16α-OH or 2-OH suggests that inter-individual differences in hormone metabolism, per se, are

insensitive to external factors. The strong relationship between surgical menopause and higher levels of 16α-OH deserves further attention. It is unknown if this reflects the indication for the hysterectomy or the lack of ovaries post-menopausally.

Table 2-1: Baseline characteristics of the SOF population and study subjects sampled from SOF

(excluding participants with prevalent breast cancer or self-reporting HT use)

	N	Sample(SD)	N	SOF (SD)	p-value[¶]
Mean age in years	279	71.49 (4.82)	7419	71.80 (5.33)	0.08
Mean body weight in kg	279	67.47 (12.01)	7291	67.51 (12.74)	0.96
Mean body mass index (kg/m^2)	279	26.65 (4.50)	7291	26.72 (4.76)	0.81
Mean age at menarche in years	259	13.13 (1.58)	6619	13.07 (1.48)	0.58
Mean age at first birth in years[†]	213	25.03 (4.84)	5541	25.33 (4.95)	0.40
Mean age at menopause in years	232	48.88 (4.79)	6082	47.89 (5.78)	0.10
% with Surgical menopause	241	11.20	6323	11.05	0.92
% Nulliparous	279	18.64	7288	16.56	0.37
% with Family history of breast cancer	237	17.72	7570	15.22	0.37
% with History of fibrocystic disease	262	11.83	6787	12.80	0.71
% with Past estrogen use	273	31.14	7289	32.10	0.79
Mean Alcohol (drinks/week)	279	1.48 (3.04)	7291	1.90 (4.18)	0.10
% Current smoker	278	6.83	7265	10.00	0.15
% who Walk for exercise	279	54.84	7290	49.45	0.09

SD=Standard Deviation

[†]Among parous women

HRT=Hormone Replacement Therapy

[¶]t-test for continuous measures, chi-square for categorical measures; Statistical test based on comparison of SOF subjects sampled vs those not sampled

Table 2-2: Pearson Pairwise correlations involving serum-based sex steroid hormone and estrogen metabolite measures[†]

	Estradiol	SHBG	2-OH	16α-OH	2:16 Ratio
Testosterone	0.323*	0.160*	0.019	0.158*	-0.092
Estradiol		-0.287*	0.046	0.171*	-0.076
SHBG			-0.086	-0.165*	0.058
2-OH				0.220*	0.661*
16α-OH					-0.538*

* Correlation is significant at the 0.01 level (2-tailed)
SHBG=Sex Hormone Binding Globulin
Sample of SOF participants (N=282)
2-OH=2-hydroxyestrone
16α-OH=16α-hydroxyestrone
[†]Sample counts vary between N=272-282 due to missing values.

Table 2-3: Simple and partial correlations between risk factor variables and estrogen metabolite measures (adjusted for log transformed estrogen, testosterone, and SHBG)

Variable	N	2-OH		16α-OH		2:16 Ratio	
		Simple Correlation	Partial Correlation	Simple Correlation	Partial Correlation	Simple Correlation	Partial Correlation
Age	282	0.054	0.081	0.058	0.081	0.013	0.081
Weight	282	0.046	-0.019	**0.122***	-0.019	-0.055	-0.019
Body Mass Index	282	0.044	-0.014	**0.162****	-0.014	-0.093	-0.014
Age at Menarche	260	-0.116	-0.123	-0.061	-0.123	-0.065	-0.123
Age at first birth	214	-0.048	-0.039	0.062	-0.039	-0.049	-0.039
Age at menopause	235	0.048	0.005	-0.066	0.005	0.062	0.005
Drinks/week	282	0.014	0.005	-0.024	0.005	-0.007	0.005

*Correlation significant at the 0.05 level (2-tailed)
**Correlation significant at the 0.01 level (2-tailed) Abbreviations-refer to table 2

Table 2-4: Geometric mean 2-hydroxyestrone (2-OH), 16α-hydroxyestrone (16α-OH), 2:16-OH ratio according to breast cancer risk factors.

Unadjusted and adjusted for estradiol, testosterone, and SHBG

			2-OH		16α-OH		2:16 Ratio	
			Unadjusted	Adjusted	Unadjusted	Adjusted	Unadjusted	Adjusted
Variable		**N**	**Geometric Mean (pg/ml)**	**Geometric Mean (pg/ml)**	**Geometric Mean (pg/ml)**	**Geometric Mean (pg/ml)**	**Geometric Mean (pg/ml)**	**Geometric Mean (pg/ml)**
Menopause	Natural	234	170	166*	222*	220*	0.75	0.73*
	Surgical	25	172	199	254	263	0.67	0.87
Ever	No	51	182	177	235	235	0.76	0.77
pregnant	Yes	218	167	168	224	224	0.74	0.73
Family	Yes	41	168	163	218	217	0.75	0.71
history of	No	228	170	171	227	228	0.74	0.75
breast cancer								
History of	Yes	31	181	166	221	220	0.82	0.73
fibrocystic	No	220	169	170	226	225	0.73	0.75
breast disease								
Past estrogen	Yes	79	176	168	219	223	0.80*	0.73
use	No	190	167	171	229	227	0.72	0.74
Ever smoke	Yes	76	169	173	231	230	0.72	0.75
	No	174	171	170	225	226	0.75	0.74
Walks for	No	123	174	168	227	224	0.76	0.73
exercise	Yes	146	167	171	225	228	0.73	0.75

*p-value significant at the <0.05 (two-tailed)
Abbreviations-refer to Table 2

Table 2-5: Multiple logistic regression

The odds of mid-tertile relative to low-tertile 2-hydroxyestrone (2-OH)/ 16α-hydroxyestrone (16α-OH) and the odds of high-tertile relative to low-tertile 2-OH/16α-OH (95 % confidence interval) according to specified change in breast cancer risk factor values.

Model	Risk Factor Change	Mid Tertile vs Low Tertile OR, (95% CI)	High Tertile vs Low Tertile OR, (95% CI)
2-OH			
Base Model	1 S.D. Unit Estradiol	1.27 (0.89-1.82)	1.01 (0.72-1.42)
	1 S.D. Unit Testosterone	0.97 (0.70-1.35)	1.18 (0.85-1.63)
	1 S.D. Unit SHBG	0.90 (0.65-1.24)	0.76 (0.54-1.06)
Base + Age	1 year of age	1.03 (0.96-1.10)	1.06 (0.99-1.12)
Base + BMI	1 BMI unit	0.99 (0.91-1.07)	0.99 (0.91-1.06)
Base + Nulliparity	Never pregnant vs ever pregnant	1.18 (0.51-2.70)	0.56 (0.27-1.16)
16α-OH			
Base Model	1 S.D. Unit Estradiol	0.97 (0.69-1.37)	1.00 (0.70-1.41)
	1 S.D. Unit Testosterone	1.24 (0.90-1.72)	1.62 (1.13-2.32)*
	1 S.D. Unit SHBG	1.10 (0.78-1.56)	0.64 (0.46-0.91)*
Base + Age	1 year of age	0.99 (0.93-1.05)	1.05 (0.98-1.12)
Base + BMI	1 BMI unit	0.99 (0.91-1.07)	0.96 (0.89-1.04)
Base + Surgical Menopause	Surgical vs Natural Menopause	1.13 (0.31-4.07)	7.37 (2.20-24.70)**

CI=Confidence Interval **significant at the 0.001 (two-tailed) level
Tertile cutpoints 2-OH= 154 pg/mL and 188 pg/m/L; 16α-OH =201 pg/mL and 253 pg/mL *significant at the 0.05 (two-tailed) level
S.D.=Standard deviation OR=odds ratio, adjusted for all other factors included in the model.
Abbreviations-refer to Table 2

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3. SECOND PAPER Breast Density and the Relationship with 2-hydroxyestrone and 16alpha-hydroxyestrone

(To be submitted for publication)

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3.1. Abstract

BACKGROUND: Breast density and measures of estrogen metabolism (blood and urinary levels of 2-hydroxyestrone (2-OH) and 16alpha-hydroxyestrone (16α-OH)) have been studied in relation to breast cancer risk. In pre- and post-menopausal women without breast cancer, we endeavored to characterize the relationship between breast density and the serum concentrations of these two major estrogen metabolites.

METHODS: We measured breast density (area measure of visibly dense breast, expressed as a percentage of the total breast area on a standard two-dimensional mammogram) and serum concentrations of 2-OH and 16α-OH in 380 (124 pre- and 256 post-menopausal) women, including 283 pre- or post-menopausal women not currently taking post-menopausal hormone medications. All subjects lacked a diagnosis of breast cancer despite screening mammography or biopsy of suspicious breast abnormalities.

RESULTS*:* In all subjects (N=380), breast density correlated weakly with log-transformed serum concentrations of 16α-OH (Pearson correlation coefficient (ρ) = 0.10, p-value < 0.1; Pearson partial correlation coefficient adjusted for body mass index (BMI) and menopausal status-specific age quartile (adjusted ρ) = 0.13, p-value < 0.05) and with log-transformed serum concentrations of 2-OH (ρ = 0.13, p-value < 0.05; adjusted ρ = 0.09, p-value < 0.1). In subjects not taking post-menopausal hormone medications (N=283), partial correlations were similar in magnitude, 0.12 (p-value < 0.1) and 0.09 (p-value = not significant) for 16α-OH and 2-OH, respectively. Stratification according to menopausal status substantially reduced or eliminated associations between breast density and the estrogen metabolite concentrations.

CONCLUSION: High serum concentrations of the 16α-OH and 2-OH correlated with breast density. The associations between the estrogen metabolites and breast density appeared

independent of post-menopausal hormone medication use. However, statistical associations disappeared upon control for menopausal status. This pattern of association is consistent with two possibilities. First, serum estrogen metabolite concentrations and breast density may share common determinants that are related to the menopause. Or, estrogen metabolite changes, occurring as a consequence of menopause, may directly contribute to the menopause-associated declines in breast density. Determination of the menopause-specific cross-sectional associations between the serum estrogen metabolites and breast density may require larger studies, with sufficient numbers of pre- and post-menopausal women.

Key Words: Breast Density, Estrogen Metabolism

3.2. Introduction

Breast cancer risk is higher in women with dense breasts(7, 8). Breast density may be a measurable manifestation of biological states or processes related to breast carcinogenesis. For example, breast cancer-relevant biological states and processes possibly associated with breast density may include sex hormone-induced breast epithelial and stromal cell hyper-proliferation(1-3, 6, 7, 11, 24, 49, 50), growth factor expression(9, 14, 26), and DNA damage(31). Breast density clearly increases in women placed on post-menopausal hormones(4, 19, 23, 36, 37, 39-41, 43, 44, 48), an exposure causally related to breast cancer(53). Other hormone-related factors associated with lower breast density include higher age, parity, and menopause(7, 34). However, breast density (expressed as a percentage of the total breast) decreases with higher body mass index (BMI)(8), a factor generally thought to increase post-menopausal endogenous estrogen exposures as a consequence of the metabolic conversion of adrenal androgens in adipose tissue(31, 54). The lower breast densities observed in women with higher BMI might simply reflect a higher fat content in the breasts of relatively more obese women.

In concert with the estrogen-breast cancer link(18), a body of evidence implicates specific estrogen metabolites with enhanced or reduced breast cancer risk. Specifically, estrogen metabolism in humans entails the formation of 2-hydroxyestrone (2-OH), 4-hydroxyestrone (4-OH), and 16alpha-hydroxyestrone (16α-OH) from estrone. Relative to 2-OH, the 16α-OH product is estrogenic and genotoxic(54, 56). Because 2-OH and 16α-OH are complementary metabolic products derived from a fixed substrate pool, any genetic or environmental factor, that increases enzymatic production of one metabolite, can be expected to reduce enzymatic production of the alternative metabolite. Taking account of the estrogenicity and genotoxicity of

16α-OH relative to 2-OH, investigators have postulated increased breast cancer risk in women with high blood or tissue levels of 16α-OH or in women who excrete relatively little 2-OH relative to 16α-OH. Although the epidemiologic evidence is not universally supportive(56), several case-control studies(21, 28, 28, 32, 33, 55) and at least one prospective study(42) are consistent with the hypothesized association between the estrogen metabolites and breast cancer risk. Notions regarding the effects of estrogen metabolites on breast cancer risk and notions regarding breast density as a biological measure of estrogen response create the justification for exploring and characterizing a possible causal association between the estrogen metabolites and breast density.

To our knowledge, only Riza et al.(45) have reported on the relationship between the estrogen metabolites and breast density. Working in northern Greece and studying participants in a mammography screening program, Riza et al. measured urinary 2-OH and 16α-OH in 70 post-menopausal women with dense (Wolfe P2 or DY parenchymal pattern) breasts and 70 post-menopausal women with non-dense (Wolfe N1 parenchymal pattern) breasts, individually matched according to year of birth, years since menopause, and date of urine collection. Relative to the women with non-dense breasts, women with dense breasts had 58% higher levels of 2-OH, 15% higher levels of 16α-OH, and a 35% higher 2-OH:16α-OH ratio. Relative to women in lowest tertile of the distribution for the 2-OH:16α-OH ratio, women in the highest tertile were much more likely to have dense (Wolfe P2/DY) as opposed to non-dense (Wolfe N1) breasts (odds ratio adjusted for potential confounders(age at first birth, current BMI, self-reported increase in body build from age 18), 6.2; 95% confidence interval (CI), 1.7-22.9). Notably, high, not the expected low ratio of 2-OH to 16α-OH associated with increased in breast density.

Taking special note of the results reported by Riza et al., we sought to characterize the relationship between breast density and serum estrogen metabolite concentrations in pre- and post-menopausal western Pennsylvania women diagnosed free of breast cancer after screening mammography or after biopsy of a breast abnormality.

3.3 Materials and Methods

3.3.1 Study Population

Subjects eligible for study included pre- or post-menopausal women with 1) no personal history of cancer (except skin) and 2) no evidence for breast cancer after mammography screening or breast biopsy. Volunteers arose from two sources: 1) women undergoing outpatient needle breast biopsy through the Breast Biopsy Service at Magee-Womens Hospital (Pittsburgh, Pennsylvania) and 2) women receiving screening mammography through Magee-Womens Hospital or through a suburban Pittsburgh Magee Womancare Center. To identify and recruit eligible subjects, a research assistant personally solicited women visiting the Breast Biopsy Service between September 2001 and May 2004 and women visiting either the Magee-Womencare Center – North (Wexford, Pennsylvania) or Magee-Womancare Center – East (Monroeville, Pennsylvania) between July 2002 and September 2003. To boost subject recruitment, Magee-Womens Hospital attached study flyers to screening result reports mailed to Magee Womancare Center patrons with negative mammography (Breast Imaging Reporting And Data (BIRAD) result levels 1 and 2) between November 2003 and May 2004.

Of approximately 750 Breast Biopsy Service patients approached, 525 (70.0% of 750) women lacked a personal cancer history, agreed to participate, completed a personal interview, and produced a blood sample. A subsequent review of breast biopsy pathology reports verified non-

breast cancer outcomes in 313 (59.6% of 525) women, including 192 (61.3% of 313) women with serum estrogen metabolite and breast density results available. Of approximately 100 Magee-Womancare Center – North and East patrons approached directly, 78 (78.0% of 100) women lacked a personal cancer history, agreed to participate, completed a personal interview, and produced a blood sample. Subsequent follow-up verified non-breast cancer outcomes in 77 (98.7% of 78) women, including 71 (92.2% of 77) women with serum estrogen metabolite and breast density results available. Finally, mailing study fliers to 6482 women produced 240 (3.7% of 6482) responses, including 228 (95.0% of 240) responses from women without a personal cancer history. One hundred thirty (130; 57.0% of 228) women signed a written consent form, completed a study visit with personal interview, and produced a blood sample. After excluding one woman found to have breast cancer after additional follow-up, 117 (90.7% of 129) women had serum estrogen metabolite and breast density results available. Therefore, the final study sample included 380 women, including 192 (50.5%), 71 (18.7%), and 117 (30.8%) women recruited from the Breast Biopsy Service, Magee-Womancare Center – North or East, and mass mailings, respectively.

Every subject included in this study signed a written informed consent document approved by the Magee-Womens Hospital Institutional Review Board.

3.4 Data Collection

Information collected at personal interview and recorded on standardized study forms included age, race, menopausal status (with menopause defined as 12 months since last menses), history of treatment with estrogen or progesterone, weight without shoes or heavy clothing (measured, in kilograms, with a standard balance beam scale), and height without shoes at full inspiration

(measured, in centimeters, with a stadiometer). Other items of information collected through standardized self-administered take-home questionnaire included reproductive history (age at menarche, age of first pregnancy lasting at least six months, and number of births), details of menopause (including age at menopause and method (surgical *vs*. natural)), and family cancer history. After editing questionnaires for completeness and consistency, a research assistant telephoned subjects, when necessary, to retrieve missing information and to resolve inconsistencies. When subjects could not be reached, a research assistant attempted to retrieve missing information through review of written medical records.

3.4.1 Breast Density

From copies of the most recent screen-film mammogram obtained before the date of study entry, a consultant reader (Ms. Martine Salane), initially trained by Wolfe, used two methods to measure breast density. For women enrolled through the Breast Biopsy Service, we sent the mammogram for the breast not biopsied. For women enrolled following a negative screening mammogram, we selected the left or right breast mammogram in a manner designed to achieve balance with the mammograms sent for a concurrent case series of women with breast cancer (subject matter for a separate manuscript). First, visually inspecting the mammogram copies, the reader placed each subject into one of the four Wolfe parenchymal pattern categories. The N1 Wolfe category included women with radiolucent (fatty) breasts containing few ducts; the P1 Wolfe category included women with breasts showing a ductal (linear) pattern occupying less than 25% of the breast area; the P2 Wolfe category included women with breasts showing a ductal (linear) pattern and nodular densities occupying more than 25% of the breast area; and the DY Wolfe category included women with breast radio-density that completely obscured the ductal pattern.

The second breast density method used the mammogram image showing the craniocaudal projection. Excluding biopsy scars, Cooper's ligaments, and breast masses, the reader used a wax pencil and transparent overlay to outline the entire breast and the portions of breast containing radio-densities. Using a compensating polar planimeter (LASICO, Los Angeles, CA), the reader traced the outline of the entire breast and outlines of dense breast to compute total breast area and dense breast area, respectively. The calculated measure of breast density was the area of visibly dense breast, expressed as a percentage of the total breast. In 28 randomly selected mammograms re-sent blindly at a later date for a second breast density determination, the intraclass correlation coefficients for intra-observer agreement were 0.86, 0.99, and 0.89 for dense breast area, total breast area, and breast density percent, respectively.

3.4.2 Laboratory Measurements

We used the laboratory of TL Klug and enzyme immunoassays (ESTRAMET^{TM} 2/16 ELISA, Immuna Care Corporation, Bethlehem, PA) to measure 2-OH and 16α-OH concentrations in serum stored at -70°C and subjected to a single freeze-thaw cycle. The ESTRAMET 2/16 ELISA assay has been validated against gas chromatography/mass spectroscopy in pooled serum spiked with known amounts of 2-OH and 16α-OH. The analytic sensitivities of the 2-OH and 16α-OH immunoassays are 20 pg/mL and 10 pg/mL, respectively. Using control sera with known 2-OH and 16α-OH concentrations, the laboratory reported concurrent within-assay coefficients of variation of 17% and 9% for 2-OH and 16α-OH concentration, respectively. The inter-assay coefficients of variation calculated from the analytic results reported for blinded duplicate serum samples from 25 postmenopausal subjects were 13.9% and 4.0% for 2-OH and 16α-OH concentration, respectively.

3.5 Statistical Analysis

To examine the association between breast density percent and questionnaire-based risk factors, we tabulated mean breast density percent according to risk factor level and used ANOVA (SAS PROC GLM) and Kruskal-Wallis tests (SAS PROC NPAR1WAY) to evaluate the statistical significance of group differences. To examine the association between serum estrogen metabolite concentrations and questionnaire-based risk factors, we tabulated geometric mean breast density percent according to risk factor level. To approximate the confidence interval for a geometric mean serum estrogen metabolite measure in a population subgroup, we exponentiated the 95% confidence interval calculated for the corresponding mean logarithm-transformed serum estrogen metabolite measure. We used analysis of variance (SAS PROC GLM) to evaluate the statistical significance of variations in a mean log-transformed estrogen metabolite measure across risk factor categories.

For study subjects as a whole and for subjects sub-grouped according to menopausal status and current use of post-menopausal hormone therapy, we used Pearson correlations to express associations between the percent breast density measure and the estrogen metabolite measures (the logarithm-transformed 2-OH concentration, the logarithm-transformed 16α-OH concentration, and the logarithm-transformed ratio between the 2-OH concentration and the 16α-OH concentration). We used Pearson partial correlation to evaluate breast density - estrogen metabolite associations adjusted for body mass index (BMI – weight in kg divided by the square of height in meters) and menopause-specific age quartile. We used menopause-specific age quartile to adjust for age in order to separate the effects of age and menopause. Finally, we used linear regression to estimate the apparent effects of 2-OH and 16α-OH (singly and together) on breast density percent, adjusted for menopausal status, current post-menopausal hormone

therapy, menopause-specific age quartile, body mass index (BMI)), and questionnaire-based risk factors with meaningful univariate associations with breast density percent or estrogen metabolites (*i.e.*, age at menarche and nulligravidity). We used the R-squared statistic and normal plots of residuals to evaluate the fit of linear regression models and linear regression after BoxCox transformation of the dependent variable to investigate the influence of minor departures of the breast density percent distribution from normality.

Statistical analysis was performed with the SAS System for Windows, Release 8.02.

3.6 Results

3.6.1 Characteristics of the study group

The study group included 124 pre-menopausal women (median age 46 years, range 39-55 years; 115 white, 6 other, and 3 unknown race; 34% overweight (BMI 25.0-29.9 kg/m^2) and 24% obese (BMI 30.0+ kg/m^2); 4 N1, 19 P1, 96 P2, and 5 DY Wolfe parenchymal pattern; median breast density 41%, inter-quartile range (IQR) 26-56%) and 256 post-menopausal women (median age 59 years, range 44-84 years; 241 white, 11 other, and 4 unknown race; 37% overweight and 31% obese; 15 N1, 66 P1, 173 P2, and 2 DY Wolfe parenchymal pattern; median breast density 32%, IQR 16-49%). Forty-six (18%) and 210 (82%) post-menopausal women had experienced surgical and natural menopause, respectively. Among 251 post-menopausal women with known history of estrogen or progesterone hormone therapy (HT), 92 (37%) reported current use.

3.6.2 Breast density percent measure

As expected, breast density percent was higher in pre-menopausal (N=124, median 41%, IQR 26-56%) than in post-menopausal women (N=256, median 32%, IQR 16-49%; $p < 0.001$ Kruskal-Wallis). As well, breast density percent was higher in post-menopausal women taking

hormones (N=92, median 37%, IQR 25-51%) than in post-menopausal women not taking hormones (N=159, median 27%, IQR 13-46%; p = 0.0105 Kruskal-Wallis). The distribution of breast density percent did not differ statistically between pre-menopausal women and post-menopausal women taking hormones (p = 0.16 Kruskal-Wallis). Figure 1 shows the distribution of the breast density percent measure in pre-menopausal women and in post-menopausal women taking and not taking hormones. The distribution of breast density percent values, particularly for post-menopausal women not taking hormones, shows some departure from normality, with observations crowding together toward lower breast densities and a surplus of observations with zero values. Because of these distributional properties, the breast density percent measure is not perfectly suited for linear regression analysis.

In subgroups defined according to menopausal status and hormone therapy, we looked for associations between breast density percent and other breast cancer risk factors, including age, race, BMI, family history of cancer, age at menarche, pregnancy history (never pregnant vs. any pregnancy lasting at least six months), age at first pregnancy lasting at least six months, number of births that followed pregnancies lasting at least six months, menopausal type (surgical vs. natural), and age at menopause. The limited number of non-white subjects in our study sample severely limited our ability to explore breast density differences according to race. Also, because detailed questions about menopause were added to study questionnaires midway through the subject recruitment period, information regarding age at menopause was available for only 127 of 256 post-menopausal subjects.

We observed, in each of the main study subgroups, the expected strong inverse association between BMI and breast density (Table 1). Breast density decreased with age (in three categories, 45-54, 55-64, and 65+ years) in post-menopausal women not taking HT (Table 1).

This observation approached statistical significance (p=0.058) in an ANOVA model that included BMI (in three categories, <25.0, 25.0-29.9, and 30.0+ kg/m^2) and age as an ordinal three-level variable (scored 0, 1, and 2 for the three categories of age, 45-54, 55-64, and 65+ years, respectively).

In addition, pre-menopausal subjects with menarche at age 13 years or later (N=53) had denser breasts than pre-menopausal subjects with earlier menarche (N=68; mean breast density 47.6% vs. 35.7%, p<0.01; Table 1). Adjustments for age (in three categories, <45, 45-49, and 50+ years) and BMI (in three categories, <25.0, 25.0-29.9, and 30.0+ kg/m^2) reduced the breast density difference between pre-menopausal women with late vs. early menarche (mean breast density 44.9% vs. 37.8%, p<0.05). The difference remained statistically significant (p<0.05) after BoxCox transformation (with λ=0.75) of the breast density percent dependent variable. Finally, nulligravid post-menopausal women not on HT (N=24) had denser breasts than post-menopausal women not on HT with at least one pregnancy lasting six months (N=129; 45.6% vs. 27.9%, p<0.001; Table 1). Again, adjustments for age (in three categories, <55, 55-64, and 65+ years) and BMI (in three categories, <25.0, 25.0-29.9, and 30.0+ kg/m^2) reduced this post-menopausal breast density difference according to pregnancy history (mean breast density 41.3% vs. 28.7%, p<0.01). Statistical significance remained after the BoxCox transformation.

3.6.3 Serum estrogen metabolite measures

Menopausal status and hormone therapy were clearly related to the serum concentrations of the estrogen metabolites. Relative to pre-menopausal women (N=124, median 422 pg/ml, IQR 374-480 pg/ml) and post-menopausal women on HT (N=92, median 412 pg/ml, IQR 356-524 pg/ml), post-menopausal women not on HT had low serum concentrations of 16α-OH (N=159, median 351 pg/ml, IQR 316-426 pg/ml; Figure 2, Panel A). Post-menopausal women on HT (median

348 pg/ml, IQR 194-472 pg/ml), pre-menopausal women (median 251, IQR 163-388 pg/ml), and post-menopausal women not on HT (median 158 pg/ml, IQR 117-218 pg/ml) had high, intermediate, and low serum concentrations of 2-OH (Figure 2, Panel B). In accordance with these differences with respect to 2-OH , post-menopausal women on HT (median 0.74, IQR 0.47-1.10 pg/ml), pre-menopausal women (median 0.53, IQR 0.40-0.83), and post-menopausal women not on HT (median 0.43, IQR 0.31-0.59) had high, intermediate, and low values for the ratio of 2-OH concentration to 16α-OH concentration (Figure 2, Panel C). For each of the three estrogen metabolite measures, the differences between post-menopausal women on and not on HT and the differences between pre-menopausal women and post-menopausal women not on HT were statistically significant ($p<0.0001$, Kruskal-Wallis). As well, pre-menopausal women and post-menopausal women on HT differed significantly with respect to 2-OH concentration and with respect to the 2-OH: 16α-OH metabolite ratio ($p<0.01$, Kruskal-Wallis). Pre-menopausal women and post-menopausal women on HT were not statistically different with respect to the 16α-OH metabolite measure.

Again, in subgroups defined according to menopausal status and hormone therapy, we looked for associations involving the serum estrogen metabolite measures and traditional breast cancer risk factors. Table 2 summarizes findings for age, BMI, age at menarche, and pregnancy history (never pregnant vs. any pregnancy lasting at least six months). The estrogen metabolite measures were unrelated to age. Among post-menopausal women on HT, increases in BMI tracked with decreases in 2-OH and with decreases in the ratio of 2-OH to 16α-OH. As a result, the previously noted higher 2-OH estrogen concentrations and estrogen metabolite ratios observed among post-menopausal hormone users relative to non-users were magnified among women with lower BMI. There was statistical evidence for significant variation in geometric

mean 16α-OH concentrations across BMI categories among pre-menopausal women and among post-menopausal women not on HT (Table 2). Within these two subgroups, obese women (BMI $\geq$ 30 kg/m^2) relative to normal BMI women (BMI < 25 kg/m^2) had a lower geometric mean 2-OH concentration, a higher geometric mean 16α-OH concentration, and lower geometric mean ratio of 2-OH concentration to 16α-OH concentration. In pre-menopausal women, the geometric mean 2-OH:16α-OH metabolite ratio was higher among women with earlier menarche than among women with later menarche. Finally, in pre-menopausal women, the geometric mean 16α-OH estrogen concentration was higher among women without prior pregnancy than among women with any prior pregnancy lasting at least six months. These latter two observations were not quite statistically significant.

3.6.4 Crude and adjusted associations between breast density and the individual estrogen metabolite measures (Figures 3 and 4)

In all subjects, breast density correlated weakly with log-transformed serum concentrations of 16α-OH (Pearson correlation coefficient (ρ) = 0.10, p-value < 0.1; Figure 3, Panel A, Plot A) and with log-transformed serum concentrations of 2-OH (ρ = 0.13, p-value < 0.05; Figure 3, Panel B, Plot A). Adjustments for BMI and menopause-specific age quartile increased the breast density correlation for 16α-OH (Pearson partial correlation coefficient (partial ρ) = 0.13, p-value < 0.05; Figure 4, Panel A, Plot A) and decreased the breast density correlation for 2-OH (partial ρ = 0.09, p-value < 0.1; Figure 4, Panel B, Plot A). Excluding post-menopausal subjects who were taking hormone medications or who were missing information about hormone medications did not materially alter the strength of association between breast density and 16α-OH (partial ρ = 0.12; p-value < 0.1; Figure 4, Panel A, Plot B) or between breast density and 2-OH (partial ρ = 0.09; p-value = not significant; Figure 4, Panel B, Plot B). Further stratification according to menopausal status (Figure 4, Panels A and B, Plots C and D) reduced or eliminated associations

between breast density and the estrogen metabolites. A weakened adjusted association between breast density and 16α-OH persisted among post-menopausal women not on hormone therapy (partial ρ = 0.07; p-value = not significant; Figure 4, Panel B, Plot D). A statistically non-significant adjusted association between breast density and 16α-OH was observed among the small number of post-menopausal women on hormone therapy (partial ρ = 0.07; p-value = not significant; Figure 4, Panel A, Plot E). The effective absence of BMI- and menopause-specific age quartile-adjusted associations between breast density and the estrogen metabolite ratio (Figure 4, Panel C) was consistent with the generally positive weak associations between breast density and the individual estrogen metabolites (Figure 4, Panels A and B).

3.6.5 Crude and adjusted associations between breast density and the 2-OH and 16α-OH measures, taken together (Table 3)

Table 3 summarizes the mutual effects of the estrogen metabolites on breast density. Table 3 expresses estrogen metabolite-breast density associations in terms of standardized beta coefficients, the estimated increase in breast density per standard deviation unit increase in log-transformed estrogen metabolite concentration (Table 3, footnote). Germane to the sub-group not taking post-menopausal hormones, both 16α-OH (BMI- and menopause-specific age quartile-adjusted standardized beta = 1.9, standard error (S.E.) = 1.3) and 2-OH (adjusted standardized beta = 1.1, S.E. = 1.3) contributed positively and independently to breast density. Upon further stratification according to menopausal status, some evidence emerged, at least in post-menopausal women not on hormone therapy, for opposing effects of 16α-OH (adjusted standardized beta = 1.7, S.E. = 1.6) and 2-OH (adjusted standardized beta = −0.7, S.E. = 1.9) on breast density. A similar pattern was observed in post-menopausal women on hormone therapy (adjusted standardized beta for 16α-OH = 2.6, S.E. = 2.0) and adjusted standardized beta for 2-OH = −1.1, S.E. = 2.1).

3.6.6 Final linear regression mode (Table 4)

Table 4 summarizes results from a single and simple linear regression breast density model (R^2=0.308) with menopausal status, current hormone therapy use, BMI, menopause-specific age quartile, 16α-OH and 2-OH included as main effects. Results from the model underscored the independent and statistically significant effects of BMI, menopause, and hormone therapy on breast density. The model ascribed a statistically non-significant 1.3 percentage point increase (S.E. = 1.1) in breast density percent with each one standard deviation unit increase in the logarithm of the serum concentration of 16α-OH. The model recognized no association between 2-OH and breast density percent (beta = −0.1, S.E. 1.1; Table 4). The residuals appeared normally distributed.

A more complete (main effect) model (R^2 = 0.327) added two factors (age at menarche and pregnancy history) connected earlier with breast density (Table 1). According to the model, pregnancy history (pregnant at least 6 months vs. never pregnant) decreased breast density percent 7.8 percentage points (S.E. = 2.5, p-value < 0.01) and late menarche (13+ years vs. <13 years) increased breast density 1.5 percentage points (S.E. = 1.9, p-value = not significant). Because of exclusions for history of pregnancies only lasting less than six months (N=11), for missing age at menarche (N=1), and for missing age at menarche and missing pregnancy history (N=5), this latter model included data from 357 instead of 374 women.

3.7 Discussion

Breast density and estrogen metabolism have both been implicated in the risk of developing breast cancer. Our main aim was to identify the relationship between breast density and serum estrogen metabolite concentrations in pre- and post-menopausal women without breast cancer.

In our overall sample, estrogen metabolites were positively correlated with breast density. These associations appeared independent of post-menopausal hormone medication use. However, statistical associations disappeared upon control for menopausal status. To our knowledge, the only other study to evaluate the relationship between estrogen metabolites and breast density is Riza et al(45). However, the sample was restricted to postmenopausal women not on HT, thereby limiting our ability to compare our results between estrogen metabolites and breast density with results reported by Riza. To our knowledge this is the first study to look at estrogen metabolites and correlates of breast density.

Mammographic breast density serves as an estimate of the proportion of fibroglandular tissue to fat in the breast. Premenopausal women, compared to postmenopausal women, generally have greater breast density secondary to a higher proportion of glandular tissue. As women age, fatty tissue replaces glandular tissue therefore decreasing breast density(12, 25, 29, 35). Consistent with this theory, reports in the literature illustrate a decrease in mammographic breast density with age, especially after the menopause(27).

In concert with the literature, we found that the mean percent breast density was higher in premenopausal women than postmenopausal women not taking HT. Furthermore, consistent with reports that have shown HT, in particular combination therapy, increases breast density(8), we also noted higher mean percent density in postmenopausal women on HT.

Consistent with the literature, we found that BMI was inversely related to breast density percent in all subgroups. Several studies have shown an inverse relationship between weight and BMI and mammographic density(7, 13, 15, 25, 30, 51). In reports, increased leanness equates with increased breast density and increased risk of breast cancer in premenopausal women and in postmenopausal women an inverse relationship exists between obesity and mammographic

density. This is suggestive that in postmenopausal women the increased risk of breast cancer in heavier women which has been associated with increased levels of estrogen (5, 7, 16, 38, 47), may not be mediated through density. In one study by Coker et al (20), African-American women had significantly lower 2-OH levels than Caucasian women among pre-and postmenopausal women and was felt to be related to ethnic differences in body mass. While we were not able to evaluate differences in BMI with respect to estrogen metabolism across ethnic groups, we did note in leaner women (BMI <25 kg/m^2) higher 2-OH levels in pre-and postmenopausal women on HT compared to heavier women. Additionally, leaner postmenopausal women on HT had higher 16α-OH levels.

Nulliparity is related to denser breast tissue and subsequently increased breast cancer risk(7, 22, 51, 52). Pregnancy is associated with a change in breast structure to more differentiated lobules with less cell proliferation with the greatest effect on breast structure obtained from the first pregnancy, and further differentiation with each subsequent pregnancy(46). Our results revealed greater percent breast density in nulligravid postmenopausal women not on HT than postmenopausal women not on HT with at least one pregnancy. This association remained statistically significant even with adjustment for age indicating that age and pregnancy independently influence breast cancer risk. With respect to the estrogen metabolites, mean 16α-OH concentrations were higher in nulligravid premenopausal women, although not statistically significant.

Menarche occurring 13 years and older yielded significantly denser breast tissue when compared with women who began menstruating at <13 years, particularly in the premenopausal population. This is in contrast to what we would expect as early age at menarche has been associated with an increased risk of breast cancer as well as increased mammographic density

suggesting the association with ovarian function(7, 17). Again, in premenopausal women, only the mean 2-OH:16α-OH ratio was associated with an earlier age at menarche vs. later menarche, although not statistically significant. Adjustment for age at menarche did not substantially impact our findings with respect to apparent effects of metabolites and density.

There are several features of the present study that warrant discussion. To our knowledge, this is the first study to examine breast density, serum estrogen metabolites, and breast cancer risk factors in pre-and postmenopausal women. The use of a single, expert breast density reader helps to reduce variability in breast density measurements. Estrogen metabolite levels in premenopausal women may be subject to fluctuations in the menstrual cycle. As we were unable to determine the phase at time of blood draw, variability may exist among pre-menopausal women with respect to the estrogen metabolite measures and the respective breast density measurement. Lastly, the use of serum vs. urine to measure estrogen metabolites may make it difficult to compare to previous studies which have predominantly used urine for metabolite measures. In a study by Bradlow et al(10), plasma and urinary levels of 2OH and 16α-OH were compared in nulliparous women aged 17-35 years. Overall, the correlation between the two methods was felt to be fair. While the comparison was only conducted in premenopausal women it is suspected that the amount of variation would be less in the postmenopausal population. The low number of HT users does not allow us to address the differences associated with HT preparations. Reproductive and lifestyle factors were obtained by self-report which may lend to recall bias, thereby attenuating our results. Finally, we cannot exclude the possibility that our findings may be due to chance or confounded by some unidentified factor.

In conclusion, this is the first study to investigate estrogen metabolites and correlates of breast density in pre-and postmenopausal women. The associations between the estrogen

metabolites and breast density appeared independent of post-menopausal hormone medication use. However, statistical associations disappeared upon control for menopausal status. Our findings suggest that serum estrogen metabolite concentrations and breast density may share common determinants that are related to the menopause. Or, estrogen metabolite changes, occurring as a consequence of menopause, may directly contribute to the menopause-associated declines in breast density. Determination of the menopause-specific cross-sectional associations between the serum estrogen metabolites and breast density may require larger studies, with sufficient numbers of pre- and post-menopausal women.

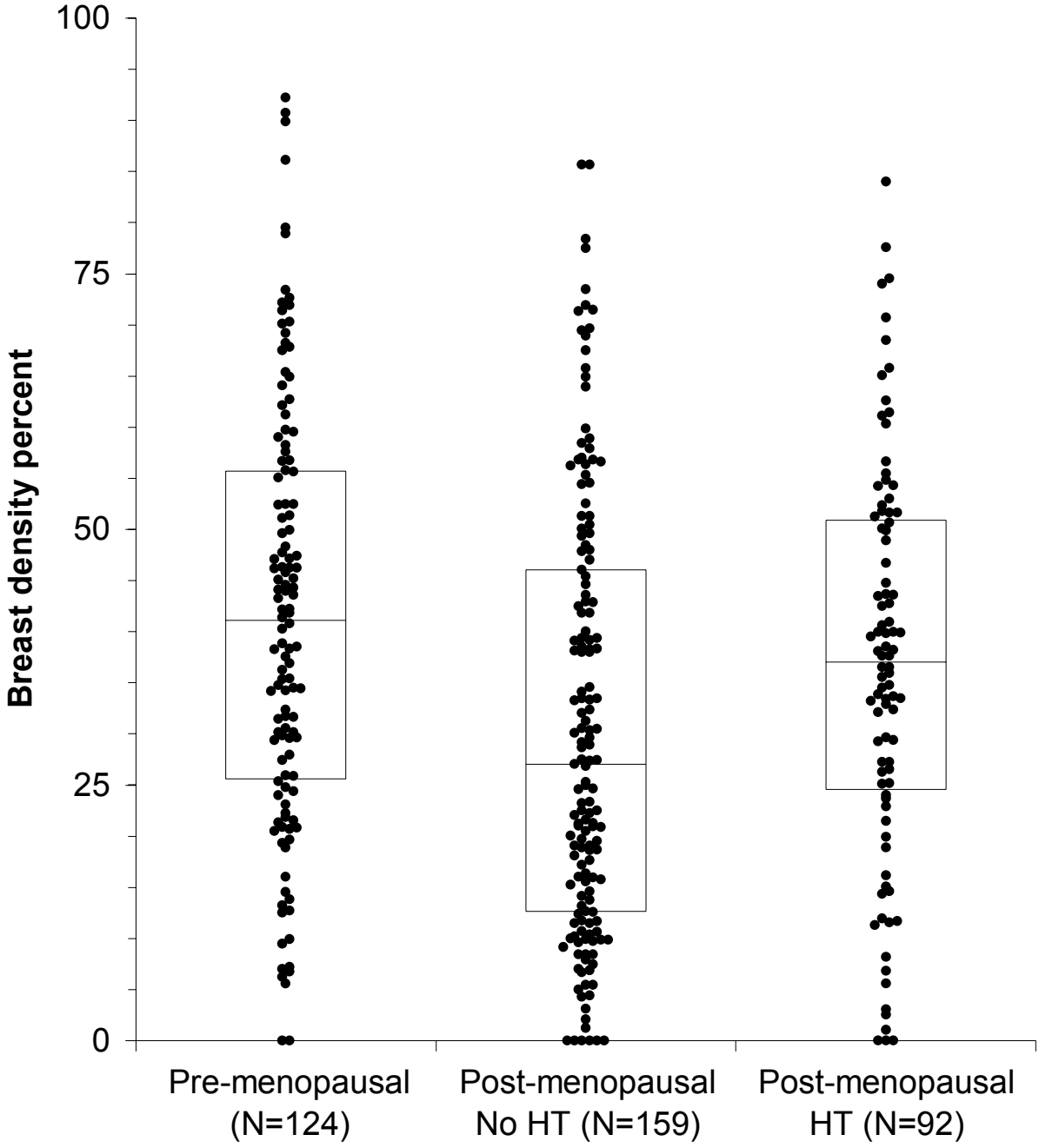

Figure 3-1 Combination dot-box plots of breast density percent

Combination dot-box plots of breast density percent in pre-menopausal subjects and in post-menopausal subjects not taking and taking hormone therapy (HT). The lower and upper boundaries of each box indicate the 25th and 75th percentiles, respectively. The line through the mid-portion of each box indicates the median.

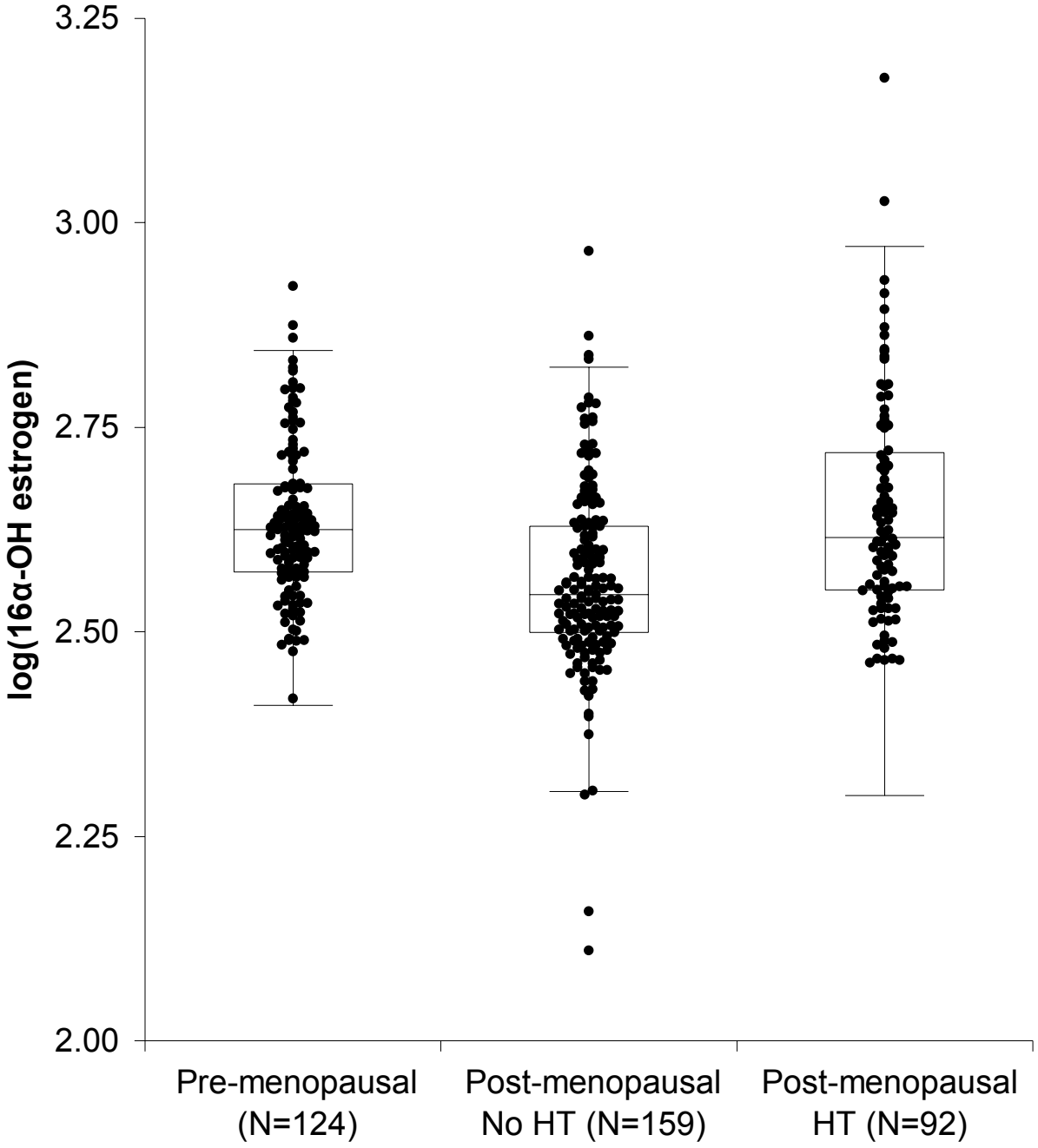

Figure 3-2: Combination dot-box plots of the log-transformed serum concentrations of 16α-OH

Combination dot-box plots of the log-transformed serum concentrations of 16α-OH (Panel A), of the log-transformed serum concentrations of 2-OH (Panel B), and the log-transformed ratios of 2-OH concentration to 16α-OH concentration (Panel C). Each panel contains a dot-box plot for pre-menopausal subjects, post-menopausal subjects not on HT, and post-menopausal subjects on HT. The lower and upper boundaries of each box indicate the 25th and 75th percentiles, respectively. The line through the mid-portion of each box indicates the median. The whiskers extend to the upper fence (75th percentile + 1.5 · IQR) and to the lower fence (25th percentile - 1.5 · IQR).

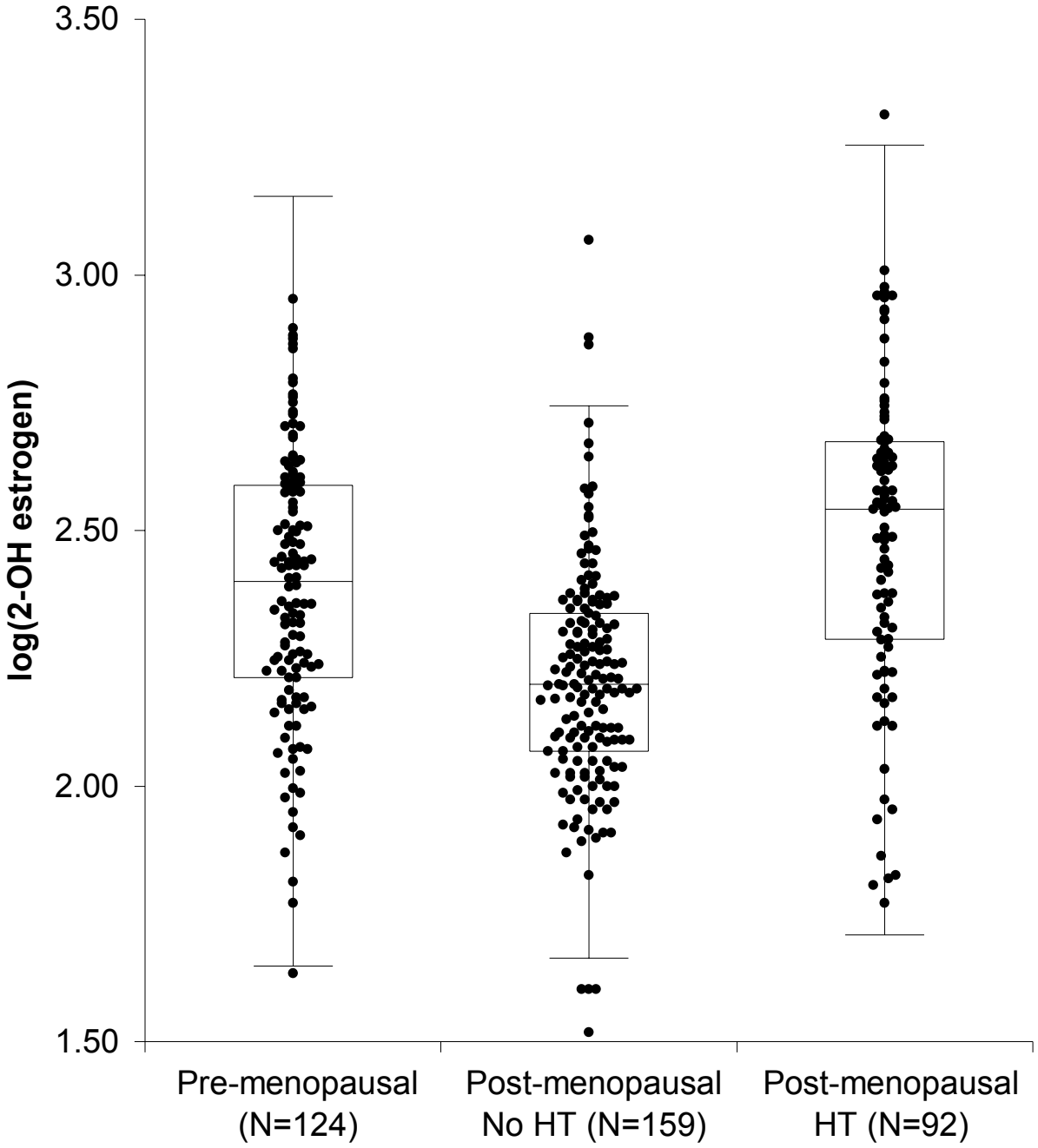

Figure 3-2 (Cont'd)

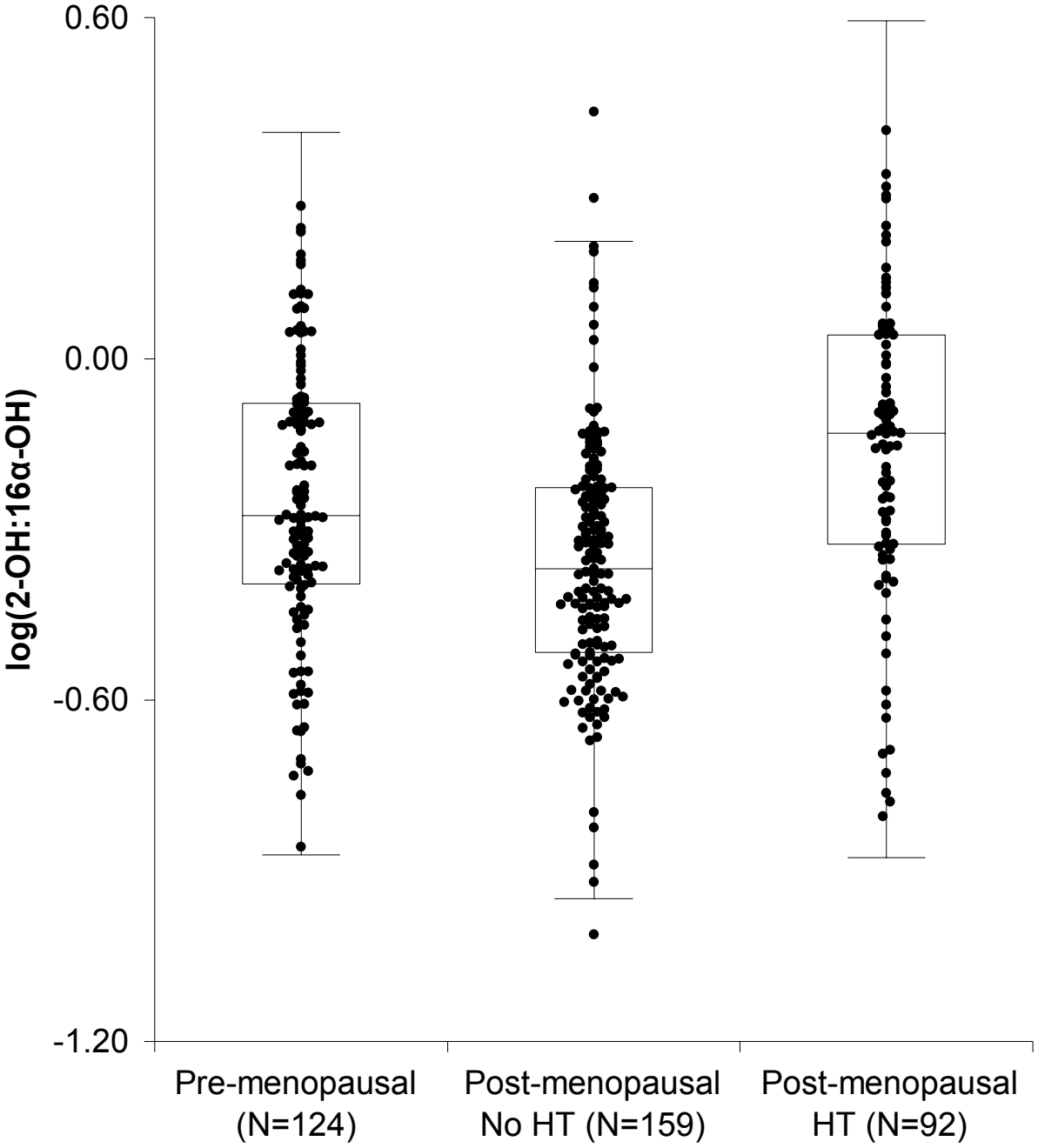

Figure 3-2 (Continued)

Figure 3-3 Description

Scatter plots of the log-transformed serum concentrations of 16α-OH (Panel A), of the log-transformed serum concentrations of 2-OH (Panel B), and the log-transformed ratios of 2-OH concentration to 16α-OH concentration (Panel C), in subgroups defined according to menopausal status and HT use. Each panel includes a table of correlations of log-transformed metabolite measure with breast density percent. Each scatter plot shows the least square regression line of breast density percent fit to the log-transformed metabolite measure. (Note: HT use is missing for five post-menopausal subjects.) Abbreviations: * − p-value <0.1, ** − p-value <0.05

Pearson correlations with breast density percent

Group	N	Correlation
A. All subjects	380	0.10 **
B. Pre-menopausal and post-menopausal, no HT	283	0.11 *
C. Pre-menopausal	124	0.09
D. Post-menopausal, no HT	159	0.03
E. Post-menopausal, HT	92	0.08

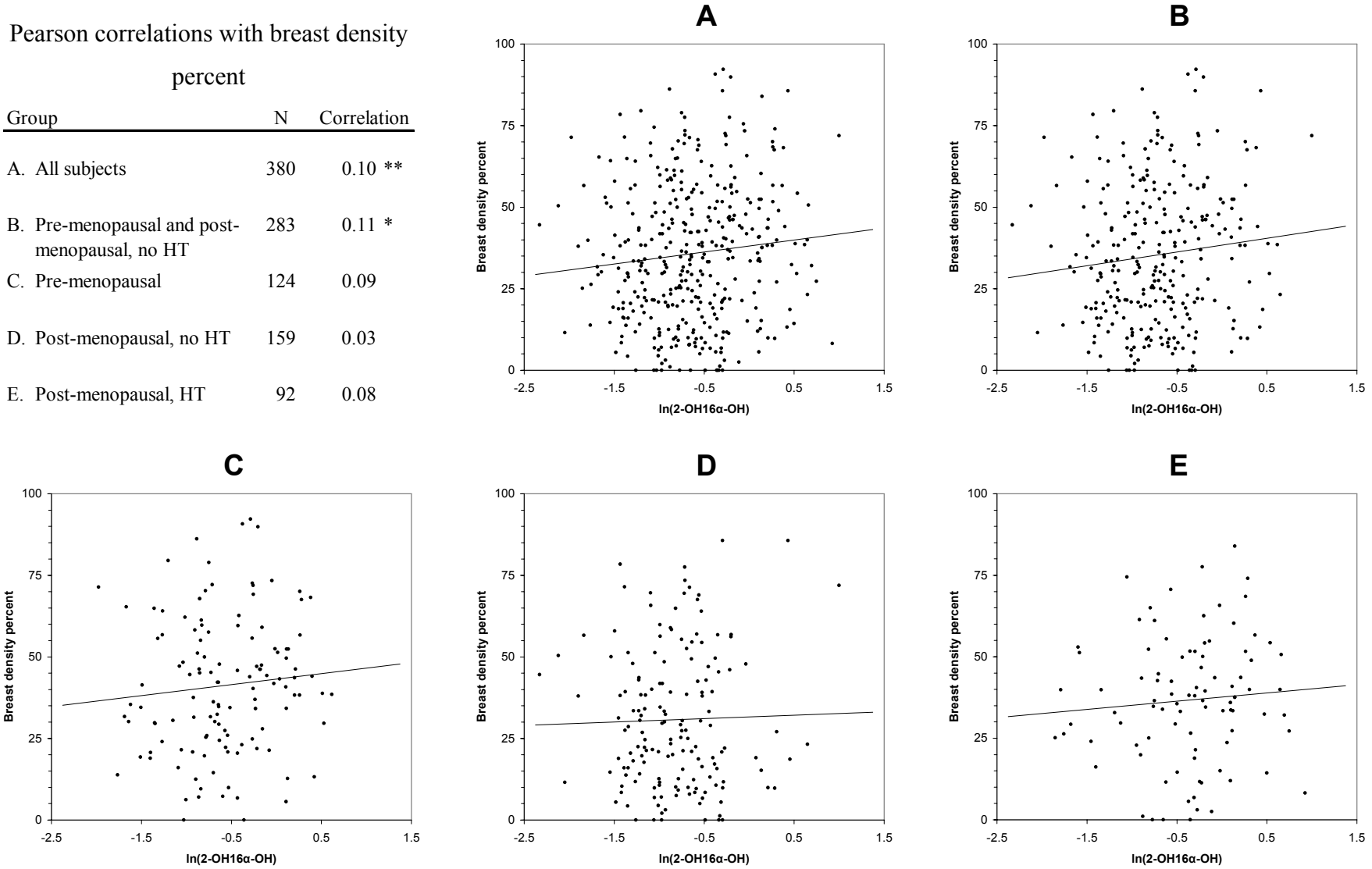

Figure 3-3-A: Scatter plots of 16α-OH

Pearson correlations with breast density percent

Group	N	Correlation
A. All subjects	380	0.13 **
B. Pre-menopausal and post-menopausal, no HT	283	0.12 **
C. Pre-menopausal	124	0.06
D. Post-menopausal, no HT	159	0.02
E. Post-menopausal, HT	92	0.14

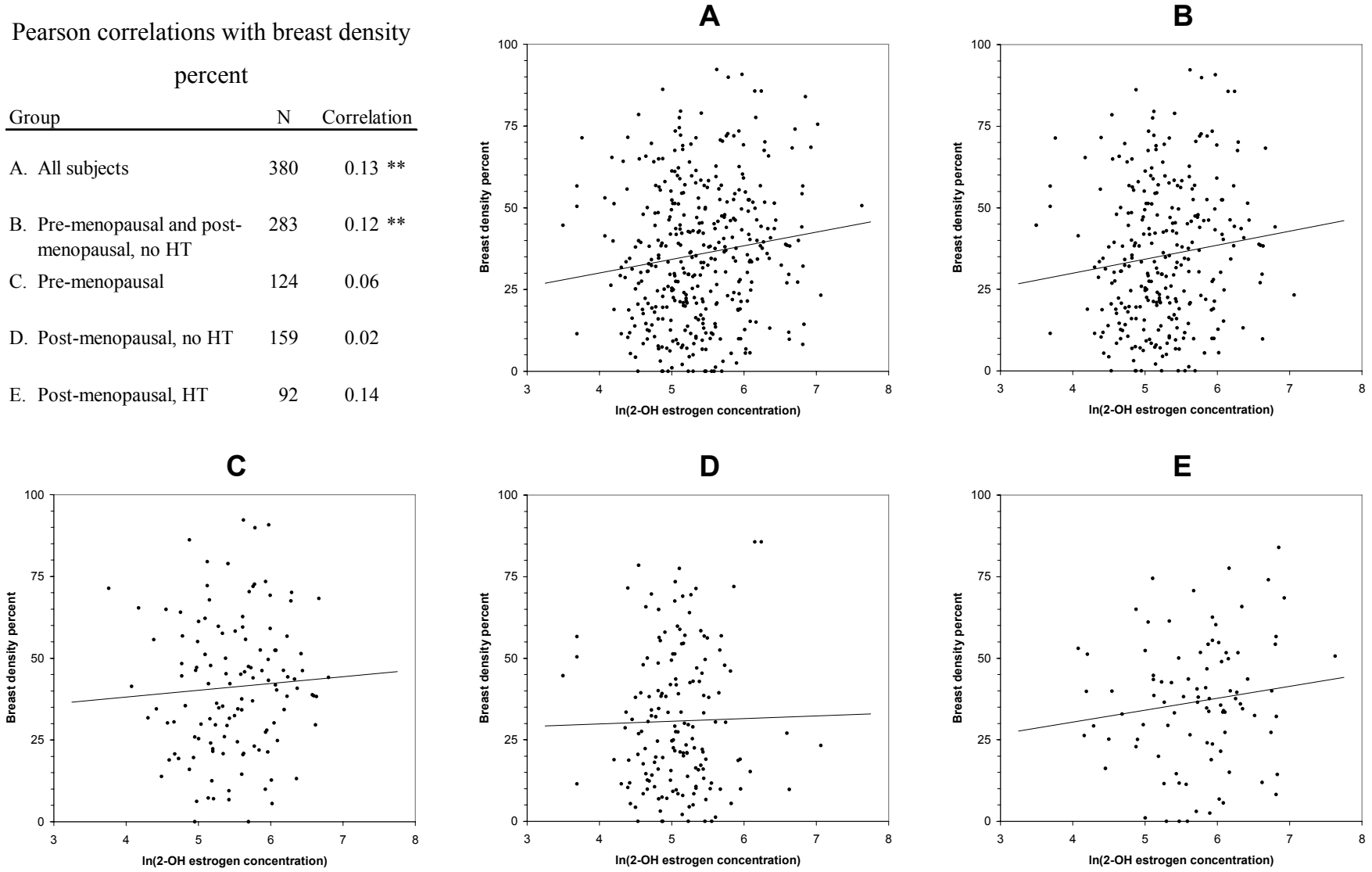

Figure3-3-B: Scatter plots of 2-OH

Pearson correlations with breast density percent

Group	N	Correlation
A. All subjects	380	0.10 **
B. Pre-menopausal and post-menopausal, no HT	283	0.11 *
C. Pre-menopausal	124	0.09
D. Post-menopausal, no HT	159	0.03
E. Post-menopausal, HT	92	0.08

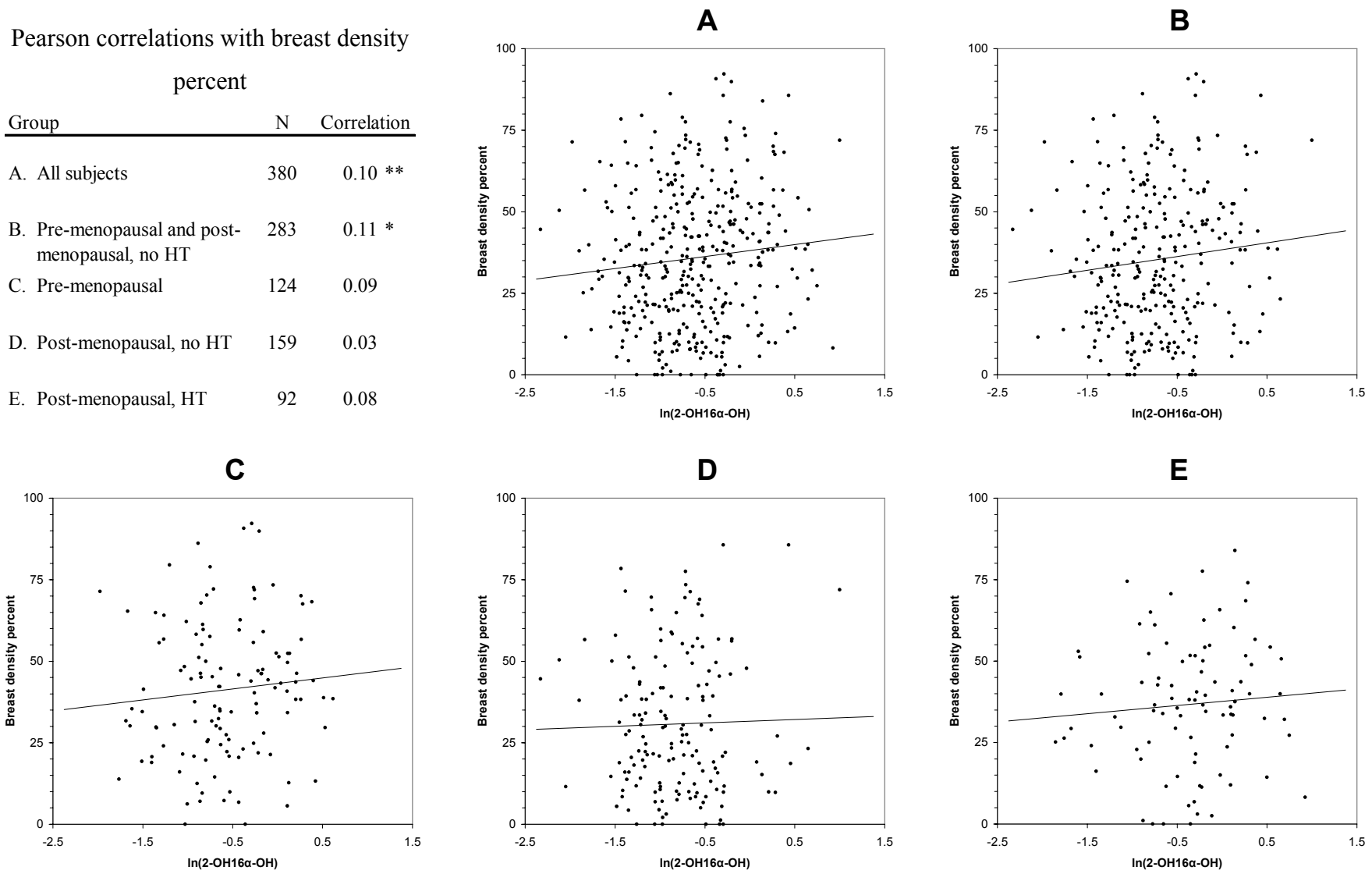

Figure 3-3-C: Scatter plots of 2:16α-OH

Figure 3-4 description

Scatter plots of the log-transformed serum concentrations of 16α-OH (Panel A), of the log-transformed serum concentrations of 2-OH (Panel B), and the log-transformed ratios of 2-OH concentration to 16α-OH estrogen concentration (Panel C), in subgroups defined according to menopausal status and HT use. Breast density percent and each of the log-transformed metabolite measures have been adjusted for log-transformed BMI and menopause-specific age quartile (treated as an integer-scored ordinal variable). Adjusted breast density percent values were set equal to zero when linear regression results produced adjusted values less than zero. This correction affected one pre-menopausal subject with adjusted density calculated at -2.0%, four no HT post-menopausal subjects with adjusted densities calculated at -11.4, -3.1, -0.9, and -2.6%, and one HT post-menopausal subject with adjusted density calculated at -0.8%.Age quartile categories are <43, 43-45, 46-48, and 49+ years and <55, 55-59, 60-63, and 64+ years for pre- and post-menopausal subjects, respectively. Each panel includes a table of correlations of the BMI- and age-adjusted log-transformed metabolite measure with BMI- and age-adjusted breast density percent. Each scatter plot shows the least square regression line of BMI- and age-adjusted breast density percent fit to the BMI- and age-adjusted log-transformed metabolite measure. (Note: HT use is missing for five post-menopausal subjects and BMI for one post-menopausal subject on HT.) Abbreviations: * value <0.1, ** − p-value <0.05

Partial correlations with breast density percent

Group	N	Correlation
A. All subjects	379	0.13 **
B. Pre-menopausal and post-menopausal, no HT	283	0.12 *
C. Pre-menopausal	124	0.00
D. Post-menopausal, no HT	159	0.07
E. Post-menopausal, HT	91	0.11

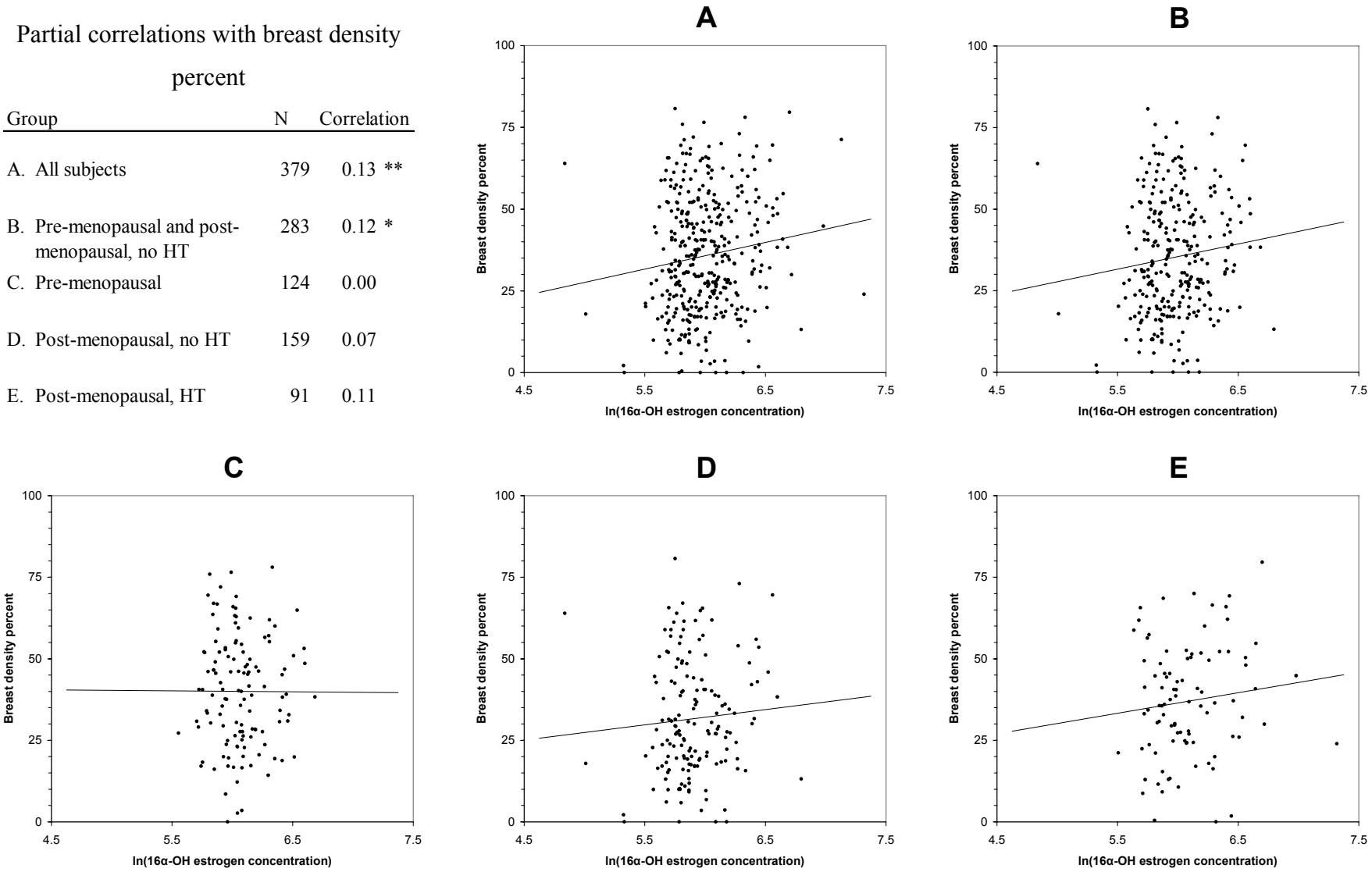

Figure 3-4-A: Scatter plots of 16α-OH

Partial correlations with breast density percent

Group	N	Correlation
A. All subjects	379	0.09 *
B. Pre-menopausal and post-menopausal, no HT	283	0.09
C. Pre-menopausal	124	0.04
D. Post-menopausal, no HT	159	-0.01
E. Post-menopausal, HT	91	0.03

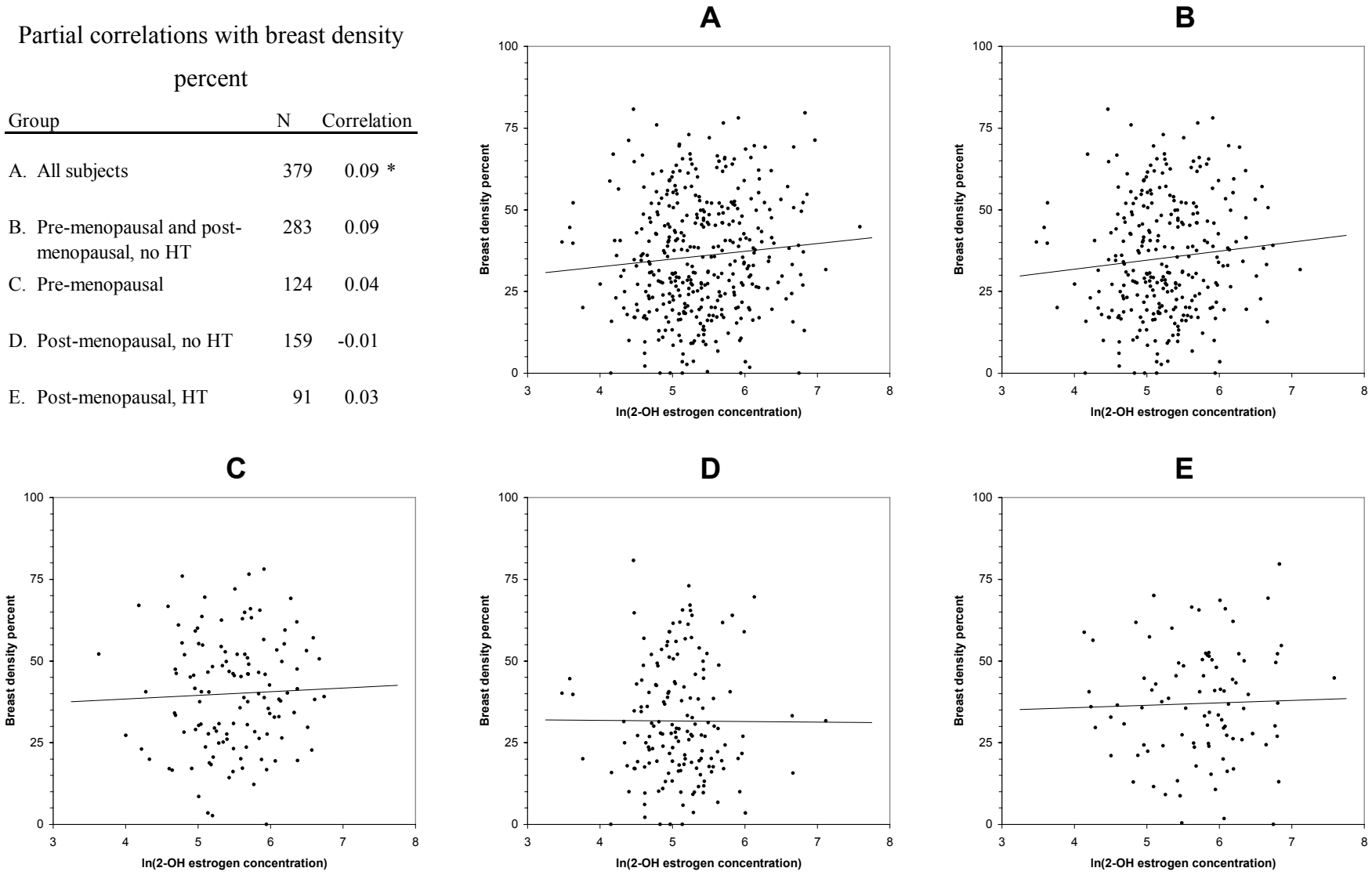

Figure 3-4-B: Scatter plots of 2-OH

Partial correlations with breast density percent

Group	N	Correlation
A. All subjects	379	0.04
B. Pre-menopausal and post-menopausal, no HT	283	0.05
C. Pre-menopausal	124	0.04
D. Post-menopausal, no HT	159	-0.04
E. Post-menopausal, HT	91	-0.02

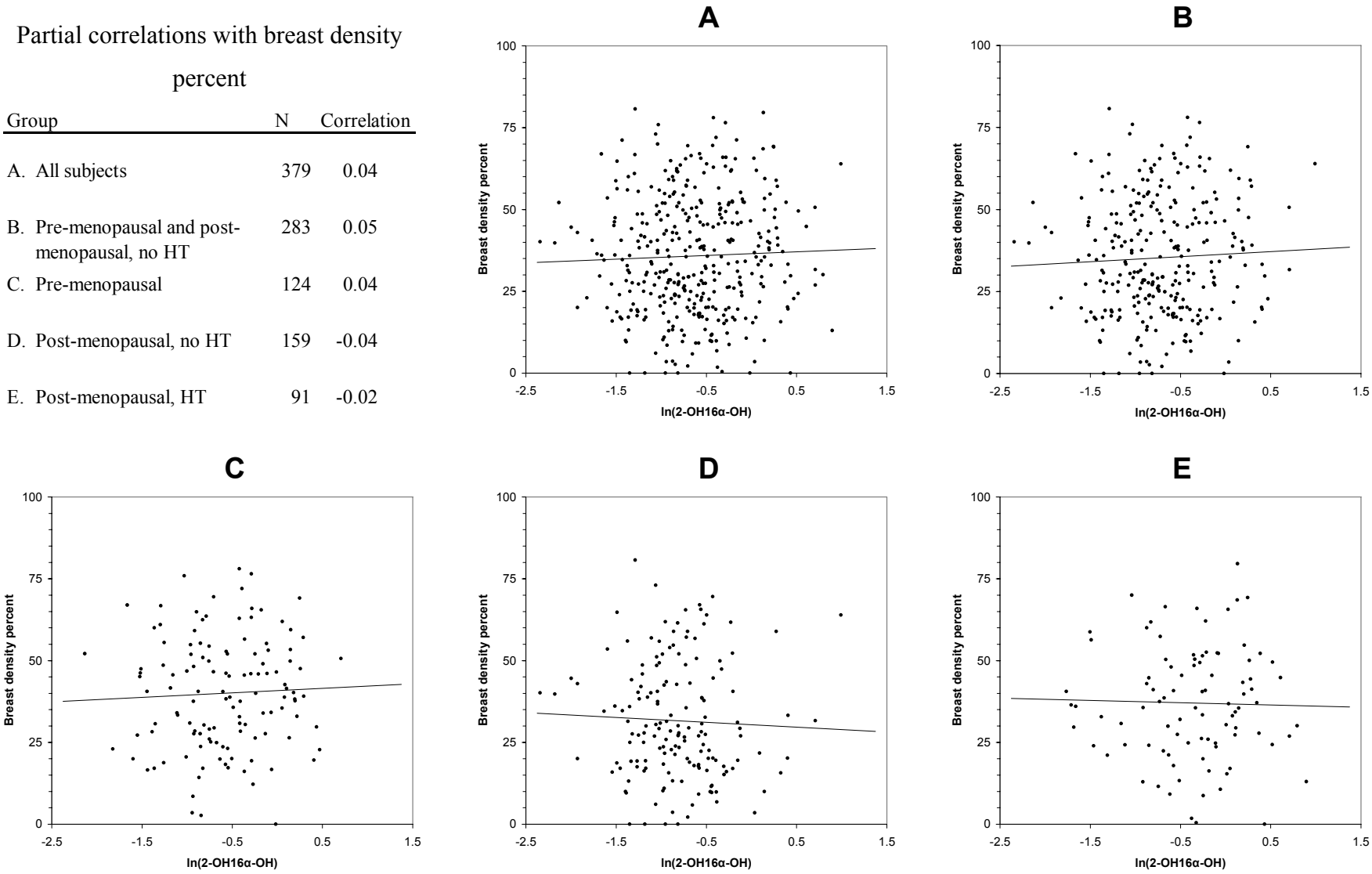

Figure 3-4-C: Scatter plots of 2:16α-OH

Table 3-1: Mean breast density percent according to breast cancer risk factor level

	Pre-menopausal (N=124)				Post-menopausal, no HT (N=159)				Post-menopausal, HT (N=92)			
Risk factor	N	Mean	95%C.I.	p-value	N	Mean	95% C.I.	p-value	N	Mean	95% C.I.	p-value
Age (years)				N.S.				N.S.				N.S.
<45	50	41.6	35.6-47.6									
45-54	73	41.4	36.6-46.2		33	34.6	26.1-43.2		27	36.1	28.3-43.9	
55-64					84	31.5	27.0-36.0		51	36.0	30.5-41.5	
65+					42	26.1	20.4-31.8		13	41.0	29.2-52.7	
Age tertile				N.S.				N.S.				N.S.
low	39	42.5	35.7-49.4		55	34.8	28.9-40.7		35	36.1	29.0-43.2	
middle	43	39.0	32.9-45.0		53	30.9	24.8-36.9		29	35.3	29.6-41.0	
upper	42	42.3	35.6-49.1		51	26.2	21.0-31.5		28	39.0	30.4-47.6	
BMI (kg/m^2)				<0.0001				<0.0001				<0.0001
<25	52	52.6	47.0-58.1		54	42.3	36.2-48.4		26	47.2	40.7-53.7	
25-29	42	36.8	31.2-42.5		53	32.6	27.9-37.2		41	37.2	31.5-43.0	
30+	30	27.7	22.1-33.3		52	16.8	12.7-21.0		24	24.8	16.9-32.6	
Menarche (years)				<0.01				N.S.				N.S.
<13	68	35.7	31.3-40.0		72	30.4	25.1-35.8		39	33.2	27.0-39.3	
13+	53	47.6	41.8-53.4		86	31.0	26.8-35.3		52	38.9	33.5-44.3	
Pregnancy history				N.S.				<0.0001				N.S.
never	19	45.2	33.4-57.0		24	45.6	36.4-54.9		14	37.9	24.9-50.9	
ever	99	39.9	36.0-43.8		129	27.9	24.4-31.4		73	35.9	31.6-40.3	

Abbreviations: HT – hormone therapy; C.I. – confidence interval; N.S. – not significant; BMI – body mass index

Notes:

1. Statistical significance of differences in mean breast density percent across risk factor category based on Kruskal-Wallis test (performed with SAS PROC NPAR1WAY).
2. Analysis according to age excludes one 55-59 year-old pre-menopausal subject and one <45 year-old post-menopausal subject on HT.
3. Low, middle, and upper age tertiles include <44, 44-47, and 48+ year-old women, if pre-menopausal, <57, 57-62, and 63+ year-old women, if post-menopausal not on HT, or <56, 56-60, and 61+ year-old women, if post-menopausal on HT.
4. BMI is unknown for one post-menopausal subject on HT.
5. Age at menarche is unknown for five subjects (three pre-menopausal, one post-menopausal not on HT, and one post-menopausal on HT).
6. Analysis according to pregnancy history excludes eleven subjects with pregnancies not lasting six months (three pre-menopausal, five post-menopausal not on HT, and three post-menopausal on HT). In addition, pregnancy history is unknown for six subjects (three pre-menopausal, one post-menopausal not on HT, and two post-menopausal on HT).

Table 3-2: Geometric mean serum 2-OH, 16α-OH and metabolite ratio according to breast cancer risk factor level

	Pre-menopausal (N=124)				Post-menopausal, no HT (N=159)				Post-menopausal, HT (N=92)			
Risk factor	N	2-OH	16α-OH	Ratio	N	2-OH	16α-OH	Ratio	N	2-OH	16α-OH	Ratio
Age (years)												
<45	50	236	441	0.53								
		201-277	415-469	0.46-0.62								
45-54	73	248	425	0.58	33	172	363	0.47	27	283	482	0.59
		213-288	403-447	0.51-0.67		141-209	326-405	0.38-0.58		202-397	428-543	0.45-0.77
55-64					84	160	371	0.43	51	321	434	0.74
						142-180	352-390	0.39-0.48		263-392	395-476	0.62-0.88
65+					42	156	361	0.43	13	324	415	0.78
						136-179	328-397	0.37-0.51		248-423	356-485	0.61-1.00
Age tertile												
low	39	242	436	0.55	55	148	364	0.41	35	288	469	0.61
		202-289	405-468	0.47-0.65		127-172	339-391	0.35-0.48		219-379	423-519	0.49-0.77
middle	43	226	421	0.54	53	174	377	0.46	29	288	420	0.69
		188-273	396-448	0.45-0.64		153-199	354-402	0.41-0.52		218-380	381-463	0.54-0.88
upper	42	262	440	0.60	51	162	358	0.45	28	359	440	0.82
		213-323	409-474	0.49-0.72		141-188	328-391	0.39-0.52		285-453	380-510	0.67-0.99
BMI			**				**			***		**
<25	52	268	431	0.62	54	161	341	0.47	26	413	471	0.88
		223-322	407-457	0.53-0.73		140-186	316-367	0.41-0.55		312-547	409-542	0.72-1.07
25-29	42	221	410	0.54	53	165	384	0.43	41	313	445	0.70
		185-265	388-433	0.46-0.64		145-189	355-416	0.37-0.50		252-388	402-492	0.58-0.86
30+	30	232	467	0.50	52	157	377	0.42	24	222	421	0.53
		188-287	421-517	0.40-0.61		134-183	353-402	0.36-0.48		165-299	377-470	0.41-0.68

Table 3-2 (Continued)

	Pre-menopausal (N=124)				Post-menopausal, no HT (N=159)				Post-menopausal, HT (N=92)			
Risk factor	N	2-OH	16α-OH	Ratio	N	2-OH	16α-OH	Ratio	N	2-OH	16α-OH	Ratio
Menarche				*								
<13	68	268	432	0.62	72	164	369	0.44	39	298	426	0.70
		232-309	408-457	0.54-0.71		144-187	351-389	0.39-0.50		246-361	394-460	0.59-0.84
13+	53	221	436	0.51	86	160	364	0.44	52	318	457	0.70
		187-262	412-462	0.44-0.59		144-177	341-388	0.39-0.49		254-398	414-506	0.58-0.84
Pregnancy			*									
never	19	250	471	0.53	24	167	380	0.44	14	307	423	0.73
		179-349	422-525	0.39-0.72		126-220	329-438	0.33-0.59		206-456	367-488	0.52-1.00
ever	99	247	427	0.58	129	160	363	0.44	73	314	449	0.70
		219-278	409-445	0.52-0.65		147-175	347-380	0.41-0.48		264-373	416-485	0.60-0.81

Abbreviations: HT – hormone therapy; 2-OH – serum 2-OH estrogen concentration (pg/ml); 16α-OH – serum 16α-OH estrogen concentration (pg/ml); ratio – ratio of 2-OH to 16α-OH; BMI – body mass index; * – $p<0.1$; ** – $p<0.05$; *** – $p<0.01$
Notes: See Table 1

Table 3-3: Multiple linear regression results

Unadjusted and adjusted effects of serum 2-OH and serum 16α-OH metabolite measures, separately and together, on breast density percent, in subject groups defined according to menopausal status and HT.

		Unadjusted					Age- and BMI-adjusted				
		2-OH		16α-OH			2-OH		16α-OH		
Subject group	Metabolite measure	B	SE	B	SE	R^2	B	SE	B	SE	R^2
All subjects (N=374)	None										0.269
	2-OH	2.6	1.1 **			0.016	1.6	0.9 *			0.278
	16α-OH			1.7	1.1	0.006			2.2	0.9 **	0.284
	2-OH and 16α-OH	2.4	1.2 *	0.5	1.3	0.016	0.7	1.1	1.9	1.1 *	0.284
All subjects, except HT (N=283)	None										0.290
	2-OH	2.8	1.4 **			0.014	1.9	1.2			0.297
	16α-OH			1.1	1.4	0.002			2.4	1.2 **	0.301
	2-OH and 16α-OH	2.8	1.5 *	0.0	1.5	0.014	1.1	1.3	1.9	1.3	0.302
Pre-menopausal (N=124)	None										0.313
	2-OH	1.4	2.0			0.004	0.7	1.7			0.314
	16α-OH			-1.8	2.4	0.005			-0.1	2.0	0.313
	2-OH and 16α-OH	2.3	2.2	-2.8	2.6	0.013	0.8	1.9	-0.5	2.2	0.314
Post-menopausal, no HT (N=159)	None										0.274
	2-OH	0.6	2.1			0.000	0.0	1.8			0.274
	16α-OH			-0.2	1.8	0.000			1.5	1.5	0.278
	2-OH and 16α-OH	0.7	2.2	-0.3	1.9	0.001	-0.7	1.9	1.7	1.6	0.279
Post-menopausal, on HT (N=91)	None										0.221
	2-OH	2.4	1.9			0.018	0.4	1.7			0.221
	16α-OH			2.7	1.9	0.023			2.0	1.7	0.234
	2-OH and 16α-OH	1.3	2.2	2.0	2.2	0.027	-1.1	2.1	2.6	2.0	0.236

Abbreviations: HT – hormone therapy; 2-OH – serum 2-OH estrogen; 16α-OH – serum 16α-OH estrogen; BMI – body mass index; B – beta coefficient; SE – standard error; * – $p<0.1$; ** – $p<0.05$

Notes:

1. Adjusted linear regression models include terms for log-transformed BMI and menopause-specific age quartile (treated as an integer-scored ordinal variable). Age quartile categories are <43, 43-45, 46-48, and 49+ years and <55, 55-59, 60-63, and 64+ years for pre- and post-menopausal subjects, respectively.
2. Beta coefficients estimate the increase in breast density percent associated with a one standard deviation unit increase in the estrogen metabolite measure (*i.e.*, 0.663 unit increase and 0.285 unit increase in natural logarithm serum 2-OH and 16α-OH estrogen concentration, respectively).
3. BMI is unknown for one post-menopausal subject on HT. HT status is unknown for five post-menopausal subjects.

Table 3-4: Final linear regression model (N=374,R^2=0.308)

Model term	Breast density percent increase in association with	beta	SE	p-value
Intercept		182.3	24.6	<0.0001
BMI	0.212 unit increase in ln(BMI)	-10.7	0.9	<0.0001
Menopause	post-menopause vs. pre-menopause	-7.9	2.2	<0.001
HT	current post-menopausal HT use vs. non-use	4.8	2.6	0.059
Age	one category increase in meno-pause-specific age quartile	-1.1	0.8	N.S.
16α-OH	0.285 unit increase in ln(16α-OH concentration)	1.3	1.1	N.S.
2-OH	0.663 unit increase in ln(2-OH concentration)	-0.1	1.1	N.S.

Abbreviations: BMI – body mass index; HT – hormone therapy; 2-OH – serum 2-OH estrogen; 16α-OH – serum 16α-OH estrogen; beta – beta coefficient; SE – standard error

Notes:

1. Age quartile categories are <43, 43-45, 46-48, and 49+ years and <55, 55-59, 60-63, and 64+ years for pre- and post-menopausal subjects, respectively.
2. Analysis excludes one subject because of unknown BMI and five subjects because of unknown HT status.
3. The beta coefficients for BMI, 16α-OH, and 2-OH convey the breast density percent change per standard deviation change in natural logarithm BMI, natural logarithm 16α-OH, and natural logarithm 2-OH, respectively.

3.8 References

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4. THIRD PAPER: Breast Density, 2-hydroxyestrone, 16α-hydroxyestrone and the Risk of Breast Cancer

(To be submitted for publication)

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4.1 Abstract

BACKGROUND: Epidemiological evidence suggests that breast density and measures of estrogen metabolism (blood and urinary levels of 2-hydroxyestrone (2-OH) and 16alpha-hydroxyestrone (16α-OH)) are associated with breast carcinogenesis. We sought to determine whether these factors are *independently* associated with breast cancer risk.

METHODS*:* Percent breast density and serum concentrations of 2-OH and 16α-OH were obtained on 176 cases (55 pre-menopausal, 121 post-menopausal) and 380 controls (124 pre-menopausal, 256 postmenopausal).

RESULTS: Logistic regression analyses showed a 3-4 fold increased risk of breast cancer among pre-menopausal women in the highest tertile of breast density compared with those in the lowest tertile of density, even with adjustment for the estrogen metabolites. A similar trend for increased risk of breast cancer in high vs. low tertile of density was observed among post-menopausal women taking hormone therapy (HT) after adjusting for estrogen metabolites, BMI, and age, but this trend was not statistically significant. Breast density did not appear to substantially increase breast cancer risk among post-menopausal women not taking HT.

CONCLUSION: A breast density-breast cancer risk relationship, that is independent of serum estrogen metabolite concentrations, exists in subgroups of women classified according to menopausal status and HT use.

Key Words: Breast Density, Estrogen Metabolism, Breast Cancer

4.2 Introduction

Breast cancer remains the most common cancer in women with an estimated 211,240 new cases in the United States in 2005(1). Mammographic breast density and estrogen metabolites, namely 2-hydroxyestrone (2-OH) and 16α-hydroxyestrone (16α-OH) have been implicated as risk factors for this disease.

Breast density is associated with an increased risk of breast cancer in both premenopausal and postmenopausal women (8, 10). The extent to which this association is a measurable manifestation of a biological state or a process related to breast carcinogenesis remains unknown. It has been postulated that sex hormone-induced breast epithelial and stromal cell hyper-proliferation(3-5, 7, 9, 12, 21, 50, 53), growth factor expression(11, 14, 24), and DNA damage(29) may be contributing to breast density. As women age, fatty tissue replaces glandular tissue therefore decreasing breast density. Breast density clearly increases in women placed on post-menopausal hormones(6, 17, 20, 32, 33, 35-37, 41, 42, 47), an exposure causally related to breast cancer(54). Evidence exists that the tissue composition of dense and non-dense breasts differ. Connective tissue, including collagen and fibroblasts, along with epithelial cells, predominate in dense breasts. Several studies have evaluated the relationship between density as it appears radiologically with the histology of breast tissue. Nine of ten studies used samples from a mastectomy or biopsy and six of these studies reported an association between density and epithelial proliferation and density and stromal proliferation(3-5, 7, 9, 12, 21, 50, 53). A concern was that these samples might not be representative of the general population. Li and colleagues reported on a study using samples from women at forensic biopsy which revealed a higher percentage of the tissue occupied by cells, glandular structures, and collagen in dense breasts(4, 10, 34).

Outside of age and mutations in BRCA1 or BRCA2, density carries the largest risk for breast cancer with a four to six-fold increase in risk in those women with densest breast tissue(9, 10). What remains unclear is the best way to measure density. Breast density can be assessed either qualitatively or quantitatively. The first qualitative method proposed by Wolfe in 1976, consists of four classifications: N1 radiolucent (fatty) breasts containing few ducts; P1 ductal (linear) pattern occupying less than 25% of the breast area; P2 ductal (linear) pattern and nodular densities occupying more than 25% of the breast area; and the DY radio-density that completely obscured the ductal pattern. The breast imaging reporting and data systems (BIRADS) is another qualitative classification. This system also utilizes four categories: extremely fatty; scattered density; heterogeneous density; and extremely dense. Several studies have found the Wolfe categories to predict breast cancer risk, and although few studies have utilized the BIRADS classification, those reported show a significant increase risk in the extremely dense category(10). Studies investigating the association between breast density and breast cancer using quantitative approaches have reported more consistent results and larger gradients of risk when compared to qualitative methods(10, 52). Current quantitative methods include computer-assisted methods, estimation by radiologists and planimetry. Despite current methods of measuring breast density, a standard approach for measurement is lacking and methodological limitations are evident. Both qualitative and quantitative methods have an element of subjectivity lending to the possibility of inter-observer and intra-observer variation. Additionally, current mammographic films are 2-dimensional, which restricts the ability to fully appreciate the volume of the breast and the subsequent dense area. Potential methods to improve density measures are underway and could help to improve the understanding between density and the risk of breast cancer(10, 26, 40).

In tandem with the evidence linking estrogen and breast cancer(16), there is a body of evidence that alludes to specific estrogen metabolites and their respective role in breast cancer risk. Specifically, estrogen metabolism in humans entails the formation of 2-hydroxyestrone (2-OH), 4-hydroxyestrone (4-OH), and 16alpha-hydroxyestrone (16α-OH) from estrone. Relative to 2-OH, the 16α-OH product possesses estrogenic and genotoxic properties(55, 57). Because 2-OH and 16α-OH are complimentary metabolic products derived from a fixed substrate pool, any genetic or environmental factor that increases enzymatic production of one metabolite can be expected to reduce enzymatic production of the alternative metabolite. It has therefore been postulated that women with high blood or tissue levels of 16α-OH or low 2-OH relative to 16α-OH have an increased breast cancer risk. Although the epidemiologic evidence is not universally supportive (15), several case-control studies(18, 27, 30, 56) and at least one prospective study(38) are consistent with the hypothesized association between the estrogen metabolites and breast cancer risk. To date, there has been one study that has evaluated the relationship between estrogen metabolism and breast density. Riza et al evaluated the role of estrogen metabolites and their relationship with high-density Wolfe mammographic parenchymal patterns (P2/DY). The study was nested within a large cross-sectional survey on determinants of mammographic patterns carried out in a population-based breast-screening program in Northern Greece. Urinary levels of 2-OH and 16α-OH were measured in a random sample of 70 postmenopausal women with P2/DY mammographic patterns and in a random sample of 70 postmenopausal women with N1 mammographic patterns, individually matched to the P2/DY women on year of birth, years since menopause and date of urine collection. Women with a P2/DY pattern had 58% higher levels of 2-OH (P = 0.002) and 15% higher levels of 16α-OH (P = 0.37) than those with an N1 pattern. The ratio of 2-OH:16α-OH was 35% higher (P = 0.005) in women with a P2/DY pattern.

Women in the highest one-third of this ratio were six times more likely to have a P2/DY pattern than those in the lowest one-third after adjusting for potential confounders (prevalence odds ratio, 6.2; 95% CI, 1.7-22.9; test for linear trend, P = 0.002). These findings seem to suggest that a high, rather than a low, 2-OH:16α-OH ratio may be associated with an increase in breast cancer risk in postmenopausal women, an increase in breast caner risk than manifests as an increase in breast density(43).

In light of the above-mentioned findings, we examined the joint distribution of breast density and serum estrogen metabolite levels in groups of women with and without breast cancer, in an attempt to understand the independent and joint effects of breast density and estrogen metabolites on breast cancer risk.

4.3 Materials and Methods

4.3.1 Study Population

Subjects eligible for study included pre- and post-menopausal women with no personal history of cancer (except non-melanoma skin cancer). Volunteers arose from three sources: 1) women undergoing outpatient needle breast biopsy through the Breast Biopsy Service at Magee-Womens Hospital (Pittsburgh, Pennsylvania), 2) women seen in the Magee-Womens Surgical Clinic for an initial evaluation after biopsy diagnosed breast cancer, and 3) women receiving screening mammography through Magee-Womens Hospital or through a suburban Pittsburgh Magee Womancare Center. To identify and recruit eligible subjects, a research assistant personally solicited women visiting the Breast Biopsy Service between September 2001 and May 2004, women visiting the Magee-Womens Surgical Clinic between June 2003 and May 2004, and women visiting Magee-Womancare Center – North (Wexford, Pennsylvania) and East

(Monroeville, Pennsylvania) between July 2002 and September 2003. To boost subject recruitment, Magee-Womens Hospital attached study flyers to screening result reports mailed to Magee-Womancare Center patrons with negative mammography between November 2003 and May 2004.

Of approximately 750 Breast Biopsy Service patients approached, 404(54% of 750) women with no personal cancer history, signed a written consent form, completed a personal interview, and produced a blood sample. A subsequent review of breast biopsy pathology reports verified a non-breast cancer outcome in 313 (77% of 404) women and a breast cancer outcome in 91 (23% of 404). Subsequently, 192 (61.3% of 313) controls and 80 (88% of 91) breast cancer cases, had serum estrogen metabolite and breast density results available. Approximately 200 women were approached in the Surgical Clinic, of which 135 women were newly diagnosed with breast cancer. Of those newly diagnosed, 121 (90%) were enrolled and 94 (78% of 121) had serum estrogen metabolite and breast density results available. At Magee-Womancare Centers – North and East, approximately 100 patrons were approached directly; 78 (78% of 100) women with no personal cancer history, signed a written consent form, completed a personal interview, and produced a blood sample. Subsequent follow-up verified non-breast cancer outcomes in 77 (98.7% of 78) women and a breast cancer outcome in one (1.3% of 78). Of these, 71 (92.2% of 77) and 1 (100% of 1) woman have serum estrogen metabolite and breast density results available. Finally, mailing study fliers to 6482 women produced 240 (3.7% of 6482) responses, including 228 (95.0% of 240) responses from women without a personal cancer history. 130 (57.0% of 228) women signed a written consent form, completed a study visit with personal interview, and produced a blood sample. Of these, 117 women (90.0% of 130) without breast cancer and 1 woman (100% of 1) with breast cancer had serum estrogen metabolite and breast

density results available. Therefore, the final study sample included 380 controls consisting of 192 (50.5%), 71 (18.7%), and 117 (30.8%) women, and 176 cases consisting of 80 (45%), 94 (53%), 1 (0.8%), and 1 (0.8%) woman from the Breast Biopsy Service, Magee-Womens Surgical Clinic, Magee-Womancare Center – North/East, and mass mailings, respectively. Breast biopsy reports were reviewed for all subjects recruited through the breast biopsy service to verify presence or absence of breast cancer.

Every subject included in this study signed a written informed consent document approved by the Magee-Womens Hospital Institutional Review Board.

4.4 Data Collection

Information collected at personal interview and recorded on standardized study forms included age, race, menopausal status (including age at menopause and cause (surgical *vs*. natural)), history of treatment with estrogen or progesterone, weight without shoes or heavy clothing (measured in kilograms with a standard balance beam scale), and height without shoes at full inspiration (measured in centimeters with a stadiometer). Additional information collected through the standardized self-administered take-home questionnaire included reproductive history (age at menarche, age of first pregnancy lasting at least six months, and number of births) and family cancer history. Questionnaires were edited for completeness and consistency. A research assistant telephoned subjects, when necessary, to retrieve missing information and to resolve inconsistencies. When subjects could not be reached, a research assistant attempted to retrieve missing information through review of written medical records.

The data from pathology reports was double extracted and recorded onto standardized study forms in order to simplify data entry. For cases, recorded data included: in situ versus invasive

cancer, primary tumor size, number of involved axillary lymph nodes, metastasis, estrogen and progesterone receptor status, and HER-2/neu oncoprotein status.

4.4.1 Breast Density

A consultant reader (Ms. Martine Salane), initially trained by Wolfe, measured both Wolfe's classification and percent breast density from copies of screen-film mammograms obtained within six months of study entry. First, visually inspecting the mammogram copies, the reader placed each subject into one of the four Wolfe parenchymal pattern categories: N1, P1, P2 and DY. The second breast density method expressed area of visibly dense breast as a percentage of the total breast area on a two-dimensional mammogram image. Using the mammogram image showing the craniocaudal projection and excluding biopsy scars, Cooper's ligaments, and breast masses, the reader used a wax pencil to outline the entire breast and the portions of breast containing radio-densities. The reader used a compensating polar planimeter (LASICO, Los Angeles, CA) and traced the outline of the entire breast and outlines of dense breast to compute total breast area and dense breast area, respectively. With respect to cases, the unaffected side was sent for evaluation. For those controls that were recruited from the biopsy clinic, the film for the breast not biopsied was used for density evaluation. All films were relabeled with a study ID to ensure that the reader remained blinded to the subject's identity and case-control status. Unknown to the reader, 28 randomly selected mammograms were re-sent at a later date to determine reproducibility of the density readings. The intraclass correlation coefficients for intra-observer agreement were 0.86, 0.99, and 0.89 for dense breast area, total breast area, and breast density percent, respectively.

4.4.2 Laboratory Measurements

We used the laboratory of TL Klug and enzyme immunoassays (Immuna Care Corporation, Bethlehem, PA; ESTRAMET^{TM} 2/16 ELISA,) to measure the 2-OH and 16α-OH estrogen concentrations in serum stored at -70°C and subjected to a single freeze-thaw cycle. The ESTRAMET 2/16 ELISA assay has been validated against gas chromatography/mass spectroscopy in pooled serum spiked with known amounts of 2-OH and 16α-OH estrogen. The analytic sensitivities of the 2-OH and 16α-OH estrogen immunoassays are 20 pg/mL and 10 pg/mL, respectively. Using control sera, the laboratory reported within-assay coefficients of variation of 17% and 9% for 2-OH and 16α-OH concentration, respectively. The inter-assay coefficients of variation calculated from the analytic results for blinded duplicate serum samples from 25 postmenopausal subjects were 13.9% and 4.0% for 2-OH and 16α-OH concentration, respectively.

4.5 Statistical Analysis

Analyses were conducted for three distinct sub-groups: pre-menopausal women, post-menopausal women not taking HT, and post-menopausal women taking HT. The statistical significance of group differences with respect to discrete and continuous measures was evaluated using the chi-square and Wilcoxon tests, respectively. Estrogen metabolite measures were logarithm-transformed to produce variables distributed more favorably for parametric statistical testing. To estimate a geometric mean and 95% confidence interval for a geometric mean metabolite measure, we took the exponential of the corresponding mean and 95% confidence interval for the mean logarithm-transformed metabolite measure. We used least square methods

(LSMEAN reported by SAS PROC GLM with OM option) to adjust mean breast density and geometric mean estrogen metabolite measures for age, BMI, and other factors of interest. We used ANOVA type III tests (as reported by SAS PROC GLM) to assess the statistical significance of case-control differences with respect to breast density and estrogen metabolite measures, unadjusted or adjusted for age, BMI, and other factors of interest.

The primary research hypothesis involved testing the association between breast density and case-control status, before and after adjustments for the estrogen metabolite measures. Crude odds ratios (OR) with 95% confidence intervals (95% CI) were calculated across breast density across tertiles using unconditional logistic regression. The tertiles were derived from the control population for each of the three separate groups: premenopausal women, postmenopausal women not taking HT, and postmenopausal women on HT. Multivariate logistic regression was used to adjust the breast density-breast cancer association for each of the estrogen metabolites, age, BMI, and other statistically significant breast cancer risk variables ($p < 0.10$) identified in univariate analyses. All tests of statistical significance were two-tailed. Probability values of ≤ 0.05 were considered statistically significant. Statistical analysis was performed with the SAS System for Windows, Release 8.02.

4.6 Results

Table 1 enumerates case and control subjects according to the major study sub-groups. In situ cancer accounted for 21.8%, 14.9%, and 60.0% of the pre-menopausal, post-menopausal, no-HT, and post-menopausal-HT cases, respectively (Table 2). Estrogen receptor status was similar among pre-menopausal and post-menopausal, no HT with 76.5% and 80.9% receptor positive respectively. Post-menopausal, HT users were predominantly estrogen receptor positive

(93.7%). Progesterone receptor status was similar across all sub-groups. With respect to age, BMI, age at menarche, and pregnancy history, distributions were similar across case-control status within the study sub-groups (Table 3). Additionally, age at first pregnancy, parity, smoking, age at menopause, type of menopause, and family history of cancer, were evaluated and were similar across case-control status and study sub-groups. Compared to controls (Table 4), cases had greater median breast density percent across all sub-groups while only significant in the pre-menopausal group (p=0.006). Lower 2-OH levels were noted in all case groups, but a statistically significant difference was noted only among pre-menopausal (p=0.032) and post-menopausal, no HT (p=0.022). The 16α-OH levels were lower in the pre-menopausal cases, but higher in both post-menopausal groups, but these associations were not statistically significant. With respect to the ratio, the pre-menopausal and post-menopausal no HT cases demonstrated a lower ratio, while only the post-menopausal no HT cases were significantly different (p=0.023). In post-menopausal HT cases, the 2:16 ratio was higher than controls, although not statistically significant.

In Table 5, the unadjusted and adjusted mean breast density and geometric mean estrogen metabolite levels are illustrated. The mean breast density percent remained greater in the pre-menopausal cases versus controls even after adjustment for age, BMI, and the estrogen metabolites (p=0.013). Additionally, lower 2-OH levels were noted in the pre-menopausal cases versus controls even after adjustment for age and BMI. In the post-menopausal, no HT users, cases had lower 2-OH and 2:16 ratio levels compared to controls even after age and BMI adjustment. No significant difference in mean breast density percent or 16α-OH was noted between cases and controls. With respect to mean breast density percent and the estrogen

metabolite levels, no significant difference was evident between cases and controls in post-menopausal HT users.

Finally, Tables 6-7 provide the odds ratio (OR) that describes the association between breast density tertile and breast cancer risk, unadjusted and adjusted for estrogen metabolites and breast cancer risk covariates. In premenopausal women (high density tertile vs. low density tertile), the OR ranged from 3.07 (95%CI 1.38-7.30) to 5.63 (95% CI 1.94-16.36) adjusting for BMI, estrogen metabolites and reproductive factors (ever pregnant, parity). Postmenopausal women not taking HT, had greater OR 1.63-1.91 inclusive of the adjustment for age, BMI, pregnancy history, parity, and estrogen metabolites in the mid vs. low tertile compared to the high vs. low tertile OR (0.85-1.27). Breast cancer associated weakly with breast density (high vs. low tertile) in postmenopausal women on HT (OR 0.88-1.64).

4.7 Discussion

Breast density and estrogen metabolism have both been implicated in the risk of developing breast cancer. Our main aim was to evaluate the extent to which adjustments for serum estrogen metabolite levels change the association between density and risk.

In this study, premenopausal women in the highest tertile of percent breast density were at a 3-4-fold increase risk of breast cancer when compared to women with the least dense breast tissue. This finding is consistent with previous reports (8, 10, 49). Moreover, we found that the association between breast density and risk of breast cancer was independent of the estrogen metabolites, age, BMI, and age at menarche.

Mammographic breast density serves as an estimate of the proportion of fibroglandular tissue to fat in the breast. Premenopausal women, compared to postmenopausal women,

generally have greater breast density secondary to a higher proportion of glandular tissue. As women age, fatty tissue replaces glandular tissue therefore decreasing breast density(13, 23, 28, 31). Consistent with this theory, reports in the literature illustrate a decrease in mammographic breast density with age, especially after the menopause(25).

Several epidemiological studies have reported breast density to be strongly associated with postmenopausal breast cancer risk (8, 10, 49). Although we did observe an increase in breast cancer risk with increase in percent density, the magnitude of the association was weak and not statistically significant in either the unadjusted or adjusted models. After adjustment for age, the trend toward increased risk with higher breast density became evident suggesting that age is confounding the association between breast density and breast cancer in the postmenopausal setting. With the addition of other breast cancer risk covariates including ever pregnant and parity, the trend for risk increased. This is consistent with the literature which has reported an association between early age at menarche, nulliparity, later age at first birth, and parity(2, 19, 22, 46, 48) with both increased breast cancer risk and greater breast density in pre- and post-menopausal women. With respect to breast density, pregnancy is associated with a change in breast structure to more differentiated lobules with less cell proliferation with the greatest effect on breast structure obtained from the first pregnancy, and further differentiation with each subsequent pregnancy(44).

The fact that in our population, breast density in postmenopausal women was not significantly associated with breast cancer risk may be a result of inadequate sample size or the inability to determine if years since menopause is confounding the association given that 39% of postmenopausal women not on HT were missing this information.

Furthermore, consistent with the literature, the use of HT was associated with increased breast density (Table 4). Several studies have reported an increase in breast density with the use of HT, in particular the combination of estrogen and progesterone(37).

It has been reported in the literature that low 2-OH levels and higher 16α-OH levels are associated with an increased risk of breast cancer(27, 30, 38, 39, 45, 56). However, not all studies have found this association(15, 51). To date, there has been only one study to report on the relationship between estrogen metabolites and breast density. Riza et al(43) evaluated the role of estrogen metabolites and their relationship with high-density Wolfe mammographic parenchymal patterns (P2/DY). The reported findings suggest that a high, rather than a low 2-OH:16α-OH ratio may be associated with an increase in breast cancer risk in postmenopausal women, which is opposite to that which would be expected.

Adjustments for 2-OH and 16α-OH produced no more than small decreases in associations observed between breast density and pre-menopausal breast cancer risk (Table 7). If breast density functioned primarily as a biological measure of estrogen metabolite status and if the metabolites acted as the proximate cause of breast cancer, adjustments for metabolite concentrations might have been expected to attenuate the breast density-breast cancer risk association to a greater extent.

There are several features of the present study that warrant discussion. Study limitations include the use of single measurement of serum estrogen metabolites and breast density. Estrogen metabolite levels in premenopausal women may be subject to fluctuations in the menstrual cycle. As we were unable to determine the phase at time of blood draw, variability may exist among pre-menopausal women with respect to the estrogen metabolite measures and the respective breast density measurement. Reproductive and lifestyle factors were obtained by

self-report which may lend to recall bias, thereby attenuating our results. Finally, we cannot exclude the possibility that our findings may be due to chance or confounded by some unidentified factor. Strengths of this study include histologically confirmed breast cancer cases, and the use of a single, expert breast density reader, which helps to reduce variability in breast density measurements and subsequent measures of effect.

In conclusion, this is the first study to investigate the association between breast density, serum estrogen metabolites, and breast cancer risk factors in pre-and post-menopausal women. A breast density-breast cancer risk relationship, that is independent of blood estrogen metabolite concentrations, exists in subgroups of women classified according to menopausal status and HT use. Additional studies are needed to clarify the relationship between breast density, estrogen metabolism and breast cancer risk.

Table 4-1: Study sub-groups according to menopausal status and hormone therapy use

	Control		Case	
Study sub-group	N	Percent	N	Percent
Pre-menopausal	124	33.1	55	31.6
Post-menopausal, no HT	159	42.4	94	54.0
Post-menopausal, HT	92	24.5	25	14.4

Abbreviations: HT – Current hormone therapy

Notes:

1. Table excludes five control and two case post-menopausal subjects with missing HT status.
2. $p = 0.009$ (chi-square test), case-control difference with respect to sub-group.

Table 4-2: Breast cancer cases according to type of cancer, stage, and estrogen/progesterone receptor status

	Pre-menopausal		Post-menopausal, no HT		Post-menopausal, HT	
	N	%	N	%	N	%
Cancer type						
In situ	12	21.8	14	14.9	15	60.0
Invasive	43	78.2	80	85.1	10	40.0
Stage						
Unknown	12		12		5	
In situ	9	20.9	12	14.6	12	60.0
I	21	48.8	50	61.0	4	20.0
II	10	23.3	17	20.7	3	15.0
III	3	7.0	3	3.7	1	5.0
IV	0	0	0	0.0	0	0.0
Estrogen Receptor						
Unknown	4		5		9	
Negative*	12	23.5	17	19.1	1	6.3
Positive**	39	76.5	72	80.9	15	93.7
Progesterone Receptor						
Unknown	4		5		9	
Negative†	13	25.5	35	39.3	4	25.0
Positive‡	38	74.5	54	60.7	12	75.0

1. Table excludes two case post-menopausal subjects with missing HT status.

2. Table excludes twelve pre-menopausal case, ten post-menopausal-no HT case, and two post-menopausal,-HT case subjects because of missing stage.

3. Table excludes four pre-menopausal case, three post-menopausal-no HT case, and nine post-menopausal,-HT case subjects because of missing estrogen receptor and progesterone receptor status.

* Includes 1 in situ, 1 in situ, and 1 in situ in pre-menopausal, post-menopausal, No HT, and post-menopausal, HT respectively

** Includes 7 in situ, 11 in situ, and 6 in situ in pre- menopausal, post-menopausal, No HT, and post-menopausal, HT respectively

† Includes 1 in situ, 3 in situ, and 2 in situ in pre- menopausal, post-menopausal, No HT, and post-menopausal, HT respectively

‡ Includes 7 in situ, 9 in situ, and 5 in situ in pre- menopausal, post-menopausal, No HT, and post-menopausal, HT respectively

Table 4-3: Cases and controls distributed according to age and three determinants of breast density

	Pre-menopausal					Post-menopausal, no HT					Post-menopausal, HT				
	Control		Case			Control		Case			Control		Case		
	N	%	N	%	P	N	%	N	%	P	N	%	N	%	P
Age (tertile)					0.923					0.085					0.214
Low	39	31.5	18	32.7		55	34.6	28	29.8		35	38.0	5	20.0	
Middle	43	34.7	20	36.4		53	33.3	23	24.5		29	31.5	9	36.0	
High	42	33.9	17	30.9		51	32.1	43	45.7		28	30.4	11	44.0	
BMI (kg/m^2)					0.499					0.721					0.687
<25	52	41.9	26	47.3		54	34.0	30	32.3		26	28.6	9	36.0	
25.0-34.9	42	33.9	20	36.4		53	33.3	28	30.1		41	45.1	9	36.0	
35.0+	30	24.2	9	16.4		52	32.7	35	37.6		24	26.4	7	28.0	
Age menarche (years)					0.353					0.528					0.055
<13	68	56.2	35	63.6		72	45.6	39	41.5		39	42.9	15	65.2	
13+	53	43.8	20	36.4		86	54.4	55	58.5		52	57.1	8	34.8	
Pregnancy history					0.885					0.321					0.347
never pregnant	19	16.1	8	17.0		24	15.7	10	11.1		14	16.1	5	25.0	
ever pregnant 6+ months	99	83.9	39	83.0		129	84.3	80	88.9		73	83.9	15	75.0	

Abbreviations: HT – Current hormone therapy; P – statistical significance (p-value; chi-square test) of case-control differences; BMI – Body mass index

Notes:

1. Table excludes five control and two case post-menopausal subjects with missing HT status.
2. BMI missing for one post-menopausal-no HT case and one post-menopausal,-HT control subject.
3. Age at menarche missing for three pre-menopausal control, one post-menopausal-no HT control, one post-menopausal-HT control, and two post-menopausal-HT case subjects.
4. Pregnancy history missing for three pre-menopausal control, three pre-menopausal case, one post-menopausal-no HT control, one post-menopausal-no HT case, two post-menopausal-HT control, and three post-menopausal-HT case subjects.
5. Ever pregnant, but never for 6+ months, for three pre-menopausal control, five pre-menopausal case, five post-menopausal-no HT control, three post-menopausal-no HT case, three post-menopausal-HT control, and two post-menopausal-HT case subjects.
6. Low, middle, and upper menopause- and HT-specific age tertiles defined according to age distribution in the control group, as follows, <44, 44-47, and 48+ year-old women, if pre-menopausal, <57, 57-62, and 63+ year-old women, if post-menopausal not on HT, or <56, 56-60, and 61+ year-old women, if post-menopausal on HT.

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Table 4-4: Breast density and estrogen metabolite measures, according to case-control status and study sub-group

	Pre-menopausal			Post-menopausal, no HT			Post-menopausal, HT		
	Control	Case	P	Control	Case	P	Control	Case	P
N	124	55		159	94		92	25	
Breast density (%)									
Median	41.1	50.3	0.006	27.0	30.8	0.827	37.1	38.2	0.634
IQR	25.6-55.7	41.4-65.9		12.7-46.1	16.9-39.9		24.6-50.9	27.5-51.4	
Range	0.0-92.2	3.5-85.9		0.0-85.7	0.0-68.1		0.0-84.0	0.0-77.2	
Mean	41.2	50.0		30.7	29.4		36.7	38.8	
95% CI	37.6-44.9	44.5-55.4		27.5-34.0	26.3-32.5		32.8-40.7	30.9-46.7	
2-OH (pg/ml)									
Median	251	187	0.032	158	140	0.022	348	287	0.800
IQR	163-388	118-308		117-218	99-183		194-472	193-638	
Range	43-897	50-680		33-1170	40-778		59-2058	71-1269	
Geometric mean	243	195		161	140		308	325	
95% CI	218-270	167-228		149-175	126-155		266-357	239-442	
16α-OH (pg/ml)									
Median	422	389	0.073	351	364	0.536	412	439	0.881
IQR	374-480	345-502		316-426	319-413		356-524	351-493	

Table 4-4 (Continued)

Range	262-836	280-659		129-923	253-734		290-1502	280-773	
Geometric mean	432	406		366	370		444	440	
95% CI	416-449	384-430		351-382	356-385		417-474	396-490	
2-OH:16α-OH ratio									
Median	0.53	0.50	0.123	0.43	0.37	0.023	0.74	0.80	0.661
IQR	0.40-0.83	0.33-0.78		0.31-0.59	0.28-0.50		0.47-1.10	0.44-1.42	
Range	0.14-1.85	0.15-1.56		0.10-2.72	0.09-2.02		0.16-2.52	0.15-1.90	
Geometric mean	0.56	0.48		0.44	0.38		0.69	0.74	
95% CI	0.51-0.62	0.42-0.55		0.41-0.48	0.34-0.42		0.61-0.79	0.56-0.97	

Abbreviations: HT – Current hormone therapy; P – statistical significance (p-value; Wilcoxon) of case-control differences; BMI – Body mass index

Notes:

1. Table excludes five control and two case post-menopausal subjects with missing HT status.

Table 4-5: Mean breast density and geometric mean estrogen metabolite measures in cases and controls

	Unadjusted			Age- and BMI-adjusted			Age-, BMI, 2-OH, and 16α-OH adjusted		
	Control	Case	P-value	Control	Case	P-value	Control	Case	P-value
Pre-menopausal									
N	124	55							
Breast density (%, mean)	41.2	50.0	0.010	41.7	48.8	0.016	41.8	48.7	0.013
2-OH (pg/ml, geometric mean)	243	195	0.027	243	194	0.022			
16α-OH (pg/ml, geometric mean)	432	406	0.086	431	407	0.108			
2-OH:16α-OH ratio (geometric mean)	0.56	0.48	0.080	0.56	0.48	0.060			
Post-menopausal, No HT									
N	158	94							
Breast density (%, mean)	30.7	29.3	0.562	30.2	30.3	0.963	30.1	30.3	0.944
2-OH (pg/ml, geometric mean)	161	139	0.031	161	139	0.037			
16α-OH (pg/ml, geometric mean)	366	371	0.694	366	372	0.638			
2-OH:16α-OH ratio (geometric mean)	0.44	0.38	0.018	0.44	0.38	0.020			
Post-menopausal, HT									
N	25	91							
Breast density (%, mean)	36.8	38.8	0.656	36.9	38.4	0.715	36.9	38.4	0.708
2-OH (pg/ml, geometric mean)	309	325	0.773	311	319	0.880			
16α-OH (pg/ml, geometric mean)	446	440	0.857	444	446	0.956			
2-OH:16α-OH ratio (geometric mean)	0.69	0.74	0.674	0.70	0.71	0.882			

Abbreviations: HT – Current hormone therapy; BMI – Body mass index

Notes:

1. Table excludes five control and two case post-menopausal subjects with missing HT status.
2. Table excludes one post-menopausal-no HT case and one post-menopausal,-HT control subject because of missing BMI.
3. Age-adjustment according to menopausal status and HT-specific age tertile category treated as a class variable, as determined by the age distribution in the control group. Low, middle, and upper age tertiles include <44, 44-47, and 48+ year-old women, if pre-menopausal, <57, 57-62, and 63+ year-old women, if post-menopausal not on HT, or <56, 56-60, and 61+ year-old women, if post-menopausal on HT.
4. BMI-adjustment according to BMI category (<25, 25-29, and 30+ kg/m2), treated as a class variable.
5. Age tertile by BMI interaction effect not statistically significant (in SAS PROC GLM) in all models examined. Therefore, age- and BMI-adjustments ignore any age by BMI interaction.
6. 2-OH and 16α-OH adjustments according to log-transformed concentration values treated as continuous variables.
7. Adjusted means and geometric means based on least square mean (LSMEAN) reported by SAS PROC GLM with OM option.
8. Statistical significance of case-control differences based on TYPE III test, as reported by SAS PROC GLM.
9. Tabulated values for the mean adjusted breast density allowed negative values for adjusted breast density. Recoding adjusted breast density percent for one pre-menopausal control (from -4.3% to 0%) increased mean adjusted breast density to 41.8%. Recoding adjusted breast density percent for three post-menopausal-no HT controls (from –4.8%, -5.5%, and –6.4% to 0%) increased mean adjusted breast density to 30.3%

Table 4-6: The odds ratio (OR) unadjusted and adjusted for age, BMA and selected breast cancer risk variables

	Breast density tertile				Breast density percent**
	Low	Mid	High	p-trend	
Pre-menopausal					
cases	10	14	31		
control	42	41	41		
		1.43	3.18		
OR (95% CI)[1]	reference	1.43 (0.57-3.59)	3.18 (1.38-7.30)	0.01	1.36 (1.07-1.72)
OR (95% CI)[2]		1.50 (0.59-3.80)	3.35 (1.43-7.87)	<0.01	1.37 (1.08-1.74)
OR (95% CI)[3]		1.51 (0.56-4.07)	4.69 (1.65-13.37)	0.05	1.33 (0.99-1.77)
OR (95% CI)[4]		1.48 (0.55-3.99)	5.63 (1.94-16.36)	0.03	1.38 (1.03-1.85)
Post-menopausal, no HT					
cases	26	44	24		
control	52	54	53		
OR (95% CI)[1]		1.63 (0.88-3.12)	0.91 (0.46-1.78)	0.60	0.95 (0.77-1.16)
OR (95% CI)[2]		1.80 (0.96-3.39)	1.19 (0.58-2.46)	0.87	0.98 (0.79-1.21)
OR (95% CI)[3]		1.90 (0.98-3.69)	1.18 (0.49-2.83)	0.78	1.03 (0.82-1.30)
OR (95% CI)[5]		1.91 (0.97-3.76)	1.27 (0.50-3.19)	0.56	1.08 (0.84-1.37)

Table 4-6 (continued)

Post-menopausal, HT					
cases	7	9	9		
control	31	31	30		
OR (95% CI)[1]		1.29 (0.43-3.89)	1.33 (0.44-4.02)	0.64	1.09 (0.77-1.53)
OR (95% CI)[2]		1.31 (0.41-4.14)	1.35 (0.44-4.16)	0.66	1.08 (0.76-1.52)
OR (95% CI)[3]		1.07 (0.28-4.07)	1.64 (0.44-6.19)	0.57	1.12 (0.76-1.67)
OR (95% CI)[5]		1.11 (0.25-4.91)	0.88 (0.15-5.16)	0.84	0.96 (0.62-1.47)

HT=Hormone therapy

1. Unadjusted, all subjects
2. Adjusted for age
3. Adjusted for age and BMI
4. Adjusted for age, BMI, and menarche
5. Adjusted for age, BMI, Ever Pregnant, Parity

*Odds of breast cancer among women with mid and high tertile breast density percent relative to women with low tertile breast density percent

**Increase in odds of breast cancer per 15% increase in breast density percent

Table 4-7: The odds ratio (OR) unadjusted and adjusted for estrogen metabolites and BMI

	Breast density tertile				Breast density percent**
	Low	Mid	High	p-trend	
Pre-menopausal					
cases	10	14	31		
control	42	41	41		
OR (95% CI)[1]	reference	1.43 (0.57-3.59)	3.18 (1.38-7.30)	0.01	1.36 (1.07-1.72)
OR (95% CI)[2]		1.56 (0.61-3.99)	3.19 (1.39-7.37)	0.01	1.37 (1.08-1.75)
OR (95% CI)[3]		1.33 (0.52-3.39)	3.07 (1.33-7.09)	0.01	1.35 (1.06-1.72)
OR (95% CI)[4]		1.43 (0.55-3.70)	3.12 (1.35-7.25)	0.01	1.36 (1.07-1.74)
OR (95% CI)[5]		1.44 (0.54-3.82)	4.35 (1.57-12.08)	0.05	1.32 (0.99-1.76)
OR (95% CI)[6]		1.51 (0.55-4.19)	4.17 (1.49-11.69)	0.06	1.32 (0.99-1.76)
OR (95% CI)[7]		1.32 (0.48-3.61)	4.14 (1.48-11.61)	0.06	1.32 (0.99-1.76)
Post-menopausal, no HT					
cases	26	44	24		
control	52	54	53		
OR (95% CI)[1]		1.63 (0.88-3.12)	0.91 (0.46-1.78)	0.60	0.95 (0.77-1.16)
OR (95% CI)[2]		1.65 (0.88-3.09)	0.93 (0.47-1.84)	0.68	0.96 (0.78-1.18)
OR (95% CI)[3]		1.64 (0.88-3.04)	0.89 (0.45-1.76)	0.59	0.95 (0.77-1.16)
OR (95% CI)[4]		1.65 (0.88-3.08)	0.92 (0.46-1.82)	0.67	0.96 (0.78-1.18)
OR (95% CI)[5]		1.67 (0.88-3.17)	0.89 (0.39-2.04)	0.94	0.99 (0.79-1.24)
OR (95% CI)[6]		1.69 (0.88-3.23)	0.90 (0.39-2.08)	0.97	0.99 (0.79-1.25)
OR (95% CI)[7]		1.69 (0.89-3.21)	0.85 (0.37-1.97)	0.92	0.99 (0.79-1.24)

Table 4-7 continued

Post-menopausal, HT					
Cases	7	9	9		
Control	31	31	30		
OR (95% CI)[1]		1.29 (0.43-3.89)	1.33 (0.44-4.02)	0.64	1.09 (0.77-1.53)
OR (95% CI)[2]		1.12 (0.36-3.49)	1.41 (0.46-4.32)	0.66	1.08 (0.77-1.53)
OR (95% CI)[3]		1.31 (0.44-3.98)	1.38 (0.45-4.20)	0.63	1.09 (0.77-1.54)
OR (95% CI)[4]		1.14 (0.36-3.56)	1.41 (0.46-4.33)	0.64	1.09 (0.77-1.54)
OR (95% CI)[5]		1.07 (0.31-3.67)	1.50 (0.41-5.46)	0.58	1.12 (0.76-1.65)
OR (95% CI)[6]		1.04 (0.30-3.61)	1.48 (0.41-5.38)	0.57	1.12 (0.76-1.65)
OR (95% CI)[7]		1.08 (0.31-3.77)	1.48 (0.41-5.37)	0.57	1.12 (0.76-1.66)

HT=Hormone therapy

BMI=Body mass index

1. Unadjusted, all subjects
2. Adjust ln(2-OH)
3. Adjust ln(16-OH)
4. Adjust ln(2-OH) and ln(16-OH)
5. Adjust BMI
6. Adjust BMI and ln(2OH)
7. Adjust BMI and ln(16OH)

*Odds of breast cancer among women with mid and high tertile breast density percent relative to women with low tertile breast density percent

**Increase in odds of breast cancer per 15% increase in breast density percent

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5. GENERAL DISCUSSION

5.1. Breast Cancer Statistics

In the year 2005, breast cancer will account for 32% (211,240 new cases) of all cancer cases in women in the United States with 41,514 deaths attributed to breast cancer(20). Assuming a life expectancy of 85 years, it is estimated that the lifetime risk of developing breast cancer is one in eight for American women with the risk rising considerably for women who have pre-existing risk factors including older age, family history of breast cancer, BRCA1 and BRCA2 gene mutations, or a history of biopsy proven precursor lesions(36). Incidence and mortality rates differ greatly among ethnic groups. Among black women in the United States, the age-standardized incidence rate is lower than the rate in white women with 119.9 per 100,000 cases and 141.7 per 100,000 respectively. Although white women have a higher incidence of breast cancer, the mortality is greater in black women (35.4 per 100,000) than white women (26.4 per 100,000)(20). In Hispanic, American Indian, and Asian women, both the incidence and mortality rates are reportedly lower than both white and black women. The disparity in incidence is believed to be secondary to more frequent mammograms, later age at first birth, and greater use of hormone therapy (HT) among white women. Higher mortality rates among black women are thought to reflect later stage at diagnosis, differences in the access for diagnosis and treatment, and biological differences, most notably an increased incidence of estrogen receptor (ER) negative tumors(20). While the incidence of breast cancer is higher in women who carry a genetic mutation, this only explains approximately two-thirds to three-fourths of familial breast cancer which itself makes up 10% of all breast cancer cases(14, 26). With greater knowledge of

factors affecting risk, an opportunity exists to identify those women who are most likely to benefit from the implementation of prevention strategies.

5.2. Breast Density

Over the past 25 years, there has been a steady increase in the incidence of breast cancer with the greatest annual percent change (3.7%) noted between 1980 and 1987 with a subsequent decrease to 0.5% between 1987 and 1999. It has been postulated that the initial increase was related to the initiation of the systematic use of mammography and the associated lead-time bias(13, 21). Mortality has decreased steadily over the past 25 years and is associated with earlier detection. Mammographic sensitivity ranges between 63-87.7% and specificity between 80-90%(3, 12, 15). Several factors can influence mammographic sensitivity and specificity including age, HT use, and density. Breast density, in particular, has gained attention in relation to the risk of breast cancer. Outside of age and BRCA1 or BRCA2, density carries the largest risk for breast cancer with a four to six-fold increase in risk in those women with dense breast tissue(7, 8). What remains unclear is the best way to measure density. The first method was proposed by Wolfe in 1976, consisting of four classifications: N1 radiolucent (fatty) breasts containing few ducts; P1 ductal (linear) pattern occupying less than 25% of the breast area; P2 ductal (linear) pattern and nodular densities occupying more than 25% of the breast area; and the DY radio-density that completely obscured the ductal pattern. Additionally, the breast imaging reporting and data systems (BIRADS) is another qualitative classification. This system also utilizes four categories: extremely fatty; scattered density; heterogeneous density; and extremely dense. Several studies have found the Wolfe categories to predict risk and although few studies have utilized the BIRADS classification, those reported show a significant increase risk in the extremely dense

category(8). Other methods of measurement include quantitative approaches which have reported consistent results and larger gradients of risk when compared to qualitative methods(8, 34). Current quantitative methods include computer-assisted methods, estimation by radiologists and planimetry. Despite current methods of measuring breast density, a standard approach for measurement is lacking and methodological limitations are evident. Both qualitative and quantitative methods have an element of subjectivity lending to the possibility of inter-observer and intra-observer variation. Additionally, current mammographic films are 2-dimensional, which restricts the ability to fully appreciate the volume of the breast and the subsequent dense area. Potential methods to improve density measures are underway and could help to improve the understanding between density and the risk of breast cancer(8, 18, 27).

The question as to whether density is associated with a causal mechanism or is just an element of masking is not new. Masking does occur and may increase the risk in the short term, but with long term follow-up and repeated screening this effect disappears(8). In a study reported by Byrne et al(11) in which 1880 out of 4000 women developed breast cancer, it was noted that some women developed breast cancer as many as 14 years after high density had been detected.

It has also been postulated that the components of breast tissue differ between dense and non-dense tissue. Connective tissue, including collagen and fibroblasts, along with epithelial cells are components of dense breast tissue. Several studies have evaluated the relationship between density as it appears radiologically with the histology of breast tissue. Nine of ten studies used samples from a mastectomy or biopsy and six of these studies reported an association between density and epithelial proliferation and density and stromal proliferation(2, 4-7, 10, 16, 32, 35). A concern was that these samples might not be representative of the general

population. Li and colleagues reported on a study using samples from women at forensic biopsy which revealed a higher percentage of the tissue occupied by cells, glandular structures, and collagen in dense breast tissue(4, 8, 22).

5.3. Estrogen and Breast Cancer

Exposure to endogenous estrogen and its role as a risk factor for breast cancer has been consistently reported in the literature(24). More recently, the metabolism of estrogens has been implicated as one of the mechanisms of carcinogenesis secondary to genotoxic and mutagenic metabolites(38, 39). It is postulated that the contribution of metabolites to breast cancer is related to the presence and formation in breast tissue(38). Supporting this theory are reports that breast tissue levels of estrogen were found to be 10-50 times the levels in blood(33) and higher estradiol concentrations were noted in malignant versus nonmalignant tissues in postmenopausal women. This is thought to be reflective of the aromatase activity in breast tissue(19, 38). Additionally, evidence that oxidative pathways are active in breast tissue was demonstrated with the detection of estrogen metabolites and conjugates ranging from 3 to 13 pmol per gram in human breast tissue(29, 38). Lastly, there is evidence to suggest a relationship between breast cancer risk and polymorphisms in genes responsible for encoding the enzymes involved in estrogen metabolism(38).

The Mammogram and Masses Study (MAMS) provided a unique opportunity to evaluate the relationship between mammographic breast density, estrogen metabolism and breast cancer risk factors in both cases and controls. Several studies have evaluated breast density or estrogen metabolism in conjunction with breast cancer risk factors, but only one has evaluated breast density and estrogen metabolism together(28). Still, many questions remain regarding the role of

estrogen metabolism, the most appropriate measurement of the metabolite levels and if risk may be modified. Additionally, measurement of density is not standardized and while improved, still has limitations. An extensive questionnaire attempted to capture the multitude of internal and external factors that are related to breast cancer risk. As with many questionnaires, recall bias is a potential issue when considering the results. Density measurements were reported for both Wolfe's method and quantitatively using planimetry to produce percent density (area of density/total area of the breast). Initially, one of the goals of the study was to evaluate the interaction between different types of HT (estrogen + progesterone and estrogen only formulations) and density and HT and estrogen metabolism. However, due to the reported increase in breast cancer risk released by the Women's Health Initiative(37), we noted a significant decline in HT users rendering the ability to effectively evaluate the association with HT use, and in particular differences among HT preparations impossible.

5.4. Article 1

In the first article, we evaluated the relationship between estrogen metabolite levels and breast cancer risk factors, independent of sex-steroid hormones, in an attempt to provide insight into the underlying biologic mechanisms. The population consisted of participants in the Study of Osteoporosis and Fractures (SOF), a longitudinal study that evaluated risk factors for osteoporosis and falls in 9,704 White women age 65 and older. They were recruited from 1986-1988 using population-based lists in Baltimore, MD, Pittsburgh, PA, Minneapolis, MN, and Portland, OR. We included only those cancer free controls who participated in the estrogen metabolite study and who also had available sex serum hormones. Our results revealed significant associations between the sex-steroid hormones and estrogen metabolite levels. The

relationship between weight and 16α-OH and BMI and 16α-OH were statistically significant in univariate analyses. However, in multivariate analyses, these relationships disappeared once we controlled for the sex-steroid hormones. In general, there were no significant relationships between the putative breast cancer risk factors and the 2-OH levels and the 2:16 ratio. We found a significant positive relationship between surgical menopause and the 16α-OH metabolite, independent of estradiol, testosterone, and SHBG. Women who had a surgical menopause had seven times the odds of having a 16α-OH level in the high versus low tertile (OR=7.37, $p<0.001$). In a study of 15,844 women, a non-significant increase in breast cancer risk was noted in women who underwent gynecologic surgery for endometriosis(30). Hence, the higher 16α-OH associated with surgical menopause may reflect the underlying hormone dysregulation associated with the indication for the hysterectomy. Lastly, in our analyses, we found no relationships between traditional risk factors (age, age at menarche, age at first birth, age at menopause) and the estrogen metabolite levels. This may have reflected the small sample size. However, if the metabolites truly cause breast cancer, it does not appear that the metabolites are mediating the breast cancer risk effects of the traditional risk factors.

5.5. Article 2

In the second article, in pre- and post-menopausal women without breast cancer, we endeavored to characterize the relationship between breast density and the serum concentrations of two major estrogen metabolites (2-OH and 16α-OH). Subjects eligible for study included pre- or post-menopausal women with 1) no personal history of cancer (except skin) and 2) no evidence for breast cancer after mammography screening or breast biopsy. Volunteers arose from two sources: 1) women undergoing outpatient needle breast biopsy through the Breast Biopsy Service

at Magee-Womens Hospital (Pittsburgh, Pennsylvania) and 2) women receiving screening mammography through Magee-Womens Hospital or through a suburban Pittsburgh Magee Womancare Center. To identify and recruit eligible subjects, a research assistant personally solicited women visiting the Breast Biopsy Service between September 2001 and May 2004 and women visiting either the Magee-Womancare Center – North (Wexford, Pennsylvania) or Magee-Womancare Center – East (Monroeville, Pennsylvania) between July 2002 and September 2003. To boost subject recruitment, Magee-Womens Hospital attached study flyers to screening result reports mailed to Magee Womancare Center patrons with negative mammography breast imaging reporting and data systems (BIRAD 1 and 2) between November 2003 and May 2004. We evaluated premenopausal women, postmenopausal women not on HT, and postmenopausal women on HT separately. We found that the mean percent breast density was higher in premenopausal women and postmenopausal women on HT than postmenopausal women not taking HT. BMI was inversely related to breast density percent in all subgroups. We did note in leaner women (BMI <25 kg/m^2) higher 2-OH levels in pre-and postmenopausal women on HT compared to heavier women, which has been previously reported(23). Additionally, leaner postmenopausal women on HT had higher 16α-OH levels(25). With respect to reproductive factors, our results revealed greater percent breast density in nulligravid postmenopausal women not on HT than postmenopausal women not on HT with at least one pregnancy. This association remained statistically significant even with adjustment for age indicating that age and pregnancy independently influence breast cancer risk. Mean 16α-OH concentrations were higher in nulligravid premenopausal women, although not statistically significant. Menarche occurring at age 13 or greater yielded significantly denser breast tissue when compared with women who began menstruatingat <13 years, particularly in the

premenopausal population. Again, in premenopausal women, only the mean 2-OH:16α-OH ratio was associated with an earlier age at menarche vs later menarche, although not statistically significant. Our results revealed associations between the estrogen metabolites and breast density, which appeared independent of post-menopausal hormone medication use. However, statistical associations disappeared upon control for menopausal status. Our findings suggest that serum estrogen metabolite concentrations and breast density may share common determinants that are related to the menopause. Or, estrogen metabolite changes, occurring as a consequence of menopause, may directly contribute to the menopause-associated declines in breast density.

5.6. Article 3

In the third article, we comparatively evaluated the association of breast density, estrogen metabolites, menopausal status and breast cancer risk factors among breast cancer cases and controls using the study as mentioned in article 2 with the addition of breast cancer cases confirmed by pathology reports for a total of 178 premenopausal (55 cases, 124 controls) and 378 post-menopausal (121 cases, 256 controls) women. .

Premenopausal women have a 3-4 fold increased risk of breast cancer in those that have high tertile breast density vs low tertile density. Breast density did not appear to substantially increase breast cancer risk among post-menopausal women not taking HT. However, a trend for increased risk of breast cancer (OR 1.41-1.64, high tertile density vs low tertile density) when adjusting for the estrogen metabolites, BMI, and age was noted. This finding is consistent with previous reports noting the increased risk with increasing density(7, 8, 31). This breast density-breast cancer association remains significant even with adjustment for the estrogen metabolites

suggesting that breast density and estrogen metabolites independently contribute to breast cancer risk. Factors associated with breast density in premenopausal women may differ from factors associated with breast density in post-menopausal women.

In premenopausal women (high density tertile vs. low density tertile), the OR ranged from 3.07 (95%CI 1.38-7.30) to 5.63 (95% CI 1.94-16.36) adjusting for BMI, estrogen metabolites and reproductive factors (ever pregnant, parity). Postmenopausal women not taking HT, had greater OR 1.63-1.91 inclusive of the adjustment for age, BMI, pregnancy history, parity, and estrogen metabolites in the mid vs. low tertile compared to the high vs. low tertile OR (0.85-1.27). Breast cancer risk associated with breast cancer in postmenopausal women on HT (OR 0.88-1.64 in high tertile vs. low tertile), revealed an association, strengthened when adjusted for the estrogen metabolites, BMI, and age.

In contrast to the association between risk of breast cancer and breast density reported among both pre- and post menopausal women(7, 8, 31), we found that among post-menopausal women breast density appeared to protect against breast cancer, while not statistically significant. This association was evident in the evaluation of breast density alone and when adjusting for the estrogen metabolites and BMI. The fact that in our population, breast density in postmenopausal women was not associated with breast cancer risk may be a result of the sample size or the inability in this dataset to determine if years since menopause is confounding the association.

There are several features of the present study – used for article 2 and 3 - that warrant discussion. To our knowledge, this is the first study to examine breast density, serum estrogen metabolites, and breast cancer risk factors. The use of a single, expert breast density reader helps to reduce variability in breast density measurements and subsequent measures of effect. However, mammographic films were only available at one time point which limits the ability to identify

changes in breast density over time with respect to age, menopausal status, as well as changes related to HT use. The use of serum to measure estrogen metabolites in particular with premenopausal women may be subject to fluctuations in the menstrual cycle and therefore may not accurately reflect the level of estrogen metabolism. In this study we utilized serum for the measurement of estrogen metabolites while most other studies have utilized urine. In a study by Bradlow et al(9), plasma and urinary levels of 2OH and 16α-OH were compared in nulliparous women aged 17-35. They concluded that the correlation varied across ethnic groups and baseline use of oral contraceptives and coffee consumption. Additionally, the time at which the samples were collected during the menstrual cycle had an impact on the levels. Overall, the correlation between the two methods was felt to be fair. While the comparison was only conducted in premenopausal women it is suspected that the amount of variation would be less in the postmenopausal population.

Lastly, reproductive and lifestyle factors were obtained by self-report which may lend to recall bias, thereby attenuating our results. Additionally, we cannot exclude the possibility that our findings may be due to chance or confounded by some unidentified factor.

5.7. Future Research

In the first study, we evaluated the determinants of estrogen metabolism in postmenopausal women. While this provided an opportunity to begin to understand the relationship between estrogen metabolism in the postmenopausal setting, the premenopausal setting deserves evaluation. Measurement of metabolism levels in the premenopausal setting will need to account for menstrual cycle variations and the subsequent fluctuations in the estrogen metabolism levels. Lastly, the most effective medium for measurement of the estrogen metabolites remains to be

determined. Currently, urine and serum assays are available and both carry limitations. Identification of tools to enhance measurement of the metabolite levels, particularly at the tissue level would be optimal. Lastly, the impact of HT and newer agents, in particular selective estrogen response modulators (SERMs) on estrogen metabolism and breast density warrants further investigation.

Evidence suggests hormonal factors may be more strongly associated with estrogen receptor (ER)+/progesterone receptor (PR)+ than ER-/PR- breast cancer risk. The presence of estrogen receptors in the primary tumor not only indicate likely response to hormonal therapy, but also confer a better prognosis(1, 17). The role of estrogen metabolism and ER/PR status has not been evaluated and may potentially lend to the understanding of the relationship between those risk factors including breast density, which appears to be hormonally responsive. As ER positive tumors confer a better prognostic factor in breast cancer, it would be useful to understand those factors, which may mediate the hormone receptor status.

With respect to density, while we have made great progress in the measurement of density, the incorporation of better methods to capture the volume of breast density is crucial to fully appreciate the underlying biology and subsequent association with breast cancer risk.

Finally, our study included predominantly white women and did not allow us to examine if similar relationships exist in the non-white population. Considering that black women have demonstrated biological differences with respect to estrogen receptor status, studies incorporating different ethnic groups would be important to identify differences in estrogen metabolism and breast density as to optimize breast cancer prevention strategies.

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6. PUBLIC HEALTH SIGNIFICANCE

This compilation of studies focused first on identifying the determinants of estrogen metabolism and then subsequently the relationship between estrogen metabolites and breast density, first in the control population and then across cases and controls. The results demonstrated that except for BMI, 16α-OH, and surgical menopause, we did not see associations between the estrogen metabolites and the traditional risk factors. Control for substrate hormones eliminated the BMI association, but not the association with type of menopause. While it appears that the substrate hormone levels appear to mediate the association between BMI and 16α-OH, the apparent lack of residual association between BMI (and other risk factors) with 16α-OH or 2-OH suggests that inter-individual differences in hormone metabolism, per se, are insensitive to external factors. Further evaluation of the association between estrogen metabolites and breast density in controls, inclusive of pre-menopausal, post-menopausal women not taking hormone therapy (HT), and post-menopausal women taking HT, revealed that high serum concentrations of the 16α-OH and 2-OH correlated with breast density. The associations between the estrogen metabolites and breast density appeared independent of post-menopausal hormone medication use. However, statistical associations disappeared upon control for menopausal status. This pattern of association is consistent with two possibilities. First, serum estrogen metabolite concentrations and breast density may share common determinants that are related to the menopause. Or, secondly, that estrogen metabolite changes, occurring as a consequence of menopause, may directly contribute to the menopause-associated declines in breast density. Determination of the menopause-specific cross-sectional associations between the serum estrogen metabolites and breast density may require larger studies, with sufficient numbers of

pre- and post-menopausal women. In the final paper, a breast density-breast cancer risk relationship, that is independent of serum estrogen metabolite concentrations, exists in subgroups of women classified according to menopausal status and HT use. Factors associated with breast density in premenopausal women may differ from factors associated with breast density in post-menopausal women.

Breast density, outside of age and BRCA1 and BRCA2 mutations, is the strongest risk factor for breast cancer. It is well known that screening mammography is the best way to reduce morbidity and mortality from breast cancer. It is also well established that breast density affects the sensitivity and specificity of mammography and therefore can reduce the benefits of screening. It has been postulated that if density were taken into account in the standard breast cancer risk assessment, up to 20% of postmenopausal women would be eligible for chemoprevention(1). Therefore, understanding factors that affect breast density and their underlying mechanism is an important public health issue. Such an understanding will help us improve breast cancer screening and may help us identify women who are at an increased risk of breast cancer and for whom prevention strategies may be useful.

6.1. References

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7. SUMMARY

The study titled "Mammograms and Masses Study" (MAMS) was designed to evaluate the role of estrogen metabolism, hormone replacement therapy (HT), body mass index (BMI), and breast density in breast cancer. The first article utilized a control population from the Study of Osteoporosis and Fractures (SOF) to answer the question regarding the determinants of estrogen metabolism. Two hundred eight-two randomly selected women without breast cancer recruited from 1986-1988 using population-based lists in Baltimore, MD, Pittsburgh, PA, Minneapolis, MN, and Portland, OR were analyzed for this report. Logistic regression was used to examine the independent association between the level of a particular estrogen metabolite and various factors including hormones (total estrogen and total testosterone concentration in blood), binding protein, and behavioral/reproductive breast cancer risk factors. Analyses were conducted with a base model consisting of standardized log transformed estradiol, testosterone, and SHBG. Age, BMI, and other statistically significant variables ($p < 0.10$), identified in univariate analyses were added to the base model singly, to evaluate the metabolite levels in mid versus low tertile as well as high versus low tertile.

Results did not show consistent associations between risk factors and estrogen metabolites except for a positive association between BMI and 16α-OH and surgical menopause and 16α-OH. When adjusting for the substrate hormones, the association between BMI and 16α-OH disappears suggesting that the hormones mediate the effect of BMI on 16α-OH. However, the association between type of menopause and 16α-OH persists after adjustments for substrate hormone concentrations. This tentative observation (higher 16α-OH in women, with surgical

menopause, not explained by differences in substrate hormone concentrations) suggests surgical menopause may identify women who metabolize estrogen differently.

The second article was designed to investigate the relationship between breast density and estrogen metabolism. We measured breast density (area measure of visibly dense breast, expressed as a percentage of the total breast area on a standard two-dimensional mammogram) and serum concentrations of 2-OH and 16α-OH in 124 pre- and 256 post-menopausal women who lacked a diagnosis of breast cancer despite screening mammography or biopsy of suspicious breast abnormalities. To identify and recruit eligible subjects, a research assistant personally solicited women visiting the Breast Biopsy Service between September 2001 and May 2004 and women visiting Magee-Womancare Center – North (Wexford, Pennsylvania) and East (Monroeville, Pennsylvania) between July 2002 and September 2003. To boost subject recruitment, Magee-Womens Hospital attached study flyers to screening result reports mailed to Magee-Womancare Center patrons with negative mammography between November 2003 and May 2004. The study group included 124 pre-menopausal women (median age 46 years, range 39-55 years; 115 white, 6 other, and 3 unknown race; 34% overweight (BMI 25.0-29.9 kg/m^2) and 24% obese (BMI 30.0+ kg/m^2); 4 N1, 19 P1, 96 P2, and 5 DY Wolfe parenchymal pattern; median breast density 41%, inter-quartile range (IQR) 26-56%) and 256 post-menopausal women (median age 59 years, range 44-84 years; 241 white, 11 other, and 4 unknown race; 37% overweight and 31% obese; 15 N1, 66 P1, 173 P2, and 2 DY Wolfe parenchymal pattern; median breast density 32%, IQR 16-49%). Forty-six (18%) and 210 (82%) post-menopausal women had experienced surgical and natural menopause, respectively. Among 251 post-menopausal women with known history of estrogen or progesterone hormone therapy (HT), 92 (37%) reported current use.

In every subgroup defined according to menopausal status and current use of hormone therapy, analyses did not show statistically significant association between any single serum estrogen metabolite measure and age. However, as previously noted by other investigators, an increase in BMI is associated, specifically in post-menopausal women taking hormone therapy, with a substantial decrease in 2-OH, 16α-OH and the 2:16α-OH ratio. Using multiple linear regression, the effects of serum 2-OH and 16α-OH on breast density were evaluated. Unadjusted, both 2-OH and 16α-OH were associated with breast density, in pre-menopausal and post-menopausal women, HT-users and HT-nonusers, considered together. This association remained even after adjustment for age and BMI. Removing HT users, there was little change in the association noted in the entire subject population indicating that the effects of estrogen metabolism on breast density are independent of HT use. However, once adjusted for menopausal status, while there is a trend for higher β 16α-OH values, no statistically significant association is noted in any of the subgroups.

As in the first paper, it would have been helpful to evaluate the sex-steroid hormones and the estrogen metabolites. However, menopausal status may serve as a proxy since the sex-steroid hormone levels are related to menopausal status.

In conclusion, there may be common determinants for both breast density and the estrogen metabolites or it is possible that menopausal status influences estrogen metabolite levels, which in turn influence breast density.

Lastly, in the third article we sought to determine whether breast density and estrogen metabolites are independently associated with breast cancer risk. The study sample included 380 controls consisting of 192 (50.5%), 71 (18.7%), and 117 (30.8%) and 176 cases consisting of 80 (45.4%), 94 (53.4%) 1 (0.6%), and 1 (0.6%), and women from the Magee-Womens Breast

Biopsy Service , Surgical Clinic, Magee-Womancare Center – North/East, and mass mailings, respectively.

Using logistic regression, the risk of breast cancer was evaluated across tertiles of density, both unadjusted and adjusted for age and BMI. As expected, the risk of breast cancer in the high tertile vs. low tertile breast density was 3-4-fold even after adjusting for age and BMI in pre-menopausal women. With respect to primary hypotheses pertaining to the independence of association, adjustments for blood estrogen metabolite concentrations did not have any meaningful effect on the observed association between breast density and breast cancer risk in pre-menopausal women.

Except perhaps for some elevation in breast cancer risk in post-menopausal women not taking HT with mid tertile relative to low tertile breast density, logistic regression analyses produced scant evidence for association between breast density and breast cancer risk in post-menopausal women, unadjusted or adjusted for estrogen metabolite concentrations. Small sample sizes and wide confidence intervals limited ability to make meaningful inferences. Other factors possibly responsible for inability to demonstrate association between density and risk in post-menopausal women include unrecognized selection bias related to recruitment of case and control subjects from diverse sources. Through observer blinding, breast density was measured in a way independent of case-controls status. Therefore, systematic bias related to breast density determination is not likely.

In conclusion, the breast density-breast cancer association remains significant even with adjustment for the estrogen metabolites, at least in pre-menopausal women, suggesting that breast density may relate to breast cancer risk through pathways not involving estrogen metabolism.

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