COMMON VARIATION IN THE FIBROBLAST GROWTH FACTOR RECEPTOR 2 (*FGFR2*) GENE, HORMONE THERAPY USE, AND MAMMOGRAPHIC BREAST DENSITY

by

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Objectives Mammographic breast density (MBD) is a strong risk factor for breast cancer. It has been hypothesized that breast cancer susceptibility loci may also be associated with breast density. Recently, two genome-wide association studies identified a single-nucleotide polymorphism (SNP), rs2981582, in intron 2 of the *FGFR2* gene to be associated with increased breast cancer risk. Further research revealed that intron 2 of *FGFR2* contains estrogen receptor transcription factor binding sites. We examined associations of four *FGFR2* SNPs (rs2981582, rs3750817, rs17542768 and rs1219643), hormone therapy (HT) use, and their interactions with MBD.

Methods We conducted a cross-sectional analysis using a subset of the Mammograms and Masses Study population. Subjects were 370 healthy postmenopausal Caucasian women. General linear models adjusted for covariates were used to evaluate the associations.

Results Overall, no statistically significant associations were observed between the four SNPs in *FGFR2* and MBD. Duration of estrogen plus progestin use, but not duration of estrogen use, and HT status were statistically significantly associated with MBD in our study population. No statistically significant interactions between genotypes and HT use were observed.

Conclusions Our results suggest that the effects of the four evaluated *FGFR2* polymorphisms on breast cancer risk are not mediated through MBD, and that the polymorphisms do not modify the effect of HT use on MBD.

Implications for public health Breast cancer is the most common cancer in women in the U.S. Identification of genes that influence MBD may provide insight into the biology of breast density and its effect on breast cancer, eventually leading to more effective prevention and treatment.

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PREFACE

First and foremost I would like to thank Dr. Brenda Diergaarde for providing me the opportunity to conduct this exciting research. I thank her for being a mentor and role model to me and her tireless effort teaching and guiding me every step of the way. I have learned so much from working on this project. I would like to thank Dr. Eleanor Feingold for her support and guidance throughout the past two and half years. Dr. Feingold has fostered my interest in genetic epidemiology through her outstanding ability to teach. I would like to thank Dr. Joel Weissfeld, who allow me to work independently while always being accessible to discuss my research.

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1.0 INTRODUCTION

Breast cancer is the most common cancer in women, and the second leading cause of cancer death among women in the U.S.[1] Based on the Surveillance Epidemiology and End Result (SEER) data, the National Cancer Institute (NCI) estimates that the lifetime risk for a woman to develop breast cancer is 12%.[2]

1.1 MAMMOGRAPHIC BREAST DENSITY, HORMONE THERAPY USE AND BREAST CANCER RISK

Extensive research on breast cancer has identified a number of risk factors, and the risk of the disease is influenced by genetic, environmental and life-style factors. High mammographic breast density (MBD) is a strong, independent risk factor for breast cancer.[3-12] Women with density in more than 75% of the breast have a four to five times greater risk of developing breast cancer than women with little or no density in the breast.[13] Compared to the frequencies of other most recognized risk factors, such as a family history of breast cancer which occurs in only 10% of women; about 30% of postmenopausal women have high breast density.[14] MBD is influenced by a number of factors related to breast cancer risk: increasing age and menopause are independent contributors to a decrease in breast density[15], higher body mass index (BMI) is associated with low breast density, whereas increased age at first birth is associated with high

breast density.[11, 16-18] In addition, postmenopausal hormone therapies (HT) that include both estrogen and progestin are associated with an increase in breast density that decreases upon discontinuation of therapy. [19-21] However, all these factors only explain about 20%-30% of the variation in MBD. MBD has a strong genetic component. Twin studies indicate that a substantial proportion (60%-75%) of the variance in MBD is due to heritability.[22-23]

There is also strong epidemiologic evidence that postmenopausal HT use, especially the use of estrogen plus progestin (E+P), is associated with increased breast cancer risk.[24-27] It has been suggested that the observed increase in breast cancer risk may be explained by the effects of HT use on breast density.[4-5] The effects of HT on breast tissue, as seen in mammography, include increases in density (focal, multifocal or diffuse), and increases in the size of cysts and fibroadenomas.[28-30]

However, the exact mechanisms by which density confers the increased risk of breast cancer remain uncertain. It is possible that the risk of breast cancer associated with increased MBD may arise from the combined effects of cell proliferation, in response to mitogens and the resulting greater number of susceptible cells, and genetic damage to cells by mutagens.[31] It has been shown that within populations of cells *in vitro* and *in vivo*, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype.[32] Epithelial hyperplasia and concomitant increases in growth factors have also been suggested[33]; several biopsy studies have shown that high-density areas are associated with epithelial hyperplasia.[34-37]

Because of the high degree of heritability of MBD and its strong association with breast cancer, it is possible that breast cancer susceptibility loci may also be associated with breast density. Investigating these associations may provide insight into the biology of breast density and its influence on breast cancer.[38]

1.2 FGFR2

Two genome-wide association studies (GWAS)[39-40] identified a single-nucleotide polymorphism (SNP), rs2981582, in intron 2 of the fibroblast growth factor receptor 2 (*FGFR2*) gene to be associated with an increased risk of breast cancer.

FGFR2 belongs to the fibroblast growth factor receptor (FGFR) family, which contributes to cell growth, invasiveness, motility and angiogenesis.[41] The association of *FGFR2* polymorphisms with breast cancer risk may be mediated through regulation of FGFR2 expression. A recent study described how two SNPs in intron 2 of *FGFR2* alter the binding of two transcription factors and cause an increase in *FGFR2* gene expression.[42] Overexpression of FGFR2 is observed in breast cancer cell lines[43], as well as in breast tumor tissues.[44] It has also been observed that aberrant *FGFR2* signaling activation induces proliferation and survival of tumor cells.[45] MBD is largely a reflection of the amount of dense stromal tissue that may provide a permissive environment for neoplastic transformation of the epithelial cells, thus it is possible that it could be influenced by variation in *FGFR2*.

Differential splicing might provide an alternative mechanism for the association of *FGFR2* and breast cancer risk.[39] *FGFR2* encodes *FGFR2b* and *FGFR2c* isoforms.[46-49] Class switch from *FGFR2b* to *FGFR2c* is accompanied by epithelial-to-mesenchymal transition (EMT) with increased potential for invasion and metastasis.[50-54]

However, the precise mechanism how SNPs in the putative enhancer region within intron 2 of *FGFR2* affect FGFR2 upregulation remains unclear. Intron 2 of *FGFR2* is highly conservative in mammals.[55] More interestingly, this region contains several estrogen receptor (ER) transcription factor binding sites.[56] It has been previously reported that FGFR2 effects are more

relevant in ER- and progesterone receptor (PR)- positive tumors than in ER- or PR- negative tumors[57-58], and that FGFR2 is differentially expressed in different breast cancer subtypes.[59]

Estrogens can influence the development of breast cancer through stimulating gene expression and cell proliferation via interaction with the estrogen receptor (ER).[60] Studies have consistently shown the presence of sex steroid metabolic enzymes and ERs in breast tissue[61-78], which suggests that local activation of estrogen to potentially reactive metabolites within breast tissue may play a role in initiating and promoting carcinogenesis.[72] In addition, progestins are more potent mitogens for breast tissue than are estrogens.[79] It is likely that functionally relevant polymorphisms in genes involved in the metabolism of sex hormones may alter a women's exposure to estrogens and progestins, and thus, affect the risk of developing breast cancer.

In fact, a recent study within the WHI clinical trial reported that two SNPs in intron 2 of *FGFR2*, rs2981582 and rs3750817, showed evidence of interaction with the HT use on breast cancer risk.[80] If the effect of *FGFR2* on breast cancer is mediated through MBD, then it is likely that the effect of HT use on MBD is also influenced by functional SNPs in *FGFR2*.

1.3 STUDY OBJECTIVES

We conducted a cross-sectional study to assess associations of four *FGFR2* SNPs (rs2981582, rs3750817, rs17542768, rs1219643), HT use, and their interactions with MBD. The SNP rs2981582 was identified in the GWAS[39-40]; and the other three SNPs (rs3750817, rs17542768, rs1219643), which also lie within intron 2, are identified through subsequent fine mapping and are not in linkage disequilibrium (LD) with rs2981582.

2.0 MATERIALS AND METHODS

We used data and samples from women who participated in the Mammograms and Masses Study (MAMS) for this analysis. The MAMS was approved by the Institutional Review Board (IRB) at the University of Pittsburgh, and all participating women provided signed, written informed consent.

2.1 STUDY POPULATION

MAMS is an unmatched case-control study of hormonal determinants of mammographic breast density.[81] In brief, women were eligible for MAMS if they were of age ≥18 years and were receiving: (a) a breast biopsy, (b) an initial surgical consultation after breast cancer diagnosis, or (c) a routine screening mammogram. Exclusion criteria were prior cancer history other than non-melanoma skin cancer, alcohol intake ≥5 drinks per day, or weight <110 lbs or >300 lbs. Women were enrolled from 2001 to 2005 through mammography and surgical clinics of Magee-Womens Hospital, Pittsburgh, PA. In total, the MAMS study population consists of 1,133 women, including 264 cases with in situ or invasive breast cancer, 313 women with benign breast disease, and 556 well controls.[82]

Only postmenopausal women with a negative routine screening mammogram (*N*= 444; "well controls") were included in the present study. We only included postmenopausal women,

because it is well accepted that some breast cancer risk factors are differentially associated with premenopausal and postmenopausal breast cancer.[83] Breast density has observed to be lower among postmenopausal women than among premenopausal women[13], and it may be the case that the specific gene contribution to density may also vary by menopausal status. We subsequently excluded all women who had no available mammogram data (*N*=32), did not complete the questionnaire (*N*=7), were not Caucasian (*N*=25), or had no available DNA (*N*=8), leaving a final total of 372 women. The number of women excluded from this study for each exclusion criterion is shown in the **Figure 1**.

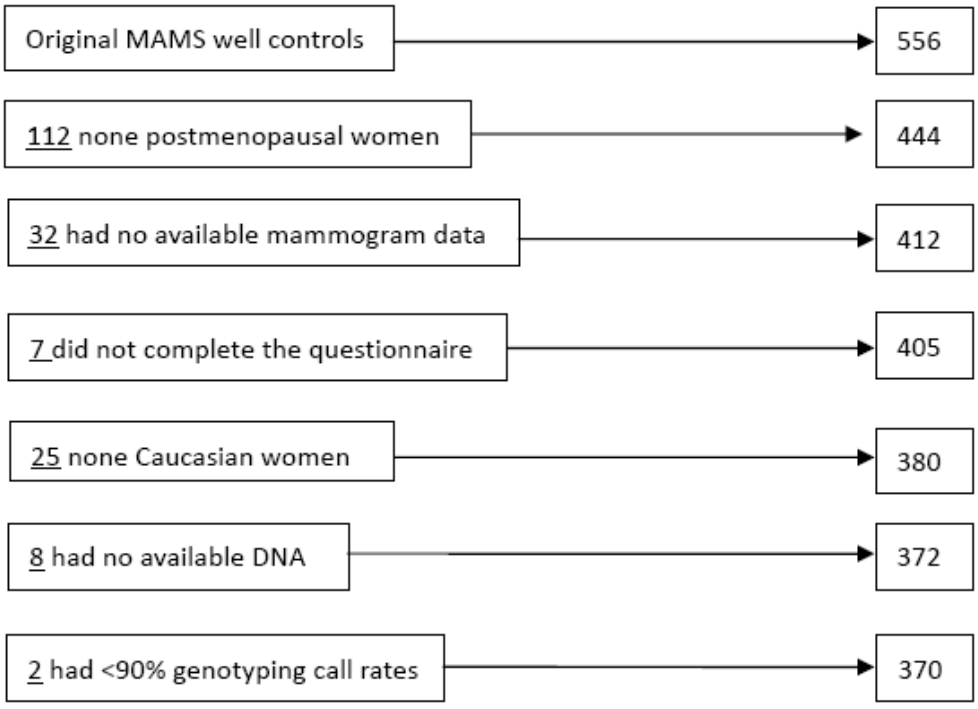

Figure 1. Study Population Flowchart

2.2 DATA COLLECTION

2.2.1 Questionnaire-based and anthropometric measures

At enrollment, participants completed a self-administered questionnaire that collected data on demographic characteristics, medical conditions and procedures, medications including hormone therapy (HT) and oral contraceptives (OCs), reproductive history, family cancer history, physical activity, smoking and alcohol use histories.[81]

Height and weight were measured by a study nurse using a stadiometer and a standard balance beam scale while participants were wearing light clothing and no shoes. Height and weight were used to calculate BMI (weight in kilograms divided by height in meters squared).[81]

Information obtained on hormone therapy use was status (never, former, current user), types of HT use, and duration of use. Years of E+P use and years of estrogen-only use were computed separately. Years of E+P use includes only those periods in which a woman used estrogen and progestin, these women could also have had periods of unopposed estrogen but these exposures were not counted in years of E+P use. Likewise, years of E-only use includes only those periods in which a woman used estrogen only, these women could also have had periods of E+P but these exposures were not counted in years of E-only use. Women who reported using estrogen but never used it in combination with progestin were classified as never E+P users. Years of E+P was set to missing for women who reported using unopposed progestin only.

Studies have shown that the effects of HT on developing denser breasts are different by subtypes[19-20, 84-88], thus we evaluated two major HT usages: duration of E+P use and duration of

E-only use. Duration of E+P use and duration of E-only use were grouped into 4 categories (Never, ≤ 1year, 1-10 years, ≥10 years).

2.2.2 Mammographic breast density measurements

Mammographic breast density is conceptualized as the percentage of the breast area on a mammogram onto which radiologically dense fibroglandular tissue (stroma and epithelium) is projected. The components of this percentage, dense breast area and total breast area, can also be considered, but only percentage breast density and dense breast area are consistently associated with breast cancer risk.[10, 13] Fibroglandular tissue attenuates X-rays more than fat and appears light in a mammogram, whereas fat appears dark.[89] Copies of original screening mammograms were obtained with the participants' permission and sent to the expert reviewer[90-92], to determine MBD. Dense breast area was measured by outlining areas of MBD on the craniocaudal view, excluding biopsy scars, Cooper's ligaments, and breast masses. Total breast area and outlined dense regions were computed using a compensating polar planimeter (LASICO). Percentage breast density was calculated by dividing the area of the outlined dense region by the total area of the breast. A subjective measure of film quality was also reported. To determine reproducibility of the readings, a random sample of 28 mammograms was re-evaluated at a later time. Measurements were very reliable (the intraclass correlation coefficient for intra-observer agreement was ρ=0.86 for the continuous measurement of dense breast area, ρ=0.99 for total area, and ρ=0.89 for percentage breast density [81]).

In the literature, percentage breast density appears to be the stronger risk factor than the absolute area of breast density.[4, 7] However, the absolute amount of dense tissue, which consists of connective and epithelial tissue, is regarded as the target tissue for breast cancer and an

important etiologic variable.[93] Therefore, we present results on both the relative and absolute measures of mammographic density, which referred to as "percentage breast density" and "dense breast area" respectively.

2.2.3 Specimen collection, DNA isolation, and genotyping

MAMS participants gave a non-fasting, 40 ml sample of peripheral blood at enrollment.[82] The sample was processed immediately after collection. Study participants were genotyped for 4 SNPs in *FGFR2* (rs2981582, rs3750817, rs17542768, and rs1219643). All genotyping was performed at the University of Pittsburgh Genomics and Proteomics Core Laboratories (Pittsburgh, PA). All SNPs were genotyped using MassARRAY® iPLEX Gold (Sequenom, Inc., San Diego, CA); the SNP specific and mass extend oligonucleotides, and assays were designed using Sequenom RealSNP (www.realsnp.com) and MassARRAY Assay Design version 3.1 (Sequenom, Inc., San Diego, CA). Sample duplicates (*N*=36) were included to monitor genotyping quality, concordance was >99%. Analyses were restricted to women with genotyping call rates of≥90%. Two study participants were excluded based on <90% call rates, leaving a total of 370 women available for analyses.

2.3 STATISTICAL ANALYSIS

Genotype and allele frequencies were calculated. Observed genotype frequencies in the study population were tested for deviation from Hardy-Weinberg Equilibrium (HWE) using the Chi-square goodness-of-fit test.

General regression analysis (GLM procedure, SAS) was used to examine the relationship between *FGFR2* SNPs and outcome variables (percentage breast density and dense breast area). Outcome variables were square root transformed to approximate a normal distribution where appropriate.

Age as a continuous variable was included as a covariate in all models. Since our goal was to investigate if breast cancer susceptibility loci are associated with MBD independent of factors known to influence breast density, known predictors of mammographic density were included in multivariate models. Based on published literature, the following covariates were considered: BMI (continuous), age at menarche (≤12, >12 years of age), previous breast biopsies (no/yes), family history of breast cancer in first-degree relatives (no/yes), age at the end of first pregnancy (<20, 20-24, 25-29, ≥30 years of age), number of live births (none, 1, 2, ≥3), OC use (never, former, current user), duration of E+P use (never, ≤ 1year, 1-10 years, ≥10 years) and HT status (never, former, current user), cigarette smoking (never, former, current smoker), alcohol intake (none, <12 grams/day, ≥12 grams/day), and physical activity (0, 0.1-10 METs/week, ≥10 METs/week). [11, 94-95]

Because the number of rare-allele homozgyotes was relatively small, we combined heterozygotes and rare-allele homozygotes in the general linear model analyses (applying dominant model).

To determine if there was a linear trend with increasing variant alleles, we calculated *P*-values including an ordinal variable for genotype (i.e., 0 to the first category, 1 to the second, and so on), regressed on square root transformed percentage breast density or dense breast area.

Similarly, the association between HT use (duration of E+P use, duration of E-only use, HT status) and MBD was tested by GLM, and dose-response was assessed using numerical scores assigned to the ordered categories of HT use as a continuous variable in the model.

We further tested the interaction of the four *FGFR2* SNPs with HT use duration (duration of E+P use and duration of E-only use). Tests for SNP interaction with HT use duration were carried out by adding the product term of HT use duration and different genotypes to the general linear model.

All significance tests were two sided; *P* values <0.05 were considered statistically significant. Data analysis was conducted using SAS statistical software version9.1.3.

3.0 RESULTS

3.1 CHARACTERISTICS OF SUBJECTS

This study examined the associations of four *FGFR2* SNPs, HT use, and their interactions with mammographic breast density in 370 healthy postmenopausal Caucasian women in the MAMS study. **Table-1** shows selected characteristics of this study population. The mean age of participants was 62.1 (SD=8.2) years. Their mean percentage breast density was 30.2% (SD=19.5%) (range: 0%-94.9%). About two thirds of the women were overweight (35.4%) or obese (31.1%). The majority of the women were former (50.8%) or current (13.2%) users of HT.

Table 1. Descriptive characteristics of the study population

Characteristic	Mean (SD)	N (%)
Age	62.1 (8.2)	
Height (m)	1.6 (0.1)	
Weight (kg)	74.1 (16.0)	
BMI (kg/m^2)	28.1 (5.9)	
BMI Category		
≤25		124 (33.5)
25-30		131 (35.4)
≥30		115 (31.1)
Age at menarche		
≤12		186 (50.3)
>12		184 (49.7)
Age at menopause (N=363)	48.6 (5.2)	
Family History of Breast Cancer		
No		314 (84.9)
≥1 first-degree relatives		56 (15.1)
Previous Breast Biopsy		
No		315 (85.1)
Yes		55 (14.9)
Ever Pregnant		
No		61 (16.5)
Yes		309 (83.5)
Age at the end of first pregnancy		
< 20		29 (7.9)
20-24		131 (35.4)
25-29		88 (23.8)
≥30		50 (13.5)
NA		72 (19.5)
Number of Live Births		
None		73 (19.7)
1		45 (12.2)
2		112 (30.3)
≥3		140 (37.8)
Oral contraceptive use		
No		154 (41.6)
Yes		216 (58.4)
Surgical menopause (N=368)		
No		318 (86.4)
Yes		50 (13.6)

(Table 1. *Cont'd*)

HT Use Status		
Never		133 (36.0)
Former		188 (50.8)
Current		49 (13.2)
Years of E+P use (N=366)		
Never		200 (54.6)
≤1 yr		29 (7.9)
1-10 years		93 (25.4)
≥10 years		44 (12.0)
Years of E use		
Never		281 (76.0)
≤1 yr		7 (1.9)
1-10 years		45 (12.2)
≥10 years		37 (10.0)
Alcohol consumption in year prior to enrollment (N=363)		
None		254 (70.0)
< 12 grams/day		68 (18.7)
≥12 grams/day		41 (11.3)
Smoking status		
Never		212 (57.3)
Former		138 (37.3)
Current		20 (5.4)
Physical Activity in METs/week		
0		47 (12.7)
0.1-10		126 (34.1)
≥10		197 (53.2)
Involved Area of Breast/Dense Breast Area (cm^2)	41.2 (26.8)	
Percentage Breast Density	30.2 (19.5)	
Total Area of Breast (cm^2)	159.4 (74.2)	
None Dense Area (cm^2)	118.2 (75.2)	

*Total number for each variable is not always 370, due to missing data in some variables.

3.2 ALLELE FREQUENCIES AND HWE

None of the four SNPs deviated significantly from HWE. (**Table-2**) Among our study population, minor allele frequencies for each of the four SNPs were similar to those of the Caucasian population (CEU) from the International HapMap Project.

Table 2. SNP Information and HWE

rs Number	n	Chr position	Allele	Allele Counts	Estimated Frequencies	Allele Frequencies in HapMap-CEU	HWE p-value*
rs2981582	355	123342307	C	440	62.0%	58.3%	
			T	270	38.0%	41.7%	0.35
rs3750817	362	123322567	C	403	55.7%	59.5%	
			T	321	44.3%	40.5%	0.03
rs17542768	364	123327804	A	629	86.4%	88.3%	
			G	99	13.6%	11.7%	0.71
rs1219643	361	123338345	G	540	74.8%	85.7%	
			T	182	25.2%	14.3%	0.01

*Goodness-of-fit Test

3.3 FGFR2 GENOTYPE AND MAMMOGRAPHIC BREAST DENSITY

Overall, no association was observed between the four *FGFR2* SNPs and percentage breast density in our study population. (**Table-3, Table-4, Table-5**)

We also examined association between these SNPs with the absolute area of mammographic density (dense breast area), but because results were similar and percentage breast density has been a stronger predictor of breast cancer risk than absolute dense breast area in many[4, 7, 9, 11] but not all studies[96], we present the results of percentage breast density as our primary analyses, and include the association with dense breast area in supplementary tables. (**Supplementary Table-1**)

Table 3. Mean of Percentage Breast Density according to *FGFR2* SNPs-Additive Model

rs number	Genotype	n(%)	Percentage Breast Density†					
			Model-1		Model-2		Model-3*	
			Mean	p	Mean	p	Mean	p
rs2981582	C/C	130 (36.6)	25.40		26.63		27.56	
(N=355)	C/T	180 (50.7)	26.83	0.78 (2 df)	26.11	0.98 (2 df)	27.46	0.78 (2 df)
	T/T	45 (12.7)	27.04	0.52 (1 df)	26.32	0.88 (1 df)	29.59	0.64 (1 df)
rs3750817	C/C	100 (27.6)	25.10		24.70		26.83	
(N=362)	C/T	203 (56.1)	27.35	0.60 (2 df)	26.83	0.48 (2 df)	27.56	0.89 (2 df)
	T/T	59 (16.3)	25.40	0.77 (1 df)	27.88	0.24 (1 df)	28.30	0.62 (1 df)
rs17542768	A/A	270 (74.2)	26.73		26.42		28.20	
(N=364)	A/G	89 (24.4)	24.80	0.61 (2 df)	25.60	0.90 (2 df)	27.04	0.85 (2 df)
	G/G	5 (1.4)	21.53	0.33 (1 df)	24.50	0.64 (1 df)	27.25	0.59 (1 df)
rs1219643	G/G	191 (52.9)	27.25		27.14		28.30	
(N=361)	G/T	158 (43.8)	25.50	0.73 (2 df)	25.60	0.70 (2 df)	26.73	0.66 (2 df)
	T/T	12 (3.3)	26.01	0.47 (1 df)	25.91	0.44 (1 df)	25.00	0.37 (1 df)

Abbreviation: df, degrees of freedom

Model-1: Adjusted for Age(continuous).

Model-2: Adjusted for Age, BMI(continuous).

Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at the End of First Pregnancy, Number of Live Birth, OC Use, Duration of E+P Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

†Results are square transformed back for easy interpretation.

*Numbers in Model-3 (345,351,353,350) are slightly lower because of missing values in additional adjustment variables.

2 df p va lue t est f or he terogeneity b etween t he m eans of pe rcentage m ammographic br east d ensity, 1df p va lue t est for dos e-response.

Table 4. Mean of Percentage Breast Density according to *FGFR2* SNPs-Dominant Model

rs number	Genotype	n(%)	Percentage Breast Density†					
			Model-1		Model-2		Model-3*	
			Mean	p	Mean	p	Mean	p
rs2981582	C/C	130 (36.6)	25.40		26.63		27.56	
(N=355)	C/T or T/T	225 (63.4)	26.94	0.48 (1 df)	26.21	0.83 (1 df)	27.88	0.87 (1 df)
rs3750817	C/C	100 (27.6)	25.10		24.70		26.83	
(N=362)	C/T or T/T	262 (72.4)	26.94	0.44 (1 df)	27.04	0.24 (1 df)	27.67	0.69 (1 df)
rs17542768	A/A	270 (74.2)	26.73		26.42		28.20	
(N=364)	A/G or G/G	94 (25.8)	24.60	0.36 (1 df)	25.50	0.66 (1 df)	26.94	0.57 (1 df)
rs1219643	G/G	191 (52.9)	27.25		27.14		28.20	
(N=361)	G/T or T/T	170 (47.1)	25.50	0.43 (1 df)	25.60	0.40 (1 df)	26.52	0.40 (1 df)

Abbreviation: df, degrees of freedom
Model-1: Adjusted for Age(continuous).
Model-2: Adjusted for Age, BMI(continuous).
Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at the End of First Pregnancy, Number of Live Birth, OC Use, Duration of E+P Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.
†Results are square transformed back for easy interpretation.
*Numbers in Model-3 (345,351,353,350) are slightly lower because of missing values in additional adjustment variables.

Table 5. Mean of Percentage Breast Density according to *FGFR2* SNPs-Recessive Model

rs number	Genotype	n(%)	Percentage Breast Density†					
			Model-1		Model-2		Model-3*	
			Mean	p	Mean	p	Mean	p
rs2981582	C/C or C/T	310 (87.3)	26.21		26.32		27.56	
(N=355)	T/T	45 (12.7)	27.04	0.79 (1 df)	26.42	0.99 (1 df)	29.70	0.48 (1 df)
rs3750817	C/C or C/T	303 (83.7)	26.63		26.11		27.25	
(N=362)	T/T	59 (16.3)	25.40	0.68 (1 df)	27.88	0.50 (1 df)	28.30	0.70 (1 df)
rs17542768	A/A or A/G	359 (98.6)	26.32		26.21		27.98	
(N=364)	G/G	5 (1.4)	21.53	0.58 (1 df)	24.50	0.82 (1 df)	27.46	0.95 (1 df)
rs1219643	G/G or G/T	349 (96.7)	26.42		26.42		27.56	
(N=361)	T/T	12 (3.3)	26.01	0.95 (1 df)	26.01	0.93 (1 df)	25.10	0.65 (1 df)

Abbreviation: df, degrees of freedom
Model-1: Adjusted for Age(continuous).
Model-2: Adjusted for Age, BMI(continuous).
Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at the End of First Pregnancy, Number of Live Birth, OC Use, Duration of E+P Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.
†Results are square transformed back for easy interpretation.
*Numbers in Model-3 (345,351,353,350) are slightly lower because of missing values in additional adjustment variables.

3.4 HT USE AND MAMMOGRAPHIC BREAST DENSITY

We observed a linear trend between duration of E+P use and percentage breast density in our age-adjusted model. Women with a longer duration of E+P use had increased percentage breast density. However, the association became borderline significant after further adjustment of BMI (P=0.05). (**Table-6**) In addition, we also observed a statistically significant trend between HT status (never, former, current user) and percentage breast density. (**Table-8**) However, we didn't find any statistically significant association between duration of E-only use and MBD. (**Table-7**)

Table 6. Mean of Percentage Breast Density according to Duration of E+P use

Duration of E+P use (N=366)	n(%)	Percentage Breast Density†					
		Model-1		Model-2		Model-3*	
		Mean	p	Mean	p	Mean	p
Never	200 (54.6)	23.81		24.60		25.50	
≤1 yr	29 (7.9)	27.98		26.73		26.83	
1-10 years	93 (25.4)	29.81	0.07 (3 df)	28.94	0.22 (3 df)	28.09	0.68 (3 df)
≥10 years	44 (12.0)	29.38	0.01 (1 df)	28.09	0.05 (1 df)	26.52	0.34 (1 df)

Abbreviation: df, degrees of freedom
Model-1: Adjusted for Age(continuous).
Model-2: Adjusted for Age, BMI(continuous).
Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.
†Results are square transformed back for easy interpretation.
*The number in Model-3 (359) is slightly lower because of missing values in additional adjustment variables.
3 df p value test for heterogeneity between the means of percentage mammographic breast density, 1df p value test for dose-response.

Table 7. Mean of Percentage Breast Density according to Duration of E-only use

Duration of E use (N=370)	n(%)	Percentage Breast Density†					
		Model-1		Model-2		Model-3*	
		Mean	p	Mean	p	Mean	p
Never	281 (76.0)	26.21		26.21		27.46	
≤1 yr	7 (1.9)	27.67		29.05		26.63	
1-10 years	45 (12.1)	26.32	0.99 (3 df)	25.00	0.80 (3 df)	24.11	0.67 (3 df)
≥10 years	37 (10.0)	27.04	0.84 (1 df)	28.52	0.70 (1 df)	28.52	0.79 (1 df)

Abbreviation: df, degrees of freedom. P values based on square root transformed percentage breast density.
Model-1: Adjusted for Age(continuous).
Model-2: Adjusted for Age, BMI(continuous).
Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.
†Results are square transformed back for easy interpretation.
*The number in Model-3 (363) is slightly lower because of missing values in additional adjustment variables.
3 df p value test for heterogeneity between the means of percentage mammographic breast density, 1df p value test for dose-response.

Table 8. Mean of Percentage Breast Density according to HT status

HT status	n(%)	Percentage Breast Density†					
		Model-1		Model-2		Model-3*	
		Mean	p	Mean	p	Mean	p
Never	133 (36.0)	23.33		24.21		25.40	
Former	188 (50.8)	27.35	0.05 (2 df)	27.04	0.13 (2 df)	26.42	0.32 (2 df)
Current	49 (13.2)	30.91	0.01 (1 df)	29.81	0.04 (1 df)	30.25	0.16 (1 df)

Abbreviation: df, degrees of freedom. P values based on square root transformed percentage breast density.
Model-1: Adjusted for Age(continuous).
Model-2: Adjusted for Age, BMI(continuous).
Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Uses, Duration of E+P Use, Smoking Status, Alcohol Intake, Physical Activity.
†Results are square transformed back for easy interpretation.
*The numbers in Model-3 (359) is slightly lower because of missing values in additional adjustment variables.
2 df p va lue t est f or he terogeneity b etween t he m eans of pe rcentage m ammographic br east d ensity, 1df p va lue t est for dose-response.

3.5 INTERACTION BETWEEN GENETIC VARIANTS AND HT USE DURATION

Subsequently, we investigated whether the different genetic variants modified the relationship between duration of HT (E+P or E-only) use and MBD. No interaction was observed between genetic variants and HT use duration in our study population. (**Table-9**)

Table 9. Duration of HT use (Never, ≤1 yr, 1-10 yrs, ≥10 yrs) and genotype interaction

	Percentage Breast Density		
	P Interaction (Duration of **E+P** Use)		
	Model-1	Model-2	Model-3
rs2981582	0.16	0.21	0.24
rs3750817	0.98	0.97	0.98
rs17542768	0.66	0.57	0.58
rs1219643	0.71	0.70	0.73

	Percentage Breast Density		
	P Interaction (Duration of **E-only** Use)		
	Model-1	Model-2	Model-3
rs2981582	0.48	0.21	0.20
rs3750817	0.72	0.78	0.92
rs17542768	0.97	0.95	0.87
rs1219643	0.65	0.73	0.38

Model-1: Adjusted for Age (continuous)
Model-2: Adjusted for Age, BMI (continuous)
Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

4.0 DISCUSSION

The rationale for this study was that breast cancer susceptibility loci may also be related to another strong risk factor, mammographic breast density. So far, mammographic breast density is the only known risk factor for breast cancer that is present in the very organ in which the disease will eventually develop. Mammographic breast density reflects the relative amounts of fibroglandular tissue as opposed to nondense fatty tissue. Fibroglandular tissue contains a mixture of fibrous connective tissue (stroma) and glandular tissue (epithelial cells). Breast cancer originates in epithelial cells, so greater areas of fibroglandular tissue may reflect a greater number of cells that are at risk of carcinogenesis and / or an increased rate of epithelial proliferation. It is hypothesized that many of the genetic and environmental factors that influence risk of breast cancer affect the proliferative activity and quantity of stromal and epithelial tissue in the breast, and that these effects are reflected in differences in MBD among women of the same age.[97] High MBD is associated with greater total nuclear area of both epithelial and nonepithelial cells.[98] A greater percentage of epithelium in benign tissue biopsies has been associated with an increased risk of hyperplasia (with or without atypia) and / or carcinoma in situ, and these histology findings are associated with increased risk of breast cancer.[99-102] Mammographic breast density may be an intermediate end point for the development of breast cancer.

Studies have shown the implication of *FGFR2* in mammary carcinogenesis. FGFR2 is a receptor tyrosine kinase; it is involved in cell proliferation, migration, and differentiation.[103-105] It is plausible that *FGFR2* influences breast cancer development through breast density.

In this study, we found that overall none of the four *FGFR2* SNPs were associated with MBD in healthy postmenopausal Caucasian women. There are several other studies[38, 106-107] that also looked at rs2981582 in *FGFR2* and MBD, but their study populations are different. None of them looked at healthy postmenopausal women in a homogenous Caucasian population. Tamimi *et al.*[38] conducted the study in the Nurses' Health Study with a sample size of 1121 women, both pre- and post-menopause. They found no statistically significant association between rs2981582 and MBD for the whole population, nor after stratifying by menopausal status. Lee *et al.*[106] included 516 premenopausal breast cancer cases; the study population was predominantly Caucasian women. They also found no association. Woolcott *et al.*[107] investigated the same *FGFR2* polymorphism within the Multiethnic Cohort study; they included both pre- and post-menopausal women. Similarly, no association was observed between rs2981582 and MBD in their study population of 262 white women. In contrast to our study, none of the above studies further explored the possible gene and HT use duration interactions.

Homozygous variants of rs2981582 or other SNPs in high LD are estimated to confer about a 60% increase in breast cancer risk relative to homozygous wildtypes.[39-40] The lack of association of the breast cancer loci in *FGFR2* with breast density suggests that *FGFR2* influences breast cancer risk independent of breast density.

Consistent with previous studies[19-21, 30, 85-88, 108-109], we observed a trend between duration of E+P use and percentage breast density; we also observed the statistically significant trend between HT status and percentage breast density. However, we found no statistically significant

association between duration of E-only use and percentage breast density in our study. Previous studies have shown that combined E+P use has a greater risk of developing denser breasts than E-only therapy.[19-20, 84-88] It has been shown that in postmenopausal women, breast epithelial proliferation was more pronounced by combined estrogen + progestin treatment than by estrogen alone.[110]

The effect of HT use on MBD may result in a reduced sensitivity and specificity of mammographic breast cancer screening.[111-112] High mammographic density can obscure subtle breast abnormalities, making it not only more difficult to diagnose small-volume breast cancer but also more likely to have a false positive mammogram reading.[113] However, the increased risk associated with greater mammographic density persists for up to 9 years after screening[114]; this argues strongly against detection bias ("masking") as the sole cause of the observed increase in breast cancer.[115] Furthermore, recent data indicate that breast density during HT is dynamic, increasing with initiation and decreasing with discontinuation.[21] It may also be that this risk factor can be changed by intervention.[116] Intervention trails have shown that decreases in breast density are associated with tamoxifen treatment[117-119], a therapy proven to decrease breast cancer risk.[120-121] Mammographic density has the potential to be used to monitor risk-lowering interventions of breast cancer.

We observed no statistically significant gene-environment interactions between the four SNPs and HT use duration. We evaluated the genetic variants and environmental interaction because there is substantial evidence that polymorphisms in candidate hormone metabolism genes may influence the disposition of exogenous hormones found in HT.[122-123] *FGFR2* contains at least one putative ER transcription factor binding site[56], thus it may relate HT effects on breast cancer and/or breast density. One possible explanation for the null result of interaction is that

maybe the effects of HT on breast density are more related to progesterone receptor (PR) than to estrogen receptor (ER). It has been consistently shown that the combined E+P therapy has a more pronounced effect in increasing breast density than E-only therapy.[19-20, 84-88] In the WHI study, Prentice *et al.*[80] evaluated variation in the *FGFR2* gene and the effects of postmenopausal HT on invasive breast cancer. They reported that SNP rs3750817 showed evidence of interaction with both the E+P (P=0.033) and E-only (P=0.046) odds ratio, whereas SNP rs2981582 showed evidence (P=0.045) of interaction with the E-only odds ratio. They further reported that the odd ratios for both E+P and E-only in the WHI hormone therapy trials depended significantly (P<0.05) on genotype for SNP 3750817, and concluded that postmenopausal women having TT genotype for SNP 3750817 have a reduced breast cancer risk and seem to experience comparatively favorable effects of HT.

The major strength of our study is the reliability of breast density measurements. Furthermore, percentage density reading is a continuous value, a more refined measure compared to categorically measurements. In addition, population stratification is not a major concern because our population was composed of 100% self-reported postmenopausal Caucasian women. Another advantage of our study is the availability of information, for example, we have a very complete database of type and duration of HT use. However, the ascertainment of HT use relies on self-report and the misclassification can occur. This may influence our results to assess genetic modification. It is also possible that the effect modification is mainly present in a specific type of hormone therapy use. We have information available on different types of hormone therapy preparation, but we chose not to make a distinction because then the sample size will become too small. Finally, we only studied four SNPs in intron 2 of *FGFR2*. It is possible that

the causal variants are not in LD with any of the four SNPs, thus we were unable to detect the association.

In conclusion, our findings support the notion that the effects of these *FGFR2* SNPs on breast cancer are not mediated by mammographic density. We did observe a statistically significant trend between duration of E+P use, HT status and percentage breast density. And there is no evidence of interaction between these genetic variants in intron 2 of *FGFR2* and duration of HT usage.

Breast cancer has been recognized as a heterogeneous diagnosis, different medical interventions[124-126] are effective primarily in subgroups with specific biological profiles.[127-128] It is important to identify markers that may assist in primary prevention of breast cancer as well as in selecting high risk individuals (e.g., women who are most susceptible to the effect of HT on MBD) of breast cancer. Further studies are needed to clarify the role of how certain factors influence the risk of breast cancer.

APPENDIX

SUPPLEMENTARY TABLES

S Table 1a. Mean of Dense Breast Area according to FGFR2 SNPs- Additive Model

rs number	Genotype	n(%)	Dense Breast Area†					
			Model-1		Model-2		Model-3*	
			Mean	p	Mean	p	Mean	p
rs2981582	C/C	130 (36.6)	36.60		37.09		38.56	
(N=355)	C/T	180 (50.7)	36.24	0.82 (2 df)	36.00	0.73 (2 df)	38.32	0.96 (2 df)
	T/T	45 (12.7)	33.76	0.60 (1 df)	33.52	0.45 (1 df)	37.09	0.80 (1 df)
rs3750817	C/C	100 (27.6)	33.64		33.52		36.60	
(N=362)	C/T	203 (56.1)	37.82	0.43 (2 df)	37.58	0.43 (2 df)	38.56	0.84 (2 df)
	T/T	59 (16.3)	36.00	0.44 (1 df)	36.72	0.34 (1 df)	37.70	0.72 (1 df)
rs17542768	A/A	270 (74.2)	36.84		36.72		40.07	
(N=364)	A/G	89 (24.4)	34.57	0.56 (2 df)	34.81	0.64 (2 df)	37.58	0.71 (2 df)
	G/G	5 (1.4)	27.46	0.32 (1 df)	28.30	0.39 (1 df)	34.34	0.41 (1 df)
rs1219643	G/G	191 (52.9)	37.95		37.95		39.44	
(N=361)	G/T	158 (43.8)	34.46	0.48 (2 df)	34.46	0.48 (2 df)	36.48	0.53 (2 df)
	T/T	12 (3.3)	35.88	0.28 (1 df)	35.76	0.28 (1 df)	33.52	0.26 (1 df)

Abbreviation: df, degrees of freedom

Model-1: Adjusted for Age(continuous).

Model-2: Adjusted for Age, BMI(continuous).

Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at the End of First Pregnancy, Number of Live Birth, OC Use, Duration of E+P Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

†Results are square transformed back for easy interpretation.

*Numbers in Model-3 (345,351,353,350) are slightly lower because of missing values in additional adjustment variables.

2 df p value test for heterogeneity between the means of mammographic breast density, 1df p value test for genotype dosage.

S Table 1b. Mean of Dense Breast Area according to FGFR2 SNPs-Dominant Model

rs number	Genotype	n(%)	Dense Breast Area†					
			Model-1		Model-2		Model-3*	
			Mean	p	Mean	p	Mean	p
rs2981582	C/C	130 (36.6)	36.60		37.09		38.56	
(N=355)	C/T or T/T	225 (63.4)	35.76	0.78 (1 df)	35.52	0.59 (1 df)	38.07	0.88 (1 df)
rs3750817	C/C	100 (27.6)	33.64		33.52		36.60	
(N=362)	C/T or T/T	262 (72.4)	37.33	0.22 (1 df)	37.45	0.20 (1 df)	38.44	0.57 (1 df)
rs17542768	A/A	270 (74.2)	36.84		36.72		40.07	
(N=364)	A/G or G/G	94 (25.8)	34.22	0.39 (1 df)	34.46	0.46 (1 df)	37.45	0.43 (1 df)
rs1219643	G/G	191 (52.9)	37.95		37.95		39.44	
(N=361)	G/T or T/T	170 (47.1)	34.57	0.23 (1 df)	34.57	0.23 (1 df)	36.24	0.28 (1 df)

Abbreviation: df, degrees of freedom

Model-1: Adjusted for Age(continuous).

Model-2: Adjusted for Age, BMI(continuous).

Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at the End of First Pregnancy, Number of Live Birth, OC Use, Duration of E+P Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

†Results are square transformed back for easy interpretation.

*Numbers in Model-3 (345,351,353,350) are slightly lower because of missing values in additional adjustment variables.

S Table 1c. Mean of Dense Breast Area according to FGFR2 SNPs-Recessive Model

rs number	Genotype	n(%)	Dense Breast Area†					
			Model-1		Model-2		Model-3*	
			Mean	p	Mean	p	Mean	p
rs2981582	C/C or C/T	310 (87.3)	36.36		36.48		38.44	
(N=355)	T/T	45 (12.7)	33.76	0.53 (1 df)	33.52	0.48 (1 df)	37.09	0.77 (1 df)
rs3750817	C/C or C/T	303 (83.7)	36.36		36.24		37.95	
(N=362)	T/T	59 (16.3)	36.00	0.91 (1 df)	36.72	0.89 (1 df)	37.82	0.97 (1 df)
rs17542768	A/A or A/G	359 (98.6)	36.36		36.24		39.56	
(N=364)	G/G	5 (1.4)	27.46	0.42 (1 df)	28.30	0.47 (1 df)	34.69	0.68 (1 df)
rs1219643	G/G or G/T	349 (96.7)	36.36		36.36		38.07	
(N=361)	T/T	12 (3.3)	35.88	0.96 (1 df)	35.88	0.95 (1 df)	33.64	0.58 (1 df)

Abbreviation: df, degrees of freedom

Model-1: Adjusted for Age(continuous).

Model-2: Adjusted for Age, BMI(continuous).

Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at the End of First Pregnancy, Number of Live Birth, OC Use, Duration of E+P Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

†Results are square transformed back for easy interpretation.

*Numbers in Model-3 (345,351,353,350) are slightly lower because of missing values in additional adjustment variables.

S Table 2a. Mean of Dense Breast Area according to Duration of E+P use.

Duration of E+P use (N=366)	n(%)	Dense Breast Area†					
		Model-1		Model-2		Model-3*	
		Mean	p	Mean	p	Mean	p
Never	200 (54.6)	34.11		34.34		37.82	
≤1 yr	29 (7.9)	41.47		41.09		41.47	
1-10 years	93 (25.4)	38.81	0.36 (3 df)	38.44	0.47 (3 df)	38.44	0.80 (3 df)
≥10 years	44 (12.0)	36.24	0.24 (1 df)	35.76	0.34 (1 df)	34.93	0.65 (1 df)

Abbreviation: df, degrees of freedom

Model-1: Adjusted for Age(continuous).

Model-2: Adjusted for Age, BMI(continuous).

Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

†Results are square transformed back for easy interpretation.

*The number in Model-3 (359) is slightly lower because of missing values in additional adjustment variables.

3 df p value test for heterogeneity between the means of percentage mammographic breast density, 1df p value test for dose-response.

S Table 2b. Mean of Dense Breast Area according to Duration of E-only use.

Duration of E use (N=370)	n(%)	Dense Breast Area†					
		Model-1		Model-2		Model-3*	
		Mean	p	Mean	p	Mean	p
Never	281 (76.0)	36.00		36.00		37.58	
≤1 yr	7 (1.9)	35.16		35.64		33.87	
1-10 years	45 (12.1)	38.44	0.95 (3 df)	38.07	0.97 (3 df)	39.31	0.95 (3 df)
≥10 years	37 (10.0)	36.48	0.71 (1 df)	36.97	0.68 (1 df)	39.19	0.70 (1 df)

Abbreviation: df, degrees of freedom. P values based on square root transformed dense breast area.

Model-1: Adjusted for Age(continuous).

Model-2: Adjusted for Age, BMI(continuous).

Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

†Results are square transformed back for easy interpretation.

*The number in Model-3 (363) is slightly lower because of missing values in additional adjustment variables.

3 df p va lue t est f or he terogeneity b etween the m eans of pe rcentage m ammographic br east d ensity, 1df p value t est for dose-response.

S Table 2c. Mean of Dense Breast Area according to HT status

HT status	n(%)	Dense Breast Area†					
		Model-1		Model-2		Model-3*	
		Mean	p	Mean	p	Mean	p
Never	133 (36.0)	33.18		33.52		34.93	
Former	188 (50.8)	37.70	0.19 (2 df)	37.58	0.25 (2 df)	38.69	0.48 (2 df)
Current	49 (13.2)	40.07	0.07 (1 df)	39.69	0.10 (1 df)	40.83	0.24 (1 df)

Abbreviation: df, degrees of freedom

Model-1: Adjusted for Age(continuous).

Model-2: Adjusted for Age, BMI(continuous).

Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Uses, Duration of E+P Use, Smoking Status, Alcohol Intake, Physical Activity.

†Results are square transformed back for easy interpretation.

*The number in Model-3 (359) is slightly lower because of missing values in additional adjustment variables.

2 df p value test for heterogeneity between the means of percentage mammographic breast density, 1df p value test for dose-response

S Table 3. Duration of HT use (Never, ≤1yr, 1-10yrs, ≥10yrs) and genotype interaction:

	Dense Breast Area		
	P Interaction (Duration of **E+P** Use)		
	Model-1	Model-2	Model-3
rs2981582	0.21	0.26	0.30
rs3750817	0.51	0.50	0.44
rs17542768	0.29	0.28	0.19
rs1219643	0.97	0.97	0.95

	Dense Breast Area		
	P Interaction (Duration of **E** Use)		
	Model-1	Model-2	Model-3
rs2981582	0.38	0.33	0.27
rs3750817	0.82	0.81	0.80
rs17542768	0.83	0.86	0.86
rs1219643	0.99	0.98	0.77

Model-1: Adjusted for Age (continuous)
Model-2: Adjusted for Age, BMI (continuous)
Model-3: Model2+ Age at Menarche, Previous Breast Biopsy, Family History of Breast Cancer in First-degree Relatives, Age at First Pregnancy, Number of Live Birth, OC Use, HT Status, Smoking Status, Alcohol Intake, Physical Activity.

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