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| 14. ABSTRACT<br>Mammographic breast density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts. Information on the etiology of breast density is currently limited. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between serum cytokine levels, genetic variation in cytokine genes, and breast density using data and samples from the Mammograms and Masses Study. This report provides information on the progress made during the third year of the grant. MAMS study participants were genotyped for common single nucleotide polymorphisms (SNPs) in or near <i>IL6</i> , <i>IL6R</i> , <i>IL6ST</i> , <i>TNF-alpha</i> , <i>TNFRSF1A</i> , and <i>TNFRSF1B</i> using MassARRAY iPLEX Gold (Sequenom). Analyses of the genotype data are ongoing. Preliminary results suggest that, in healthy postmenopausal women, common variation in <i>IL6R</i> and <i>IL6ST</i> is associated with percentage breast density. None of the evaluated SNPs in <i>IL6</i> and <i>TNF-alpha</i> were significantly associated with percentage breast density in this study population. |                  |                          |                                          |                                                          |
| 15. SUBJECT TERMS<br>Breast cancer; breast density; cytokines; genetic variation                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                  |                          |                                          |                                                          |
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## INTRODUCTION

Mammographic breast density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts (1, 2). Information on the etiology of breast density is currently limited, and the biological mechanism by which mammographic breast density is associated with breast cancer risk is unclear. Various evidence suggest that exposure to sex hormones, estrogens in particular, may be an important factor in breast density. Changes in density have been observed in response to hormone replacement therapy use and use of tamoxifen (3, 4). Pro-inflammatory cytokines, specifically tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6, have emerged as critical regulators of estrogen synthesis in breast tissues (5), and may so affect breast density and breast cancer risk. In line with this, polymorphisms in *IL6* have been found associated with breast cancer risk and to modify the association between estrogen and aspirin and breast cancer risk (6). To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between serum cytokine levels, genetic variation in cytokine genes, and breast density. Existing data and banked specimens from women who participated in a recently completed, cross-sectional study on hormones and breast density, the Mammograms and Masses Study (MAMS), are used.

## BODY

### ▪ Cytokine serum levels and mammographic breast density

As reported in our previous annual report, serum levels of IL-6, soluble IL-6R and TNF- $\alpha$  were measured for all 722 study participants. Our preliminary results suggested that there is no significant association between IL-6 and TNF- $\alpha$  serum levels and breast density. Due to issues with the ELISA assays (results not reproducible) we had been unable to measure soluble TNF-R1 and TNF-R1 levels. Since then we have investigated other assays, and we are currently in the process of measuring soluble TNF-R1 and TNF-R1 levels (in duplicate) using Luminex Technology (Cytokine Human TNF-RI Single-Plex kit and Cytokine Human TNF-RII Single-Plex kit, respectively) in the Immunologic Monitoring Laboratory of the University of Pittsburgh Cancer Institute (Pittsburgh, PA). Both sTNF-RI and sTNF-RII are known to modulate TNF- $\alpha$  activity by decreasing TNF- $\alpha$  levels, and for completeness we plan to publish the results for these receptors together with those for IL-6 and TNF- $\alpha$ .

### ▪ Genetic variation and mammographic breast density

DNA was extracted from buffy coat samples and plates were prepared for genotyping in Dr. Robert Ferrell's laboratory in the Department of Human Genetics of the University of Pittsburgh (Pittsburgh, PA).

Study participants were genotyped for, in total, 45 single nucleotide polymorphisms (SNPs) located in or near *IL6* (9 SNPs), *IL6R* (12 SNPs), *IL6ST* (7 SNPs), *TNF- $\alpha$*  (1 SNP), *TNFRSF1A*

(7 SNPs), and *TNFRSF1B* (9 SNPs). The *IL6ST* gene was included because IL-6 acts by binding to IL-6R which must associate with gp130 (coded for by *IL6ST*) in order for signal transduction to occur. Putative functional SNPs were selected using public databases such as the Genome Variation Server (<http://gvs.gs.washington.edu/GVS/>) and dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), and literature search. Additionally, for each gene except *TNF-α*, tagSNPs capturing common variants in the gene region were selected using data from the International HapMap project ([www.hapmap.org](http://www.hapmap.org); CEU population) and Haploview's Tagger (7, 8) [criteria used: minor allele frequency (MAF) > 0.05 and pairwise correlation  $r^2 \geq 0.80$ ]. All genotyping was performed at the University of Pittsburgh Genomics and Proteomics Core Laboratories (Pittsburgh, PA). SNP rs1800629 (*TNF-α* -308 G/A), was assessed using TaqMan (Assay ID: C\_\_7514879\_10; Applied Biosystems, Foster City, CA). All other 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](http://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; no discrepant genotypes were observed. Analyses were restricted to women with genotyping call rates of  $\geq 90\%$ .

The current preliminary analyses are restricted to Caucasian postmenopausal women with a negative routine screening mammogram, no previous cancer, available mammogram and questionnaire data, and genotyping call rates of  $\geq 90\%$ ;  $N=369$ . Selected characteristics of the study population are presented in Table 1. The mean age of the study participants was 62.1 years, the mean body mass index (BMI) was 28.1 kg/m<sup>2</sup>, and the average percentage breast density was 30.2%.

Deviation from Hardy-Weinberg equilibrium was assessed with the Chi-square goodness-of-fit test. With the exception of rs2228576 in *TNFRSF1A* ( $P=0.0002$ ) and rs653667 in *TNFRSF1B* ( $P=0.0013$ ), all SNPs were in Hardy Weinberg equilibrium. Linear regression models (GLM procedure in SAS) were used to examine the relationship between each SNP and percentage breast density. Because the number of rare-allele homozygotes in some cases was relatively small, heterozygotes and rare-allele homozygotes were combined in the analyses (common allele homozygotes were used as the reference group). Percentage breast density was square-root transformed to normalize the distribution. For ease of interpretation, the presented means were transformed back to the original scale. To determine if there was a linear trend with increasing variant alleles,  $P$  values were also calculated with a linear regression model based on the number of copies of rare alleles (0, 1, 2). All models were adjusted for age (continuous), BMI (continuous), hormone therapy use (never, past, current), NSAID use (no, yes), pregnancy for at least 6 months (no, yes), and previous biopsy (no, yes). All significance tests were two-sided;  $P$  values < 0.05 were considered statistically significant. All analyses were performed with use of the SAS® statistical software package (SAS version 9.2, SAS Institute Inc., Cary, NC). Results are presented in Table 2.

Table 1: Selected characteristics of the study population

|                                                                                                                                                                           | All study participants<br>$N_{total}=369$<br>N(%)             |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
| Age (in years):<br>- younger than 50<br>- 50-59<br>- 60-69<br>- 70 or older                                                                                               | 3 (0.8)<br>164 (44.4)<br>128 (34.7)<br>74 (20.1)              |
| Body mass index (kg/m <sup>2</sup> ):<br>- normal (less than 25.0)<br>- overweight (25.0-<30.0)<br>- obese (30.0 or more)                                                 | 124 (33.6)<br>130 (35.2)<br>115 (31.2)                        |
| Smoking:<br>- never<br>- former<br>- current                                                                                                                              | 211 (57.2)<br>138 (37.4)<br>20 (5.4)                          |
| Current NSAID use                                                                                                                                                         | 182 (49.3)                                                    |
| First-degree relative with breast cancer <sup>a</sup>                                                                                                                     | 55 (15.0)                                                     |
| Previous biopsy                                                                                                                                                           | 55 (14.9)                                                     |
| Older than 12 years of age at menarche                                                                                                                                    | 184 (49.9)                                                    |
| Ever been pregnant                                                                                                                                                        | 308 (83.5)                                                    |
| Age at first pregnancy lasting $\geq 6$ months (in years):<br>- younger than 20<br>- 20-24<br>- 25-29<br>- 30 or older<br>- never pregnant/no pregnancies $\geq 6$ months | 28 (7.6)<br>131 (35.5)<br>88 (23.9)<br>50 (13.6)<br>72 (19.5) |
| Younger than 50 years of age at menopause <sup>b</sup>                                                                                                                    | 147 (40.6)                                                    |
| Type of menopause <sup>c</sup> :<br>- natural menopause<br>- hysterectomy without oophorectomy<br>- hysterectomy with uni- or bilateral oophorectomy                      | 263 (74.1)<br>41 (11.6)<br>51 (14.4)                          |
| Postmenopausal hormone therapy use:<br>- never<br>- former<br>- current (within previous 3 months)                                                                        | 133 (36.0)<br>186 (50.4)<br>50 (13.6)                         |

<sup>a</sup> Variable not available for 3 participants; <sup>b</sup> Variable not available for 7 participants; <sup>c</sup> Variable not available for 14 participants.

Table 2: Mean percentage mammographic density by genotype

| GENE               | SNP        | Genotype                  | Mean % density <sup>a</sup> | P <sup>b</sup> | P <sub>TREND</sub> |
|--------------------|------------|---------------------------|-----------------------------|----------------|--------------------|
| <b><i>IL6</i></b>  |            |                           |                             |                |                    |
|                    | rs1800795  | G/G (N=132)               | 24.94                       | 0.23           | 0.57               |
|                    |            | G/C (N=165) or C/C (N=56) | 27.22                       |                |                    |
|                    | rs2069827  | G/G (N=298)               | 26.81                       | 0.33           | 0.4                |
|                    |            | G/T (N=50) or T/T (N=4)   | 24.33                       |                |                    |
|                    | rs7801617  | G/G (N=268)               | 26.73                       | 0.43           | 0.43               |
|                    |            | G/A (N=81) or A/A (N=10)  | 25.12                       |                |                    |
|                    | rs2069840  | C/C (N=163)               | 26.86                       | 0.63           | 0.65               |
|                    |            | G/C (N=160) or G/G (N=32) | 25.98                       |                |                    |
|                    | rs2069861  | C/C (N=295)               | 26.2                        | 0.72           | 0.85               |
|                    |            | C/T (N=61) or T/T (N=3)   | 27.06                       |                |                    |
|                    | rs12700386 | C/C (N=245)               | 26.56                       | 0.75           | 0.91               |
|                    |            | G/C (N=100) or G/G (N=11) | 25.93                       |                |                    |
|                    | rs2069837  | A/A (N=305)               | 26.42                       | 0.78           | 0.67               |
|                    |            | G/A (N=51) or G/G (N=3)   | 25.72                       |                |                    |
|                    | rs7805828  | G/G (N=140)               | 26.72                       | 0.84           | 0.8                |
|                    |            | A/G (N=160) or A/A (N=53) | 26.33                       |                |                    |
|                    | rs2069860  | A/A (N=354)               | 26.33                       | 0.89           | 0.89               |
|                    |            | A/T (N=5)                 | 25.23                       |                |                    |
| <b><i>IL6R</i></b> |            |                           |                             |                |                    |
|                    | rs6427627  | T/T (N=138)               | 23.57                       | 0.01           | 0.22               |
|                    |            | C/T (N=170) or C/C (N=47) | 28.39                       |                |                    |
|                    | rs11265608 | G/G (N=299)               | 25.45                       | 0.03           | 0.02               |
|                    |            | A/G (N=56) or A/A (N=4)   | 30.88                       |                |                    |
|                    | rs1386821  | A/A (N=230)               | 25.04                       | 0.05           | 0.16               |
|                    |            | C/A (N=113) or C/C (N=16) | 28.76                       |                |                    |
|                    | rs4240872  | T/T (N=215)               | 27.57                       | 0.11           | 0.23               |
|                    |            | C/T (N=127) or C/C (N=17) | 24.58                       |                |                    |
|                    | rs10159236 | C/C (N=234)               | 25.58                       | 0.25           | 0.24               |
|                    |            | C/A (N=105) or A/A (N=16) | 27.84                       |                |                    |
|                    | rs4072391  | C/C (N=242)               | 26.88                       | 0.37           | 0.57               |
|                    |            | C/T (N=104) or T/T (N=13) | 25.17                       |                |                    |
|                    | rs2228145  | A/A (N=117)               | 27.42                       | 0.4            | 0.34               |
|                    |            | C/A (N=187) or C/C (N=48) | 25.8                        |                |                    |
|                    | rs11265622 | G/G (N=143)               | 27.11                       | 0.47           | 0.79               |
|                    |            | A/G (N=156) or A/A (N=60) | 25.8                        |                |                    |
|                    | rs2054855  | C/C (N=256)               | 26.06                       | 0.65           | 0.76               |
|                    |            | C/T (N=92) or T/T (N=11)  | 26.98                       |                |                    |
|                    | rs6684439  | C/C (N=124)               | 26.96                       | 0.77           | 0.51               |
|                    |            | C/T (N=178) or T/T (N=47) | 26.41                       |                |                    |
|                    | rs4845618  | T/T (N=125)               | 26.09                       | 0.85           | 0.7                |
|                    |            | G/T (N=178) or G/G (N=56) | 26.44                       |                |                    |
|                    | rs4601580  | A/A (N=106)               | 26.24                       | 0.99           | 0.87               |
|                    |            | A/T (N=189) or T/T (N=62) | 26.27                       |                |                    |

| <b>Table 2 cont.</b>   |            |                           |                                   |                      |                          |
|------------------------|------------|---------------------------|-----------------------------------|----------------------|--------------------------|
| <b>GENE</b>            | <b>SNP</b> | <b>Genotype</b>           | <b>Mean % density<sup>a</sup></b> | <b>P<sup>b</sup></b> | <b>P<sub>TREND</sub></b> |
| <b><i>IL6ST</i></b>    |            |                           |                                   |                      |                          |
|                        | rs11574780 | A/A (N=311)               | 27.09                             | 0.03                 | 0.03                     |
|                        |            | A/G (N=34)                | 20.67                             |                      |                          |
|                        | rs10940495 | A/A (N=189)               | 27.66                             | 0.13                 | 0.32                     |
|                        |            | G/A (N=150) or G/G (N=20) | 24.93                             |                      |                          |
|                        | rs1063560  | C/C (N=341)               | 26.64                             | 0.14                 | 0.14                     |
|                        |            | C/G (N=15)                | 20.35                             |                      |                          |
|                        | rs10471417 | A/A (N=165)               | 25.54                             | 0.4                  | 0.58                     |
|                        |            | C/A (N=147) or C/C (N=43) | 27.08                             |                      |                          |
|                        | rs6870870  | C/C (N=136)               | 27.19                             | 0.51                 | 0.42                     |
|                        |            | C/A (N=168) or A/A (N=51) | 25.93                             |                      |                          |
|                        | rs2228043  | C/C (N=289)               | 26.35                             | 0.99                 | 0.82                     |
|                        |            | C/G (N=63) or G/G (N=5)   | 26.39                             |                      |                          |
|                        | rs1900173  | T/T (N=309)               | 26.38                             | 1                    | 0.77                     |
|                        |            | T/A (N=37) or A/A (N=2)   | 26.37                             |                      |                          |
| <b><i>TNFRSF1A</i></b> |            |                           |                                   |                      |                          |
|                        | rs4149584  | G/G(N=338)                | 26.13                             | 0.26                 | 0.26                     |
|                        |            | G/A (N=18)                | 30.95                             |                      |                          |
|                        | rs4149577  | T/T (N=94)                | 25.31                             | 0.49                 | 0.98                     |
|                        |            | T/C (N=179) or C/C (N=83) | 26.74                             |                      |                          |
|                        | rs4149579  | G/G (N=313)               | 26.16                             | 0.65                 | 0.65                     |
|                        |            | G/A (N=45) or A/A (N=1)   | 27.4                              |                      |                          |
|                        | rs2228576  | G/G (N=171)               | 26.85                             | 0.71                 | 0.96                     |
|                        |            | G/A (N=162) or A/A (N=11) | 26.15                             |                      |                          |
|                        | rs4149570  | G/G (N=131)               | 26.06                             | 0.79                 | 0.8                      |
|                        |            | G/T (N=167) or T/T (N=52) | 26.57                             |                      |                          |
|                        | rs11064145 | T/T (N=103)               | 26.02                             | 0.82                 | 0.85                     |
|                        |            | G/T (N=180) or G/G (N=70) | 26.48                             |                      |                          |
|                        | rs4149578  | G/G (N=300)               | 26.31                             | 0.83                 | 0.65                     |
|                        |            | G/A (N=51) or A/A (N=4)   | 26.87                             |                      |                          |
| <b><i>TNFRSF1B</i></b> |            |                           |                                   |                      |                          |
|                        | rs652284   | T/T (N=99)                | 23.71                             | 0.06                 | 0.34                     |
|                        |            | T/C (N=182) or C/C (N=78) | 27.39                             |                      |                          |
|                        | rs1201157  | C/C (N=139)               | 27.68                             | 0.25                 | 0.5                      |
|                        |            | C/T (N=165) or T/T (N=52) | 25.53                             |                      |                          |
|                        | rs5746016  | C/C (N=318)               | 26.08                             | 0.39                 | 0.38                     |
|                        |            | T/C (N=34) or T/T (N=1)   | 28.78                             |                      |                          |
|                        | rs590977   | A/A (N=238)               | 26.89                             | 0.42                 | 0.44                     |
|                        |            | C/A (N=115) or C/C (N=15) | 25.38                             |                      |                          |
|                        | rs5746001  | G/G (N=236)               | 26.8                              | 0.51                 | 0.49                     |
|                        |            | G/A (N=105) or A/A (N=15) | 25.51                             |                      |                          |
|                        | rs653667   | A/A (N=88)                | 27.18                             | 0.62                 | 0.31                     |
|                        |            | C/A (N=207) or C/C (N=59) | 26.14                             |                      |                          |
|                        | rs683240   | T/T (N=189)               | 26.66                             | 0.65                 | 0.65                     |
|                        |            | C/T (N=147) or C/C (N=22) | 25.85                             |                      |                          |
|                        | rs1061622  | T/T (N=193)               | 26.66                             | 0.78                 | 0.73                     |
|                        |            | G/T (N=140) or G/G (N=22) | 26.15                             |                      |                          |
|                        | rs816060   | T/T (N=102)               | 26.58                             | 0.81                 | 0.33                     |
|                        |            | C/T (N=178) or C/C (N=77) | 26.09                             |                      |                          |

| <b>Table 2 cont.</b> |            |                         |                                   |                      |                          |
|----------------------|------------|-------------------------|-----------------------------------|----------------------|--------------------------|
| <b>GENE</b>          | <b>SNP</b> | <b>Genotype</b>         | <b>Mean % density<sup>a</sup></b> | <b>P<sup>b</sup></b> | <b>P<sub>TREND</sub></b> |
| <i>TNF-α</i>         |            |                         |                                   |                      |                          |
|                      | rs1800629  | G/G (N=278)             | 26.85                             | 0.29                 | 0.31                     |
|                      |            | A/G (N=77) or A/A (N=4) | 24.6                              |                      |                          |

<sup>a</sup> Mean percentage breast density adjusted for age, BMI, hormone therapy use, NSAID use, pregnancy for at least 6 months and previous biopsy.

<sup>b</sup> P value for difference in mean percentage breast density adjusted for age, BMI, hormone therapy use, NSAID use, pregnancy for at least 6 months and previous biopsy.

None of the evaluated SNPs in *IL6* and *TNF-α* were significantly associated with percentage mammographic density in our study population. However, two SNPs in *IL6R*, rs11265608 and rs64227627, and one in *IL6-ST*, rs11574780, were statistically significantly associated with percentage mammographic density. For both rs11265608 and rs64227627, mean percentage mammographic density was significantly higher among women with at least one rare allele than among women homozygous for the common allele ( $P=0.01$  and  $P=0.03$ , respectively). For rs11574780, mean percentage mammographic density was significantly higher among women homozygous for the common allele ( $P=0.03$ ).

Interactions with BMI, NSAID use and hormone therapy use have not yet been assessed but will be investigated.

## KEY RESEARCH ACCOMPLISHMENTS

Progress was made during the third year of this grant. However, not all work was completed during the approved project period due to the grant not having a Principal Investigator and not being able to use grant money from the time Dr. Modugno left until January 2008 when Dr. Diergaarde officially took over. Additional time beyond the established expiration date to ensure adequate completion of the originally approved project was requested and granted.

The preliminary results from the SNP data suggest that common variation in *IL6R* and *IL6ST* is associated with percentage mammographic density in healthy Caucasian postmenopausal women.

## REPORTABLE OUTCOMES

An abstract on the relation between common genetic variants in *IL6*, *IL6R*, *IL6ST*, *TNF*, *TNFRSF1A* and *TNFRSF1B*, and mammographic breast density has been submitted to the 2010 American Society of Preventive Oncology (ASPO) meeting. A manuscript on this topic is in preparation as is a manuscript on the relation between cytokine levels and mammographic breast density (regarding the latter, we are currently waiting on the measurement of sTNF-RI and sTNF-RII, see above).

## CONCLUSION

Polymorphisms in *IL6*, *TNF- $\alpha$*  and the genes that code for their receptors may alter exposure to estrogens and so affect mammographic breast density. In line with this, our preliminary results suggest that common variation in *IL6R* and *IL6ST* is associated with percentage mammographic density in healthy Caucasian postmenopausal women. Identification of the genes (and within the genes the functional polymorphisms) that affect mammographic breast density will likely provide insights into the biology of the breast and may identify potential targets for breast cancer (chemo)prevention.

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## APPENDICES

None