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Lusine Yaghjyan  
*University of Florida, Gainesville*

Andreas Pettersson  
*Harvard School of Public Health*

Graham A. Colditz  
*Washington University School of Medicine in St Louis*

Laura C. Collins  
*Harvard Medical School*

Stuart J. Schnitt  
*Harvard Medical School*

*See next page for additional authors*

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Authors
Lusine Yaghjyan, Andreas Pettersson, Graham A. Colditz, Laura C. Collins, Stuart J. Schnitt, Andrew H. Beck, Bernard Rosner, Celine Vachon, and Rulla M. Tamimi

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Postmenopausal mammographic breast density and subsequent breast cancer risk according to selected tissue markers

Lusine Yaghjian1, Andreas Pettersson2,3, Graham A Colditz4,5, Laura C Collins6, Stuart J Schnitt6, Andrew H Beck6, Bernard Rosner7, Celine Vachon8 and Rulla M Tamimi*,2,7

1Department of Epidemiology, College of Public Health and Health Professions and College of Medicine, University of Florida, 2004 Mowry Road, Gainesville, FL 32610, USA; 2Department of Epidemiology, Harvard School of Public Health, 181 Longwood Avenue, Boston, MA 02115, USA; 3Department of Medicine Solna, Clinical Epidemiology Unit, Karolinska Institutet, 171 76 Solna, Stockholm, Sweden; 4Division of Public Health Sciences, Department of Surgery, Washington University in St Louis School of Medicine, 660S. Euclid Avenue, St Louis, MO 63110, USA; 5Institute for Public Health, Washington University in St Louis, St Louis, MO, USA; 6Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA; 7Channing Division of Network Medicine, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115, USA and 8Department of Health Sciences Research, Mayo Clinic College of Medicine, Charlton 6-239, 200 First Street Southwest, Rochester, MN 55905, USA

Background: This study aimed to determine if associations of pre-diagnostic percent breast density, absolute dense area, and non-dense area with subsequent breast cancer risk differ by the tumour’s molecular marker status.

Methods: We included 1010 postmenopausal women with breast cancer and 2077 matched controls from the Nurses’ Health Study (NHS) and the Nurses’ Health Study II (NHS II) cohorts. Breast density was estimated from digitised film mammograms using computer-assisted thresholding techniques. Information on breast cancer risk factors was obtained prospectively from biennial questionnaires. Polychotomous logistic regression was used to assess associations of breast density measures with tumour subtypes by the status of selected tissue markers. All tests of statistical significance were two sided.

Results: The association of percent density with breast cancer risk appeared to be stronger in ER− as compared with ER+ tumours, but the difference did not reach statistical significance (density ≥ 50% vs < 10% odds ratio (OR) = 3.06, 95% confidence interval (CI) 2.17–4.32 for ER+; OR = 4.61, 95% CI 2.36–9.03 for ER−, \( P_{\text{heterogeneity}} = 0.08 \)). Stronger positive associations were found for absolute dense area and CK5/6− and EGFR− as compared with respective marker-positive tumours (\( P_{\text{heterogeneity}} = 0.002 \) and 0.001, respectively). Stronger inverse associations of non-dense area with breast cancer risk were found for ER− as compared with ER+ tumours (\( P_{\text{heterogeneity}} = 0.0001 \)) and for AR+, CK5/6+, and EGFR+ as compared with respective marker-negative tumours (\( P_{\text{heterogeneity}} = 0.03, 0.005, \) and 0.009, respectively). The associations of density measures with breast cancer did not differ by progesterone receptor and human epidermal growth factor receptor 2 status.

Conclusions: Breast density influences the risk of breast cancer subtypes by potentially different mechanisms.

*Correspondence: Dr RM Tamimi; E-mail: nhrmt@channing.harvard.edu

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Mammographic breast density is a well-established and strong predictor of breast cancer risk (McCormack and dos Santos Silva, 2006). Appearance of the breast on the mammogram is a reflection of the amount of fat, connective tissue, and epithelial tissue in the breast (Tamimi et al., 2007). Women with 75% or greater percent breast density (proportion of the total breast area that appears dense on the mammogram) are at four- to six-fold greater risk of breast cancer compared with women with predominantly fatty breasts (Boyd et al., 1995, Byrne et al., 1995, Tamimi et al., 2007). Absolute dense area of the breast that represents fibroglandular tissue has been shown to be positively associated with breast cancer risk while findings for the association between non-dense area of the breast (representing adipose tissue) and breast cancer risk have been conflicting (Petersson et al., 2014).

Breast cancer is a heterogeneous disease; different molecular subtypes of breast cancer have distinct clinico-pathological features and biomarker expression that influence patient survival (Arpino et al., 2004, Potti et al., 2005, Phips et al., 2010). We have recently reported a stronger association of percent breast density with the risk of more unfavourable oestrogen receptor-negative breast tumours (ER−) as compared with oestrogen receptor-positive tumours (ER+) among postmenopausal women (Yaghjyan et al., 2011). Also, a large pooled analysis showed a stronger association of percent density with ER− tumours among younger women suggesting that high density may play an important role in tumour aggressiveness (Bertrand et al., 2013). Whether the associations of breast density with breast cancer risk differ by status of other tissue markers implicated in breast cancer aetiology and prognosis is unknown. Understanding these differences might help to identify biological pathways linking high density to specific breast cancer subtypes. We analysed prospective data in postmenopausal women from the Nurses’ Health Study (NHS) and the Nurses’ Health Study II (NHS II) cohorts to determine if there are differences in the associations of percent breast density, absolute dense area, and non-dense area with subsequent risk of breast cancer according to the tumour’s status of the following tissue markers involved in cellular proliferation, differentiation, and angiogenesis that have been shown to influence tumour aggressiveness: oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), proliferation marker Ki67, androgen receptor (AR), cytokeratins 5/6 (CK5/6), epidermal growth factor receptor (EGFR), and vascular endothelial growth factor (VEGF).

Mammographic breast density. The screening mammogram before the reference date was used for density measurements. The average time between the mammogram date and the date of breast cancer diagnosis was 4.8 years (interquartile range (IQR) = 2–7 years) for NHS I and 3.0 years (IQR = 1–5 years) for NHS II. The average time between mammogram and the reference date of controls was 4.2 years (IQR = 1–7 years) for NHS I and 1.5 years (IQR = 0–5 years) for NHS II. All mammograms in the study were film (analogue) mammograms. To quantify mammographic density, the craniocaudal views of both breasts were digitised at 261 µm per pixel with a LumiSys 85 laser film scanner (LumiSys, Sunnyvale, CA, USA). The Cumulus software (University of Toronto, Toronto, Canada) was used for computer-assisted determination of the absolute dense area, non-dense area, and percent density (Byng et al., 1996). During this assessment, the observer was blinded to the participant’s case-control status. As reported previously, the measure of mammographic breast density from NHS I mammograms was highly reproducible (within-person intraclass correlation coefficient was 0.93) (Tamimi et al., 2007). All NHS II images were read by a single reader in two batches of mammograms ~3 years apart. Cases and their matched controls were included in the same batch. Although within batch reproducibility was high (interclass correlation coefficient 0.90 and greater) (Petersson et al., 2011), density measures varied across the two NHS II batches. We included a small subset of identical mammograms in both batches to account for batch drift in density measurement readings. The density measures from the second batch of NHS II mammograms were adjusted to account for the effect of batch on density measurements, as previously described (Bertrand et al., 2012).

Because breast densities of the right and left breast for an individual woman are strongly correlated (correlation coefficient = 0.92–0.96 for density estimated from right vs left craniocaudal views) (Byng et al., 1996), the average density of both breasts was used in this analysis.

Breast cancer tissue markers. Information on the tumour’s ER, PR, and HER2 status was obtained primarily from pathology reports and medical records. For breast cancers with missing receptor data from pathology reports, the receptor status was obtained from immunohistochemical staining performed on paraffin sections of the tumour tissue microarray (TMA) according to a standard protocol (Tamimi et al., 2008). Of all cases in the study, information on ER, PR, and HER2 status was available for 94% (n = 933), 93% (n = 916), and 71% (n = 701), respectively. Among women with receptor status information, ER, PR, and
HER2 receptor status was available from the pathology reports for 94%, 93%, and 44% of the tumours, respectively. Information on Ki67, AR, CK5/6, EGFR, and VEGF status was obtained from TMA only and was available for 16% (n = 160), 41% (n = 406), 29% (n = 285), 30% (n = 297), and 18% (n = 182) of cases, respectively.

Variation in the number of cases with available marker data is explained by the source of marker data (pathology reports and TMA (ER, PR, HER2) or TMA only (all other markers)) as well as funding support for quantification for each of the markers. Currently, tissue blocks have been obtained and TMA samples created for ~0% of all eligible cases in the cohort. However, as quantification of each of the markers is supported by a different grant, not every TMA are stained and scored at the same time for all of these markers resulting in variation in the number of cases with available marker data across the markers.

Immunostained TMA sections were reviewed by an expert pathologist and visually scored for the percentage of cells that stained positively for each of the markers. For ER and PR, positivity from TMA was defined as any staining in the cytoplasm of tumour cells (Hefti et al., 2013). For AR, positivity from TMA was defined as >10% of tumour cell nuclei staining. (Tamimi et al, 2008) Moderate (2+) or strong (3+) membrane staining (as opposed to low (1+) or none (0)) for HER2 in more than 10% of the tumour cells was used as the cut-off for tumour's HER2 positivity (Tamimi et al, 2008). Cases were considered CK5/6-positive or EGFR-positive if any staining was detected in the tumour cells, including only focal (Collins et al, 2011). VEGF-positivity was defined as any staining in the cytoplasm of tumour cells (Liu et al, 2011).

We quantified Ki67 expression with the aid of computational image analysis, implemented with Definiens Tissue Studio (Munich, Germany). For each core, we computed the number of total nuclei and the number of Ki67-positive nuclei. We aggregated counts of Ki67-positive nuclei and total nuclei across a patient’s TMA cores, and cases were considered Ki67-positive if the sum of the percentage of Ki67-positive nuclei across cores was 14% or greater (Cheang et al, 2009).

**Covariates.** Information on breast cancer risk factors was obtained from the biennial questionnaires before the reference date. Women were considered to be postmenopausal if they reported: (1) no menstrual periods within the 12 months before blood collection with natural menopause, (2) bilateral oophorectomy, or (3) hysterectomy with one or both ovaries retained and were 54 years or older for ever smokers or 56 years or older for never smokers (Willett et al, 1983).

**Statistical analysis.** We used Wilcoxon–Mann–Whitney test to analyse the difference in breast density distributions in cases and controls. We used unconditional logistic regression to analyse the association between breast density measures and breast cancer risk, while adjusting for the following potential confounders in the fully adjusted logistic regression models: age at diagnosis (continuous, years), body mass index (continuous, kg m\(^{-2}\)), alcohol consumption (0, <5, 5 to <15, ≥15 g per day), and study cohort (NHS, NHS II). Percent breast density was categorised as <10% (referent), 10–24%, 25–49%, and ≥50%. Absolute dense and non-dense areas were categorised as quartiles based on their distribution in controls (absolute dense area: <19 cm\(^2\) (referent), 19–<35 cm\(^2\), 35–<61 cm\(^2\), ≥61 cm\(^2\); non-dense area: <83 cm\(^2\) (referent), 83–<138 cm\(^2\), 138–<208 cm\(^2\), ≥208 cm\(^2\)).

Differences in the association of breast density with receptor-specific tumour subtypes were investigated using polychotomous logistic regression (Tamimi et al, 2007). In this analysis, the outcome has three levels that include controls and two breast cancer subtypes defined based on the receptor status (receptor-
positive and receptor-negative, for e.g., ER+ and ER− breast cancer). In additional analysis, we also compared these associations in ER+/PR+ and ER−/PR− tumours. We did not analyse the association between breast density and breast cancer risk by combined ER, PR, and HER2 (ER/PR/HER2) status or by combined CK5/6/EGFR status because the small number of tumours in some subsets did not provide sufficient power to draw meaningful conclusions. For example, defining subtypes based on ER, PR, and HER2 resulted in the following numbers: ER+/PR+/HER2− n = 356; ER+/PR+/HER2+ n = 82; ER−/PR−/HER2+ n = 39, ER−/PR−/HER2− n = 57. The numbers further became much smaller when CK5/6, EGFR, and Ki67 were considered. We used a likelihood ratio test to compare a model with separate mammographic density slopes in each case group with a model with a common slope. This method has been described in detail elsewhere (Yaghjian et al, 2011). In this analysis, the density measures were modelled using respective medians within each of the density categories. For all analyses, the level of statistical significance was assessed at α = 0.05. All tests were two sided.

Because the ER and PR status were moderately correlated, in a secondary analysis we used previously published method to examine associations of percent density with breast cancer by ER status while adjusting for PR (Rosner et al, 2013). All analyses except the test of heterogeneity were performed using SAS software (version 9.2, SAS Institute, Cary, NC, USA). The test of heterogeneity from polychotomous logistic regression models was done using STATA version 11.0 (Stata Corp, College Station, TX, USA).

### RESULTS

In this nested case-control study of 1010 postmenopausal women diagnosed with breast cancer and 2077 matched controls, cases had a higher median percent breast density (28.1% vs 21.6%, $P<0.0001$), higher median absolute dense area (45.1 vs 35.4 cm$^2$, $P<0.0001$) and smaller median area of non-dense breast tissue (120.5 vs 138.5 cm$^2$, $P<0.0001$) as compared with controls. Distribution of breast cancer risk factors in this study population is presented in Table 1. Cases were more likely to have a history of postmenopausal hormone use (76.1% vs 71.6%; $P=0.001$), a family history of breast cancer (19.6% vs 14.4%; $P<0.001$), a confirmed benign breast disease (33.4% vs 25.8%; $P<0.0001$), and older age at natural menopause (48.9 vs 48.4; $P=0.02$) as compared with controls. Cases and controls did not differ significantly with respect to other risk factors.

In the multivariable analysis, higher percent density was associated with breast cancer risk increase ($\geq 50$% vs $<10$%, odds ratio (OR) = 3.18, 95% confidence interval (CI) 2.32–4.35, $P<0.0001$), larger absolute dense area was associated with an increase in breast cancer risk (4th vs 1st quartile, OR = 1.93, 95% CI 1.52–2.45, $P_{\text{trend}}<0.0001$), and older age at natural menopause (OR = 0.58, 95% CI 0.40–0.68, $P_{\text{trend}}<0.0001$) (Supplementary table 1).

### Breast density and breast cancer risk according to the marker status

Correlations of tissue markers with each other are presented in Table 2. We observed a positive moderate correlation of ER with PR (correlation coefficient $r = 0.54$, $P<0.0001$) and AR ($r = 0.41$, $P<0.0001$) and negative moderate correlation with EGFR ($r = -0.44$, $P<0.0001$). CK5/6 was moderately correlated with EGFR ($r = 0.58$, $P<0.0001$). None of the other markers were correlated with each other.

### Percent breast density

Percent density was positively associated with all marker-defined subtypes of breast cancer (Table 3). The association of percent density with breast cancer risk appeared to be stronger in ER− tumours as compared with ER+ tumours, as we reported previously (Yaghjian et al, 2011) This difference in associations by ER status, however, did not reach statistical significance (density $\geq 50$% vs $<10$%; OR = 3.06, 95% CI 2.17–4.32 for ER+ tumours; OR = 4.61, 95% CI 2.36–9.03 for
Table 2. Correlations between eight tissue markers evaluated among 1010 cases included in the study

| Tissue Marker Pair | Spearman correlation coefficient (P-value), n | PR (n = 916) | HER2 (n = 701) | AR (n = 406) | CK5/6 (n = 285) | EGFR (n = 297) | VEGF (n = 182) | Ki67 (n = 160) |
|-------------------|---------------------------------------------|--------------|---------------|--------------|----------------|----------------|----------------|---------------|
| ER (n = 933)      | 0.54 (<0.0001)                              | 915          |               | 0.41 (<0.0001) | 382            | 0.44 (<0.0001) | 299            | -0.31 (0.0001) |
| PR                | 0.19 (<0.0001)                              | 71           | 0.12 (0.03)   | 0.12 (0.03)   | 0.24 (<0.0001) | 0.03 (0.73)   | 0.21 (0.01)   |
| HER2              | -0.16 (<0.0001)                             | 657          | -0.06 (0.31)  | 0.14 (0.02)   | 0.01 (0.89)   | 0.02 (0.83)   |               |
| AR                | -0.08 (0.10)                               | 405          | 0.08 (0.27)   | 0.13 (0.06)   | 0.19 (0.01)   | 0.29 (0.0007) |               |
| CK5/6             | -0.08 (0.10)                               | 405          | -0.13 (0.06)  | 202           | 0.08 (0.0001) | 274           |               |
| EGFR              | -0.08 (0.10)                               | 405          | -0.19 (0.01)  | 177           | 0.58 (<0.0001) | NE*           | 0.13 (0.24)   |
| VEGF              | -0.08 (0.10)                               | 405          | -0.19 (0.01)  | 177           | 0.58 (<0.0001) | NE*           | 0.13 (0.25)   |

Abbreviations: AR = androgen receptor; CK5/6 = cytokeratins 5/6; EGFR = epidermal growth factor receptor; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2; Ki67 = proliferation marker; PR = progesterone receptor; VEGF = vascular endothelial growth factor.

*Non estimable: all tumours CK5/6.

bNon estimable: all tumours EGFR– and VEGF.

Table 3. Association of percent breast density with the risk of marker-defined subtypes of breast cancer in postmenopausal women

| Tumour Marker | Percent density category | No. cases/controls | OR (95% CI) | No. cases/controls | OR (95% CI) | P-value |
|---------------|------------------------|-------------------|-------------|-------------------|-------------|---------|
|               | Total                  | 766/2052          | 165/2052    |                   |             |         |
| ER            | <10%                   | 112/461           | 1.00 (referent) | 17/461            | 1.00 (referent) | 0.08    |
|               | 10–24%                 | 224/727           | 1.36 (1.04–1.77) | 48/727            | 1.78 (1.00–3.17) |         |
|               | 25–49%                 | 312/651           | 2.39 (1.82–3.14) | 65/651            | 2.75 (1.53–4.95) |         |
|               | >50%                   | 120/213           | 3.06 (2.17–4.32) | 35/213            | 4.61 (2.36–9.03) |         |
|               | P_trend                |                   | <0.0001     |                   | <0.0001     |         |
| PR            | <10%                   | 94/461            | 1.00 (referent) | 32/461            | 1.00 (referent) | 0.63    |
|               | 10–24%                 | 176/727           | 1.27 (0.95–1.69) | 96/727            | 1.91 (1.25–2.94) |         |
|               | 25–49%                 | 261/651           | 2.37 (1.77–3.18) | 102/651           | 2.37 (1.52–3.72) |         |
|               | >50%                   | 102/213           | 3.04 (2.10–4.40) | 53/213            | 3.95 (2.34–6.65) |         |
|               | P_trend                |                   | <0.0001     |                   | <0.0001     |         |
| HER2          | <10%                   | 21/461            | 1.00 (referent) | 76/461            | 1.00 (referent) | 0.43    |
|               | 10–24%                 | 51/727            | 1.56 (0.91–2.68) | 161/727           | 1.38 (1.01–1.87) |         |
|               | 25–49%                 | 74/651            | 2.89 (1.68–4.98) | 210/651           | 2.16 (1.57–2.96) |         |
|               | >50%                   | 18/213            | 2.39 (1.16–4.89) | 90/213            | 2.99 (2.02–4.42) |         |
|               | P_trend                |                   | 0.001       |                   | <0.0001     |         |
| Ki67          | <10%                   | 5/461             | 1.00 (referent) | 14/461            | 1.00 (referent) | 0.47    |
|               | 10–24%                 | 10/727            | 1.43 (0.47–4.39) | 32/727            | 1.69 (0.88–3.26) |         |
|               | 25–49%                 | 18/651            | 3.48 (1.17–10.39) | 55/651            | 3.74 (1.95–7.20) |         |
|               | >50%                   | 9/213             | 6.74 (1.90–23.83) | 17/213            | 3.96 (1.76–8.93) |         |
|               | P_trend                |                   | 0.0003      |                   | <0.0001     |         |
| CK5/6         | <10%                   | 9/461             | 1.00 (referent) | 30/461            | 1.00 (referent) | 0.46    |
|               | 10–24%                 | 13/727            | 1.05 (0.44–2.55) | 74/727            | 1.58 (1.00–2.49) |         |
|               | 25–49%                 | 23/651            | 2.43 (1.03–5.78) | 94/651            | 2.47 (1.55–3.93) |         |
|               | >50%                   | 5/213             | 2.26 (0.67–7.62) | 37/213            | 3.29 (1.85–5.84) |         |
|               | P_trend                |                   | 0.03        |                   | <0.0001     |         |
Table 3. (Continued)

| Tumour Marker | Percent density category | No. cases/controls | OR (95% CI) | No. cases/controls | OR (95% CI) | P-valueb |
|---------------|--------------------------|-------------------|-------------|-------------------|-------------|---------|
| AR            | Total                    | 301/2052          | 1.00 (referent) | 105/2052          | 1.00 (referent) | 0.35    |
|               | <10%                     | 49/461            | 1.10 (0.75–1.61) | 18/461            | 0.99 (0.53–1.86) |         |
|               | 10–24%                   | 82/727            | 1.83 (1.24–2.70) | 26/727            | 1.70 (0.90–3.21) |         |
|               | ≥50                      | 51/213            | 2.53 (1.56–4.10) | 25/213            | 4.27 (2.06–8.86) |         |
| P_heterogeneity | <0.0001                 |                   |              |                   |              |         |
| EGFR          | Total                    | 48/2052           | 1.00 (referent) | 249/2052          | 1.00 (referent) | 0.36    |
|               | <10%                     | 8/461             | 1.37 (0.57–3.32) | 35/461            | 1.36 (0.88–2.10) |         |
|               | 10–24%                   | 16/727            | 1.97 (0.79–4.93) | 73/727            | 2.48 (1.60–3.86) |         |
|               | ≥50                      | 5/213             | 1.89 (0.55–5.55) | 105/213           | 2.97 (1.50–5.19) |         |
| P_heterogeneity | 0.18                    |                   |              |                   |              |         |
| VEGF          | Total                    | 145/2052          | 1.00 (referent) | 37/2052           | 1.00 (referent) | 0.35    |
|               | <10%                     | 26/461            | 1.08 (0.64–1.84) | 4/461             | 1.10 (0.32–3.85) |         |
|               | 25–49%                   | 40/727            | 1.53 (0.89–2.61) | 8/727             | 2.52 (0.76–8.33) |         |
|               | ≥50                      | 28/213            | 2.68 (1.41–5.12) | 7/213             | 3.01 (0.74–12.29) |         |
| P_heterogeneity | 0.0009                  |                   |              |                   |              |         |

Abbreviations: AR = androgen receptor; CI = confidence interval; CK5/6 = cytokeratins 5/6; EGFR = epidermal growth factor receptor; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2; Ki67 = proliferation marker; OR = odds ratio; PR = progesterone receptor; SE = standard error; VEGF = vascular endothelial growth factor.

*Polychotomous multinivariable logistic regression model adjusted for age (continuous), body mass index (continuous), age at menarche (<12, 12, 13, or >13 years), parity, age at first birth (nulliparous, 1–4 children with age at first birth <25 years, 1–4 children with age at first birth of 25–29 years, 1–4 children with age at first birth of ≥30 years), ≥5 children with age at first birth of ≥25 years, or ≥5 children with age at first birth of ≥25 years). The risk of breast cancer subtypes defined by VEGF status.

ER−tumours, P_heterogeneity = 0.08. In the analysis of associations by ER status adjusted for PR status (915 cases), the difference in the association by ER status did not reach statistical significance (P_heterogeneity = 0.20). In the additional analysis comparing ER+/PR+ and ER−/PR− tumours, the association of percent density appeared to be stronger in ER−/PR− tumours (OR = 4.59, 95% CI 2.15–9.83 vs OR = 3.03, 95% CI 2.07–4.43); the difference, however, did not reach statistical significance (P_heterogeneity = 0.35).

No differences across the subtypes were observed for any of the other markers (Table 3).

Absolute dense area. In the analyses by the tumour’s ER, PR, and HER2 status, absolute dense area was positively associated with increased breast cancer risk in both marker-positive and marker-negative tumours and these subset-specific associations were similar (Table 4). The association of absolute dense area was similar while comparing ER+/PR+ and ER−/PR− tumours (P_heterogeneity = 0.32).

In the analysis by AR, CK5/6, and EGFR, the differences in subset-specific associations were significant for CK5/6 (P_heterogeneity = 0.002) and EGFR (P_heterogeneity = 0.001) and were marginally significant for AR (P_heterogeneity = 0.07).

Associations of absolute dense area with Ki67+ and Ki67− tumours were similar. Absolute dense area was not associated with the risk of breast cancer subtypes defined by VEGF status.

Non-dense area. In the analyses by tumour ER and PR status, non-dense breast area was inversely associated with breast cancer risk in both marker-positive and marker-negative tumours (Table 4). The magnitude of the association was stronger in ER− (4th vs 1st quartile OR = 0.26, 95% CI 0.14–0.46) as compared with ER+ tumours (OR = 0.60, 95% CI 0.45–0.80; P_heterogeneity <0.0001). In the additional analysis comparing ER+/PR+ and ER−/PR− tumours, the association of percent dense area was stronger in ER−/PR− tumours (4th quartile vs 1st quartile: OR = 0.30, 95% CI 0.16–0.56 vs OR = 0.56, 95% CI 0.41–0.77, P_heterogeneity = 0.0073).

The difference in the associations of non-dense area with breast cancer in Ki67+ and Ki67− tumours did not reach statistical significance, but the risk estimates suggested a possible stronger inverse association in Ki67− tumours (4th vs 1st quartile OR = 0.44 for Ki67+ and OR = 0.26 for Ki67− tumours). The association between non-dense area and breast cancer risk was stronger in AR− (4th vs 1st quartile OR = 0.27, 95% CI 0.17–0.43) as compared with AR+ tumours (OR = 0.62, 95% CI 0.33–1.19, P_heterogeneity = 0.03). We found significant differences in the association of non-dense area with breast cancer by status of CK5/6 and EGFR (P_heterogeneity = 0.005 and 0.009, respectively) (Table 4). In the analyses by tumour’s PR, HER2, and VEGF status, the inverse associations of non-dense breast area with breast cancer risk were similar.

In an additional analysis, we mutually adjusted the risk estimates for absolute dense and non-dense areas by including both of the density measures in the model simultaneously. The risk estimates for both absolute dense area and non-dense area remained essentially unchanged suggesting independent nature of the observed associations (data not shown). We found no correlation between absolute dense area and non-dense area (correlation coefficient = 0.02).

In secondary analyses, we excluded women diagnosed with breast cancer within 2 years of their mammogram from the current analysis; the results were not affected. We also used ASCO/CAP clinical definition of HER2 positivity (as 3+ membrane staining as opposed to 2+, 1+ or none) in additional analysis by HER2 status; the results were similar (data not shown). Finally, the results for all three density measures were similar when the analysis was restricted to invasive cancers only (data not shown).

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| Tumor marker | Density category | OR (95% CI) | P-value |
|--------------|------------------|------------|---------|
| ER           | Total            | 0.27       | 0.0001  |
|              | 1st quartile     | 1.00 (referent) | 0.07    |
|              | 2nd quartile     | 1.09 (0.83–1.43) | 0.46    |
|              | 3rd quartile     | 1.75 (1.35–2.25) | <0.0001 |
|              | 4th quartile     | 2.06 (1.59–2.57) | <0.0001 |
| PR           | Total            | 0.00       | 0.0001  |
|              | 1st quartile     | 1.00 (referent) | 0.07    |
|              | 2nd quartile     | 1.05 (0.79–1.41) | 0.46    |
|              | 3rd quartile     | 1.74 (1.29–2.34) | <0.0001 |
|              | 4th quartile     | 2.16 (1.73–3.35) | <0.0001 |
| HER2         | Total            | 0.49       | 0.0001  |
|              | 1st quartile     | 1.00 (referent) | 0.07    |
|              | 2nd quartile     | 1.42 (0.83–2.42) | 0.46    |
|              | 3rd quartile     | 1.56 (1.18–2.05) | <0.0001 |
|              | 4th quartile     | 1.18 (0.92–1.53) | 0.46    |
| Ki67         | Total            | 0.43       | 0.0001  |
|              | 1st quartile     | 1.00 (referent) | 0.07    |
|              | 2nd quartile     | 0.96 (0.57–1.61) | 0.46    |
|              | 3rd quartile     | 0.80 (0.59–1.12) | <0.0001 |
|              | 4th quartile     | 0.42 (0.30–0.60) | 0.46    |
| CK5/6        | Total            | 0.43       | 0.0001  |
|              | 1st quartile     | 1.00 (referent) | 0.07    |
|              | 2nd quartile     | 1.11 (0.68–1.80) | 0.46    |
|              | 3rd quartile     | 0.79 (0.51–1.27) | <0.0001 |
|              | 4th quartile     | 0.43 (0.26–0.72) | 0.46    |
| AR           | Total            | 0.76       | 0.0001  |
|              | 1st quartile     | 1.00 (referent) | 0.07    |
|              | 2nd quartile     | 0.66 (0.59–1.51) | 0.46    |
|              | 3rd quartile     | 1.18 (0.97–1.45) | <0.0001 |
|              | 4th quartile     | 0.43 (0.26–0.69) | 0.46    |

Table 4. Association of absolute dense area and non-dense area with the risk of marker-defined subtypes of breast cancer in postmenopausal women.
Table 4. (Continued)

| Tumor marker | Density category | Marker-negative subtype | Marker-positive subtype | P-value<sup>b</sup> | Non-dense area | Marker-negative subtype | Marker-positive subtype | P-value<sup>b</sup> |
|--------------|------------------|-------------------------|-------------------------|------------------|------------------|-------------------------|-------------------------|------------------|
| EGFR | Total | 48/2052 | 249/2052 | 0.001 | 48/2052 | 249/2052 | 0.009 |
| | 1<sup>st</sup> quartile | 13/510 | 1.00 (referent) | 38/510 | 1.00 (referent) | 16/518 | 1.00 (referent) | 51/518 | 1.00 (referent) |
| | 2<sup>nd</sup> quartile | 11/503 | 0.84 (0.37–1.92) | 32/503 | 0.83 (0.51–1.37) | 18/506 | 1.05 (0.51–2.15) | 70/506 | 1.26 (0.85–1.87) |
| | 3<sup>rd</sup> quartile | 13/524 | 1.03 (0.46–2.31) | 69/524 | 1.71 (1.11–2.62) | 6/512 | 0.36 (0.13–0.99) | 58/512 | 0.98 (0.64–1.50) |
| | 4<sup>th</sup> quartile | 11/515 | 1.18 (0.51–2.74) | 110/515 | 2.72 (1.80–4.11) | 8/516 | 0.45 (0.16–1.25) | 70/516 | 1.09 (0.69–1.71) |
| | P<sub>trend</sub> | | | | | | | 0.58 | 0.10 |
| VEGF | Total | 145/2052 | 37/2052 | 0.17 | 145/2052 | 37/2052 | 0.09 |
| | 1<sup>st</sup> quartile | 35/510 | 1.00 (referent) | 11/510 | 1.00 (referent) | 70/518 | 1.00 (referent) | 22/518 | 1.00 (referent) |
| | 2<sup>nd</sup> quartile | 47/503 | 1.41 (0.88–2.25) | 7/503 | 0.52 (0.20–1.40) | 40/506 | 0.57 (0.37–0.89) | 10/506 | 0.52 (0.22–1.20) |
| | 3<sup>rd</sup> quartile | 46/524 | 1.28 (0.80–2.06) | 9/524 | 0.66 (0.26–1.67) | 23/512 | 0.29 (0.17–0.51) | 4/512 | 0.20 (0.06–0.66) |
| | 4<sup>th</sup> quartile | 17/515 | 0.64 (0.35–1.18) | 10/515 | 1.00 (0.40–2.53) | 12/516 | 0.13 (0.06–0.28) | 1/516 | 0.05 (0.01–0.43) |
| | P<sub>trend</sub> | | | | | | | 0.69 | 0.0005 |

Abbreviations: AR = androgen receptor; CI = confidence interval; CK5/6 = cytokeratins 5/6; EGFR = epidermal growth factor receptor; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2; Ki67 = proliferation marker; OR = odds ratio; PR = progesterone receptor; SE = standard error; VEGF = vascular endothelial growth factor.

<sup>a</sup>Polytomous multivariable logistic regression model adjusted for age (continuous), body mass index (continuous), age at menarche (<12, 12, 13, or >13 years), parity and age at first birth (nulliparous, 1–4 children with age at first birth <25 years, 1–4 children with age at first birth of 25–29 years, 1–4 children with age at first birth of ≥30 years, 5 or more children with age at first birth of <25 years, 5 or more children with age at first birth of 25–29 years), age at menopause (<46, 46–<50, 50–<55, ≥55, unknown), postmenopausal hormone use (never, ever, unknown), family history (yes or no), confirmed history of benign breast disease (yes or no), alcohol consumption (0, <5, 5 to <15, or ≥15 g per day), and NHS cohort (NHS II vs NHS I). Quartiles are defined as follows: absolute dense area: 1<sup>st</sup>: <19 cm<sup>2</sup>, 2<sup>nd</sup>: 19–<35 cm<sup>2</sup>, 3<sup>rd</sup>: 35–<61 cm<sup>2</sup>, 4<sup>th</sup>: ≥61 cm<sup>2</sup>; non-dense area: 1<sup>st</sup>: <83 cm<sup>2</sup>, 2<sup>nd</sup>: 83–<138 cm<sup>2</sup>, 3<sup>rd</sup>: 138–<208 cm<sup>2</sup>, 4<sup>th</sup>: ≥208 cm<sup>2</sup>.

<sup>b</sup>P-values for the test of heterogeneity of odds ratios were calculated using a two-sided likelihood ratio test.
**DISCUSSION**

In this nested case-control study, we investigated the associations of percent breast density, absolute dense area, and non-dense area with breast cancer risk according to the status of selected tissue markers among 1010 postmenopausal breast cancer cases and 2077 matched controls. The strength of the associations of absolute dense area and non-dense area with breast cancer risk varied by ER, CK5/6, AR, and EGFR status. Stronger positive associations were noted for absolute dense area and CK5/6+ and EGFR+ tumours. Stronger inverse associations of non-dense area with breast cancer risk were observed in ER−, AR+, CK5/6+, and EGFR+ tumours. We did not find differences in the associations of density measures with breast cancer risk according to the status of other markers.

We have previously reported a stronger association of percent breast density with ER− tumours (Yaghjyan et al, 2011, Bertrand et al, 2013). Similar to our previous results, we observed suggestive stronger associations in ER− than ER+ tumours with percent density, though this difference did not reach statistical significance. Findings on the association of breast density with receptor status of the tumour from previous studies are inconsistent (Yaghjyan et al, 2011, Antoni et al, 2013, Pollan et al, 2013), although one of the largest studies to date showed a stronger association of percent density with ER− than ER+ breast cancer among younger women (Bertrand et al, 2013). Our findings suggest that regulatory factors other than oestrogen may play an important role in the origins of ER− tumours in denser breasts. Finally, it appears that this association is being driven in large part by non-dense area rather than absolute dense area.

We found a stronger inverse association between non-dense breast area and ER− tumours compared with ER+ tumours. Similarly, a recent pooled analysis of 4095 breast cancers and 8558 controls have reported a stronger inverse association of non-dense area with breast cancer risk by combined ER/PR/HER2 status or by combined CK5/6/EGFR status because the small numbers of tumours in the subsets did not provide sufficient statistical power to draw meaningful conclusions. Finally, as the marker data for Ki67, AR, CK5/6, EGFR, and VEGF were available on a small subset of cases, these exploratory findings should be interpreted with caution.

Breast cancer is a heterogeneous disease. Previous studies suggested aetiological heterogeneity of ER+ and ER− tumours (Colditz et al, 2004, Rusiecki et al, 2005, Yang et al, 2007, Kwan et al, 2009). Some recent studies suggest that clinical outcomes and patient survival can differ by tumour CK5/6, EGFR, VEGF, and AR status (Collins et al, 2011, Dawood et al, 2011, Hu et al, 2011, Liu et al, 2011). While expression of CK5/6, EGFR, and VEGF involved in cellular proliferation, differentiation, and angiogenesis has been linked to a more aggressive tumour phenotype and poorer survival, AR+ tumours seem to have more favourable outcomes (Collins et al, 2011, Hu et al, 2011), perhaps due to the inhibition of growth of breast cancer cells through the androgen signalling pathway (Birrell et al, 2007). Identifying risk factors specific to certain molecular subtypes of breast tumours would help researchers to understand the aetiology of those breast cancer subtypes and develop subtype-specific risk prediction models.

In conclusion, we investigated the association of breast density with tissue marker-defined breast cancer subtypes in postmenopausal women. Our results suggest that breast density influences the risk of breast cancer subtypes by potentially different mechanisms. Further studies are warranted to explain underlying biological processes and elucidate the possible pathways from high breast density to the specific subtypes of breast carcinomas.

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**CONFLICT OF INTEREST**

GAC has advisory role with Orion Genomics and royalties from UpToDate. The remaining authors declare no conflicts of interest.
AUTHOR CONTRIBUTIONS

GAC, RMT, and LY designed the study. LCC, SJS, and AHB assessed tissue markers. LY, RMT, GAC, and BR analysed the data and LY performed the statistical analyses. LY and RMT wrote the manuscript with final approval from all authors.

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