



Associations of mammographic breast density with breast stem cell marker-defined breast cancer subtypes

Lusine Yaghjian¹ · Ashwini K. Esnakula² · Christopher G. Scott³ · Akemi T. Wijayabahu¹ · Matthew R. Jensen³ · Celine M. Vachon⁴

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Abstract

Purpose High mammographic breast density is a strong, well-established breast cancer risk factor. Whether stem cells may explain high breast cancer risk in dense breasts is unknown. We investigated the association between breast density and breast cancer risk by the status of stem cell markers CD44, CD24, and ALDH1A1 in the tumor.

Methods We included 223 women with primary invasive or in situ breast cancer and 399 age-matched controls from Mayo Clinic Mammography Study. Percent breast density (PD), absolute dense area (DA), and non-dense area (NDA) were assessed using computer-assisted thresholding technique. Immunohistochemical analysis of the markers was performed on tumor tissue microarrays according to a standard protocol. We used polytomous logistic regression to quantify the associations of breast density measures with breast cancer risk across marker-defined tumor subtypes.

Results Of the 223 cancers in the study, 182 were positive for CD44, 83 for CD24 and 52 for ALDH1A1. Associations of PD were not significantly different across marker-defined subtypes (51% + vs. 11–25%: OR 2.83, 95% CI 1.49–5.37 for CD44+ vs. OR 1.87, 95% CI 0.47–7.51 for CD44–, *p*-heterogeneity = 0.66; OR 2.80, 95% CI 1.27–6.18 for CD24+ vs. OR 2.44, 95% CI 1.14–5.22 for CD24–, *p*-heterogeneity = 0.61; OR 3.04, 95% CI 1.14–8.10 for ALDH1A1+ vs. OR 2.57, 95% CI 1.30–5.08 for ALDH1A1–, *p*-heterogeneity = 0.94). Positive associations of DA and inverse associations of NDA with breast cancer risk were similar across marker-defined subtypes.

Conclusions We found no evidence of differential associations of breast density with breast cancer risk by the status of stem cell markers. Further studies in larger study populations are warranted to confirm these associations.

Keywords Mammographic breast density · Breast cancer risk · Breast stem cell markers · CD44 · CD24 · ALDH1A1

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✉ Lusine Yaghjian
lyaghjian@ufl.edu

Ashwini K. Esnakula
esnakula@ufl.edu

Christopher G. Scott
Scott.Christopher@mayo.edu

Akemi T. Wijayabahu
akemiwijayabahu@ufl.edu

Matthew R. Jensen
Jensen.Matthew@mayo.edu

Celine M. Vachon
vachon.celine@mayo.edu

¹ Department of Epidemiology, College of Public Health and Health Professions and College of Medicine, University of Florida, 2004 Mowry Rd, Gainesville, FL 32610, USA

² Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, 1600 SW Archer Road, Gainesville, FL 32610, USA

³ Division of Biomedical Statistics and Informatics, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905, USA

⁴ Division of Epidemiology, Department of Health Sciences Research, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905, USA

Background

Mammographic breast density is a well-established and strong breast cancer risk factor that reflects the amount of adipose, connective, and epithelial tissue in the breast [1]. Although several risk factors were examined in relation to the risk of high breast density [2–6], the mechanisms by which breast density alters breast cancer risk remain poorly understood. Breast tissue undergoes significant structural changes throughout a woman's life [7]. The breast tissue architecture is maintained by a population of stem cells with self-renewal capacity, which are essential for tissue repair and remodeling throughout the woman's life [8]. The mammary stem cells are a rapidly cycling cell population in the normal adult [9]. According to the stem cell hypothesis of breast carcinogenesis, larger mammary gland mass is expected to have a larger pool of mammary cells and be correlated with the number of mammary stem cells [10] and subsequent breast cancer risk [11], which could also be reflected in the degree of mammographic breast density.

In previous studies, well-characterized stem cell markers CD44, CD24, and aldehyde dehydrogenase family 1 member A1 (ALDH1A1) have been linked to younger age at diagnosis, higher odds of unfavorable tumor characteristics, including triple-negative receptor status (estrogen, progesterone, and HER2), poor prognosis, chemotherapy resistance, and distant metastasis [12–17]. Our recent study using a unique source of breast core biopsy tissue from healthy women compared, for the first time, the expression of stem cell markers CD44, CD24, and ALDH1A1 in areas of dense and non-dense tissue within the same woman [18]. For all three markers, we found higher levels of staining extent in dense as compared to non-dense tissue, though for CD24 and ALDH1A1 the difference did not reach statistical significance (CD44: 6.3% vs. 2.0%, $p < 0.001$; CD24: 8.0% vs. 5.6%, $p = 0.10$; and ALDH1A1: 0.5% vs. 0.3%, $p = 0.12$). Thus, our findings suggested an increased presence and/or activity of stem cells in dense as compared to non-dense breast tissue. To further examine the potential interrelationship of stem cell markers with density-associated increase in breast cancer risk, we investigated the association of breast density with the risk of breast cancer subtypes defined by the status of stem cell markers CD44, CD24, and ALDH1A1.

Materials and methods

Study design and population

The participants for this investigation were selected from Mayo Clinic Mammography Study (MCMAM), a

retrospective case–control study nested within the Mayo Clinic mammography screening practice in Rochester, Minnesota [19, 20]. Briefly, breast cancer cases were women 50 years or older diagnosed with primary invasive or in situ cancer between 1997 and 2001 who had at least two prior screening mammograms 2 years before diagnosis and lived within a 120-mile radius of the clinic. Two controls with no prior history of breast cancer from the screening practice were matched to each case on age (within 5 years), final screening exam date, menopausal status at final exam date (pre or post), time between baseline and final mammogram, number of prior screening mammograms, and county of residence [19, 20]. We included in this study invasive cases who had tumor tissue samples available for tissue microarray (TMA) construction (74% of invasive cases). Cases with and without TMAs were similar with respect to the distribution of breast cancer risk factors. The final study sample included 223 cases and 399 matched controls. This study was approved by the Mayo Clinic and University of Florida Institutional Review Boards.

Assessment of mammographic breast density

The earliest pre-diagnostic screening mammogram during the preceding 10-year period, but at least 2 years before breast cancer or corresponding exam date for matched controls, was used for breast density assessment. The average time between mammogram and reference date was 7.0 years (range 2.1–10.4) and was similar for cases and control. Left and right mediolateral oblique (MLO) and cranio-caudal (CC) views of all mammograms were digitized on a Lumiscan 75 scanner with 12-bit grayscale depth and $0.13 \times 0.13 \text{ mm}^2$ pixel size. Percent breast density (percentage of the breast area occupied by fibroglandular elements), absolute dense and non-dense areas (in cm^2) were estimated using computer-assisted thresholding technique [21–23]. As previously described, first the cases and controls were randomly grouped into batches, order of woman was randomly assigned, and then mammograms with different views and sides were randomly ordered within each woman. Each batch contained 5% repeat set of images to assess reliability of the readings. In this reproducibility study, reliability in breast density assessment was very high ($r > 0.93$) [19].

Tissue microarray construction and immunohistochemistry

Two breast tumor TMAs were constructed using 2 or 3 cores per woman as well as control samples from benign breast tissue, placenta, liver, ovary and tonsils (total of 714 tumor cores and 42 controls). Immunohistochemical analysis of the markers TMAs was performed at the

University of Florida Pathology Core Lab according to the previously described standard protocol and using commercially available antibodies (DAKO AutostainerPlus, CD44 [DAKO] 1:25 dilution; CD24 [Abcam] 1:200 dilution and ALDH1A1 [Abcam] 1:300 dilution) [18]. Staining for each of the tissue markers was performed on a separate TMA. Briefly, slides were de-paraffinized with xylene and re-hydrated through decreasing concentrations of ethanol to water, including an intermediary step to quench endogenous peroxidase activity (3% hydrogen peroxide in methanol). Slides were transferred to 1X TBS-T (Tris-buffered saline-Tween). For heat-induced antigen retrieval, sections were heated in a steamer while being submerged in Citra (Biogenex, Fremont, CA) or Trilogy (Cell Marque, Rocklin, CA) for 30 min. Slides were subsequently rinsed in 1XTBS-T and incubated with a universal protein blocker Sniper (Biocare Medical, Walnut Creek, CA), for 15 min and then rinsed in 1XTBS-T and co-incubated in primary antibody ALDH1A1 or CD24 or CD44 for 1 h. Next, slides were rinsed in 1XTBS-T followed by application of conjugated secondary antibody: Mach 2 goat anti-rabbit horse (or mouse) radish peroxidase-conjugated (Biocare Medical, Walnut Creek, CA) for 30 min. Detection of antibodies was achieved by incubating slides in 3'3' diaminobenzidine (Vector Laboratories Inc., Burlingame, CA) for 4 min. Slides were counterstained with hematoxylin (Biocare Medical, Walnut Creek, CA) 1:10 for 3 min and mounted with Cytoseal XYL (Richard-Allen Scientific, Kalamazoo, MI).

The percentage of staining extent for each marker was assessed by a single pathologist using Olympus BX43 microscope. Any cellular staining pattern (e.g., nuclear, cytoplasmic or membranous) was considered positive. Staining extent was quantified as percentage of the area occupied by positively stained cells out of the total tissue area in the slide. Cases with one or more percent of positive staining on any of the cores were considered marker-positive. We also calculated the average marker expression across all available cores for a woman. The composition of the breast tissue was evaluated as proportion of epithelial, stromal, and adipose tissue elements.

Covariates

Weight, height, and use of postmenopausal hormones (PMH) at the time of the index mammogram were abstracted from the Mayo Clinic medical records. Weight and height were used to calculate body mass index (BMI) in kg/m². Information on all other covariates was obtained from a clinical database of self-reported information collected at each visit.

Statistical analysis

Mammographic percent breast density was categorized as 0–10%, 11–25% (reference), 26–50%, and ≥ 51%, consistent with previous analyses [24, 25]. Absolute dense and non-dense areas were defined as quartiles based on the distribution in controls (absolute dense area: 0.0–18.9; 2nd: 19.0–33.9; 3rd: 34.0–54.6; 4th: > 54.7 cm²; non-dense area: 1st: 4.2–75.5; 2nd: 75.6–132.2; 3rd: 132.3–200.4; 4th: ≥ 200.5 cm²). Density measures were also modeled as continuous variables. We used polytomous logistic regression to describe the associations of breast density measures with breast cancer risk by the status of each of the stem cell markers. The risk estimates were adjusted for age (continuous) and body mass index (continuous). We further examined the associations by the number of markers that stained positively for each woman (i.e., none, one, or two–three). Finally, in additional analysis, we modeled each of the density variables a binary using the median in controls as a cut-point (21%, 34 cm², and 132.5 cm² for percent density, absolute dense, and non-dense areas, respectively).

Contrasts were used to construct a test of association of breast density with breast cancer risk by marker status (*p*-heterogeneity) within the polytomous regression framework to investigate whether there was statistical evidence of differences in associations for marker-defined tumor subtypes. For these heterogeneity tests, breast density was modeled using an ordinal trend across quartiles in order to increase power.

Spearman correlation coefficient was used to examine correlations of stem cell marker expression with clinico-histological tumor characteristics (tumor grade, size, nodal involvement, and statuses of estrogen receptor, progesterone receptor, and HER2). Analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA). For all analyses, the level of statistical significance was assessed at 0.05 level. All tests were two-sided.

Results

Characteristics of breast cancer cases and controls in this study are presented in Table 1. Compared to controls, cases had a higher percent density (29.8% vs. 24.0%, *p* < 0.001) and a larger dense area (53.2 cm² vs. 40.5 cm², *p* < 0.001). The mean age at mammogram was similar in cases and controls (61.8 and 61.6 years, respectively, *p* = 0.81). As compared to controls, cases appeared to have slightly greater BMI (27.9 vs. 27.1 kg/cm², *p* = 0.05). Distributions of other risk factors were similar in cases and controls. The average expression of stem cell markers in the tumor samples is presented in Table 2.

Table 1 Characteristics of study participants, by breast cancer status

	Cases (<i>n</i> =223)	Controls (<i>n</i> =399)	<i>p</i> for difference
Mean (SD)			
% Mammographic density	29.8 (18.6)	24.0 (17.7)	<0.001
Dense area (cm ²)	53.2 (35.8)	40.5 (30.6)	<0.001
Non-dense area (cm ²)	141.5 (91.2)	147.4 (91.4)	0.37
Age at mammogram (years)	61.8 (10.6)	61.6 (10.3)	0.81
Age at diagnosis (years)	68.8 (10.5)	–	
Body Mass Index (kg/m ²)	27.9 (5.2)	27.1 (4.9)	0.09
Number (%)			
Body mass index categories (kg/m ²)			
<25	71 (31.8%)	154 (38.6%)	0.05
25–29	80 (35.9%)	137 (34.3%)	
30–34	41 (18.4%)	69 (17.3%)	
35+	21 (9.4%)	30 (7.5%)	
Unknown	10 (4.5%)	9 (2.3%)	
Parity			
Nulliparous	20 (9.0%)	49 (12.3%)	0.34
Parous	203 (91.0%)	349 (87.5%)	
Unknown		1 (0.3%)	
Family history			
No	184 (82.5%)	343 (86%)	0.25
Yes	39 (17.5%)	56 (14%)	
Menopause			
Pre	34 (15.2%)	64 (16.0%)	0.78
Post	188 (84.3%)	332 (83.2%)	
Unknown	1 (0.4%)	3 (0.8%)	
HRT among postmenopausal			
Not current	129 (68.6%)	224 (67.5%)	0.16
Current	41 (21.8%)	89 (26.8%)	
Unknown	18 (9.6%)	19 (5.7%)	

HRT hormone replacement therapy, SD standard deviation

Table 2 Distribution of the stem cell markers expression in study samples

Marker expression measure	N	Mean (STD)	Median	Range	Interquartile range	Number (%) of positive tumors
CD44	223	8.3 (10.1)	5.00	0.0–80.0	1.67–11.67	182 (82%)
CD24	223	8.0 (14.6)	0.00	0.0–73.3	0.00–10.00	83 (37%)
ALDH1A1	223	1.0 (2.7)	0.00	0.0–23.3	0.00–0.00	52 (23%)

Average marker expression across all available cores for a woman

Among 628 cores for CD44, our results show the mean staining extent of 8.5% (range 0–90%), among 632 cores for CD24 the mean staining extent of 8.3% (range 0–90%), and among 635 cores for ALDH1A1 the mean expression of 1.0% (range 0–40%). Comparison of the staining for each marker across all cores for a woman (2 to 3) showed high correlation across the cores (intraclass correlation [ICC]=0.82 [95% CI 0.78–0.86], 0.78 [95% CI 0.72–0.83] and 0.58 [95% CI 0.48–0.67] for CD44, CD24, and

ALDH1A1, respectively). Of the 223 cancers in the study, 182 (82%) were positive for CD44, 83 (37%) for CD24 and 52 (23%) for ALDH1A1. As expected, percent breast density and absolute dense area were positively associated with breast cancer in this subset of the case–control study (OR 2.72, 95% CI 1.47–5.05 for >51% vs. 11–25% and OR 1.78, 95% CI 1.12–2.83 for absolute dense area fourth vs. second quartile) (Table 3). Association of breast density with stem cell marker-defined breast cancer subtypes is presented in

Table 3 Association of breast density with breast cancer risk in the study population (odds ratios and 95% confidence intervals)

Density category or quartile ^a	Percent density (categories)		Absolute dense area (quartiles)		Non-dense area (quartiles)	
	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)
1st	34/108	0.45 (0.27, 0.75)	24/99	0.48 (0.27, 0.85)	65/100	1.78 (1.08, 2.90)
2nd	73/135	1.00 (REF)	47/100	1.00 (REF)	48/101	1.00 (REF)
3rd	84/121	1.65 (1.08, 2.53)	68/101	1.37 (0.86, 2.19)	59/98	1.07 (0.66, 1.74)
4th	32/35	2.72 (1.47, 5.05)	84/99	1.78 (1.12, 2.83)	51/100	0.70 (0.41, 1.22)
Continuous	223/399	1.69 (1.39, 2.05)	223/399	1.44 (1.22, 1.69)	223/399	0.71 (0.56, 0.90)

CI confidence interval, OR odds ratio, REF reference category

Adjusted for age and BMI

^aDefined categories for percent density (0–10%, 11–25%, 26–50%, and ≥51%) and quartiles of controls for absolute dense and non-dense areas

Table 4. Percent breast density was positively associated with all three marker-defined breast cancer subtypes. Associations of percent breast density with CD24 and ALDH1A1-defined subtypes were similar in marker-positive and marker-negative tumors (percent density ≥51% vs. 11–25%: OR 2.80, 95% CI 1.27–6.18 for CD24+ vs. OR 2.44, 95% CI 1.14–5.22 for CD24–, *p*-heterogeneity=0.61; OR 3.04, 95% CI 1.14–8.10 for ALDH1A1 vs. OR 2.57, 95% CI 1.30–5.08 for ALDH1A1–, *p*-heterogeneity=0.94). Even though the difference in the association of percent breast density with breast cancer risk in CD44+ and CD44– tumors was not statistically significant, the risk estimates appeared stronger in CD44+ subtypes (percent density ≥51% vs. 11–25%: OR 2.83, 95% CI 1.49–5.37 vs. OR 1.87, 95% CI 0.47–7.51, respectively, *p*-heterogeneity=0.66).

Absolute dense area was also positively associated with all marker-defined breast cancer subtypes (Table 4). Association of absolute dense area appeared to be stronger in CD44+ as compared to CD44– tumors but the difference did not reach statistical significance (fourth vs. second quartile: OR 1.95, 95% CI 1.18–3.21 for CD44+ vs. OR 1.22, 95% CI 0.51–2.93 for CD44–, *p*-heterogeneity=0.36). The association of the absolute dense area by CD24 and ALDH1A1 status was similar in marker-positive and marker-negative tumors (fourth vs. second quartile: OR 1.75, 95% CI 0.90–3.38 for CD24+ vs. OR 1.82, 95% CI 1.05–3.15 for CD24–, *p*-heterogeneity=0.99; OR 1.86, 95% CI 0.83–4.16 for ALDH1+ vs. OR 1.77, 95% CI 1.06–2.94 for ALDH1A1–, *p*-heterogeneity=0.82).

Absolute non-dense area was inversely associated with breast cancer risk in most of the marker-defined tumor subtypes. We found no significant differences in the associations of non-dense area with breast cancer risk by the status of any of the three markers (*p*-heterogeneity=0.29, 0.69, and 0.49 for CD44, CD24, and ALDH1A1, respectively) (Table 4).

Correlations between stem cell marker expression and selected clinico-histological tumor characteristics, though significant, were negligible in its magnitude (Supplementary Tables 1 and 2).

When considering combined expression of the markers, we did not find any differences in associations in those with one or two–three positive markers as compared to no positivity (Supplementary Table 3). Similarly, we found no differences in associations of density with breast cancer risk across marker-defined subtypes when density measures were modeled as binary (Supplementary Table 4).

Discussion

We present the first study to examine breast density with breast cancer subtypes defined by stem cell markers. This preliminary investigation found no evidence of differential associations of breast density measures with breast cancer risk by the status of stem cell markers.

Breast tissue is very dynamic and undergoes significant remodeling and structural changes during puberty, pregnancy, lactation, and involution [7]. Breast stem cells are essential for the continuous tissue remodeling and maintenance of the breast tissue architecture. The association between stem cells and breast density is supported by findings from previous studies that demonstrate consistent associations of epidemiologic risk factors that influence breast stem cell activity (birthweight, early-life body size, and parity) with breast density [2, 6, 26–41]. In our recent investigation using paired core biopsy samples from healthy women, we found greater expression of stem cell markers in areas of dense as compared to non-dense breast tissue [18]. Previous literature suggests that stem cell markers are expressed predominantly in epithelium and stroma [42] and as expected, the observed differences were attenuated after additional adjustment for proportion of epithelial or stromal elements and further attenuated after adjustment for combined area of for epithelial and stromal tissue. In our study, expression of all three markers was more apparent in tissue sections with a larger proportion of epithelium though the staining for all three markers was detected even in some sections with no epithelium, but large proportion of stromal tissue suggesting

Table 4 Association of breast density with stem cell marker-defined breast cancer subtypes

Density category or quartile ^a	Percent density (categories)			Dense area (quartiles)			Non-dense area (quartiles)		
	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
CD44+									
1st	28	108	0.49 (0.29, 0.85)	22	99	0.58 (0.32, 1.07)	57	100	1.66 (0.99, 2.79)
2nd	57	135	1.00 (REF)	36	100	1.00 (REF)	43	101	1.00 (REF)
3rd	68	121	1.62 (1.03, 2.55)	53	101	1.40 (0.84, 2.33)	43	98	0.89 (0.53, 1.51)
4th	29	35	2.83 (1.49, 5.37)	71	99	1.95 (1.18, 3.21)	39	100	0.64 (0.35, 1.15)
Continuous	182	399	1.70 (1.39, 2.09)	182	399	1.44 (1.22, 1.70)	182	399	0.72 (0.55, 0.93)
CD44–									
1st	6	108	0.31 (0.11, 0.85)	2	99	0.16 (0.03, 0.76)	8	100	2.44 (0.74, 8.06)
2nd	16	135	1.00 (REF)	11	100	1.00 (REF)	5	101	1.00 (REF)
3rd	16	121	1.83 (0.83, 4.03)	15	101	1.29 (0.56, 2.99)	16	98	2.52 (0.87, 7.31)
4th	3	35	1.87 (0.47, 7.51)	13	99	1.22 (0.51, 2.93)	12	100	1.25 (0.38, 4.10)
Continuous	41	399	1.60 (1.10, 2.34)	41	399	1.43 (1.08, 1.88)	41	399	0.67 (0.43, 1.05)
<i>p</i> -heterogeneity			0.66			0.36			0.29
CD24+									
1st	9	108	0.36 (0.16, 0.82)	8	99	0.44 (0.18, 1.06)	31	100	2.15 (1.06, 4.36)
2nd	27	135	1.00 (REF)	18	100	1.00 (REF)	16	101	1.00 (REF)
3rd	30	121	1.33 (0.73, 2.45)	25	101	1.32 (0.67, 2.59)	23	98	1.39 (0.68, 2.84)
4th	17	35	2.80 (1.27, 6.18)	32	99	1.75 (0.90, 3.38)	13	100	0.70 (0.29, 1.69)
Continuous	83	399	1.64 (1.26, 2.13)	83	399	1.37 (1.10, 1.71)	83	399	0.72 (0.50, 1.04)
CD24–									
1st	25	108	0.50 (0.28, 0.89)	16	99	0.51 (0.26, 1.01)	34	100	1.51 (0.84, 2.72)
2nd	46	135	1.00 (REF)	29	100	1.00 (REF)	32	101	1.00 (REF)
3rd	54	121	1.86 (1.13, 3.07)	43	101	1.41 (0.81, 2.45)	36	98	0.93 (0.52, 1.64)
4th	15	35	2.44 (1.14, 5.22)	52	99	1.82 (1.05, 3.15)	38	100	0.68 (0.36, 1.29)
Continuous	140	399	1.71 (1.36, 2.15)	140	399	1.48 (1.23, 1.77)	140	399	0.70 (0.53, 0.92)
<i>p</i> -heterogeneity			0.61			0.99			0.69
ALDH1A1+									
1st	7	108	0.57 (0.22, 1.50)	7	99	0.65 (0.24, 1.76)	23	100	2.61 (1.12, 6.08)
2nd	14	135	1.00 (REF)	11	100	1.00 (REF)	10	101	1.00 (REF)
3rd	21	121	1.76 (0.83, 3.75)	14	101	1.25 (0.54, 2.92)	10	98	0.95 (0.37, 2.43)
4th	10	35	3.04 (1.14, 8.10)	20	99	1.86 (0.83, 4.16)	9	100	0.75 (0.26, 2.15)
Continuous	52	399	1.73 (1.27, 2.37)	52	399	1.40 (1.08, 1.82)	52	399	0.60 (0.38, 0.97)
ALDH1A1–									
1st	27	108	0.42 (0.24, 0.73)	17	99	0.43 (0.23, 0.83)	42	100	1.49 (0.86, 2.59)
2nd	59	135	1.00 (REF)	36	100	1.00 (REF)	38	101	1.00 (REF)
3rd	63	121	1.62 (1.02, 2.57)	54	101	1.41 (0.85, 2.35)	49	98	1.10 (0.65, 1.85)
4th	22	35	2.57 (1.30, 5.08)	64	99	1.77 (1.06, 2.94)	42	100	0.69 (0.38, 1.25)
Continuous	171	399	1.67 (1.35, 2.06)	171	399	1.45 (1.22, 1.72)	171	399	0.73 (0.57, 0.95)
<i>p</i> -heterogeneity			0.94			0.82			0.49

Adjusted for age and body mass index

^aPercent density categories: 0–10%, 11–25%, 26–50%, and ≥51%; absolute dense area quartiles: 1st: 0.0–18.9; 2nd: 19.0–33.9; 3rd: 34.0–54.6; 4th: >54.7 cm²; non-dense area quartiles: 1st: 4.2–75.5; 2nd: 75.6–132.2; 3rd: 132.3–200.4; 4th: ≥200.5 cm²

that expression in the stroma is also important. These findings support the hypothesis that breast density-associated increase in breast cancer could potentially result from higher presence of cells with stem-like properties in dense breast tissue. Findings from our current exploratory study, though

not significant, suggest that the magnitude of the association of breast density with breast cancer risk may be greater for stem cell marker-positive tumors as compared to marker-negative subtypes. The association of percent density with the risk of tumor subtypes also appears to be driven by the

absolute dense area, consistent with predominant expression of these markers in epithelial and stromal tissue. However, given the small sample in this pilot investigation, these results should be interpreted with caution and warrant confirmation in future larger studies.

We examined, for the first time, the association of mammographic breast density with the risk of breast cancer subtypes defined by the status of CD44, CD24, and ALDH1A1 markers. Our case–control study had several strengths, including the close matching on important covariates, mammograms from at least 5 years before breast cancer for >90% of cases, ascertainment of weight, height and data on breast cancer risk factors with medical records for all cases and controls generally at time of mammogram, and the quantitative assessment of three breast density phenotypes [19]. Our study had a few limitations. From the previous nested case–control study, TMAs for staining were available only for 74% of breast cancer cases. However, women with and without TMA were similar with respect to important characteristics, including age, BMI, reproductive variables, hormone use, and a family history of breast cancer. Marker staining was assessed by a single expert pathologist. Validation studies have demonstrated a very high inter-observer agreement in staining assessment by different experts as well as between automated and pathologist readings [43–45]. Consistently, in clinical practice, reading of the staining results for several markers is performed by only one pathologist and not by consensus of multiple pathologists. Further, use of a single reader eliminates inter-rater variability.

Even though this study used the clinic-based design and was lacking ethnical diversity in the study population, because of the restricting eligibility to the 120-mile radius and requiring serial mammograms on all women, the study population was closer to a community-based population rather than a referral or high-risk population [19]. Finally, in this preliminary investigation, we did not have sufficient power to examine associations by menopausal status or to define subtypes using combination of the markers.

Conclusions

We found no evidence of differential associations of breast density measures with breast cancer risk by the status of stem cell markers. However, our power was limited and further studies in larger study populations are warranted to examine these associations in a larger sample.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent This study was compliant with the U.S. Health Insurance Portability and Accountability Act and was approved by the Mayo Clinic Institutional Review Board (IRB) which issued a waiver of informed consent as well as the University of Florida IRBs. The Mayo Clinic patients provided a general authorization for use of medical record information for research purposes.

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