

# Serum calcium levels, TRPM7, TRPC1, microcalcifications, and breast cancer using breast imaging reporting and data system scores

Shravya Mandavilli<sup>1</sup>

Brij B Singh<sup>2</sup>

Abe E Sahmoun<sup>1</sup>

<sup>1</sup>Department of Internal Medicine, University of North Dakota School of Medicine and Health Sciences, Fargo, ND, USA; <sup>2</sup>Department of Biochemistry and Molecular Biology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND, USA

**Background:** An association between higher serum calcium ( $\text{Ca}^{2+}$ ) levels and breast cancer has been previously reported. However, little is known regarding the relationship between serum  $\text{Ca}^{2+}$  levels and the expression of  $\text{Ca}^{2+}$  channels in the presence of breast microcalcifications.

**Methods:** A retrospective analysis of women newly diagnosed with breast microcalcifications was performed based on the Breast Imaging Reporting and Data System (BI-RADS). The expression of TRPC1, TRPC3, and TRPM7 using normal biopsy without microcalcifications (controls) and infiltrating ductal carcinoma with microcalcifications was evaluated.

**Results:** Data on 138 women were analyzed. Seventy percent of women had a BI-RADS score (1–3) corresponding to benign disease. Seventy-six percent of women with a BI-RADS score (4 or 5) were diagnosed with breast cancer, 56% were cancers in situ, and 93% were infiltrating ductal carcinomas. No difference in the distribution of corrected serum  $\text{Ca}^{2+}$  levels between BI-RADS scores (1–3) and BI-RADS scores (4–5) ( $P = 0.82$ ) was observed. Serum  $\text{Ca}^{2+}$  levels were similar in women without cancer and women diagnosed with breast cancer ( $P = 0.94$ ). However, the expression of TRPM7 and TRPC1, but not TRPC3,  $\text{Ca}^{2+}$  channels were increased in infiltrating ductal carcinoma samples with microcalcifications when compared with age-matched controls without calcification or cancer.

**Conclusion:** We observed an increase in the expression of TRPM7 and TRPC1  $\text{Ca}^{2+}$  channels in infiltrating ductal carcinoma samples with microcalcifications, whereas no change in serum  $\text{Ca}^{2+}$  levels was observed. Together these data suggest that increased expression of these channels might lead to an increase in intracellular  $\text{Ca}^{2+}$  levels thereby restoring serum  $\text{Ca}^{2+}$  levels, but these can contribute to the breast microcalcifications. However, future studies exploring the intracellular  $\text{Ca}^{2+}$  levels as well as the role of TRPM7 and TRPC1 function according to BI-RADS scores are needed.

**Keywords:** breast microcalcifications, BI-RADS,  $\text{Ca}^{2+}$ , ductal carcinoma, TRPC1, TRPM7

Correspondence: Brij B Singh  
Room 2711, School of Medicine and Health Sciences, University of North Dakota, 501 N. Columbia Road, Grand Forks, ND 58201, USA  
Tel +1 701 777 0834  
Fax +1 701 777 2382  
Email brij.singh@med.und.edu

Abe E Sahmoun  
Department of Internal Medicine, UND School of Medicine and Health Sciences, 1919 Elm Street N Fargo, ND 58102, USA  
Tel +1 701 293 4131  
Fax +1 701 293 4145  
Email abe.sahmoun@med.und.edu

## Introduction

Mammary microcalcifications occur in 30% to 50% of breast cancer patients,<sup>1</sup> which are frequently associated with a poor survival.<sup>2</sup> The presence of microcalcifications in the breasts has been correlated with an increased risk of synchronous and ensuing breast carcinoma;<sup>3</sup> however, the mechanism is not known. Elevated serum calcium ( $\text{Ca}^{2+}$ ) levels have been shown to increase the risk of breast cancer. For example, Almquist et al<sup>4</sup> found that high serum  $\text{Ca}^{2+}$  increased the risk of breast cancer among overweight and postmenopausal women. A follow-up of this cohort revealed higher serum  $\text{Ca}^{2+}$  levels were associated with cancer aggressiveness.<sup>5</sup> Consistent with these studies, in a large case control study, we found that women newly diagnosed with breast cancer had significantly higher serum  $\text{Ca}^{2+}$  levels than women without cancer.<sup>6</sup>

In addition, our results also show that postmenopausal women with breast tumors (>2 cm) have higher serum  $\text{Ca}^{2+}$  levels than women with smaller tumors,<sup>7</sup> suggesting that  $\text{Ca}^{2+}$  levels play an important role in either the initiation or progression of breast cancer.

In breast cancer cells, intracellular  $\text{Ca}^{2+}$  levels are maintained via two separate classes of  $\text{Ca}^{2+}$  channels that have distinct modes of activation. One of the mechanisms is activated by store depletion *per se*, where release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (ER) initiates  $\text{Ca}^{2+}$  influx, which is termed as store operated  $\text{Ca}^{2+}$  entry (SOCE). Importantly, both TRPC1 and Orai1 have been identified as SOCE channels in different cells; however, their function in breast cancer, especially with microcalcification, is not clear. The other  $\text{Ca}^{2+}$  entry mechanism is more diverse and is mediated by various mechanisms that include receptor activation followed by the generation of second messengers, or they can be activated just by a decrease in serum magnesium levels. Some members of the TRPC channels, especially TRPC3 have been shown to be activated by receptor activation, whereas TRPM7 is activated by changes in serum magnesium levels. In addition, some of these TRP channels have been reported to be correlated with cell proliferation and survival.<sup>8,9</sup> TRPM7 and TRPC1 channels have been shown to be altered in breast cancer cell lines,<sup>10,11</sup> and thus could play a role in breast carcinogenesis; however, its expression in breast cancer tissues with microcalcifications remains unknown.

Several studies have examined microcalcifications from the pathological and radiological perspectives; however, the association between serum  $\text{Ca}^{2+}$  levels and the presence of breast microcalcifications is still unknown. The aim of this study was to investigate whether serum  $\text{Ca}^{2+}$  levels correlate with the Breast Imaging Reporting and Data System (BI-RADS) scores, and to examine the expression of these TRP channels in tissue samples from control or infiltrating ductal carcinoma with microcalcifications.

## Methods

### Study design

We reviewed the medical charts of women with incident breast microcalcifications between February 2004 and August 2011. Patients were identified using International Classification of Diseases-9 code 793.81 for mammographic microcalcifications at Sanford Health, North Dakota, USA. The study was approved by the Institutional Review Boards of the Hospital and the University of North Dakota.

## Data collection

Information on age at diagnosis of microcalcifications, date of microcalcification diagnosis, BI-RADS score ascertained from the pathology report, presence of carcinoma, histology, serum  $\text{Ca}^{2+}$ , albumin, sodium, vitamin D, and the date of serum calcium measurement were obtained from the electronic medical records.

The exclusion criteria consisted of male gender, diagnosis of any cancer, chronic kidney disease, primary hyperparathyroidism, thiazides drug use, date of serum  $\text{Ca}^{2+}$  was more than 12 months before microcalcifications diagnosis, and diagnosis outside the study period.

Eligible cases were 219 women with confirmed breast microcalcifications from the pathology report. Thirty-six women were excluded; 14 women had missing values for serum  $\text{Ca}^{2+}$ , 11 had a missing BI-RADS score, 11 were missing both, and 45 serum calcium measurements were done more than a year from microcalcifications diagnosis, which left 138 eligible patients.

If the serum albumin level was  $<4$  g/dL, the total serum  $\text{Ca}^{2+}$  was adjusted by a standard formula:<sup>15</sup>

$$\text{Corrected serum calcium} = \text{Total calcium} + 0.8 * (4 - \text{albumin}) \quad (1)$$

If the serum albumin was  $\geq 4$  g/dL or if the albumin was not measured, then the total serum  $\text{Ca}^{2+}$  was used.

We used the BI-RADS from the American College of Radiology to categorize the types of breast microcalcifications.<sup>12–14</sup> BI-RADS scores (1, 2, and 3) correspond to benign disease and (4 and 5) to high-risk of breast cancer. Bent et al<sup>3</sup> have shown that the positive predictive value for breast carcinoma based on BI-RADS valuation groups were: category 2 and 3 (0%), category 4A (13%), category 4B (36%), category 4C (79%), and category 5 (100%).

## Immunohistochemistry and imaging

Paraffin embedded tissues from age-matched controls without cancer and infiltrating ductal carcinomas with microcalcifications were obtained, and 10–12  $\mu\text{m}$  thick cryosections were performed. Hematoxylin and eosin staining was performed on the sections using the standard procedure (Sigma-Aldrich, St Louis, MO, USA). For fluorescent confocal imaging, sections from respective samples were permeabilized at room temperature with 0.1% Triton<sup>TM</sup> X-100 (Sigma-Aldrich) in phosphate buffered saline (pH 7.4), blocked (10% donkey serum and 5% bovine serum albumin in phosphate buffered saline), and were probed overnight with respective

primary antibodies in a hydrated chamber maintained at 4°C. Following incubation with primary antibodies, the slides were washed and reacted with fluorophore-conjugated respective secondary antibodies for 1 hour at room temperature. Thereafter, the slides were washed and coverslip mounted using VECTASHIELD® HardSet Mounting Media with DAPI (Vector Laboratories, Inc, Burlingame, CA, USA). Images were acquired at 63× magnifications using a confocal laser-scanning LSM 510 Meta Confocal Microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). Total fluorescence from each section was quantified using the ImageJ program (NIH, Bethesda, MD), and increase in fluorescence range was determined.

## Quantitative real-time polymerase chain reaction

TRPC1 and TRPM7 messenger ribonucleic acid (mRNA) expressions were assessed with real-time polymerase chain reaction (PCR) using commercially available primers (OriGene Technologies, Inc, Rockville, MD, USA). Total ribonucleic acid (RNA) was isolated from 10 µm thick pooled paraffin embedded tissues (two each) from each condition (control and breast cancer with microcalcification) using the QIAGEN kit (Qiagen, USA). Individual complementary deoxyribonucleic acid (cDNA) was transcribed from 0.5 µg of total RNA with iScript cDNA (Bio-Rad Laboratories, Hercules, CA, USA). An equal amount of cDNA template was added to iQ SYBR green super mix together with the appropriate primers at 0.2 µM each. Quantitative PCR was performed using an iCycler iQ™ (Bio-Rad Laboratories) real-time detection system following the specifications of the manufacturer. The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. Glyceraldehyde 3-phosphate dehydrogenase was used for normalization of the transcripts. Specificity of PCR product formation was confirmed by monitoring melting peaks.

## Statistical analysis

Crude mean values were computed for all continuous variables and frequency distributions were calculated for all categorical variables. We compared women who were diagnosed with breast microcalcifications based on their BI-RADS scores (1, 2, and 3) versus (4 and 5) on demographic and other variables using the Wilcoxon signed-rank test for continuous variables and the chi-square test for categorical variables. Logistic regression was used to assess whether serum Ca<sup>2+</sup>, as a continuous variable, predicts higher BI-RADS score. All

statistical tests were two-tailed with  $P < 0.05$  considered to be significant. Statistics were performed using SAS (version 9.3 Users Guide; SAS Institute Inc, Cary, NC, USA).

## Results

Participants were 138 women with newly-diagnosed breast microcalcifications (Table 1). The majority (70%) had a BI-RADS score of 1, 2, or 3, whereas the rest had a BI-RADS score of 4 or 5. Importantly, 76% of women with high BI-RADS scores (4 or 5) were diagnosed with breast cancer, suggesting that higher BI-RADS scores correlated with breast cancer. Additionally, 56% of these breast cancers were cancers in situ, and 93% of the cancers were infiltrating ductal carcinomas. We did not observe a significant difference in the distribution of corrected serum Ca<sup>2+</sup> between BI-RADS scores 1, 2, or 3, and BI-RADS scores 4 or 5 ( $9.54 \pm 0.4$  versus  $9.56 \pm 0.5$ ;  $P$ -value = 0.82), or between women who were diagnosed with breast cancer versus women who were cancer free ( $P$ -value = 0.94). Comparing women with BI-RADS scores of 4 or 5 to women with BI-RADS scores of 1, 2, or 3, the age-adjusted odds ratio for corrected serum Ca<sup>2+</sup> was 1.05 (95% confidence interval: 0.46–2.41) (Table 1). Furthermore, the level of serum 25-hydroxyvitamin D, which is important for the absorption of Ca<sup>2+</sup>, was increased in higher BI-RADS scores (4, 5) compared to women with BI-RADS scores of 1, 2, or 3, suggesting that perhaps an increase in intracellular Ca<sup>2+</sup> is the reason that no alteration in serum Ca<sup>2+</sup> is observed.

Since Ca<sup>2+</sup> entry is dependent on various Ca<sup>2+</sup> channels, we studied the expression/localization of various Ca<sup>2+</sup> channels that have distinct modes of activation. In tissue samples with no cancer, localization of the endogenous TRPC1 protein displayed a uniform plasma membrane along with some subplasma membrane staining of both acinar and ductal cells (Figure 1). Conversely, a significant increase (~30%) in TRPC1 expression was observed in the infiltrating ductal carcinoma tissue samples that had microcalcification, whereas no increase in actin (used as control) staining was observed. Tissues without the TRPC1 antibody showed no staining (Figure 1). To further establish the expression of receptor operated Ca<sup>2+</sup> channels, expression of TRPC3 was observed. As indicated in Figure 1, no change in expression levels of TRPC3 was observed in control or infiltrating ductal carcinoma with microcalcification. TRPM7, which has been shown to be activated by decrease in serum magnesium levels and is increased in breast cancer, was also evaluated. As indicated in Figure 1, increased staining of TRPM7 was also observed in infiltrating ductal carcinoma

**Table 1** Descriptive characteristics of newly diagnosed breast microcalcifications by BI-RADS scores

Variables	BI-RADS 1–3		BI-RADS 4–5		P-value
	n	%	n	%	
Total (n = 138)	96	70	42	30	
Age at diagnosis					0.15
51–64 (n = 41)	33	34	8	19	
65–79 (n = 56)	37	39	19	45	
80–93 (n = 41)	26	27	15	36	
Median (range)	71 (51–93)		75 (53–91)		
Diagnosis of breast cancer					0.0001
Yes	–	–	32	76	
No	96	100	10	24	
Types of malignancy (n = 32)					
DCIS	–	–	16	50	
DCIS and IDC	–	–	3	9	
DCIS mixed	–	–	1	3	
IDC	–	–	9	28	
IDC mixed	–	–	1	3	
ILC	–	–	1	3	
LCIS	–	–	1	3	
Serum albumin, g/dL median (range)	4.2 (2.8–4.9)		4.2 (3.3–4.8)		0.97
Corrected serum calcium mg/dL	9.5 (8.2–10.6)		9.6 (8.6–10.8)		0.82
Sodium, mmol/L	141 (129–145)		141 (130–149)		0.45
Serum 25-hydroxyvitamin D, ng/mL	37 (18–57)		42 (33–61)		0.13
Age adjusted odds ratio for serum Ca <sup>2+</sup> (95% confidence interval)	Reference		1.05 (0.46–2.41)		0.90

**Note:** Albumin calculation is based on 76 and 39 observations, and 25-hydroxyvitamin D calculation is based on 15 and six observations, respectively.

**Abbreviations:** BI-RADS, Breast Imaging Reporting and Data System; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma in situ.

patients that exhibited microcalcification (~40% increase) when compared with control samples, further suggesting that these channels can play a role in breast cancer associated with microcalcifications. To validate these results, RNA was isolated from pooled samples (two each) from control and breast cancer and cDNA was obtained. Finally real-time PCR was performed to confirm the expression of TRPC1, which showed a twofold increase in expression in breast cancer cells. Similarly, a fourfold increase in TRPM7 expression was observed in breast cancer samples when compared with controls.

## Discussion

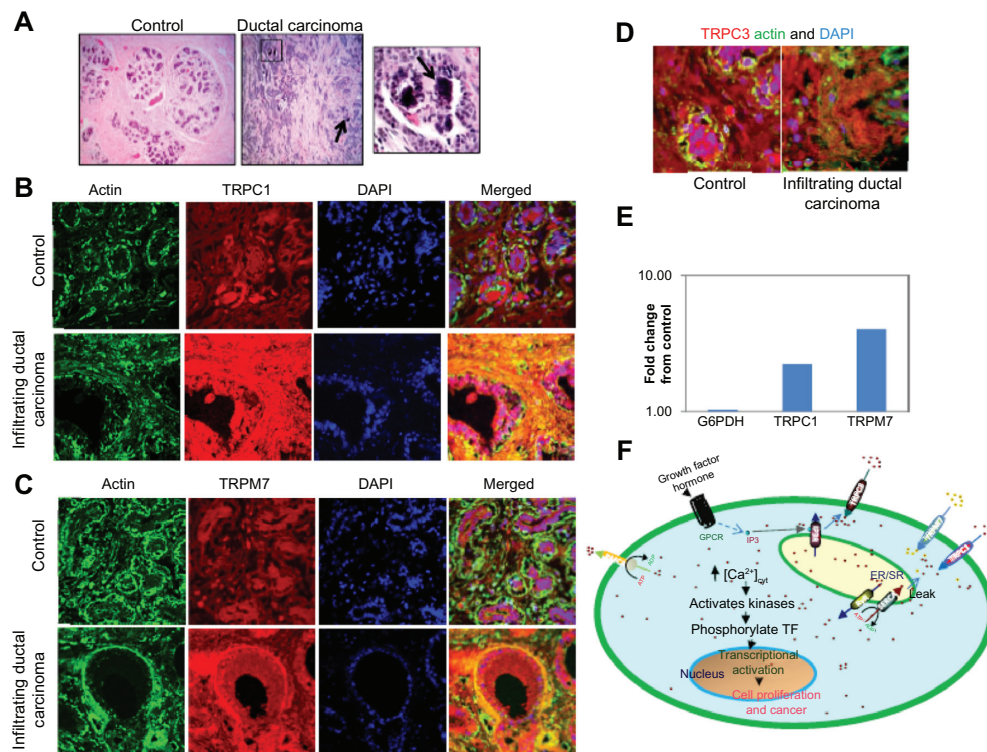
In this study of women diagnosed with incident breast microcalcifications that were confirmed in the pathology report, we did not find an association between serum Ca<sup>2+</sup> levels and BI-RADS scores, or breast cancer with microcalcifications. Our data also showed that the overall incidence of breast cancer was 23.2%, which is in accordance with the positive predictive value for breast cancer reported by other investigators (21%–42%).<sup>3,16–19</sup> Of the malignancies found, 62% involved infiltrating ductal carcinoma, whereas the remaining 34% represented invasive cancer. The proportion of these

histopathologic findings is similar to those of previously published reports.<sup>15,17,18</sup>

To understand why no alterations in serum Ca<sup>2+</sup> levels were observed, we focused our attention on various Ca<sup>2+</sup> channels. Importantly, Cheung et al<sup>20</sup> reported that in cell cultures, the deposition of calcification crystals on the cell membrane leads to an acute Ca<sup>2+</sup> response by increasing intracellular Ca<sup>2+</sup> concentration, suggesting that microcalcification stimulates Ca<sup>2+</sup> channels on the membrane. Additionally, the latter on a sustained elevation in intracellular Ca<sup>2+</sup> levels was also observed, which could be due to crystal ingestion and perhaps its dissolution. Last but not least, experiments where cells are treated with crystals that are too large to endocytose only revealed a transient rise in intracellular Ca<sup>2+</sup>,<sup>20</sup> indicating that crystal deposition per se causes an influx of extracellular Ca<sup>2+</sup>. This is consistent with our findings, which showed that expression of TRPM7 and TRPC1, but not TRPC3 channels were increased in infiltrating ductal carcinoma samples when compared with age-matched controls without cancer.

In response to many extracellular signals, mammalian cells can trigger an elevation of intracellular Ca<sup>2+</sup> by increasing the entry of Ca<sup>2+</sup> from the extracellular through the Ca<sup>2+</sup> channels, thereby decreasing serum Ca<sup>2+</sup> levels. Intracellular Ca<sup>2+</sup>





**Figure 1** TRPC1 and TRPM7 expression is increased in infiltrating ductal carcinomas. Hematoxylin and eosin staining of control and ductal carcinoma samples. Respective images were taken at 10 $\times$  zoom; microcalcification has been shown with an arrow, and also a magnified image showing microcalcification is provided on the right (**A**). Confocal images showing endogenous TRPC1 (**B**), TRPM7 (**C**), TRPC3 (**D**), and actin staining in age-matched controls (without cancer or microcalcifications) and infiltrating ductal carcinoma samples with microcalcifications were observed. Anti-TRPC1, Anti-TRPC3, or Anti-TRPM7 antibodies followed by rhodamine-conjugated secondary antibodies were used to detect endogenous TRPC1, TRPC3, and TRPM7 protein. Antiactin antibodies followed by FITC-labeled secondary antibodies were used to detect actin levels. The images provided are from at least two to three individual sections, and total fluorescence was evaluated using the Zeiss software (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). Fold change in the expression of G6PDH, TRPC1, and TRPM7 in controls, as well as breast cancer samples were observed (**E**). A model for the activation of various channels and their role in increasing cytosolic calcium that leads to cancer is shown (**F**).

**Abbreviations:** DAPI, 4',6-diamidino-2-phenylindole; GPCR, G-protein coupled receptors; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; TF, tissue factor; G6PDH, glucose-6-phosphate dehydrogenase; FITC, fluorescein isothiocyanate.

could also be released simultaneously from intracellular stores as a consequence of 1,4,5-inositol triphosphate generation.<sup>21,22</sup> Binding of 1,4,5-inositol triphosphate to its receptor in the ER triggers a release of  $\text{Ca}^{2+}$  from the internal ER stores that further activates plasma membrane  $\text{Ca}^{2+}$  channels, a phenomenon known as receptor operated  $\text{Ca}^{2+}$  entry (Figure 1F).<sup>23</sup> Although the ion channels that regulate the receptor operated calcium channels in breast cancer cells are unknown, previous studies have shown that TRPC3 can be activated by this mechanism. In addition, store depletion per se has been shown to activate SOCE, and both Orai1 and TRPC1 channels can function as SOCE channels in different cells.<sup>24</sup> Thus, an increase in the expression of these channels could facilitate an increase in intracellular  $\text{Ca}^{2+}$  that can lead to breast cancer. Since the expression of TRPC1 was increased in these cells, we hypothesize that TRPC1 could significantly contribute to increased intracellular  $\text{Ca}^{2+}$  to induce cell proliferation. TRPM7 channel has also been linked to cell proliferation and survival of breast cancer cells, which is consistent with our results, where

increased expression of TRPM7 was observed in the ductal carcinoma samples with microcalcifications.

Holme et al<sup>25</sup> hypothesized that an abnormality in  $\text{Ca}^{2+}$  pumps or  $\text{Ca}^{2+}$  regulatory proteins might result in the deposition of microcalcifications. Consistent with these results, calcium-sensing receptors (CaR) have also been suggested to be involved in calcifications in the vascular smooth muscle cells.<sup>26</sup> Importantly, CaR has been shown to play a key role in the regulation of extracellular  $\text{Ca}^{2+}$  homeostasis in various tissues including breast tissues.<sup>27</sup> Although the role of CaR in microcalcifications of breast tissues has not yet been established, it can be hypothesized that it may have an impact on microcalcification genesis. Alteration in this  $\text{Ca}^{2+}$  homeostasis, due to aberrant activation of CaR, can lead to microcalcification, and more research is needed to establish this link. However, an increase in microcalcification can be ultimately responsible for the intake of  $\text{Ca}^{2+}$  via the TRPC1 channels that will lead to an increase in cell proliferation.

We acknowledge some limitations; this study was restricted to women with recent data on  $\text{Ca}^{2+}$ , which raises the possibility of selection bias. However, extending the period for  $\text{Ca}^{2+}$  measurement from the microcalcifications diagnosis did not result in an association between serum  $\text{Ca}^{2+}$  and BI-RADS scores. It is possible that our results could be influenced by differences in vitamin D levels. Although serum  $\text{Ca}^{2+}$  levels might increase with serum levels of vitamin D, this correlation is most evident in the setting of vitamin D insufficiency and excess.<sup>28</sup> Conversely, this is the first study to assess serum  $\text{Ca}^{2+}$  levels in the presence of breast microcalcifications and to quantify two distinct  $\text{Ca}^{2+}$  channels in ductal carcinoma samples in the presence of microcalcifications. Finally, although TRPC1 and TRPM7 channels were overexpressed in this study, their activity is still unknown. Future studies should explore the function of these channels using wet tissue samples and measure intracellular  $\text{Ca}^{2+}$  levels according to BI-RADS scores. This information will be critical in understanding the etiology of ductal carcinoma and in exploring potential therapeutic interventions to treat breast cancer.

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## Disclosure

The authors report no conflicts of interest in this work.

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