

# Microcalcifications in breast cancer: from pathophysiology to diagnosis and prognosis

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## Keywords

Breast cancer; Microcalcifications; Mammography; Mineralisation; Tumour microenvironment

## Abstract

The implementation of mammographic screening programmes in many countries has been linked to a marked increase in early detection and improved prognosis for breast cancer patients. Breast tumours can be detected by assessing several features in mammographic images but one of the most common are the presence of small deposits of calcium known as microcalcifications, which in many cases may be the only detectable sign of a breast tumour. In addition to their efficacy in the detection of breast cancer, the presence of microcalcifications within a breast tumour may also convey useful prognostic information. Breast tumours with associated calcifications display an increased rate of HER2 overexpression as well as decreased survival, increased risk of recurrence, high tumour grade and increased likelihood of spread to the lymph nodes. Clearly, the presence of microcalcifications in a tumour is a clinically significant finding, suggesting that a detailed understanding of their formation may improve our knowledge of the early stages of breast tumorigenesis, yet there are no reports which attempt to bring together recent basic science research findings and current knowledge of the clinical significance of microcalcifications. This review will summarise the most current understanding of the formation of calcifications within breast tissue and explore their associated clinical features and prognostic value.

## Abbreviations

ALP, alkaline phosphatase; BI-RADS, Breast Imaging Reporting and Data System; BMP2, bone morphogenetic protein 2; BSP, bone sialoprotein; CA1, carbonic anhydrase I; COX2, cyclooxygenase-2; DCIS, ductal carcinoma *in situ*; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HA, hydroxyapatite; IL-1 $\beta$ , interleukin 1 beta; MGP, matrix gla protein; MMP, matrix metalloproteinase; NLRP3, NLR family pyrin domain containing 3; OSN, osteonectin; OPN, osteopontin; Pi, phosphate; Pit-1, sodium-dependent phosphate transporter 1; PPI, pyrophosphate; PFS, progression-free survival; PGE2, prostaglandin E2; RUNX2, runt-related transcription factor 2; SPCA, secretory pathway Ca<sup>2+</sup>-ATPase; TGF- $\beta$ , transforming growth factor beta; TRPC1,

transient receptor potential channel 1; TRPM7, transient receptor potential cation channel, subfamily M, member 7; VSMC, vascular smooth muscle cells

## 1. Introduction

Breast cancer survival rates have increased significantly in recent years (1), due to a combination of improved treatment options and increased detection of early-stage tumours. As with other forms of cancer, patients whose breast tumours are detected at an early stage will typically respond much better to treatment: 5-year survival rates for stage-I breast cancer are close to 100%, compared to approximately 20% for patients with a stage-IV diagnosis (2).

To aid in this crucial endeavour of early detection, many countries now offer X-ray based mammography screening programmes to women in high-risk age brackets, typically beginning between 40–50 years and continuing until 65-75 years, varying from country to country (3). Many studies have demonstrated a significant improvement in breast cancer survival following introduction of mammography screening. A meta-analysis of 11 large-scale studies (all with a cohort size of at least 50,000) by the Independent UK Panel on Breast Cancer Screening demonstrated a reduction in relative risk of breast cancer mortality of 20% in patients who had undergone screening versus those who had not (4). Other studies have reached similar conclusions (5-11) .

However, the adoption of mammography screening is not without controversy. Many of the lesions detected through mammography are small, benign growths unlikely to progress to malignant breast cancer and pose little threat. A meta-analysis by the Cochrane collaboration also found a 20% decrease in mortality but reached very different conclusions. Pointing out issues with inadequate randomisation and bias associated with reporting cause of death, they concluded that several studies should be excluded from consideration. When these studies, deemed inadequate, were removed from the analysis, the benefit of screening declined from an initial relative risk value of 0.81 to 1.02 (12). These findings have been challenged by several groups (13, 14), although other studies have been more supportive. For instance, a recent study argued that in many countries, breast cancer mortality had already started to decline before the implementation of screening, likely due to improved treatment regimens (15). The clinical efficacy of screening mammography is also hampered by a low positive-predictive value (16), leading to a significant drive in efforts to further improve the diagnostic power of breast imaging techniques (17). It is worth noting, that although some disagreement may exist over the efficacy of mammography in population screening, its effectiveness in a diagnostic capacity, where it is usually combined with a clinical exam and biopsy analysis is widely accepted, with sensitivity and specificity of this “triple-assessment” approaching 100% (18-20).

Despite the controversy, mammographic imaging remains a vital tool in early detection of breast cancer in many countries, with most utilizing the Breast Imaging Reporting

and Data System (BI-RADS), a standardized system for classifying and reporting clinical findings from mammography. Mammograms are scored on a number of features including density, architectural distortions and calcifications, and placed into one of 7 BI-RADS categories (Table 1), ranging from “Incomplete Assessment” (Category 0) up to “Known Biopsy-Proven Malignancy” (Category 6), each with a recommended course of action (21, 22). One of the most commonly detected mammographic abnormalities are microcalcifications. First identified in 1951 (23), they have long been a highly useful marker of breast cancer, with between 30 and 50% of non-palpable tumours found in screening identified solely due to the presence of microcalcifications (16, 24). They are also present in the majority of ductal carcinoma *in situ* (DCIS) cases (25). This review will summarise the most current understanding of the formation of calcifications within breast tissue and explore their associated clinical features and prognostic value.

**Table 1.** Breast imaging and reporting data system (BI-RADS) categories for mammography and its association with microcalcification appearance.

Category	Description	Management	Likelihood of malignancy
0	Incomplete	Additional imaging required	Not applicable
1	Negative	Continue routine screening	0%
2	Benign	Continue routine screening	0%
3	Probably benign	Short interval follow up	0 – 2%
4			
A	Low suspicion	May require biopsy	2 – 10%
B	Intermediate suspicion		10 – 50%
C	High suspicion		50 – 95%
5	Highly suggestive of malignancy	Biopsy required	>95%
6	Biopsy proven malignancy	Begin treatment	100%
			<b>BI-RADS category</b>
<b>Calcifications</b>	Typically benign	Skin Vascular Dystrophic Eggshell Large rod-like Popcorn-like	2 or 3
	Suspicious	Amorphous Coarse heterogeneous Fine pleomorphic Fine linear (casting) Fine linear (casting),segmental distribution	4B 4B 4B 4C 5

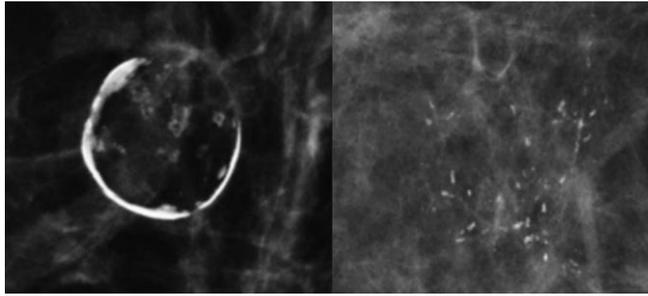
## 2. Mammographic characterisation of microcalcifications

Calcifications, when detected by mammography, can be characterised based on a number of attributes, including morphology, size and distribution. Based on these features, radiographers will then assign calcifications to a BI-RADS category indicative of their likelihood of malignancy. Calcification morphologies typically considered low-risk include “popcorn-like”, eggshell or dystrophic, whilst calcifications of a coarse heterogeneous or fine linear morphology convey a significantly increased risk of

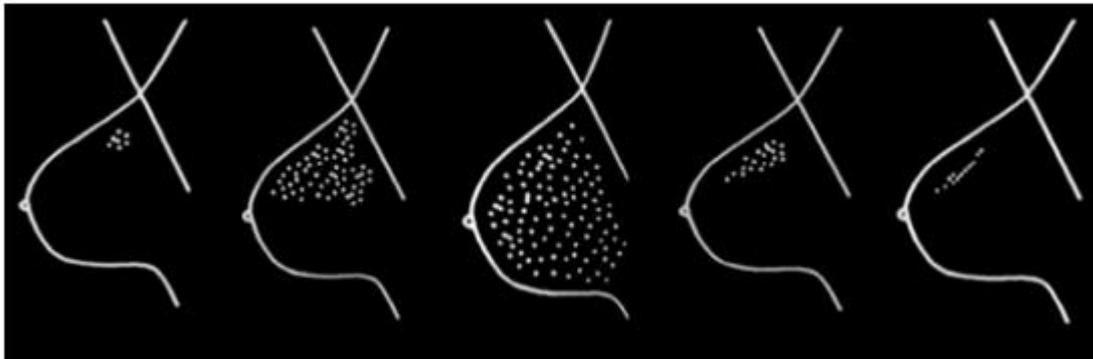
malignancy (Fig. 1A). A clear understanding of the relationship between different calcification morphologies and their formation can help inform clinical opinion as to which are likely to indicate the presence of cancerous tissue. For example, a benign eggshell calcification, consisting of a thin, spherical structure, usually forms as the result of calcium deposition on the surface of an oil cyst, and is totally unrelated to breast cancer. In contrast, the long, thin branching structure of fine linear (often referred to as casting) calcifications indicates spread of calcium deposition along the lumen of a breast duct, and is highly suggestive of malignancy (26). A meta-analysis of 40 studies representing a total of 10,665 patients with mammographically detected calcifications demonstrated pooled malignancy rates of 13% for coarse heterogeneous, 27% for amorphous or indistinct, 50% for pleomorphic, and 78% for fine-linear (27). Other studies have found similar results, with fine linear calcifications regarded as extremely suspicious (28, 29).

In addition to morphology, the spatial distribution of microcalcifications (Fig. 1B) within the breast tissue can also inform clinical opinion (29, 30). Calcifications grouped in a linear (representing spread of calcified deposits along a duct) or segmental pattern (calcium deposition within a duct and associated branches, following the shape of a breast lobe) are considered high-risk, and are significantly more likely to represent a malignancy than calcifications in a clustered distribution (5 calcifications within an area of 1 cm<sup>2</sup>). In turn, clustered calcifications (an intermediate category) are considered more suspicious than calcifications in a diffuse (random distribution within the breast) or regional pattern (calcifications spread in a larger volume >2 cm<sup>2</sup>). Some calcifications (e.g. vascular or thick linear) can form a linear pattern, yet still be considered likely to be benign based on their underlying morphology (31). This highlights the necessity to consider both morphology and distribution of calcifications when assessing likelihood of malignancy.

(A)



(B)



**Figure 1. Morphology and distribution patterns of breast microcalcifications.** Representative mammogram images (A) of a benign eggshell calcification (left) and suspicious fine-linear/casting calcifications (right). Adapted and reproduced from (26). Commonly observed distribution patterns (B) of mammographically detected breast calcifications, in order of increasing likelihood of malignancy. Order is as follows: diffuse, regional, clustered, segmental, and linear. Adapted and reproduced from (31).

### 3. Chemical composition of microcalcifications

In addition to their morphology and distribution pattern, some studies have also grouped microcalcifications into two simplified categories based on their chemical composition and association with benign or malignant lesions. Type I calcifications, composed of calcium oxalate [ $\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ ], are generally found solely in benign tumours whilst type II calcifications, consisting of calcium phosphate in the form of hydroxyapatite [ $(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2)$ ], may be found in either benign or malignant tumours (32, 33). In an analysis of microcalcifications taken from 25 patients, Frappart et al. found type II calcifications in all cases of infiltrating carcinomas and intraductal adenocarcinomas and in 50% of benign tumours whilst type I calcifications were only present in benign samples (34). Similarly, Winston et al. examined biopsy samples from 55 women and found that in the 8 patients presenting with exclusively calcium oxalate calcifications, all had benign growths. In contrast, 40% of patients with calcifications of hydroxyapatite (HA) had carcinoma (35). Although many of these studies were relatively small-scale, more recent studies with larger sample sets and more advanced analysis techniques have reached similar conclusions (33, 36).

Due to the inability of current standard clinical imaging techniques to reliably differentiate type I from type II calcifications, the chemical nature of breast calcifications is not routinely determined. However, such a detection capability could

potentially reduce the number of patients requiring invasive biopsy procedures, making translation of these findings to the clinic an active area of current research (37-39).

Although mammography cannot distinguish type I and type II calcifications, Raman spectroscopy, which measures energy shifts in inelastically scattered light to generate a molecule-specific spectrum, has been used to analyse biopsy samples, successfully distinguishing type I and type II calcifications (33, 40, 41). Although loss of light by scattering typically limits Raman spectroscopy to a relatively shallow depth, recent developments have allowed for analysis at a depth of up to 40 mm of tissue (39). This raises the possibility of utilising Raman analysis as a non-invasive, clinical imaging technique to identify calcifications likely to be representative of a malignancy. A recent study even found that Raman analysis of whole blood samples could detect Raman shifts characteristic of calcium oxalate and hydroxyapatite, which were elevated in blood from breast cancer patients (42).

In recent years, the application of sophisticated imaging and analysis techniques has advanced our understanding of the chemical differences between benign and malignant calcifications significantly beyond a simple type I and type II categorisation. Using Raman spectroscopy to examine the degree of carbonate substitution in the hydroxyapatite crystal structure, Haka et al. found a significant difference between calcification samples taken from benign and malignant lesions (33). This was followed by a study by Baker et al. using Fourier transform mid-infrared (FTIR) spectroscopy, which demonstrated that the carbonate content of calcifications is inversely correlated to malignancy, with calcifications representative of invasive, *in situ* and benign disease possessing a mean carbonate content of 1.41, 1.83 and 2.08% respectively (43). Carbonate content was also found to be informative of tumour grade, with progressively decreasing levels in grade 1-3 invasive tumours and in high versus low-grade DCIS. When the ratio of protein matrix to mineral was analysed, the opposite pattern was observed, with increased protein content going from benign to *in situ* to invasive. Combining both these metrics yielded a linear discriminant model capable of identifying malignancy lesions with a sensitivity and specificity of 90% and 96%, respectively.

More recently, a study using energy dispersive X-ray microanalysis identified significant amounts of magnesium within the crystal lattice of malignancy associated microcalcifications (36). A subsequent study using X-ray diffraction suggested that this crystal phase was likely to consist of a mix of whitlockite and magnesium substituted hydroxyapatite (44). The percentage of whitlockite in samples increased significantly from benign to *in situ* lesions to invasive tumours. The biological relevance of the magnesium content of microcalcifications remains unclear but its apparent association with malignant lesions may prove highly valuable alongside carbonate percentage as a guide to distinguishing benign from malignant growths.

#### **4. Microcalcifications and patient prognosis**

In addition to their utility in the detection of breast cancer, the presence of calcifications on a mammogram may also be indicative of patient prognosis. In a study of 343 patients with small, screen-detected breast tumours, Tabar et al. observed far higher mortality rates in patients with casting-type calcifications than would typically be expected for such small tumours (20-year survival of 55%), suggesting that these small, calcification associated tumours behaved as if they were much larger (45). This was followed by a second study by Tabar et al. with a larger cohort of women diagnosed with small, invasive tumours which found significant variations in outcome, including a 9-fold increased risk of mortality in patients with casting-type calcifications versus those with stellate tumours with no associated calcifications (46). The findings of Tabar are supported by a study of 96 cases of invasive breast cancer by Thurfjell et al, which found a clear link between the presence of casting calcifications and decreased survival (47). Similarly, Peacock et al. compared 50 women diagnosed with small invasive tumours and associated casting-type calcifications with tumour size and lymph node matched controls. There were 5 deaths in the calcification group but none in the control, a difference that was found to be statistically significant (48).

In a study of 498 patients diagnosed with invasive breast cancer, Tsau et al. found that the presence of casting calcifications was associated with a 3.47-fold increased hazard ratio for mortality compared to other mammographic findings (49). Similarly, Ling et al. found that women presenting with calcifications had a 2-fold increase in risk of relapse and a 2.4-fold increased risk of dying from breast cancer compared with women without calcifications (50). Zhang et al. also found calcifications to be associated with poor progression-free survival (PFS), although not overall survival (51). The most recent study identified for this review found patients with mammographic calcifications to have risk ratios of 2.46 for local recurrence, 2.24 for metastasis and 2.5 for mortality following breast-conserving therapy (52).

It is worth noting that not all studies are in agreement over the prognostic value of calcifications. In a study of 515 women with 1–15 mm tumours, Månsson et al. observed a trend towards decreased survival in patients with casting calcifications (odds ratio of 1.63 relative to stellate tumours) although this was not found to be significant (53). However, another form of malignancy-associated calcification (fine-pleomorphic or “crushed-stone”) did display a significant association with prognosis. James et al. found the presence of casting-type calcifications to be associated with small, high-grade tumours but not with survival (54). Evans et al. also did not find any association between patient survival and calcifications (55).

Many studies have also highlighted links between microcalcifications and increased risk of recurrence, with most studies finding between a 2- to 5- fold increase in risk. In a study of 409 patients treated with breast conserving surgery, Qi et al. found a 2.46-fold increased rate of local recurrence in patients with mammographically detected calcifications (52). Patients with calcifications in a linear or segmental distribution were particularly likely to suffer a relapse. Rauch et al. found a 5.2-fold increase in local

recurrence in patients presenting with fine linear microcalcifications (56). Holmberg et al. also found a significant increase in risk of recurrence in patients with casting calcifications compared to those without, although interestingly when cases of invasive carcinoma and DCIS were analysed separately, this increase was found to be mainly confined to the DCIS sub-cohort. (57). Increased risk of recurrence is supported by recent studies using the Oncotype DX assay, a diagnostic assay which uses a 21-gene expression signature to estimate likelihood of recurrence. Patients presenting with calcifications were shown to be significantly more likely to be assigned to the “high-risk” category than those without (58-60). In contrast, in a study of 937 cases of invasive carcinoma, Naseem et al. did not find any association between the presence of calcifications and recurrence (61).

Calcifications have also shown a significant association with high tumour grade. Rauch et al. found tumour grade to be significantly associated with calcification morphology, with the highest risk conveyed by fine-linear calcifications (3.4-fold increase relative to calcifications of a punctate/amorphous morphology (56). Naseem et al. observed a borderline-significant ( $P=0.057$ ) trend toward increased tumour grade in calcification associated tumours, with the rate of calcification increasing from 30.7% for grade I to 39.8% for grade II and 41.3% for grade III (61). A recent study involving over 8,000 cases of invasive breast cancers found calcifications to be associated with 1.43-fold increased risk of high tumour grade (62). Similar results were found in an analysis of mammography data from over 7,000 patients in China (63).

Calcifications may also be associated with tumour invasion into the lymphatic system, although not all studies are in agreement. Tabar et al. found the presence of casting calcifications to confer a 3.29-fold increased risk of positive lymph node status, relative to stellate lesions with no calcifications (46). Several other studies have also found an increased rate of lymph node involvement in patients with calcifications (50, 63), although others have failed to find an association (61, 64). Similarly inconsistent results were found with tumour size, with studies finding calcifications to be associated with either increased (50, 65) or decreased tumour size (62), or not associated at all (52, 61, 66). Calcifications may also predict response to neoadjuvant therapy although the evidence thus far is relatively weak (67-69).

Perhaps the most consistent finding in these studies is a strong association between the presence of calcifications and HER2 overexpression. A recent microarray analysis found the *ERBB2* gene to be amongst the most differentially expressed between patients with highly suspicious calcifications versus those without (64). In addition, a meta-analysis encompassing 17,745 breast cancers found that the presence of calcifications carried a pooled odds ratio of HER2 overexpression of 3.14, regardless of whether the detected calcification was found with or without an associated mass (70). Although studies show a strong link between HER2 and microcalcifications, the exact nature of this relationship and any possible link between HER2 expression and the formation of microcalcifications remains unexplored.

Despite some disagreement within the literature, the majority of evidence thus far does seem to indicate an important role for microcalcifications, and in particular, those of a casting-type morphology, in predicting prognosis, risk of malignancy, likelihood of recurrence and numerous other clinically important attributes (Table 2). Many of these discrepancies could likely be resolved through the usage of large-scale meta-analysis, which has been done for some clinical factors relevant to mammographic calcifications, but not all. For instance, the relationship between calcification morphology and risk of malignancy was analysed by a meta-analysis encompassing over 10,000 patients and provided strong evidence of a high degree of risk associated with calcifications of a pleomorphic or fine-linear morphology (27).

**Table 2. Key studies on the association between microcalcifications and prognosis, recurrence and HER2 status in breast cancer patients.**

	Patient cohort	Effect	Ref.
<b>Prognosis</b>	721 patients with invasive ductal carcinoma	2.41-fold increased mortality	(50)
	343 screen detected invasive cancers, 1-14 mm size	20-year survival of 55% for patients with casting calcifications versus 87% for patients without	(45)
	714 screen detected invasive cancers, 1-14 mm size	9.19-fold increased mortality for patients with casting calcifications compared with stellate tumours	(46)
	409 patients with breast carcinoma treated with breast conserving surgery	2.5-fold increased mortality in patients with calcifications	(52)
	498 patients with invasive ductal carcinoma	Casting calcifications associated with 3.47-fold increased hazard ratio for mortality	(49)
<b>Recurrence</b>	721 patients with invasive ductal carcinoma	1.98-fold increased risk of relapse	(50)
	1,657 patients with DCIS and calcifications	5.2-fold increased risk of local recurrence associated with casting vs. benign calcifications	(56)
	409 patients with breast carcinoma treated with breast conserving surgery	2.46-fold increased risk of local recurrence in patients with calcifications	(52)
	408 patients with invasive breast cancer	Increased risk of recurrence risk as assessed by Oncotype DX assay in patients with calcifications	(60)
	267 patients with ER+, HER2- invasive breast cancer	Presence of calcification in a mass associated with high Oncotype DX score	(58)
<b>HER2 status</b>	7,317 breast cancer patients	2.5-fold higher rate of HER2 positivity in patients with calcifications (22.34 vs. 8.93%)	(63)
	937 cases of breast cancer	Calcifications significantly more prevalent in HER2+ tumours (52.9 vs. 33.8%)	(61)
	Meta-analysis, total of 17,745 cases of breast cancer	Pooled odds ratio of 3.14 of HER2 positivity in patients with calcifications	(70)
	985 cases of invasive breast cancer	Calcifications significantly more prevalent in HER2+ tumours (51 vs. 22%)	(71)

## 5. Biological effects of microcalcifications

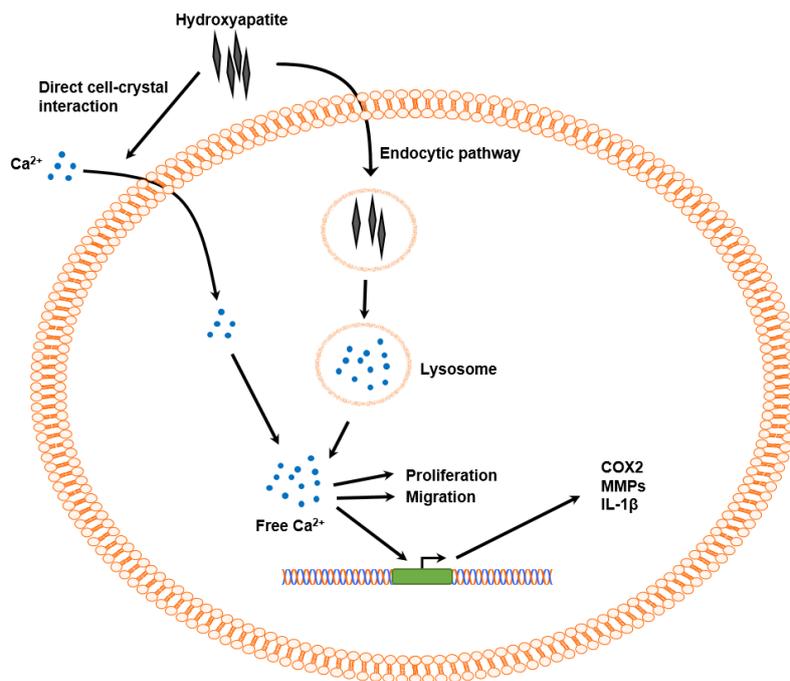
With a significant body of evidence to demonstrate a link between calcifications and a range of prognostic factors, it is interesting to speculate how the presence of calcifications could potentially be directly influencing tumour behaviour. The first studies to investigate the potential biological significance of calcifications on breast cancer found that stimulation of MCF-7 and Hs578T breast cancer cell lines with synthetic hydroxyapatite (HA) particles (representative of malignancy-associated type II microcalcifications) led to increased mitogenesis (72). Pathi et al. also observed increased proliferation in both MDA-MB-231 and MCF-7 cells grown on HA-mineralised scaffolds as compared to non-mineralised scaffolds (73). In addition to increased proliferation, previous results from our lab also demonstrated increased migration by scratch-wound assay in HA stimulated murine 4T1 cells (74). Although the process by which HA influenced migration was not explored, it is interesting to note that stimulation with calcium oxalate (representative of benign, type I calcifications) did not elicit any such increase. The precise mechanism of HA stimulation in breast cancer cells has not been fully elucidated. However results from other cell types suggest a two-fold effect involving a rapid but transient spike in intracellular  $\text{Ca}^{2+}$  mediated by influx of  $\text{Ca}^{2+}$  from the extracellular environment triggered by direct cell-crystal interactions (Fig. 2). This is followed by phagocytosis and crystal dissolution, yielding a second, gradual rise in intracellular  $\text{Ca}^{2+}$  (75, 76). Thus far, these studies have been limited to *in vitro* studies and it is unclear if similar mechanisms would occur *in vivo*.

In addition to stimulating proliferation and migration, HA crystals also exert a potent inflammatory effect in breast cancer cells. HA stimulated MCF-7 cells display increased levels of prostaglandin E2 (PGE2) (72). This increase is likely mediated by increased expression of (cyclooxygenase-2) COX2, as HA crystals upregulate COX2 expression (75), and blocking COX2 activity with aspirin led to a decrease in PGE2 in HA stimulated cells (72). COX2 is a significant promoter of breast tumour development and is upregulated in breast cancer, with one study even finding a link between increased immunohistochemical staining for COX-2 and the presence of mammographic calcifications (77). Long term aspirin use decreases risk of developing breast cancer (78) and may improve patient survival (79). In addition, COX-2 expression is associated with tumour size and grade (80) and promotes epithelial-mesenchymal transition (EMT) and invasion (81). Considering the multiple tumour-promoting effects associated with COX-2 expression in breast tissue, the link between microcalcifications and COX-2 may play a role in the unusually aggressive nature of calcification-associated tumours observed in some studies (45, 46).

HA was also found to upregulate production and activity of matrix metalloproteinases (MMPs) in both normal and malignant breast cells (72, 75). Multiple studies have also shown HA to upregulate MMP expression in other cell types (82-85). MMPs are multi-functional promoters of breast tumour progression. In addition to their well-known activity in promoting invasion via extracellular matrix (ECM) degradation, MMPs can also activate and release precursor growth factors from surrounding tissue, decrease

apoptotic signalling through cleavage of Fas ligands, promote EMT and modulate tumour immune surveillance via proteolytic activation of TGF- $\beta$  (86, 87).

Finally, HA stimulation can increase expression of IL-1 $\beta$  (75). This effect may involve activation of the NLRP3 inflammasome, which responds to crystalline structures and has been shown to mediate IL-1 $\beta$  upregulation in response to HA particles in other cell types (88), although this has not been examined in breast cancer cells.



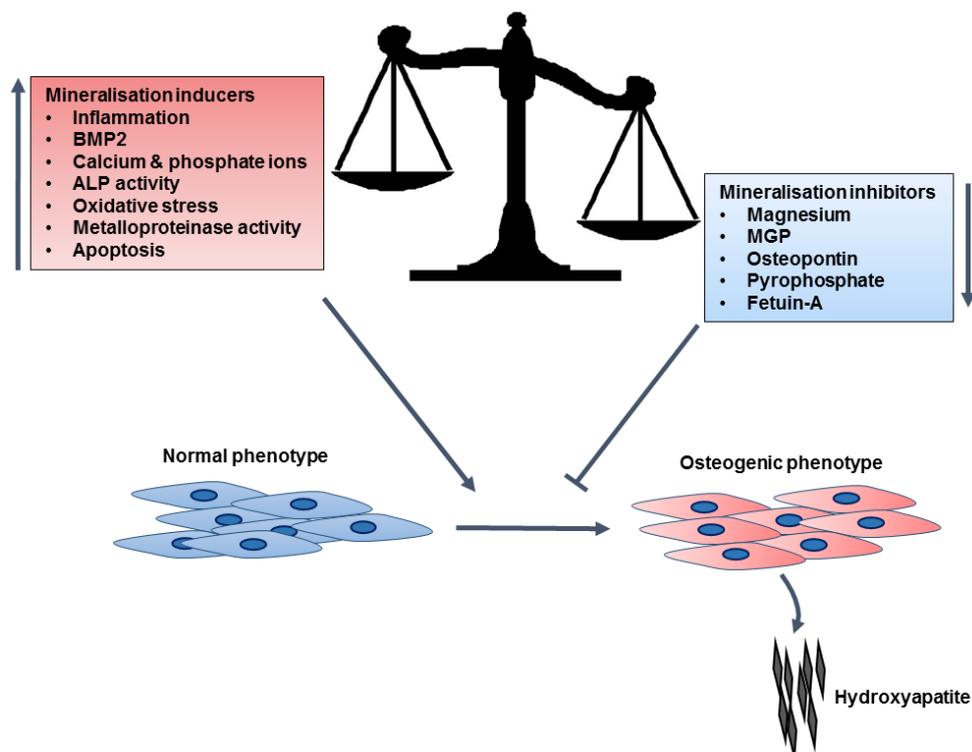
**Figure 2. Tumour-promoting signalling of hydroxyapatite crystals.** Hydroxyapatite (HA) crystals may stimulate entry of Ca<sup>2+</sup> from the extracellular medium by direct cell-crystal interaction, causing a rapid but transient spike in intracellular Ca<sup>2+</sup>. HA crystals may also be taken up by the cell, entering the lysosomal pathway. Crystal dissolution and subsequent release of free Ca<sup>2+</sup> into the cytosol creates a slow, but sustained raise in intracellular Ca<sup>2+</sup>. Together, these two mechanisms cause a biphasic Ca<sup>2+</sup> increase, leading to activation of proliferative and migratory signalling pathways, and upregulation of inflammatory mediators.

## 6. Formation of microcalcifications – lessons from other physiological mineralisation mechanisms

HA is also present under normal physiological conditions, forming the primary inorganic component of bone, teeth and other calcified structures. Evidence suggest that formation of HA microcalcifications in breast tumours may follow a similar process as physiological mineralisation. A crucial factor in regulating this process is the balance between phosphate (Pi) and pyrophosphate (PPi), and the enzymes responsible for their production. PPi, which consists of two molecules of Pi joined by an ester bond, acts as a vital endogenous inhibitor of mineralisation, primarily through

adsorption to the forming HA crystals and interfering with nucleation (89). The inhibitory effect of PPI is counteracted by the activity of the alkaline phosphatase (ALP) enzyme which hydrolyses PPI to Pi, simultaneously removing a source of inhibition and producing free Pi for HA formation (90). This double-action makes ALP activity a powerful driver of both physiological and pathological mineralisation.

In addition to Pi-PPI balance, a range of other inhibitors have been described that tightly regulate calcium deposition to specific locations in the body, including the vitamin K-dependent calcification inhibitor matrix gla protein (MGP), the liver produced plasma protein fetuin-A, and  $Mg^{2+}$  ions (91-93). Together, these various mineralisation inhibitors tightly regulate deposition of calcium and prevent the pathological mineralisation of soft tissue (Fig. 3). However, this careful balance is perturbed in a number of diseases, resulting in formation of ectopic calcifications. A well-studied example is vascular calcification, in which disruption of the various inhibitory processes previously described results in deposition of calcium within the collagenous matrix of the intimal or medial layers of the arterial wall. Vascular calcification may be triggered by a variety of conditions including inflammation and oxidative stress (94), kidney dysfunction leading to elevated serum Pi (95), aging and diabetes (96).



**Figure 3. Development of hydroxyapatite calcifications as an imbalance between calcification promoters and inhibitors.** Under normal physiological conditions, soft tissue mineralisation is prevented through a careful balance of mineralisation promoting factors (e.g.  $Ca^{2+}$ /Pi concentrations, inflammatory cytokines, apoptosis) and mineralisation inhibitors (e.g.  $Mg^{2+}$ , osteopontin). This balance can be knocked out of place by various causes including injury to the vessel wall, diabetes, cardiovascular disease or impaired kidney function, resulting in transdifferentiation of vascular smooth muscle cells (VSMCs) to an osteoblastic-like phenotype, capable of promoting mineralisation of the local extracellular matrix.

Similar to physiological mechanisms of mineralisation, studies in vascular calcification have demonstrated the role of an active, cell regulated process in the formation of calcium deposits within the vasculature. Vascular smooth muscle cells (VSMCs) grown under mineralisation promoting conditions undergo phenotypic conversion to an osteogenic state, upregulating mineralisation associated genes including ALP, bone morphogenetic protein 2 (BMP2) and the osteogenic transcription factor runt-related transcription factor 2 (RUNX2) (97).

As a well-established form of pathological calcification, it is possible that results from studies of vascular calcification may provide clues to the origin of breast microcalcifications. Using the same reagents and protocols from established mineralisation studies (98), our group developed the first *in vitro* model of breast microcalcification formation (74, 99). Formation of microcalcifications was found to be highly dependent on ALP activity, as addition of the ALP inhibitor levamisole completely blocked mineralisation. Mineralisation was also blocked by addition of phosphonoformic acid, an inhibitor of Na(+)-dependent Pi transporters. Upregulation of several markers of mineralisation was also observed, suggesting a transition to an osteogenic state. These findings indicate that, similar to vascular calcification, the deposition of calcium within breast lesions seems to result from an active, regulated process of mineral deposition with many similarities to physiological mineralisation.

Since its development, other groups have also utilised our model to probe the molecular mechanisms underlying formation of mammary calcifications. Zheng et al. examined the role of carbonic anhydrase I (CA1) in mineralising 4T1 cells (100) and found that inhibition of CA1 activity by the small molecule inhibitor acetazolamide resulted in decreased CA1 expression and calcium deposition. Although the precise mechanism by which CA1 promotes formation of microcalcifications is unclear, CA1 belongs to a family of enzymes that catalyse the interconversion of carbon dioxide and water to bicarbonate and has previously been shown to promote the formation of calcium carbonate which may facilitate formation of microcalcifications by acting as a seed for subsequent hydroxyapatite formation (101).

More recently, Dang et al. highlighted a role for the secretory pathway  $\text{Ca}^{2+}$ -ATPases SPCA1 and SPCA2 in development of microcalcifications (102). Expression of SPCA1 and SPCA2 was increased during microcalcification formation and transfection with a SPCA2 containing plasmid resulted in significant increases in calcium deposition. Transfection with a catalytically inactive SPCA2 mutant failed to increase calcification, demonstrating the importance of an active  $\text{Ca}^{2+}$  pumping process.

Other calcium transport proteins may also be involved in the formation of microcalcifications. Zhang et al. analysed a miR-367 binding site in the 3'UTR of ryanodine receptor 3 (RYR3) gene, an important regulator of calcium homeostasis, and identified an A→G SNP that was associated with increased rates of breast calcification (51). Breast carcinomas with associated calcifications have also been shown to express high levels of the transient receptor potential (TRP) ion channels TRPC1 and TRPM7 (103). The functional relationship between these channels and

microcalcifications has not been explored, although both channels have previously been shown to be significantly upregulated in breast carcinomas and to play a role in migration, invasion and proliferation (104-106). In addition, the TRPM7 channel was recently shown to promote development of vascular calcification (107).

## **7. Osteomimicry of breast cancer cells**

In a recent review of Ca<sup>2+</sup> signalling pathways in breast cancer, Cross et al. proposed a mechanism of microcalcification formation involving dysregulation of Ca<sup>2+</sup> transport pathways in conjunction with “abnormal expression of bone matrix proteins” (108). Although the number of calcium transport pathways that have been studied in the context of breast calcification remains low, a significant number of studies have correlated expression of bone matrix proteins with the presence of microcalcifications. Expression of bone-associated proteins in breast tumours is a well-established phenomenon and this “osteomimicry” appears to act as a significant promoter of tumourigenesis. BMP2 is expressed in both primary breast tumours and many breast cancer cell lines (109), and can promote EMT and development of cell-stemness via the Rb and CD44 signalling pathways (110). RUNX2 is highly expressed in triple-negative breast tumours and may act as a marker within this subtype (111, 112). Osteomimicry of breast tumours may also influence breast cancers propensity for forming bone metastases (113, 114).

Several groups have noted an association between expression of mineralisation-associated genes and the presence of microcalcifications, suggesting a possible mechanistic role for these genes in the formation of microcalcifications (Table 3). Bellahcène & Castronovo found increased immunostaining for osteonectin (OSN) and osteopontin (OPN) in *in situ* and invasive breast carcinomas relative to normal breast tissue (115). They also noted that microcalcifications, when present, were usually found in areas with high immunoreactivity for the two bone proteins. Increased expression of bone sialoprotein was also observed in breast carcinomas, with particularly high staining in samples with associated microcalcifications (116). In a study of 141 cases of invasive carcinoma, Wang et al. found that microcalcifications were significantly more common in OPN positive than in OPN negative tumours (54.3% vs 30.6%) and were particularly frequent when calcifications were found in combination with a mass (72.7% for OPN positive tumours vs 18.2% for OPN negative tumours) (117, 118). The majority of calcifications found in OPN positive tumours were of a pleomorphic morphology, a form of calcification significantly associated with malignancy (28). Scimeca et al. also found significantly increased OPN in infiltrating carcinomas with microcalcifications compared to those without, and also observed a focal staining pattern, with high OPN signal around calcifications (36). Similar levels of upregulation were also observed for BMP2 and the mesenchymal marker vimentin, leading to the authors’ conclusion that formation of microcalcifications results from an EMT process and acquisition of an osteogenic phenotype. OPN is a highly-phosphorylated protein, ubiquitously expressed but found in high concentrations in areas of mineralised tissues including ectopic calcifications (119).

OPN is an important regulator of calcium deposition in soft-tissue (120) and is believed to act as an inducible inhibitor (121). Therefore, the increased expression observed in calcification-associated tumours may reflect a protective response and an attempt to limit further calcification.

**Table 3. Mineralisation associated genes and their role in breast cancer and formation of microcalcifications.**

Gene	Role in mineralisation	Role in breast cancer	Association with breast microcalcifications
<b>ALP</b>	Hydrolysis of pyrophosphate to free phosphate (89).	Serum ALP increased in patients with metastatic disease (122).	Inhibition of ALP activity blocks <i>in vitro</i> mineralisation (74).
<b>BMP2</b>	BMP2 activates pro-mineralisation signalling pathways (123).	Remains unclear. Has been associated with both altered proliferation, apoptosis and migration (109, 124).	Highly expressed in microcalcification associated breast tumours (36). Exogenous BMP2 increases <i>in vitro</i> mineralisation (99). BMP2 overexpression induces breast calcification in rat model (125).
<b>TRPC1 TRPM7</b>	No known role for TRPC1. TRPM7 may inhibit mineralisation by promoting Mg <sup>2+</sup> influx (126, 127) or promote it via interaction with IL-18 signalling (107).	TRPC1 regulates non-stimulated Ca <sup>2+</sup> influx and cellular response to hypoxia (128, 129). TRPM7 regulates EMT, proliferation and metastasis (104, 106, 130).	Highly expressed in microcalcification associated breast tumours (103).
<b>SPCA1 SPCA2</b>	No known role in mineralisation	SPCA1 upregulated in basal tumours. Regulates IGF1R processing and proliferation (131). SPCA2 upregulated in breast tumours and promotes Orai1 mediated Ca <sup>2+</sup> influx (132).	Upregulated during <i>in vitro</i> mineralisation. siRNA knockdown inhibits mineralisation (102).
<b>OPN</b>	Inducible inhibitor of mineralisation (120, 121)	Overexpression promotes lymphatic invasion (133). High expression associated with decreased DFS and OS (134).	Highly expressed in microcalcification associated breast tumours (36, 115, 135).
<b>OSN</b>	Regulates osteoblast differentiation and promotes mineralisation (136-138).	Remains unclear. Has been associated with both positive (139) and negative (140, 141) prognosis factors.	Highly expressed in microcalcification associated breast tumours (115).
<b>BSP</b>	Promotes hydroxyapatite nucleation (142).	High BSP expression associated with bone metastasis (143, 144).	Highly expressed in microcalcification associated breast tumours (116).
<b>Pit-1</b>	Promotes mineralisation by facilitating phosphate transport (145, 146).	Pit1 expression associated with decreased survival in ER+ breast cancer (147).	Inhibition of Pit-1 activity suppresses <i>in vitro</i> mineralisation (74).
<b>CA1</b>	Deposition of calcium carbonate by CA1 may promote by acting as a seed for subsequent hydroxyapatite formation (101).	Increased CA1 expression in breast cancer (100).	CA1 expression increased in mineralising breast cancer cells. CA1 inhibition suppresses <i>in vitro</i> mineralisation (100).

## **8. Conclusion**

Despite their long history of clinical use in the detection of breast cancer, many questions remain regarding both the formation and prognostic significance of breast microcalcifications. However, significant progress is underway. Studies from our own group and others has begun to unravel their formation process and seems to indicate an active, cell-regulated mechanism with similarities to both physiological and pathological mineralisation. Our understanding of the prognostic relevance of microcalcifications has also seen significant improvement recently, with several recent studies with larger cohorts of patients as well as meta-analyses providing strong evidence for a diminished prognosis in patients presenting with calcification-associated breast tumours. Studies of HA stimulation in both breast cancer and other cell types also suggests that this decrease in survival may result from a direct influence of calcifications on tumour behaviour. Considering the high rate of detection of calcifications in early-stage, small tumours, a more detailed understanding of the formation process of microcalcifications could help inform clinical understanding of the early events of breast tumourigenesis and could perhaps identify new targets for therapeutic intervention or prognostic markers to further predict outcomes.

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