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INVITED REVIEW

From latency to overt bone metastasis in breast cancer: potential for treatment and prevention

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Abstract

Bone metastasis is present in a high percentage of breast cancer (BCa) patients with distant disease, especially in those with the estrogen receptor-positive (ER^+) subtype. Most cells that escape primary tumors are unable to establish metastatic lesions, which suggests that target organ microenvironments are hostile for tumor cells. This implies that BCa cells must achieve a process of speciation to adapt to the new conditions imposed in the new organ. Bone has unique characteristics that can be exploited by cancer cells: it undergoes constant remodeling and comprises diverse environments (including osteogenic, perivascular, and hematopoietic stem cell niches). This allows colonizing cells to take advantage of numerous adhesion molecules, matrix proteins, and soluble factors that facilitate homing, survival, and, eventually, metastatic outgrowth. However, in most cases, metastatic lesions enter into a latency state that can last months, years, or even decades, before forming a clinically detectable macrometastasis. This dormant state challenges the effectiveness of adjuvant chemotherapy. Detecting which tumors are more prone to metastasize to bone and developing new specific therapies that target bone metastasis represent urgent clinical needs. Here, we review the biological mechanisms of BCa bone metastasis and provide the latest options of treatments and predictive markers that are currently in clinical use or are being tested in clinical assays.

Keywords: breast cancer; metastasis; latency; bone; bisphosphonates; dormancy; biomarker

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Introduction

Breast cancer (BCa) comprises multiple diseases with a complex mutational landscape; currently, 22 morphological variants are recognized [1]. For clinical management, these can be divided into breast cancers that express (or not) estrogen receptor (ER), progesterone receptor (PR), and/or human epidermal growth factor receptor 2 (HER2; also termed ERBB2). However, classification based on these markers' expression is not 'fixed' and can change between primary and metastatic BCa. Molecular and gene expression profiles can further classify BCa into four different intrinsic subtypes, which have biological and clinically different outcomes: luminal A, luminal B, HER2-enriched ($HER2^+$), and basal-like (note, though, that the luminal A and B categories are not highly robust and may overlap when it comes to single-sample predictors) [2]. Understanding these molecular distinctions is necessary for understanding (1) how misfunction of these pathways leads to BCa, and (2) how to effectively target these pathways for therapeutic treatments. Currently, blocking ER signaling with tamoxifen or aromatase inhibitors and HER2 signaling with trastuzumab represents the main targeted therapy for ER^+ and $HER2^+$ BCa patients, respectively.

Mammary glands comprise basal cells and luminal cells (LCs) (which are ER^+ or ER^-), among other lineages. Whereas the origin of basal-like BCa is still unclear, the current hypothesis sustains that either bipotent progenitors or luminal progenitors give rise to both luminal and basal-like cancers. Molecularly, estrogen interacts with ERα, and the resulting dimer then binds estrogen-responsive elements, thereby recruiting transcription co-factors, inducing transcription and resulting in breast epithelial cell division. Another major player is HER2. *HER2* gene amplification and oncogenic

mutations constitutively activate the HER2 homodimeric tyrosine kinase activity and reduce the growth factor dependence of *HER2*-amplified cells, through prolonged stimulation of the AKT/mitogen-activated protein kinase pathway. *HER2* amplification increases hypersensibility to the EGF family and contributes significantly to tumor progression. Although pharmacological inhibition of HER2 using a monoclonal antibody (trastuzumab) can effectively treat $HER2^+$ BCa [3], patients with recurrence are usually refractory to treatment and most will die from the disease [4,5]. Of the different subtypes, the ER^- $HER2^+$ subtype clearly correlates with a more aggressive disease [6,7].

The diversity of BCa metastasis

Metastasis is the major cause of death in BCa patients and usually manifests asynchronously with the primary tumor, with variable timing to clinical detection. This lag depends on the volume, stage, and molecular subtype of the primary tumor [8]. Luminal tumors (which are usually ER^+) may recur after a long period, named latency, marked by the absence of clinical symptoms. The mechanisms enabling BCa cells to exit from latency and to genetically evolve towards overt metastasis are only poorly understood [9]. Metastasis of ER^+ BCa tumors is usually slow, suggesting that BCa cells must accumulate metastatic traits under the selective pressure of organ microenvironments [8,10]. Different BCa types show distinct metastatic organ tropism. Unfortunately, however, the systematic use of therapies targeting specific molecular pathways can also change the course and tissue specificity of metastasis in some BCa subtypes; for instance, metastastis can occur later and specifically in the brain in $HER2^+$ BCa patients post-therapy [8,10].

Metastatic lesions from disseminated tumor cells (DTCs), or micrometastases after a period of latency, retain most molecular alterations (80–85%) initially described at the primary site [11]. In contrast (and as mentioned above), the intrinsic molecular subtype of BCa can change during metastatic progression. For instance, luminal A/$HER2^-$ tumors can acquire a luminal B or $HER2^+$ profile; this switch can be observed by immunohistochemistry (IHC) as well as molecular profiling [11–13]. Thus, important but subtle loss of molecular differentiation changes can arise during metastatic progression, and dormancy may be an endowed state [14]. For example, marked BCa luminal differentiation prevents metastatic progression [15]. It remains unclear whether the heterogeneity of luminal-derived tumors and metastasis post-treatment arises from a pre-existing heterogeneity within luminal cells. While these molecular changes may reflect tumor evolution, it is unclear whether they are passenger differentiation changes or if they have functional consequences on latency and overt metastasis. Moreover, the origin of the genetic changes necessary for tumor evolution and metastasis is an open question.

Metastatic progression relies on specific biological steps that need to be targeted to improve current therapeutic strategies. Chemotherapy targets high-proliferating tumor cells rather than the low-proliferating metastastic tumor cells, which can then spread from the primary tumor to distant sites, where they resist conventional treatments, proliferate, and cause vital organ failure [16]. Strikingly, different BCa types show distinct metastatic organ tropism, and acquisition of metastasis may vary from one tumor type to another [17]. Simplifying metastasis into an orderly sequence of basic steps – local invasion, intravasation, survival in circulation, extravasation, and colonization – has helped to rationalize the complex set of biological properties required for metastatic disease [18]. However, the steps of the kinetics and mechanisms that regulate tissue-specific metastasis remain poorly understood [16,19]. Cancer cells must orchestrate diverse cellular functions to overcome the difficulties of transiting the metastatic cascade; these functions are limited to cell-autonomous traits and are highly dependent on the interactions between the metastatic cell and the tumor and host stroma [19]. Several functions can be required to implement a single step, or a single function may influence multiple steps. This speciation is reflected by the distinct kinetics of cancer relapse to different sites in the same patient, and by the co-existence of malignant cells that differ in organ tropism in patient-derived samples.

Physiological function of bone

Bone is among the pre-eminent organs targeted by metastatic cells. In prostate and breast cancers, over 65–70% of patients with advanced disease develop skeletal metastasis [20]. In BCa, ER^+ tumors are more prone to metastasize into bone, while ER^- tumors tend to go to visceral organs (e.g. lungs, liver, or brain) [21], indicating that tumor cell-intrinsic characteristics and the primary tumor microenviroment influence bone metastasis.

Bone is a dynamic organ that is remodeling continuously by the coordinated activity of specific cells. Normal homeostasis balances the functions of osteoblasts (bone matrix formation and mineralization) and osteoclasts (bone resorption) [22,23]. Osteoblasts secrete the receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which activate osteoclasts that acidify the media and secrete collagenases and other proteases that demineralize the bone matrix [24–26]. These spaces are filled by osteoblasts, which replenish the bone matrix [27]. When embedded in the matrix, osteoblasts differentiate into osteocytes that further control bone remodeling [28]. The bone microenvironment also comprises hematopoietic, immune, endothelial cells, and adipocytes.

In normal homeostasis, the balance sustaining this dynamic structure is governed by systemic estrogen

in females pre-menopause and by paracrine factors post-menopause. ERα and ERβ are highly expressed in both osteoblasts and osteoclast lineages and are thought to be pivotal in bone cell differentiation [29]; post-menopause, paracrine bone morphogens and activins take over [30]. Further, ERα expression is high in pre-osteoblasts and pre-osteoclasts but low in mature populations, suggesting that the stimulus is relevant for differentiation purposes but not for established mature functions. Osteocytes can undergo senescence (due to aging, reduced physical activity, or other factors), leading to osteoporosis. Although ERα and ERβ receptors are structurally similar, they have different functions [31]. ERα knockout mice exhibit reproductive organ alterations and shorter longitudinal bone growth [32], while ERβ knockout mice have longer bone growth and alterations in trabecular bone formation [33]. Double ERα/β knockout mice have intermediate bone size, implying that the ER receptors have compensatory effects. Some of these effects have been attributed to the ability of estrogen to trigger osteoblast division from periosteal bone.

Factors involved in bone remodeling and homeostasis are also associated with mammary gland formation and BCa progression. RANK/RANKL signaling (responsible for osteoclast differentiation) accounts for lobuloalveolar development during pregnancy [34]. Overexpression of RANK stimulates epithelial cell proliferation, causes reduced apoptosis, and compromises differentiation [34,35]. Physiologically, RANKL expression peaks during pregnancy to expand the mammary stem cell compartment and its blockade reduces pregnancy-dependent tumorigenesis in mouse models. RANKL sequestration reduces tumor growth in *BRCA1*-deficient mice [36]. Similarly, RUNX2 (a central regulator of osteogenesis) contributes to mammary gland alveologenesis [37].

Bone metastasis: from seeding to overt metastatic colonization

Bone metastasis homing

The discontinuous endothelium of bone makes this organ especially permissive to DTCs, and bone maintenance and cell growth are critical steps supporting bone metastasis [38]. The osteogenic niche plays a pivotal role in supporting survival and latency, but also promotes bone colonization by DTCs. DTC homing to bone is controlled by the specific microenvironment, chemokines, and adhesion molecules expressed both in DTCs and in bone tissue. For instance, C-X-C motif chemokine receptor 4 (CXCR4) is expressed at the DTC surface, whereas its ligand, CXCL12, is expressed in mesenchymal cells or pericytes inside bone [39]. Interestingly, CXCR4 belongs to the gene signature predicting BCa metastasis to bone [40]. Moreover, recent work postulated that HIF signaling in osteoprogenitor cells increases blood levels of CXCL-12, favoring bone metastasis [41]. Inhibition of CXCR4 interactions between cancer cells and bone stroma may increase the effect of standard chemotherapy in prostate cancer [42]. Tumor cells also express different combinations of integrins that interact with molecules expressed in bone, such as bone sialoprotein (BSP), fibronectin, osteopontin (OPN), or VCAM1 [43–48]. The heterotypic adherent junctions between E-cadherin and N-cadherin are important for early-stage bone colonization [49]. Moreover, RANK-expressing cells can be attracted to osteolytic areas in which high levels of RANKL are produced, favoring cancer cell migration and bone metastasis [50] (Figure 1).

Metastasis latency

Although bone marrow hosts residual disease of multiple cancer types, the majority of these DTCs will never develop metastasis [51]. Micrometastases and/or DTCs in the bone marrow have the capacity to maintain themselves at low numbers after primary tumor resection, which is critical for tumor latency and may explain how the disease can resist treatment and reappear after long asymptomatic periods (of up to decades). This state of dormancy is characterized by an arrest in tumor growth, during which DTCs are not adapted to the bone microenvironment, thus avoiding disease progression. Several mechanisms have been proposed to maintain the state of dormancy of DTCs or micrometastasis, including cell autonomous mechanisms and angiogenic and immunological processes. DTCs enter quiescence either by the actions of stromal signals [52], such as hypoxia [53], with TGFβ and BMP as inhibitors of DTC growth [54–56], or by preventing WNT-mediated niche support for cell proliferation as part of normal tissue homeostasis [57]. Inhibition of the PI3K–AKT signaling pathway is characteristic of a dormant phenotype in DTCs isolated from BCa patients. Under nutritional stress, cancer cells inhibit PI3K signaling, inducing quiescence and cell autophagy [58,59]. Stress signals stemming from the microenvironment have been proposed to induce dormancy by modulating the ratio of ERK and p38 MAPKs in DTCs [51]. In particular, TGFβ2 produced in bone marrow controls tumor dormancy by increasing p38 MAPK activity [54]. However, several clinical trials have revealed that the presence of circulating tumor cells (CTCs) in blood has prognostic relevance with respect to metastasis progression [60]. This observation suggests that solitary cell dormancy or quiescence is not a unique feature of latent metastatic lesions, and that a combination of proliferative and apoptotic activities is required to sustain the release of CTCs. For instance, the kinase MSK1 (RPS6KA5), a downstream effector of p38, regulates tumor metastatic latency: reduced MSK1 levels impair cellular differentiation and increase the bone homing capacity of metastatic cells through loss of histone acetylation at promoters that regulate the expression of luminal differentiation genes (including those for the GATA3 transcription factor, which prevent the progression of ER^+ BCa towards metastasis) [61]

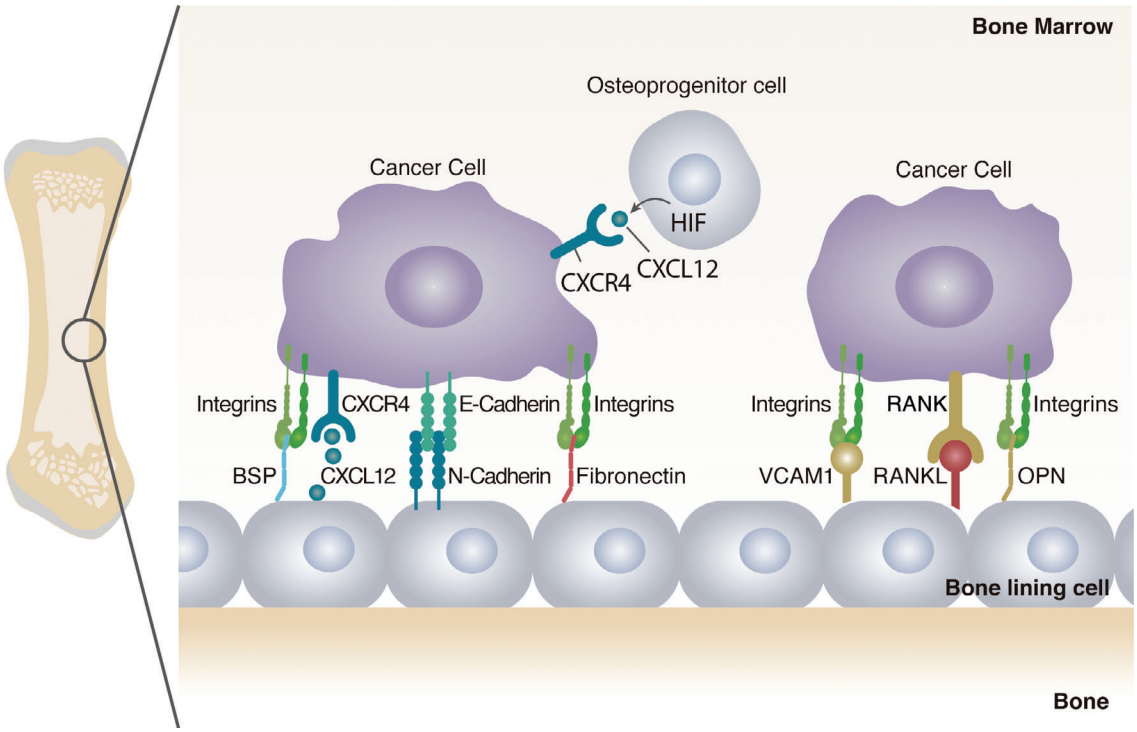

Figure 1. Interactions supporting breast cancer cell bone homing. Tumor cells use a wide repertoire of molecules to facilitate the first steps of bone colonization. These include CXCR4 interactions with CXCL12; different combinations of integrins (e.g. $\alpha_v\beta_3$, $\alpha_5\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$) that bind to BSP, fibronectin, OPN, and VCAM1; association between RANK and RANKL; and interactions of different adhesion molecules such as E-cadherin and N-cadherin.

(Figure 2). Whether MSK1-related chromatin remodeling affects other processes relevant in dormancy, such as angiogenesis and immune surveillance, is still unknown.

Micrometastatic lesions secrete angiogenic factors, including vascular endothelial growth factor (VEGF), which attracts endothelial progenitor cells and facilitates metastasis outgrowth. However, inhibitory signals from perivascular niches, such as thrombospondin 1 (TSP1) secretion, maintain tumor cells in a dormant state by inhibiting angiogenesis [62]. Another mechanism that is critical for controlling dormancy is the actions of the immune system, with cytotoxic T cells and natural killer (NK) cells as two pivotal players (Figure 2). Bone marrow aspirates from BCa patients with DTCs present higher proportions of $CD8^+$ and $CD4^+$ T cells, macrophages, and NK cells than those from BCa patients without DTCs or from healthy donors [63]. Depletion or pharmacologic inhibition of these cell populations promotes metastasis in mice [57,64,65].

Bone metastastic outgrowth

Obtaining the capacity to initiate macrometastatic growth at a secondary site can be stochastic and depend on newly established interactions between DTCs and the target microenvironment, such as VCAM-1 expression by DTCs and its local effect on osteoclast activation [66]. Alternatively, this capacity can already be encoded in the arriving DTCs, e.g. by attenuating the signaling cascades emanating from the environment cues or by allowing DTCs to bypass the natural immune response [57]. Cancer cells develop in a co-evolving microenvironment that suppresses immune surveillance. As support is not immediately available to DTCs, most of these cells die. NK cell activity is suppressed in patients with advanced metastatic disease, and is tightly regulated by stimulatory and inhibitory signals from a panel of NK cell receptors (NKRs) expressed at the cell surface. The activation or inhibition of NK and T cells is regulated by proteins that are expressed on the surface of tumor cells, including class I HLA and programmed death ligand-1 (PDL-1), whose relevance has already been demonstrated in clinics [67,68].

Thus, control of dormant cell reactivation is a complex process that is still not fully understood which combines cell autonomous (intrinsic) mechanisms favoring metastasis-initiating capacity with non-tumor cell (extrinsic) processes, involving angiogenic, hematopoietic stem cell (HSC), and osteogenic niches, to produce overt metastatic colonization. The factors that provide new genetic or epigenetic changes favoring exit of dormancy are not fully known, although chromosome instability may play an important role [69,70].

Growth of metastatic lesions generally implies aberrant bone remodeling activities, which eventually account for the skeletal-related events [71]. At clinical presentation, bone metastasis is associated with increased levels of calcium and alkaline phosphate, and with pain. In overt bone metastatic colonization, cancer cells modify the bone microenvironment to activate osteoclasts and suppress bone formation. This is achieved by paracrine crosstalk among cancer

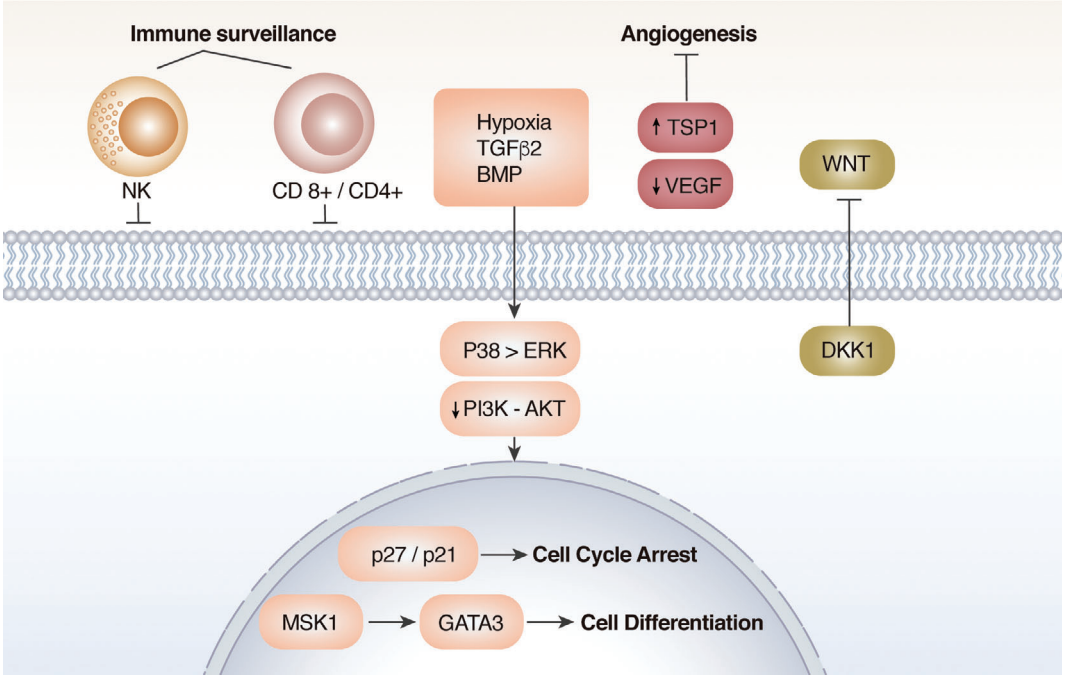

Figure 2. Breast cancer metastasis dormancy. Maintenance of the latent state of DTCs involves both cell autonomous mechanisms and interactions with other components of bone and tumoral stroma. The variety of signals that are relevant to control this state reveals that tumor dormancy is a complex feature that likely involves not only solitary cell dormancy but also tumor mass dormancy, thereby balancing mitotic and apoptotic events. The innate and adaptive immune systems play a key role in controlling latency. In this setting, inhibition of autocrine Wnt signaling promotes immune evasion and a slow cycling state of the tumor cells. Inhibition of the angiogenic switch also provides an important checkpoint of the dormant state. Numerous cell signaling pathways (including those with MAPKs and PI3K) that respond to exogenous factors (such as hypoxia, TGFβ2, and BMP) mediate a cell response that results in latency maintenance with cell cycle arrest or cell differentiation.

cells, osteoblasts, osteoclasts, and the bone matrix. Cancer cells secrete osteolytic factors that activate bone-resorbing osteoclasts. To activate osteoblasts, metastatic cells produce cytokines and growth factors, including parathyroid hormone-like protein (PTHrP), interleukin (IL)-11, IL-6, IL-8, VEGF, and tumor necrosis factor (TNF-α) (reviewed in refs 72 and 73). As a result, osteoblasts release soluble RANKL and inactivate its antagonist osteoprotegerin (OPG). The ratio of RANKL to OPG is critical for osteoclast activation, as OPG prevents RANKL from binding to its receptor RANK, located at osteoclasts' membrane. Once activated upon ligand binding, the multinucleated osteoclasts attach to the bone surface and release acid and proteolytic enzymes, such as cathepsin K and matrix metalloproteinases (MMPs), to resorb the bone matrix (Figure 3). Osteolysis releases growth factors stored in the matrix, including TGFβ, insulin-like growth factors (IGFs), and BMPs, as well as calcium ions, into the bone microenvironment. In addition to tumor growth enhancement, TGFβ activates both Smad-dependent and Smad-independent signaling pathways to induce PTHrP [74,75] in metastatic cells. Therefore, tumor growth is stimulated, leading to the production of additional osteolytic and osteoblastic factors and resulting in a vicious cycle of bone metastasis. In addition, bone resorption can be promoted by the Notch signaling pathway, which results in IL-6 secretion upon binding of tumor-derived JAGGED-1 (JAG-1) to osteoblasts [76] (Figure 3). Strikingly, chemotherapy-induced JAG-1 expression in osteoblasts creates a pro-survival niche for DTCs and early lesions. Recent pre-clinical data using antibodies against JAG-1 targeting individual Notch receptor signaling activities in combination with chemotherapy in pre-clinical models suggest the potential of eliminating these cells [77].

Bone metastasis therapeutic options

The majority of breast cancers are ERα-positive [78]. Hormone therapy inhibits ERα activity and improves patients' overall survival [79]. Unfortunately, resistance often occurs and patients eventually relapse. Activating mutations in the *ESR1* gene are common particularly in the advanced metastatic setting, with those that occur upon selective pressure of aromatase inhibition especially observed in bone metastasis, implying a potentially causal role [80,81]. Two main categories of drugs are used routinely in clinical practice to reduce bone metastasis morbidity, in combination with standard therapy for advanced breast cancer (hormone therapy alone or in combination with CDK4/6 inhibitors, or chemotherapy). These classes of drugs, namely bisphosphonates (zoledronic acid, ibandronate, palmidronate, clodronate) and anti-RANKL antibody (denosumab), modify the bone microenvironment and effectively control bone metastasis-associated

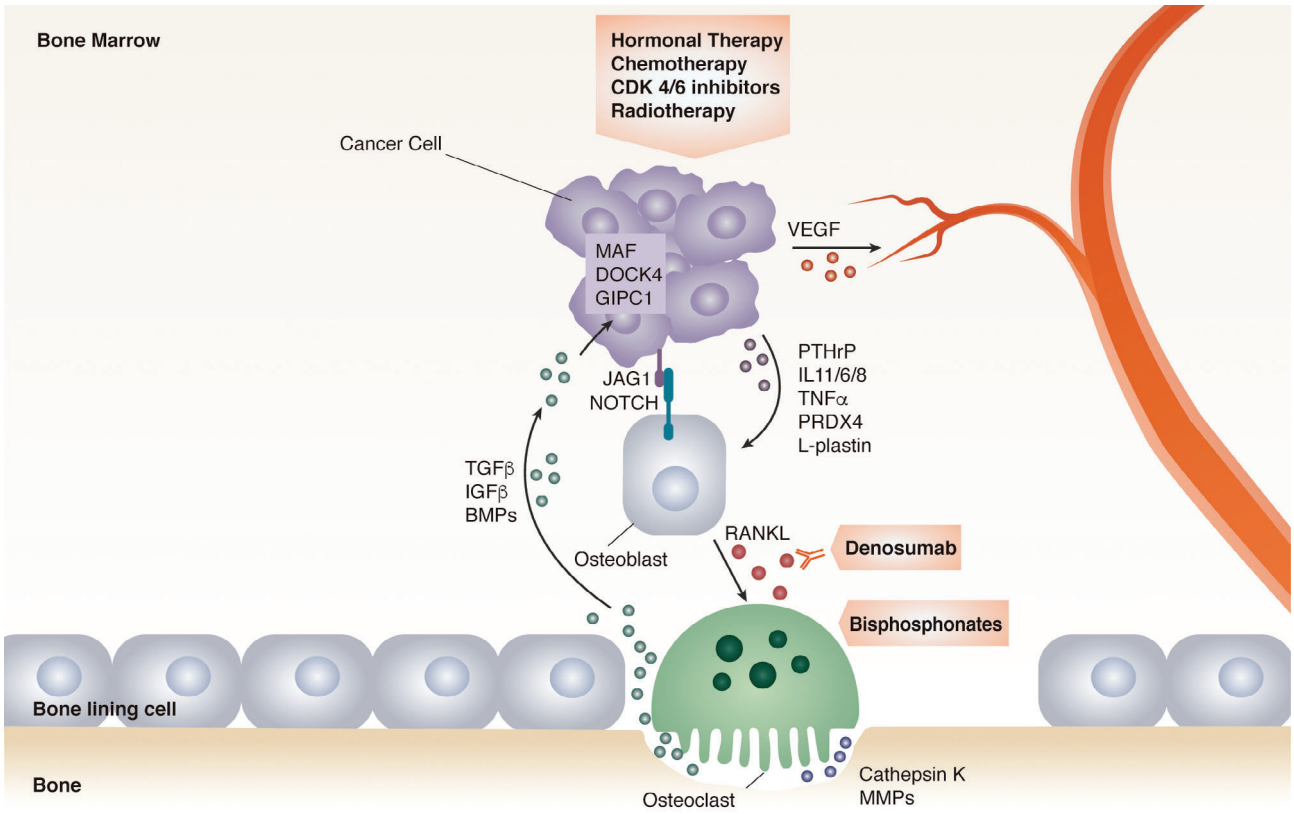

Figure 3. Bone metastasis outgrowth. Awakening dormant tumor cells within bone and secretion of chemokines and other factors, including PTHrP, IL-11, IL-6, IL-8, VEGF, and TNF-α, leads to osteoblast activation. Release of RANKL by osteoblasts contributes to osteoclast differentiation, which in turn produces proteolytic enzymes that degrade the bone matrix. Release of growth factors (e.g. TGFβ, IGFs, and BMPs) promotes tumor growth and stimulates the production of chemokines by breast cancer cells, thus involving a vicious cycle. Therapeutic approaches targeting BCa bone metastatic cells are based on the use of hormone therapy, chemotherapy, CDK4/6 inhibitors, and radiotherapy, together with bone remodeling-related drugs (e.g. denosumab and bisphosphonates). Use of these agents in the adjuvant setting is still not included in the clinics.

skeletal-related events [20,82] (Figure 3). These compounds do not target cancer cell autonomous functions but rather the bone stroma. However, some studies suggest that bisphosphonates may also have a direct effect on the growth of tumor cells and angiogenesis [83]. Alternatively, inhibition of cathepsin K, a cysteine protease expressed in active osteoclasts, is as effective as zoledronic treatment [84], while sclerostin, secreted by osteocytes to inhibit bone formation, has also been postulated to be targeted in bone metastatic patients [85]. However, targeting these molecules by specific antibodies increases the risk of severe secondary effects, thus discarding their use in a clinical setting. Radiopharmaceuticals, including radium-223, or inhibition of recepteur d'origine nantais (RON), a tyrosine kinase that induces bone destruction independently of RANKL, are currently being tested in clinical trials with bone metastatic patients [85,86] and may improve patient outcome with advanced metastasis. Similarly, the approval of everolimus in combination with exametasane for patients with advanced ER^+ BCa metastasis ameliorates symptoms. Bone metastasis treatments benefit from a multidisciplinary approach to control the expansion of the disease; unfortunately, patients with bone metastasis still suffer skeletal-related events, reducing quality of life and survival. While combining these systemic treatments with loco-regional approaches (e.g. radiation therapy and orthopedic surgery) can improve patient management to a degree [87], there is a significant unmet need for identifying cures for bone metastasis.

Biomarkers to predict relapse and response to bone-modifying agents in the adjuvant setting in BCa

BCa molecular profiling

With the advent of gene expression profiling, bioinformatic classification of breast tumors into 'molecular subtypes' is progressively being implemented into the clinic. Each molecular subtype is associated with a certain risk of relapse. Complementary efforts have also provided 'poor-prognosis' gene-expression 'signatures', of which several have been turned into commercial assays that are used to spare low-risk patients from aggressive chemotherapy treatment (i.e. Oncotype Dx, Mamaprint, EndoPredict, Mammostrat, or Prosigna tests) [88,89]. Unfortunately, the majority of these tools are only appropriate for a

subgroup of patients with particular clinical features. Nonetheless, these tumor taxonomy tools are based on gene-expression events as markers and do not provide information about the molecular mediators of tumor progression or metastasis for each BCa subtype. Albeit of large clinical utility, these tools do not support the guidance of metastasis-specific treatments beyond standard chemotherapeutic agents targeting cell division.

Bone metastasis preventive treatments

Preventing metastasis in high-risk patients would be far better than having to treat it. Bone microenvironment-modifying agents (such as bisphosphonates or the anti-RANK ligand antibody denosumab) have the theoretical potential to prevent bone metastasis, although data from clinical trials are as yet inconclusive in unselected patient populations [90,91]. Intravital multiphoton microscopy in mice now provides access to cellular and molecular mechanisms of bone metastasis. Specifically, miniaturized tissue-engineered bone constructs have been used in nude mice with a skin window to non-invasively and repetitively monitor prostate cancer lesions in three dimensions [92]. After growing tumors inside these bone cavities and inducing niches of osteoclast activation, interventional bisphosphonate therapy reduced osteoclast kinetics and osteolysis, but unexpectedly did not perturb tumor growth [92]. This report probes dissociation of the tumor–stroma axis from the tumor growth dynamics and highlights the need to therapeutically target both processes at the same time. Registration trials comparing adjuvant bisphosphonates (clodronate, ibandronate, palmidronate, or zoledronate) to placebo for women (both pre-menopausal and post-menopausal) with different stages of BCa have not confirmed clinical utility. To investigate the available evidence in a more robust and precise manner, the Early Breast Cancer Trials Collaborative Group (EBCTCG) conducted a formal individual patient data meta-analysis of data from 18 766 women involved in 26 randomized trials of adjuvant bisphosphonates for early breast cancer. Strikingly, in post-menopausal women ($n = 11\,767$), bisphosphonates reduced not only recurrence in bone but also overall BCa recurrence and mortality, leading to recommendation for use in clinical practice [93–95]. These reductions were similar irrespective of ER status or grade of the primary tumor, axillary lymph node involvement, and use/non-use of chemotherapy, suggesting that menopause status should be the main criterion for patient selection for adjuvant bisphosphonates in prevention of metastases. How these tumor populations become sensitive to the treatment, and which are the contextual determinants that switch the drug response, is unclear. This can be partially explained by the fact that ER induces apoptosis in osteoclasts [96], therefore inhibiting bone resorption. This indicates that estrogen and bisphosphonates may act in redundant ways, suggesting that bisphosphonates are only effective in low-estrogen patients (ovariectomized or post-menopausal patients) [96,97]. Importantly, whether there are benefits for any subpopulations of pre-menopausal BCa patients, for whom there are limited treatment options, remains to be addressed. Notably, the overall trials did not show any trend for outcome improvement, despite the potential benefits for post-menopausal women. Thus, there is a high unmet medical need for a diagnostic tool to aid in identifying a patient population with a positive benefit–risk ratio for adjuvant bisphosphonates treatment (and as an extension, for denosumab), which could have immediate clinical application [20,98]. Moreover, developing new strategies to evaluate the bone resorptive activity of bisphosphonates and the subjacent mechanism in pre-clinical models is critical for better understanding their therapeutic potential [92,99].

Bone turnover biomarkers

To correlate bone turnover with clinical outcome (e.g. for patients undergoing bone-modifying therapies), several retrospective analyses have been investigated in various tumor types. These included serum BALP and serum or urine NTX levels, which are associated with risk reduction for death and pathological fractures upon treatment with bisphosphonates [100–105]. Patients with high serum levels of P1NP, CTX, and 1-CTP shortly after diagnosis were shown to be at high risk of bone metastasis during the course of the disease [106], confirming previous indications in breast and lung cancers. This latter evidence came from a retrospective analysis of serum from a subset of patients from a large phase III trial, the AZURE trial [107]. Interestingly, the presence of these markers reflects the same metabolic processes, and points towards bone turnover independently of the menopause status as a means to support bone dissemination. Changes in this bone microenvironment may provide the adequate niche for BCa cell homing and development of skeletal metastasis. These markers are specific for bone recurrence but not distant recurrence and, although with low-to-moderate bone prognostic potential (Harrell's *c*-index 0.57), were not predictive for zoledronate in adjuvant treatment response [106] (Table 1). The extent to which these factors predict response to the inclusion of bone-modifying drugs in the adjuvant regime is unclear and requires more analyses. The molecular explanation is also unknown, although a confounding effect by the intrinsic nature of the anti-resorptive agent or the hormone treatments can be anticipated. Interestingly, some of these biomarkers hold promise as a surrogate for treatment efficacy rather than a biomarker for initial treatment decision-making [104].

Bone metastasis prognostic biomarkers

To overcome the limitations of bone turnover biomarkers, several endeavors have been taken to support early identification of patients at risk of skeletal metastasis who could adopt personalized adjuvant

Table 1. Bone metastasis markers in breast cancer

Protein	Predictive marker	Potential marker to include adjuvant bisphosphonates	Detection	References
BALP	High SRE	No	Serum	[100–105]
NTX	High SRE	No	Serum/urine	[100–105]
P1NP	Bone-specific recurrence	No	Serum	[106]
CTX	Bone-specific recurrence	No	Serum/urine	[106]
1-CTP	Bone-specific recurrence	No	Serum	[106]
IL-1b	Bone-specific recurrence	…	IHC	[108,109]
CAPG/GIPC1	Bone-specific recurrence	Yes	IHC	[110–114]
PRLR	Bone-specific recurrence	…	IHC	[115]
BSP	Bone-specific recurrence	…	IHC/serum	[116]
PRDX4	Metastasis	…	mRNA	[117]
PAK4	Bone-specific recurrence (ER^+)	…	IHC	[118]
MAF	Bone-specific recurrence/extra-skeletal recurrence *	Yes	FISH	[119–121]
DOCK4	Bone-specific recurrence	Yes	IHC	[122]

BALP, bone alkaline phosphatase; NTX, N-telopeptide of type I collagen; P1NP, N-terminal propeptide of procollagen type 1; CTX, C-telopeptide of type I collagen; 1-CTP, pyridinoline crosslinked carboxy-terminal telopeptide of type-1 collagen; IL-1b, interleukin 1 beta; CAPG, macrophage-capping protein; GIPC1, PDZ domain-containing protein GIPC1; PRLR, prolactin receptor; BSP, bone sialoprotein; PRDX4, peroxiredoxin-4; PAK4, p21-activated kinase 4; MAF, V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog; DOCK4, dedicator of cytokinesis protein 4; FISH, fluorescence *in situ* hybridization; SRE, skeletal-related event; IHC, immunohistochemistry.
*Upon zoledronic treatment in non-post-menopausal patients.

treatments, using biologically-driven unbiased discovery approaches. Several potential markers of bone metastasis, and their potential clinical implications, have been described. Experimental xenograft mouse models were developed to derive and select BCa cells prone to causing bone metastasis based on ER^+ MCF7 cells or ER^- MDA-MB-231 BCa cells. Cell xenografts were chosen as they have the potential to cause bone metastasis in only a fraction of mice, making their use tailored for metastatic enrichment [40,66,123,124]. These mice were subjected to gene expression, miRNA, or proteomics analysis to provide relevant candidates. In a bone-homing clone of MDA-MB-231 cells, IL-1β was selectively up-regulated as compared to the initial non-specific population and was significantly correlated with bone metastasis development in 150 BCa primary tumors [108] (Table 1 and Figure 3). Interestingly, inhibitors of IL-1β prevent bone events in pre-clinical experimental mouse models [109]; no molecular explanations are currently available. Using the bone-homing variant of MDA-MB-231 cells, DOCK4 was identified as a biomarker of bone metastasis in early BCa; this finding was clinically validated using the control group of the AZURE trial [122].

Several molecules, including peroxiredoxin-4 (PRDX4) and L-plastin, have been postulated as osteoclastogenesis mediators that facilitate bone metastasis through cancer-derived exosomes [117,125]. The PAK4–ERα axis mediates bone metastasis by targeting leukemia inhibitory factor receptor (LIFR) in ER^+ BCa [118] (Table 1). Non-coding microRNAs (miRNAs) have also been investigated. Although the expression of several miRNAs (miR-10b, miR-373, miR520c, miR206, miR-126, and miR-335) is associated with metastatic capabilities such as invasiveness and migration [126–128], only a few have been specifically associated with bone metastasis: (1) miR-218 controls osteoblast differentiation and cancer cells' osteomimicry [129] and regulates collagen deposition by osteoblasts [130]; (2) miR-214-3p levels are increased in BCa primary tumors with osteolytic bone metastasis [128]; and (3) miR-124 inhibits bone metastasis by repressing IL-11 [131]. Although biologically interesting, these observations lack confirmation in large clinical trial sample sets.

Predictive biomarkers for bone metastasis preventive treatment

Metastatic speciation driven by a Darwinian selection process in mice identified CAPG and GIPC1 proteomically as significantly associated in a bivariate analysis with development of bone metastasis (Figure 3); the combination of these in primary tumor samples (identified by immunohistochemistry) was the strongest prognostic indicator [110]. In patients with high expression of both CAPG and GIPC1, the inclusion of adjuvant zoledronate reduced distant recurrence in bone (by 90%) compared with standard care; no differences were observed upon the inclusion of adjuvant bisphosphonate for patients with low expression of both [110]. Critically, however, both proteins have been associated with increased metastatic potential and poor outcome in some cancers [111–113]. A deeper understanding about the biology of CAPG beyond its regulation of cytoplasmatic and nuclear structures [114], and of GIPC1 at the peripheral membrane, is necessary to understand bone-specific features. For instance, is modulation of AKT/MDM2 and p53 axis downstream of GIPC1 signaling [114] bone-specific? What is their association with systemic estrogen levels and the menopause status?

Through a similar unbiased experimental approach, MAF (encoded from a gene within the 16q23 genomic gain) was shown to drive the molecular processes of bone colonization in ER^+ BCa cells (Figure 3). Association of 16q23 gain and relapse was retrospectively validated in a prospective randomized control arm of the AZURE clinical trial [119,120]. In particular, the 16q23

amplicon, which leads to high MAF levels, was found to be a prognostic factor for poor invasive disease-free survival (IDFS) in post-menopausal patients (Table 1). MAF is a transcription factor that regulates the expression of genes that collectively may support several steps of BCa cell metastasis, particularly to bone, through a series of cell-autonomous and niche-related functions [121]. These results imply that MAF transcriptionally controls functions required for bone metastasis – mainly adhesion to bone marrow-derived cells and osteoclast differentiation [121]. Collectively, these observations point to MAF as a molecular target for the prevention or treatment of bone metastasis, as MAF accumulation (16q23 gain) has a hierarchical role in bone colonization. Unexpectedly, the subgroup of AZURE patients with MAF^{-} tumors (according to MAF-test FISH; 79% of total patients) had a reduced risk of invasive disease progression [$HR_{IDFS} = 0.75$ (0.58–0.97)] and increased overall survival (OS) [$HR_{OS} = 0.69$ (0.50–0.94)] after adjuvant treatment with zoledronate, irrespective of menopause status [120]. In contrast, the patient subgroups with MAF^{+} tumors (and especially non-post-menopausal patients) developed visceral metastases, with significantly worse IDFS and OS, after adjuvant zoledronate treatment compared with those untreated [119].

As a nuclear protein with no enzymatic activity and an intrinsically disordered structure, MAF may be an extremely difficult therapeutic target. However, MAF downstream targets have the potential to become therapeutically actionable. Thus, several questions remained: (1) Are MAF downstream target gene products causal drivers of bone metastasis? (2) Are gene products transcriptionally controlled by MAF actionable? And (3) does targeting the aforementioned gene products prevent or reduce bone metastasis *in vivo*?

The MAF 'biomarker' explains for the first time (1) the association of anti-resorptive bone agents in the adjuvant setting and outcome, (2) benefits observed in post-menopausal patients, (3) the apparent lack of effect when considering all patients together in the AZURE trial outcome analyses, and (4) the very significant and unexpected adverse effects of zoledronate in MAF^{+} non-post-menopausal patients observed clinically. Remarkably, 79% of BCa patients who are MAF^{-} benefit from zoledronate, including those who are pre-menopausal. At the molecular level, further studies are needed to confirm these observations, which could drastically change the standard of care. It is tantalizing to speculate that in the very aggressive MAF^{+} tumors, bone nesting is restrained, yet cells are capable of nesting elsewhere, translating into increased extra-skeletal metastasis and poor clinical outcome [119].

Concluding remarks

Multidisciplinary approaches have been shown superior to control the expansion of bone metastasis; unfortunately, patients still suffer skeletal-related events, reducing quality of life and survival. Thus, there is an unmet need for identifying new treatments for patients who have already succumbed to advance disease. Remarkably, to increase the OS of patients with cancer, preventing metastasis is a more logical therapeutic strategy than managing advanced metastatic disease. Unfortunately, many patients are still at risk of relapse despite the significant improvements in surgical and systemic therapeutic approaches. Newer bone anti-resorptive treatment biomarkers could support treatments that prevent metastasis, by preventing expansion of DTCs at the distant site. Importantly, reports highlight that the use of specific biomarkers positively impacts OS when combined with adjuvant bisphosphonate treatment to prevent bone metastasis, pioneering a path to improve patient management. Careful patient selection for groups to be treated with bone resorptive therapies in the adjuvant setting will be critical, as trials in unselected populations failed to report benefits. Ideally, confirmation of these findings in an independent trial is needed. The capacity to predict the treatment response of these biomarkers might also be extended to other bone-modifying and targeted agents (i.e. anti-RANKL, anti-JAGGED, or Rad223 approaches). Mechanistically, systematic analyses are needed to better understand (1) the degree of overlap between the reported different biological findings, and (2) to what extent they are common with other tumor types that colonize bone. Further, different biomarkers may be interrelated within the same pathways or functional groups. The period with asymptomatic residual disease reflects the capacity of DTCs or micrometastases to endure long periods after tumor resection at very low numbers. Understanding the molecular basis responsible for DTC latency is required in order to deliver effective drugs that target core bone metastasis and not only the stroma. In the future, continuous efforts for identifying cancer patients at risk of distant metastasis are critical for defining new strategies of preventing secondary events.

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Author contributions statement

RRG conceived the review structure and themes. FS, ALL, and RRG wrote the manuscript.

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