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Review Article

Application of "omics" sciences to the prediction of bone metastases from breast cancer: State of the art

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ABSTRACT

Breast cancer (BC) is the most frequent malignancy and the first cause of cancer-related death in women.

The majority of patients with advanced BC develop skeletal metastases which may ultimately lead to serious complications, termed skeletal-related events, that often dramatically impact on quality of life and survival.

Therefore, the identification of biomarkers able to stratify BC patient risk to develop bone metastases (BM) is fundamental to define personalized diagnostic and therapeutic strategies, possibly at the earliest stages of the disease.

In this regard, the advent of "omics" sciences boosted the investigation of several putative biomarkers of BC osteotropism, including deregulated genes, proteins and microRNAs.

The present review revisits the current knowledge on BM development in BC and the most recent studies exploring potential BM-predicting biomarkers, based on the application of omics sciences to the study of primary breast malignancies.

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Contents

List of Abbreviations: ADAMTS1, a disintegrin-like and metalloproteinase with thrombospondin type 1; ALP, alkaline phosphatase; BALP (BSAP), bone-specific alkaline phosphatase; BC, breast cancer; BM, bone metastases; BTM, bone turnover markers; BOLCs, breast osteoblast-like cells; CAPG, capping-protein; CCN3, cellular communication network factor 3; CDH11, cadherin-11; CNV, copy number variation; CTGF, connective tissue-derived growth factor; CTSK, cathepsin K; CTX, C-telopeptide; CXCL, C-X-C-ligand; CXCR, C–X–C motif chemokine receptor; DEGs, differentially expressed genes; DOCK4, dedicator of cytokinesis protein 4; DPD, deoxypyridoline; DTC, disseminated tumour cells; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; ERRα, estrogen-related receptor alpha; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FST, follistatin; GIPC1, PDZ domain-containing protein member 1; Her, human epidermal growth factor; HR, hazard ratio; ICAM-1, intercellular adhesion molecule 1; IGF, insulin-like growth factor; IHC, immunohistochemistry; IL, interleukin; LC/MS/MS, liquid chromatography/mass spectrometry/mass spectrometry; MAF, v-maf avian muscolo aponeurotic fibro-sarcoma oncogene homolog; MDA-MB, MD Anderson metastatic BC; miRNAs, microRNAs; MMP1, matrix metalloproteinase-1; ncRNAs, noncoding RNA; NTX, N-telopeptide; OPG, osteoprotegerin; PDGF, platelet-derived growth factor; P1CP, pro-collagen type I C-terminal; P1NP, pro-collagen type I N-terminal; PlGF, placental growth factor; PgR, progesterone receptor; PRG1, proteoglycan-1; PTH-rP, parathyroid hormone-related protein; PYD, pyridoline; RANK, receptor activator of nuclear factor κ-B; RT-PCR, real time-PCR; SILAC-MS, stable isotope labelling by amino acids in cell culture-mass spectrometry; SNPs, single nucleotide polymorphisms; SPP1, osteopontin; SREs, skeletal-related events; TCGA, the cancer genome atlas; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor-α; TRACP-5b, tartrate resistant acid phosphatase-5b; VEGF, vascular endothelial growth factor; ZNF217, zinc-finger protein 217.

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1. Introduction

Breast cancer (BC) is the most frequent female malignancy worldwide, representing about one third of cancer diagnoses and the first cause of cancer-related death in women. In the United States of America, as many as 276,480 new cases and 42,170 deaths were estimated for 2020 [1], while 404,920 new BC in women were estimated in Europe for 2018 [2], with an age-adjusted annual incidence of 113.7/100,000 [3].

Despite these figures, BC mortality has progressively decreased in western countries, due to both the widespread diffusion of screening programs, leading to earlier diagnoses, and the therapeutic advances with innovative drugs which enabled more personalized treatments [4]. Indeed, the age-standardized mortality rate for BC in Europe has improved from 16.44 to 13.36 in the last decade [5].

The natural history of BC is frequently characterized by tumour cell seeding in the skeleton which, indeed, represents one of the most common sites of distant metastases. At the time of diagnosis, approximately 5–6% of BC patients exhibit bone metastases (BM), while up to 65–75% of women with advanced hormone receptor-positive breast tumours experience skeletal dissemination during the course of the disease [6,7].

It has been demonstrated that approximately 30% of BC patients host disseminated tumour cells (DTC) within the bone marrow, even in the absence of any clinical and radiological signs, and these metastatic niches constitute the reservoir for subsequent tumour recurrence [8].

BM may be either asymptomatic or symptomatic, with bone pain representing the most frequent clinical presentation. Moreover, several patients develop serious complications termed skeletal-related events (SREs) which include hypercalcaemia, pathological fractures, spinal cord injuries and intractable pain requiring palliative radiotherapy or surgery. Besides interfering with patients' quality of life, SREs severely affect their autonomy and negatively impact on survival [9,10].

Randomized clinical trials have demonstrated that the addition of bisphosphonates to standard adjuvant treatments significantly reduces the risk of BM onset in a sub-population of BC patients [11–15], although their administration did not improve hard end-points such as survival, not to take into account that these agents are not free from potential side effects and require adequate patient monitoring [14].

In this context, the discovery of biomarkers suitable to define the risk of BM since the time of BC diagnosis would enable the timely identification of high-risk patients, with appropriate implementation of diagnostic and therapeutic strategies. Several authors attempted the identification of specific bone turnover markers (BTM) potentially correlated with the onset of BM and their evolution during treatment. However, BTM levels are influenced by a number of factors, including patients' age, food intake, circadian and seasonal fluctuations, as well as concomitant diseases and medications [16], making their interpretation often unaffordable in clinical practice, though intriguing.

More recently, "omics" sciences have been applied to the identification of molecules and/or genomic aberrations of primary tumours as potential prognostic biomarkers; however, their wide-scale clinical application has to be established yet [17].

Here we review the most compelling research in BC aimed at identifying such potential biomarkers of osteotropism, based on the development of omics technologies in the "precision medicine" era.

2. Pathogenesis of BM in BC

According to their clinical behavior, metastatic breast malignancies can be classified into three main categories, namely tumours seeding the skeleton only, which exhibit a more favorable outcome, those giving origin to extra-skeletal metastases, and neoplasms seeding in both skeletal and visceral sites [18].

In women with early BC, several clinico-pathological features have been proposed as risk factors for subsequent development of BM, including primary tumour size larger than 2 cm, the expression of estrogen and/or progesterone receptors (ER and PgR, respectively), the presence of more than four metastatic lymph nodes and young age at the time of diagnosis [19].

Besides these factors, invasive ductal histotype as well as high concentration of alkaline phosphatase (ALP) and serum tumor markers (e.g. CA 15.3 and CA 125), together with low hemoglobin levels, have been closely related to the development of BM [20].

BM from BC are usually lytic, whereas approximately 15–20% of patients exhibit osteoblastic or mixed lesions [6]. The axial skeleton is the most frequent site of BC seeding in bone [21].

BC osteotropism is regulated by different gene signatures and signaling pathways activated in malignant epithelial cells. Indeed, the development of BM from BC is a multi-step process (Fig. 1) in which, on the one hand, BC cells undergo epithelial-to-mesenchymal transition (EMT), namely a reversible process characterized by the loss of intercellular junctions, the acquisition of spindle-like shape and high motility and invasiveness, as well as the up-regulation of mesenchymal markers at the expense of epithelial ones [22].

On the other hand, BC cells release exosomes, growth factors (e.g. transforming growth factor beta, TGF-β; vascular endothelial growth factor, VEGF; placental growth factor, PlGF) and cytokines, such as tumour necrosis factor-α (TNF-α), that recruit bone marrow-derived stromal cells to prepare and establish the "pre-metastatic niches", to sustain metastatic tumour cell growth and proliferation.

Chemokine/chemokine receptor axes and some integrin complexes, such as αvβ3 and α4β1, are involved in tumour cell tropism towards bone. Interestingly, C–X–C motif chemokine receptor-4

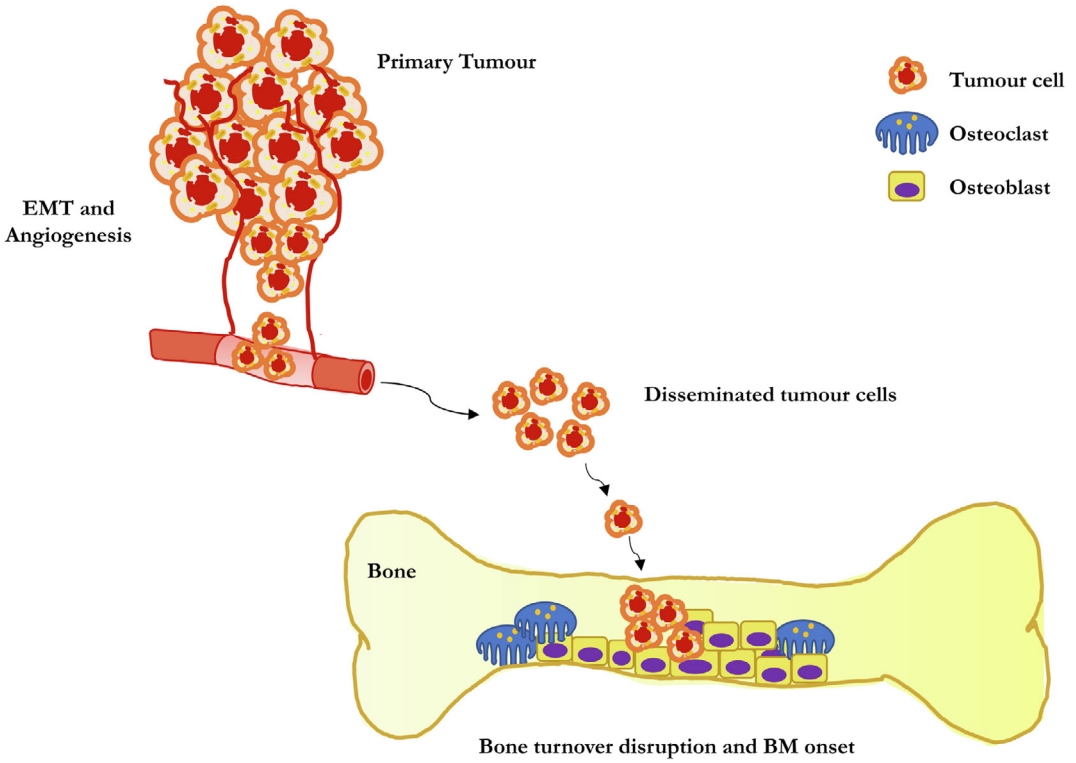

Fig. 1. The multi-step process leading to the development of BM in BC. Within the primary tumour, BC cells may undergo EMT while releasing exosomes, cytokines and growth factors to recruit bone marrow-derived stromal cells. The latter participate in the establishment of "pre-metastatic niches", resulting from increased vascular permeability, remodeling of the ECM and induced immunosuppression. Months or years after their seeding in the skeleton, DTC may start proliferating inside the niches, acquiring the phenotypic characteristics of bone cells (osteomimicry) and causing a progressive alteration of the physiological turnover, shifting its balance towards excessive osteolysis or osteogenesis, which ultimately lead to the development of clinically detectable BM.

(CXCR-4)/CXC-ligand-12 (CXCL-12) axis has been demonstrated to drive the bone homing process of several malignancies, including BC [23,24]. More recently, CXCR-2 and its ligands CXCL-5, CXCL-6 and CXCL-8 have been found capable to sustain the processes of bone homing and proliferation in BC cells [25,26]. In addition, the expression of calcium sensing receptor and the receptor activator of nuclear factor κ-B (RANK) have been shown to participate in BC cell attraction towards osteolytic areas [27,28].

Once nested in bone, BC cells undergo the so-called "osteomimicry" process, namely the acquisition of typical bone cell-markers (e.g. intercellular adhesion molecule 1, ICAM-1; cadherin-11, CDH11; osteopontin, SPP1; bone sialoproteins; cellular communication network factor 3, CCN3; etc) with consequent evasion of immune response [29–31].

DTC may start proliferating inside the niches several months or years after bone colonization, causing a progressive alteration of its physiological turnover. In particular, tumour cells produce pro-osteoclastogenic factors (e.g. TNFα; interleukin 8, IL-8; parathyroid hormone-related protein, PTH-rP, etc) which enhance bone resorption. The latter, in turn, perpetuates this circle through the release of growth factors physiologically stored in the bone matrix, such as TGF-β, insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) that further sustain the proliferation of cancer cells [30].

The mechanisms supporting sclerotic bone formation in BC are not completely elucidated, although a number of tumour-derived growth factors, including TGF-β, bone morphogenetic proteins and fibroblast growth factor (FGF) have been found capable to enhance the differentiation of mesenchymal progenitors into osteoblasts, shifting the balance of bone turnover in favor of osteogenesis [7,32].

3. Current knowledge on BTM role in the management of bone-metastatic BC

3.1. Physiological bone turnover and BTM release

Physiologically, bone turnover results from the balanced activities of osteoclasts and osteoblasts. The former derive from monocyte-macrophages and are responsible for bone resorption by releasing protons and enzymes, such as tartrate resistant acid phosphatase 5b (TRACP-5b) and cathepsin K (CTSK). During this process, type I collagen is cleaved and its degradation peptides (e.g. N-telopeptide, NTX; C-telopeptide, CTX; Pyridoline, PYD; Deoxypyridoline, DPD) become detectable in both blood and urine [33]. By contrast, the latter derive from mesenchymal stem cells and are deputed to bone tissue formation and production of pro-collagen, whose cleavage at C- and N-terminals releases serum pro-collagen type I C-terminal (P1CP) and N-terminal (P1NP) pro-peptides [34]. Osteoblasts secrete also bone-specific alkaline phosphatase (BALP or BSAP) that contributes to bone matrix mineralization [30].

Bone turnover is regulated by RANK/RANK-ligand (RANK-L)/osteoprotegerin (OPG) axis. In particular, stromal cells and osteoblasts release RANK-L that, by interacting with its receptor (RANK) expressed by pre-osteoclasts and osteoclasts, primes their differentiation and activation. In order to prevent excessive bone resorption, OPG acts as a soluble decoy receptor for RANK-L. Several systemic factors, including sex hormones, vitamin D, parathyroid hormone and calcitonin contribute to the maintenance of such a delicate equilibrium [35].

3.2. Clinical studies investigating BTM applications in bone metastatic BC

Several authors explored the potential applications of BTM in the management of skeletal metastases from BC. In this context, both bone formation and bone resorption markers were investigated for their putative diagnostic role, due to the significantly higher levels found in bone metastatic BC patients, as compared to those without BM [36,37], and their correlation with the extent of skeletal disease [38].

Moreover, elevated levels of either serum or urine NTX were associated with increased risk of SREs, disease progression and death [39,40] while, among bone formation markers, BALP correlated with subsequent skeletal complications [40].

Interestingly, Elfar and coworkers attempted to integrate serum levels of RANK-L and OPG into a single parameter (RANK-L/OPG ratio), which yielded a 73% sensitivity and 72% specificity for BM detection [41]; Lumachi and colleagues described similar data after combining TRAcP-5b with BALP and P1NP [42]. Such results confirmed that the alteration of both bone turnover phases critically contributes to the establishment of BC-related bone disease. In this regard, P1NP and CTX higher serum levels have recently emerged as prognostic for subsequent BM development ($p < 0.05$ in both instances) in patients enrolled in the AZURE clinical trial of chemotherapy and/or hormone therapy with or without zoledronate, in women with stage II or III BC [43].

The clinical application of BTM has also been attempted in bone metastatic patients receiving anti-resorptive drugs, with the purpose to develop surrogate biomarkers of response to treatment. Interestingly, urine NTX levels not only reflected the response to bisphosphonates [44] but also predicted their efficacy in patients with highly aggressive metastatic bone disease [45]. Moreover, the persistence of either urine NTX or serum BALP levels above the normal range after three months of anti-resorptive treatment was associated with poor clinical outcome, in terms of overall survival, risk of disease progression at any site and risk of progression in bone [46].

However, the high intra- and inter-individual variability found in BTM measurement, due to both patient features and technical issues [30], have limited their routine clinical application, especially for diagnostic purposes, underlying the need for more reliable and reproducible biomarkers.

4. Application of "omics sciences to the early identification of osteotropic breast malignancies

In the past decade, a rapid development of omics sciences applied to cancer research has provided improved knowledge of both tumour biology and genes involved in cell proliferation and metastasis [47]. This enabled the discovery of druggable molecular targets and dramatically improved the clinical management of several solid malignancies, paving the way to the so called "precision medicine" era [48].

The "omics" sciences include the study of genes (genomics), epigenetic processes (epigenomics), transcripts (transcriptomics), proteins (proteomics) and metabolites (metabolomics) that are expressed by a cell, organ or a whole organism (Fig. 2). Among omics science applications in oncology, genomics includes the study of cancer germline and somatic DNA sequence variants, such as mutations, single nucleotide polymorphisms (SNPs) and copy number variations (CNV) [49]. Non-small cell lung cancer is an emblematic example of neoplasm whose natural history has radically changed since driver gene mutations were identified and targeted therapeutic agents were developed [50]. A similar advance

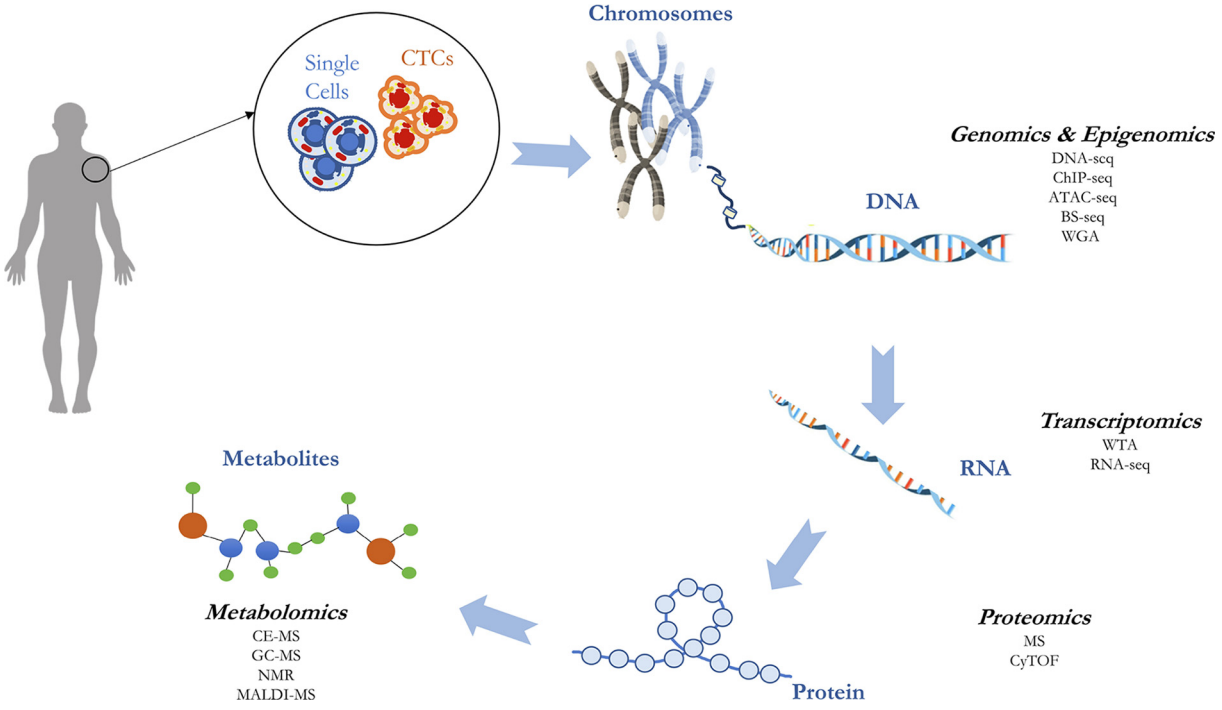

Fig. 2. "Omics" technology portrait. The figure provides an overview of the omics technologies currently applicable to cancer research, based on the analysis of primary/secondary tumour samples and circulating tumour cells (CTCs). Four major categories of these technologies, based on high-throughput methods (listed in the picture), are commonly performed, namely genomics and epigenomics (i.e. analysis of the DNA sequence), transcriptomics (analysis of transcribed RNA), proteomics (analysis of proteins) and metabolomics (identification and quantification of all cell metabolites). List of abbreviations: DNA-Seq, DNA sequencing; ChIP-seq, chromatin immunoprecipitation sequencing; ATAC-seq, assay for transposase-accessible chromatin using sequencing; BS-seq, bisulfite sequencing; WGA, Whole Genome Amplification; WTA, Whole Transcriptome Amplification; RNA-seq, RNA sequencing; CyTOF, cytometry by time of flight; MS, mass spectrometry; CE-MS, Capillary electrophoresis mass spectrometry; GS-MS, Gas chromatography mass spectrometry; NMR, nuclear magnetic resonance; MALDI-MS, Matrix Assisted Laser Desorption/Ionization mass spectrometry.

Table 1
Main studies on genomic alterations related to BC osteotropism.

PUTATIVE BIOMARKERS	TECHNIQUE OF BIOMARKER INVESTIGATION	VALIDATION ON CELL LINES OR CLINICAL SAMPLES	MAJOR MECHANISMS OF BM REGULATION	REFERENCES
ND	NGS mutational analysis of 50 commonly mutated cancer-related genes	389 BC patients	ND	[54]
MAF	Oligonucleotide Array Assays and FISH	Bone-homing human BC cell lines 334 primary BC	Control of tumour cell/bone stroma interaction; regulation of cell adhesion, migration and osteoclast differentiation; regulation of	[55]
	FISH	1739 BC patients enrolled in AZURE clinical trial	PTH-rP expression.	[56]
102-gene signature, including *CXCR4, FGF5, CTGF, IL11, MMP1, FST, ADAMTS1, PRG1 (fold change > 4)*	Microarray analysis	MDA-MB-231 human BC cell subclones divided in weakly and highly metastatic to bone	Modulation of tumour cell invasion, angiogenesis, bone-homing, osteoclastogenesis and osteoclast activation	[57]
31-gene signature	Microarray analysis	Human B02 BC cells derived from BM caused by MDA-MB-231 cells	Osteoblast differentiation	[58]
APOPEC3B, ATL2, C6orf61, C6orf167, KCNS1, MFAP3L, NIP7, NUP155, PALM2, PGD5, SFT2D2, STEAP, NAT1, BBS1, PH-4.	Microarray analysis	157 metastatic BC patients + a dataset of 376 BC used as validation cohort	EMT, invasion/migration, bone-homing	[47]
IL-1B	RT-PCR IHC	Bone-homing sub-clone of MDA-MB-231 cells; 150 primary BC	Bone homing	[59]
	RT-PCR	MDA-MB-231 human BC cell line; 1300 patients included in AZURE clinical trial	promotion of EMT, invasion, migration, and bone homing	[60]
IL-6 gene signature	Microarray analysis RT-PCR	Co-culture of MDA-MB231 BC cell line with osteoblast; 295 early stage BC from Netherlands Cancer Institute	osteoclast activation, BC stem cell biology	[62]
ZNF217 gene signature	RT-PCR	113 women with primary breast tumours	tumour cell invasion/migration, EMT, osteogenesis, bone remodeling and metastasis	[64]
FOXF2	RT-PCR	118 primary BC tissues	EMT, bone homing, epithelial-to-osteomimicry transition	[65]
ESR1, PGR, BCL2, REPS2, NAT1, GATA3, ANXA9, C9orf116	Nanostring Gene expression analysis	92 BC patients with and without BM	stimulation of cell proliferation, apoptosis inhibition and modulation of metastatic profile acquisition	[66]
66-gene signature	Computational Analysis	Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) genomics data repository	extracellular matrix (ECM)-receptor interaction, calcium signaling, Wnt, PI3K/AKT signaling and focal adhesion kinase (FAK)	[67]

Abbreviations not mentioned in the main text: ND; Not described; NGS, next generation sequencing; FISH, Fluorescent in situ hybridization;

was reached in other diseases, such as melanoma, colorectal and breast cancers [48]. Other applications of omics sciences in oncology include the study of cancer transcriptome and gene expression profile [49,51], the search for peptides and epigenetic modifications able to act as prognostic and predictive biomarkers, as well as novel therapeutic targets [52].

The next sections will summarize recent attempts made by researchers to define BC osteotropism, by applying the above mentioned approaches.

4.1. Identification of genomic alterations related to BC osteotropism

BC is a heterogeneous family of malignancies, whose therapeutic management needs to be adjusted to molecular tumour features. Indeed, based on the expression of ER and PgR and/or the presence of human epidermal growth factor (Her)-2 amplification, different targeted therapies can be proposed, both in the early disease as well as in the metastatic setting. Furthermore, commercial tests (i.e. Oncotype Dx, MammaPrint and PAM50) have recently been developed to identify the risk of relapse in the early disease setting [53].

With respect to extensive mutational analyses performed in the "osteotropism" field of research, 389 primary BC samples were screened for the presence of hot spot somatic mutations within 50 common cancer-related genes (including *ERBB2*, *PIK3CA*, *TP53* and others) by next-generation sequencing, without finding any significant associations between gene alterations and the onset of bone-only metastases as first relapse [54].

On the other hand, the application of genomics techniques led to the identification, in bone-homing BC cell lines, of a 16q23 gain copy number aberration encoding the transcription factor v-maf avian muscolo aponeurotic fibro-sarcoma oncogene homolog (MAF) that regulates the expression of several genes involved in BC spreading to bone, including PTH-rP. Interestingly, this CNV was also tested in an independent validation set of 334 primary BC patient samples, and the presence of at least 1.5 copies of the region, normalized to the CEP16 centromeric probe, significantly correlated with BM occurrence at any time (hazard ratio, HR = 14.5, 95% CI = 6.4 to 32.9, $p < 0.001$) [55]. Moreover, MAF over-expression was associated with high-risk primary tumour features and worse prognosis in pre-menopausal BC patients enrolled in the zoledronate arm of AZURE clinical trial, further confirming the potential utility of this molecular alteration in the management of BC [56] (Table 1).

One of the first attempts made to identify a gene expression profile associated with BC osteotropism was described by Kang and coworkers who, by using microarray analysis on several subclones of MD Anderson metastatic BC (MDA-MB)-231 cell line, divided in two groups based on their degree of osteotropism, identified 102 differentially expressed genes (DEGs) between the two cell categories. Among over-expressed genes found in bone-homing cells, those with a fold change greater than 4 were *CXCR4*, *FGF5*, connective tissue-derived growth factor (*CTGF*), *IL11*, matrix metalloproteinase-1 (*MMP1*), follistatin (*FST*), a disintegrin-like and metalloproteinase with thrombospondin type 1 (*ADAMTS1*) and proteoglycan-1 (*PRG1*). This signature included genes encoding both secreted and cell surface proteins, playing roles in different steps of BM onset, such as tumour cell invasion and homing towards the skeleton, as well as osteoclastogenesis and osteoclast activation [57] (Table 1). In addition, functional studies performed on female BALB/c-nu/nu nude mice showed that the osteoclastic and angiogenic capacities of IL-11 and CTGF were increased by the activity of the pro-metastatic cytokine TGFβ [57].

Similarly, Bellahcène and co-authors, by using microarray analysis, performed a transcriptomic study on B02 cells, namely a subclone derived from the MDA-MB-231 cell line and characterized by high capacity to form BM in vivo. The analysis demonstrated the presence of a cluster of 31 DEGs (20 up- and 11 down-regulated) related to the osteoblastic differentiation process [58].

Later, Savci-Heijink and coworkers identified by a similar microarray analysis a smaller signature including 15 genes expressed in up to 82.4% of bone metastatic breast malignancies, on a series of 157 primary BC samples and on a public dataset of 376 breast tumours, used as validation cohort. In this panel, 12 down-regulated genes (*APOPEC3B*, *ATL2*, *C6orf61*, *C6orf167*, *KCNS1*, *MFAP3L*, *NIP7*, *NUP155*, *PALM2*, *PGBD5*, *SFT2D2* and *STEAP*) encoded several membrane-bound proteins and peptides, while the overexpressed *NAT1*, *BBS1* and *PH-4* ones were involved in the EMT process [47]. However, despite such a huge panel, none of the genes of this putative bone signature overlapped the DEGs previously identified by Kang et al. [57].

In subsequent studies, IL-1 β gene expression was found by Real Time-PCR (RT-PCR) significantly up-regulated in another bone-homing sub-clone of MDA-MB-231 cells. On this basis, 150 primary BC cores, belonging to patients undergone long-term follow-up after adjuvant anti-cancer treatment, were analyzed for IL-1 β expression by immunohistochemistry (IHC), observing a statistically significant correlation between the levels of this biomarker in the primary tumour and the subsequent development of BM ($p < 0.0001$) [59].

These data have been recently confirmed on 1,300 patients included in AZURE clinical trial [60] and have led to the successful pre-clinical evaluation of Anakinra, namely an IL-1 β receptor antagonist, and Canakinumab, a monoclonal anti-IL-1 β antibody, for the prevention and treatment of BM in murine models of BC [60,61]. Interestingly, the administration of either Anakinra or Canakinumab significantly reduced the number of circulating BC cells and the size of bone skeletal lesions in mice, confirming the role of IL-1 β signaling in the BM process [60].

In a similar fashion, Rajski and co-workers investigated the presence of a specific gene cluster named "IL-6 gene signature", consisting of 72 DEGs, first detected in a co-culture of MDA-MB231 cells with osteoblasts and subsequently identified in 295 early BC specimens from the Netherlands Cancer Institute. The authors described a correlation between the presence of such signature and BM-free survival at 10 years (74% vs 83% in patients with *high* vs *low* gene expression levels, respectively; $p = 0.048$) [62]. Interestingly, a more recent study has showed that a monoclonal antibody against the human IL-6 receptor (Tocilizumab) reduced lytic BM development in a murine model of BC, by interfering with tumour cell proliferation, neoangiogenesis, and RANK signaling [63].

Bellanger and co-workers evaluated the zinc-finger protein 217 (ZNF217) gene expression in 113 samples from primary BC and observed that higher mRNA levels were associated with shorter metastasis-free survival ($p = 0.023$) and bone-only metastases ($p = 0.005$). Subsequent transcriptomic analyses on MDA-MB-231 cells stably transfected with ZNF217 demonstrated that increased levels of ZNF217 were associated with the presence of a cluster of 67 DEGs, correlated with the functions of osteogenesis, bone remodeling and metastasis [64]. Interestingly, 17 out of 67 genes (25%) and 28 of 67 genes (42%) overlapped those detected in the gene expression signatures previously described by Kang [57] and Bellahcene [58], respectively.

More recently, Wang and co-workers have demonstrated, by investigating data sets of human BC cell lines and then primary breast tumour mRNAs, that high levels of forkhead box F2 (*FOX2*) were related to BM. The biological process underlying the role of this molecule, also ascertained with in vivo studies, relies in the capability of FOXF2, as a master transcription factor, to induce cancer cells to develop into BM seeds through the "epithelium-to-ost

Table 2
Main studies on miRNAs related to BC osteotropism.

PUTATIVE BIOMARKERS	TECHNIQUE OF BIOMARKER INVESTIGATION	VALIDATION ON CELL LINES OR CLINICAL SAMPLES	MAJOR MECHANISMS OF BM REGULATION	REFERENCES
miR-373, miR-520c	RT-PCR	MCF-7 human BC cell line; 11 pairs of matched primary breast cancer and lymph node metastasis tumour samples	promotion of BC cell migration and invasiveness by suppression of CD44	[69]
miR10b	RT-PCR	HMECs, MCF-10A, MCF-7, MDA-MB-231, HEK293T, SUM149, SUM159, SUM1315, 67NR, 168FARN, 4TO7 and 4 T1 cell lines; 18 metastatic BC patients	promotion of BC cell migration and invasiveness	[70]
miR218	RT-PCR	MC3T3-E1 murine and MCF10A human cell line	control on Wnt signaling to promote the osteomimicry of metastatic cancer cells	[71]
miR218-5p	RT-PCR	MCF-10, MCF-7, MDA-MB-231-a, MDA-MB-231-b cells; tissue biopsies from BM of BC patients	promotion of proliferation, increase of Wnt PTHrP signaling and osteclast differentiation	[72]
miR19a, miR93, miR106a	Computational Analysis Small RNA sequencing	Dataset of 1051 BC patients from TCGA 20 BC patient samples	promotion of tumour cell survival and proliferation, stimulation of angiogenesis	[73]
miR126, miR335, miR206	Array-based miRNA profiling RT-PCR	Parental and subclones MDA-MB-231 human BC cell line; 20 primary breast tumors	mir126 reduces tumour proliferation; miR-335 and mir206 suppress tumour cell migration and metastasis	[74]
miR124	ISH	Human BC cell lines (BT-549, MDA-MB-231, Hs578T, MDA-MB-468, MDA-MB-436, MCF7, T47D, BT-474, RAW264.7 and MC3T3-E1);79 pairs of primary BC tissues and para-tumour tissues + 34 BM samples from BC patients	inhibition of tumour cell migration and invasion	[75]
miR-135, miR203	RT-PCR	MCF-10A, MCF-7, MDA-MB-231-a, MDA-MB-231-b cell lines; tissue biopsies derived from primary tumors and BM of patients with BC	decreased expression of Runx2 and target genes as- IL11, MMP-13, and PTHrP;suppression of tumour cell migration and proliferation	[76]
miR-429	ISH, H&E, WB, RT-PCR	MDA-MB-231-a, MDA-MB-231-b cell lines. 21 BM specimens including 7 paired BM tissue and primary BC tissue samples	regulation of bone microenvironment; suppression of CrkL and MMP-9	[77]
miR30 family	RT-PCR	109 BC patients	inhibition of invasion, osteomimicry, and bone destruction by directly targeting multiple BM- associated genes	[78]

Abbreviations not mentioned in the main text: ISH, In situ hybridization; H&E, Hematoxylin and eosin staining; WB, Western Blotting.

Table 3
Main studies on proteins involved in BC osteotropism.

PUTATIVE BIOMARKERS	TECHNIQUE OF BIOMARKER INVESTIGATION	VALIDATION ON CELL LINES OR CLINICAL SAMPLES	MAJOR MECHANISMS OF BM REGULATION	REFERENCES
ICAM-1, cadherin-11, osteoactivin, bone sialoprotein, CCN3, IL-11, CCL2, CITED2, CXCR4, CTGF, OPN, CX3CR1, TWIST1, adrenomedullin, Enpp1	multiple techniques	not applicable (review)	cell proliferation, differentiation and adhesion, chemokine signaling and bone mineralization	[79]
CAPG, G1PC1	LC/MS/MS IHC	Metastatic variants of the human BC cell line MDA-MB-231 homing to bone (BM1, BM2); 724 BC pts enrolled in AZURE trial	cell cycle regulation, cell adhesion and migration	[80]
DOCK4	SILAC-MS	Metastatic variants of the human BC cell line MDA-MB-231 homing to bone (BM1); 689 patients enrolled in AZURE trial	promotion of cancer cell invasiveness	[81]
TGF-β, vimentin	IHC	64 breast infiltrating carcinomas, 50 breast benign lesions, and 10 biopsies of BM	promotion of EMT	[82,83]
ERRα	IHC	100 BC tissue samples + 446 specimens from unilateral invasive radically-resected BC patients	RANK axis regulation and promotion of tumour migration/ proliferation	[85]

eomimicry transition", namely the acquisition of osteomimetic features through the ectopic expression of genes related to the early stages of bone differentiation [65].

In a recent study by Cosphiadi et al, 22 genes have been found differentially expressed between BC samples derived from patients with BM (with/without other extra-skeletal recurrence) and tumour samples belonging to women without skeletal lesions. Once focusing on the comparison between BM-only associated tumours and non-bone metastatic BC, 17 DEGs were identified. In particular, the two differential analyses shared eight DEGs, namely *ESR1*, *PGR*, *BCL2*, *REPS2*, *NAT1*, *GATA3*, *ANXA9* and C9orf116, which may deserve validation in prospective clinical studies as putative osteotropism biomarkers [66].

Finally, Chen and co-workers have used a novel computational approach to identify the molecular mechanisms associated with BC osteotropism. The authors have analyzed a microarray gene expression profile dataset relative to patients with BC from the Gene Expression Omnibus (GEO) data repository and applied a Significance Analysis of Microarrays (SAM) to obtain a specific signature of 66 DEGs. Through a functional protein association network construction, various pathways have been identified, such as extracellular matrix (ECM)-receptor interaction, calcium signaling, Wnt, PI3K/AKT signaling and focal adhesion kinase (FAK) ones, closely associated with the ability of BC to metastasize to the skeleton [67].

Clearly, the huge number of data emerged from such an extensive research confirms the importance of this topic and the high complexity and heterogeneity of breast malignancies, whose molecular characterization is, probably, far from being completed. None of the mentioned signatures has presently entered the routine clinical practice, suggesting the need for further, prospective investigation.

4.2. Role of microRNAs (miRNAs) in the establishment of BM from BC

Even if not encoding protein products, noncoding RNA (ncRNAs) are critical regulators of cellular processes. In particular, miRNAs are small (approximately 22 base pairs) ncRNAs that act as epigenetic modulators by targeting the turnover of specific mRNAs, through which they regulate a huge number of processes, including signal transduction, cell cycle, cell proliferation and metabolism, all of them playing key roles in cancer [68,69]. miRNAs have been investigated for their capability to participate in the bone homing process and act as potential biomarkers of BC skeletal colonization (Table 2).

In this regard, preclinical studies showed that the up-regulation of miR-10b, miR-373 and miR-520c was correlated with migration and invasiveness of BC cells, both in vitro and in vivo [69,70], while miR-218 acted on Wnt signaling to promote the osteomimicry of metastatic cancer cells [71].

In the latter case, it has also been proven that the overexpression of miR-218-5p in BC cells is able to reduce the Wnt inhibitors SOST and sFRP-2, resulting in the activation of Wnt signaling that, in turn, influences the process of metastatization to bone through both autocrine and paracrine mechanisms. Indeed, anti-miR-218-5p delivery to MDA-MB-231 cells impaired their growth in bone microenvironment and interrupted the vicious cycle of osteolytic BM in vivo [72].

More recently, a miRNA signature score (including miR19a, miR-93 and miR 106a) has been developed by using a bioinformatics approach applied to a dataset of 1051 BC patients from The Cancer Genome Atlas (TCGA) network. The *high* miRNA-based score, determined in primary tumour samples and further validated in three independent cohorts, significantly correlated with poor BC patient prognosis ($p = 0.0005$) and risk of BM onset ($p = 0.0052$) [73].

On the other hand, miR-126, miR-335 and miR-206 have been shown to restrain tumour cell proliferation, migration and invasiveness. An array-based miRNA profiling of MDA-MB-231 cell sub-clones with different organotropism showed down-regulation of those miRNAs in bone-homing cells, while their up-regulation, induced by retroviral transduction, significantly impaired BM onset in mice. Moreover, low expression of miR-335, miR-126 or miR-206 in primary BC samples correlated with shorter median time to metastasis [74].

Similarly, Cai and coworkers described by in situ hybridization the down-regulation of miR-124 in a bone metastatic sub-clone of MDA-MB-231 cells, as well as in primary BC and BM specimens [75]. Based on their results, the authors argued that low levels of miR-124 determine aggressive clinical characteristics and shorter BM-free survival. Notably, lentivirus-induced stable miR-124 expression in tumour cells impaired BM formation in a murine model of BC, while the administration of a specific inhibitor exerted an opposite effect. The authors also described a negative

correlation between the miRNA levels and IL-11 expression, in both BC cell lines and human metastatic bone tissues, suggesting this pro-osteoclastogenic cytokine as a potential downstream target of miR-124 [75].

Interestingly, the aberrant expression of Runx2, namely a transcription factor which participates to BM development by modulating the expression of bone-related genes, including *IL-11, MMP-13* and *PTHrP*, has been associated with the loss of miR-135 and miR-203 in osteotropic BC cells [76].

Other miRNAs that have emerged as inhibitors of the BM process include miR-429, that has been recently shown to counteract osteoclast differentiation while promoting osteoblastogenesis in vitro, and to restrain skeletal destruction in murine models of BC, by negatively regulating the expression of V-crk sarcoma virus CT10 oncogene homolog-like (CrkL) and MMP-9 genes [77].

In a similar fashion, members of miR-30 family have been recently defined as suppressors of BC homing to bone through the regulation of multiple processes including osteomimicry and tumour cell invasion; moreover, among the target genes of miR-30 family, *CTGF*, *ITGB3*, *ITGA5*, *DKK-1*, *RUNX2, IL-8*, *TWIF1/IL-11*, and *CDH11* emerge for their critical role in BM development. In agreement with these data, low expression of mi-R30s in primary BC samples was associated with poor relapse free survival [78].

The results of all these studies suggest that miRNAs could act not only as potential prognostic biomarkers for skeletal colonization, but also promising therapeutic targets to restore physiological bone homeostasis and counteract cancer-induced skeletal destruction, although further clinical investigation is needed before any clinical application of these molecules.

4.3. Proteomics studies applied to the investigation of BC osteotropism

Several peptides have been investigated as potential BM-predicting biomarkers in BC (Table 3), to be theoretically employed for patient stratification and selection for adjuvant bisphosphonate treatment.

Indeed, a systematic review by Awolaran and coworkers summarized the state of the art up to 2016 and collected data on 15 proteins whose over-expression in BC cells had been correlated with BM onset in vivo. This panel included molecules involved in key cell processes (e.g. proliferation, differentiation and adhesion) as well as chemokine signaling and bone mineralization [79].

In a quantitative proteomic analysis performed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) on both parental and bone-homing sub-populations of MDA-MB-231 cells, a number of peptides, including the macrophage capping-protein (CAPG) and PDZ domain-containing protein member 1 (GIPC1), were found significantly up-regulated in the latter and further investigated as prognostic biomarkers [80]. In particular, the expression of both CAPG and GIPC1 was subsequently investigated and clinically validated by IHC on 427 primary BC samples belonging to patients enrolled in the already mentioned AZURE clinical trial [12,14] and a significant correlation was described between the up-regulation of both proteins and the risk of BM onset (HR = 4.5, 95% CI = 2.1 to 9.8, $p < 0.001$). Interestingly, high-risk patients ($CAPG^{high}/GIPC1^{high}$) substantially benefited from adjuvant zoledronate administration in terms of HR reduction for first recurrence in bone (10-fold reduction vs placebo, $p = 0.008$) [80].

By using a quantitative proteomics approach with stable isotope labelling by amino acids in cell culture-mass spectrometry (SILAC-MS), Westbrook and co-workers identified another protein up-regulated in the bone-homing MDA-MB-231 sub-clone, namely high dedicator of cytokinesis protein 4 (DOCK4). In a successive clinical validation step, DOCK4 over-expression in early BC samples from AZURE trial was significantly correlated with first recurrence in the skeleton in control (HR 2.13, 95%CI 1.06–4.30, $p = 0.034$), but not in zoledronate arm (HR 0.812, 95%CI 0.176–3.76, $p = 0.790$), confirming the capability of the bisphosphonate to prevent BM in high-risk patients; notably, DOCK4 up-regulation showed no correlation with extra-skeletal dissemination ($p = 0.08$) [81].

Scimeca et al. have recently demonstrated that the appearance of casting-type calcifications within breast tissues could be related with the formation of osteoblast-like cells (BOLCs) which might play a role also in the development of BM from BC [82]. In a subsequent work, the authors have detected by IHC a significant up-regulation of TGF-β and vimentin in BC samples with high BOLC count ($p < 0.0001$ in both instances), suggesting that the hyper-activation of the EMT process could be crucial in the progression of such malignancies [83].

In addition, the expression of estrogen-related receptor alpha (ERRα) has been recently correlated with tumour cell invasiveness, RANK expression by cancer cells and spontaneous formation of bone micrometastases in a murine model of BC. Moreover, ERRα levels have been evaluated by IHC on primary BC samples derived from patients enrolled in the ABCSG-6 trial [84] demonstrating a significant correlation between its over-expression and poor distant-recurrence free survival. A transcriptomic analysis on BC samples has further confirmed this association, leading to the pre-clinical pharmacological inhibition of ERRα through a specific inverse agonist, which has successfully reduced both primary tumour growth and bone micro-metastases formation in mice [85].

5. Conclusions and future perspectives

BM represent a common and feared complication of BC, responsible for significant quality of life impairment, poor prognosis and socio-economic consequences, especially when accompanied by the onset of SREs [7].

Once DTC colonize the skeleton, curative aims cannot be pursued, and BC patients usually receive both anti-cancer drugs and bone-targeting agents for symptom palliation and prevention of disease progression [7]. On the other hand, the administration of bisphosphonates in the adjuvant setting has been shown to reduce the risk of BM development in postmenopausal BC patients and young women undergoing ovarian suppression [11–15], and has been recently introduced in European guidelines for BM prevention [86].

In this context, there is still an unmet need for reliable and reproducible means to quantify BM risk since the earliest stages of the disease, in order to plan appropriate and personalized follow-up and therapeutic strategies, potentially able to modify the course of the disease. Moreover, elucidation of the cellular and molecular mechanisms underlying BC homing towards the skeleton could contribute to the development of further therapeutic agents to manage and, hopefully, prevent BM.

Several authors have pursued this aim, by applying omics technologies to the study of primary BC samples, with the purpose to identify prognostic and predictive biomarkers. A huge number of molecules has been identified so far, including proteins, miRNAs and genes, whose aberrant structure and/or expression have been correlated with several steps of BM development. Among these, *MAF* CNV as well as CAPG/GIPC1 and DOCK-4 over-expression in the primary tumour have been found to correlate not only with subsequent BM onset, but also with adjuvant zoledronate effectiveness [56,80,81]. Moreover, based on results of pre-clinical and clinical studies [59–63,72], high intra-tumour levels of IL-1 β, IL-6 or miR-218-5p could not only provide prognostic information, but also potentially be targeted by novel therapeutic agents.

Prospective clinical trials are awaited to validate these markers before their introduction in the routine clinical practice.

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Author contributions

Conceptualization: SD; Data curation: MG, AC, DL, RP, SD; Original draft writing: MG, AC, SD; Review and editing: CP, FS, SD.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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