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1 **Bone Metastasis: mechanisms, therapies and biomarkers.**

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4

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11

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15

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84 **Abstract:**

85 Skeletal metastases are frequent complications of many cancers, causing bone complications
86 (fractures, bone pain, disability), which negatively affect the patient's quality of life. Here, we first
87 discuss the burden of skeletal complications in cancer bone metastasis. We then describe the
88 pathophysiology of bone metastasis. Bone metastasis is a multistage process; long before the
89 development of clinically detectable metastases, circulating tumor cells settle and enter a dormant state
90 in normal vascular and endosteal niches present in the bone marrow, which provide immediate
91 attachment and shelter, and only become active years later as they proliferate and alter the functions of
92 bone-resorbing (osteoclasts) and bone-forming (osteoblasts) cells, promoting skeletal destruction. The
93 molecular mechanisms involved in mediating each of these steps are described and we also explain
94 how tumor cells interact with a myriad of interconnected cell populations in the bone marrow, including a
95 rich vascular network, immune cells, adipocytes and nerves. We discuss metabolic programs that tumor
96 cells could engage with to specifically grow in bone. We also describe the progress and future directions
97 of existing bone-targeted agents and report emerging therapies that have arisen from recent advances
98 in our understanding of the pathophysiology of bone metastases. Finally, we discuss the value of bone
99 turnover biomarkers in detection and monitoring of progression and therapeutic effects in patients with
100 bone metastasis.

101

102 I. INTRODUCTION

103 During metastatic dissemination, cancer cells from the primary tumor must first undergo epithelial-
104 to-mesenchymal transition (EMT) to invade the surrounding tissue and enter the microvasculature
105 (intravasation) of the blood and/or lymphatic systems (49, 268). Once in the bloodstream, cancer cells
106 may disseminate to distant organs, exit from blood vessels (extravasation) and settle in the foreign
107 microenvironment where they enter a dormant state or proliferate to subsequently form macroscopic
108 secondary tumors (metastases) (49). It has been estimated that only 0.02% of cancer cells entering the
109 circulation produce clinically detectable metastases (217). Metastasis formation is therefore a highly
110 inefficient process. However, when metastases do occur, they are responsible for 90% of cancer-
111 associated mortality (49). There is therefore an urgent need to increase our understanding of the
112 cellular and molecular mechanisms associated with metastasis formation, in order to develop therapies
113 that will improve patient outcome.

114 Bone metastases occur in more than 1.5 million patients with cancer worldwide (361). They are
115 frequent complications of many cancers but are especially common from tumors arising in the breast
116 and prostate. Weakened bones due to skeletal metastases can lead to occurrence of skeletal-related
117 events, such as fractures, spinal cord compression, bone pain and disability, contributing substantially
118 to both morbidity and mortality in patients with advanced cancer (150, 361). In adults, the bone mass is
119 maintained by continuously shaping and reshaping the overall bone structure through a process called
120 bone remodeling, which is a balance between the resorption of mineralized bone by bone-resorbing
121 cells (osteoclasts) and formation of new bone by bone-forming cells (osteoblasts) (76). Bone remodeling
122 is tightly regulated by systemic and local factors in order to maintain this balance at its physiological
123 steady state (76, 150). The late Greg Mundy pioneered the field of cancer and bone, demonstrating that
124 skeletal-related complications associated with bone metastasis were a consequence of a distortion in
125 bone remodeling caused by interactions between cancer cells and cells within the bone
126 microenvironment (236).

127 In this review, we provide a broad overview of the current understanding of cancer-associated
128 bone metastasis. We first review the incidence of bone metastasis in different cancer types and discuss
129 the burden of skeletal complications in cancer bone metastasis. Current knowledge of the
130 pathophysiology of bone metastasis is then described in detail. Bone metastasis is a stepwise sequence
131 of events that include tumor cell colonization of the bone marrow, adaptation to the microenvironment,
132 construction of a cancer niche, disruption of normal bone homeostasis through tumor cell interactions
133 with bone cells (osteoclasts, osteoblasts and osteocytes, the latter being osteoblasts that have
134 undergone a dramatic morphological transformation into stellate cells) and the release of signals from
135 the resorbed bone matrix that promote skeletal tumor growth. We describe molecular mechanisms that
136 are involved in mediating each of these steps and explain how bone marrow cells (*e.g.* immune cells,
137 endothelial cells, adipocytes, and nerve cells) contribute to tumor development through multiple
138 interactions. We also highlight metabolic adaptations of cancer cells that facilitate tumor progression in
139 bone. Finally, we review current and future therapies for the treatment and prevention of bone
140 metastasis and discuss the clinical utility of bone turnover biomarkers to predict the risk of disease
141 relapse in patients with cancer. Given the vast collection of literature existing on the pathophysiology of
142 bone metastasis we focus here on cellular and molecular mechanisms that are the most relevant to
143 human cancer. However, it is important to note that we also cover emerging research areas where
144 many mechanisms are derived from model systems, which still remain to be validated in human
145 systems but could ultimately yield clues for better understanding and prevention of bone metastases.

146

147 **II. BONE METASTASIS INCIDENCE AND CONSEQUENCES FOR BONE HEALTH**

148 **A. Common Ground – Bone Metastasis in Different Cancer Types**

149 Bone is one of the most common sites for metastasis in cancer. Much of the work performed
150 to describe the natural history of bone metastases is based on autopsy studies and large case series

151 from single institutions conducted several decades ago. Although bone is a frequent location for
152 metastases from many malignancies, there are specific types of cancers that have a predilection for
153 metastasis to the skeleton (150, 361). In particular, bone metastases are frequent complications of
154 breast (especially estrogen receptor positive) and prostate cancer. In their retrospective study, Coleman
155 and Rubens found in breast cancer a bone metastasis incidence of around 70% (68). These findings
156 were consistent with the post-mortem examination from Galasko (115), who reported bone metastasis
157 incidences of 73% and 68% of bone metastasis in breast and prostate cancer, respectively. Autopsies
158 allowed the identification of a second group of osteophilic tumors with a postmortem prevalence of bone
159 metastases of 60% in thyroid cancer, 30-40% in lung cancer, 40% in bladder cancer, 20-25% in renal
160 cancer and 14-45% in melanoma (65). Apart from osteoblastic bone metastases in prostate cancer,
161 bone metastases from other cancers are mainly osteolytic or a mix of lytic and blastic changes to the
162 bone structure (Figure 1).

163 With the exception of a few relatively rare malignancies such as high-grade lymphoma or
164 germ cell tumors affecting bone, metastatic bone disease is currently incurable. However, for many
165 patients the median prognosis after development of bone metastasis is measurable in years, especially
166 in those patients with metastatic breast or prostate cancers or multiple myeloma who, with modern
167 treatment approaches, can often be expected to survive more than 5 years after bone involvement is
168 diagnosed (65). Furthermore, new drugs, such as tyrosine kinase inhibitors and immune checkpoint
169 inhibitors, have prolonged primary disease control in patients considerably, resulting in longer survival
170 and consequently living long enough for bone metastasis to become clinically relevant (338). Thus, the
171 epidemiology of bone metastases is evolving. In the coming years we may therefore expect an onset of
172 bone metastases in patients who would have never developed clinically detectable bone metastases
173 some years ago because they would have died from their cancer at a time when they only had (sub-
174 clinical) bone marrow micrometastases. As a result, the prevalence of bone metastasis is increasing

175 and, in many cancers, the dominant site of disease requires specialist expertise and multidisciplinary
176 management (72).

177

178 **B. Cancer-Related Skeletal Complications**

179 Bone metastases may be identified when asymptomatic through imaging tests such as
180 computerized tomography (CT), ¹⁸F-fluorodeoxyglucose (FDG)-positron emission tomography (PET)
181 scanning, or radionuclide bone scanning. However, most patients with bone metastasis present with
182 bone pain (150, 236). Usually the onset of pain is insidious, may be localized or multifocal, and is often
183 confused with benign causes such as osteoarthritis. With time the pain typically worsens and becomes
184 persistent, frequently reaching a severe level that may not be relieved by opioids (150, 236).

185 Bone metastasis is associated with impaired quality of life, reduced physical function and
186 loss of autonomy (68, 150). Because of the proximity between bone and neurological structures (spinal
187 cord and nerve roots), bone metastasis often causes neurological pain, such as paresthesia and tingling
188 or burning sensations induced by epiduritis (68). Fractures are major complications of bone metastases
189 and are commonly a result of osteolytic lesions in the vertebrae and weight-bearing bones, such as the
190 proximal femur (68). The humerus is also at risk because of the forces applied through the arm use in
191 daily life. Once pathological fractures have occurred, bone healing is compromised, and surgical
192 intervention often required. Pathological fractures can be devastating complication for cancer patients,
193 typically worsening their quality of life and increasing mortality (68, 150).

194 Hypercalcemia is an important metabolic complication of bone metastases (337). Symptoms
195 include a wide spectrum of presentations from subtle changes in mood and gastrointestinal symptoms
196 of nausea and constipation to a life-threatening state with vomiting and dehydration, acute renal
197 insufficiency, disordered consciousness and ultimately coma (337). In bone metastases, hypercalcemia
198 usually results from increased osteoclastic bone resorption but may be exacerbated by the

199 paraneoplastic secretion of parathyroid hormone-related peptide (PTHrP) or an abnormal activation of
200 25-OH vitamin D (345). The use of systemic anti-resorptive drugs has considerably reduced the number
201 of patients with hypercalcemia (61, 345).

202 Most clinical studies use the composite endpoint Skeletal-Related Events (SREs) to establish
203 the efficacy of systemic anti-resorptive drugs (61, 345). SREs are defined as pathologic fractures, spinal
204 cord compression and the requirement for radiation therapy and/or surgery to bone; episodes of
205 hypercalcemia may also be considered within the definition (220). Early placebo-controlled
206 bisphosphonate clinical trials estimated that 50 to 56% of patients with bone metastases from solid
207 tumors suffer from at least one SRE during follow-up on standard anti-cancer treatments without the
208 addition of a bone targeted treatment (157, 279). SREs can occur quite early and indeed can be the
209 presenting event in a patient with bone metastasis. In these trials, the median time to occurrence of the
210 first SRE ranged from 5 to 7 months (157, 279). Moreover, patients with a first SRE are at increased risk
211 for subsequent events, strengthening the importance of primary and secondary SRE prevention in
212 cancer patients with bone metastases (68, 72). In addition to reducing a patient's quality of life and
213 social and functional independence, the management of SREs consumes considerable health care
214 resources (68, 72).

215 Besides analgesics and anticancer treatments, bone metastases benefit from systemic anti-
216 resorptive treatments (bisphosphonate or denosumab) and local treatments such as radiotherapy,
217 surgery or interventional radiology (cementoplasty, radiofrequency, ablation, cryotherapy). Optimal care
218 should be discussed in a Bone Metastasis Multidisciplinary Board in order to reach a personalized
219 strategy for every patient (72). Bone-targeted agents such as bisphosphonates and denosumab have
220 been shown to be very effective in preventing and reducing SREs and are now the standard of care for
221 the treatment of patients with malignant bone disease (see sections IX-A.1 and A.2 for further
222 discussion).

223

224 III. TAKING OVER THE NEIGHBORHOOD - BONE COLONIZATION BY TUMOR 225 CELLS

226 Bone colonization by tumor cells is a stepwise sequence of events that include (i) the formation of
227 a pre-metastatic niche in the bone marrow to attract circulating tumor cells, (ii) the extravasation of
228 these tumor cells from the circulation and homing to the pre-metastatic niche, and (iii), following tumor
229 cell engraftment, the evolution of this pre-metastatic niche into a metastatic niche, the latter being
230 conducive to the survival of these tumor cells. Each of these events is discussed below (Figure 2).

231 A. Preparing the Soil – the Concept of the Premetastatic Niche

232 The concept of premetastatic niche was first described by Dr Lyden and colleagues showing that
233 vascular endothelial growth factor (VEGF)-A and placental growth factor (PIGF) secreted from primary
234 tumors mobilize bone marrow-derived VEGF receptor 1 (VEGFR-1)-positive hematopoietic cells to the
235 lungs before the arrival of tumor cells (169). Furthermore, an upregulation of fibronectin in resident
236 fibroblasts at these premetastatic sites subsequently supports adhesion of VEGFR-1-positive cells
237 (169). This localized accumulation of bone marrow-derived hematopoietic cells and stromal fibronectin
238 creates docking sites for the future engraftment of tumor cells in lungs (169). Since then, many other
239 tumor-derived factors, including cytokines, chemokines, extracellular matrix components, small
240 noncoding RNAs and tumor-shed extracellular vesicles have been shown to act as systemic signals that
241 trigger the formation of premetastatic niches in lung, liver or lymph nodes in different preclinical models
242 (149, 261). Clinical evidence for the existence of premetastatic tissues comes from patients with
243 meningioma (a benign brain tumor) who later progress with tumor-to-meningioma metastasis of breast,
244 lung or renal cancer (88, 264, 270). It is suggested that the presence of pro-inflammatory macrophages
245 and the high microvascular density in meningioma contribute to metastasis formation (88). Similarly, the
246 existence of a premetastatic tissue in sentinel lymph nodes resected from patients with solid tumors has
247 been reported (302). Thus, there is preclinical and clinical evidence that primary tumors may remotely
248 induce the formation of a permissive environment within distant organs for future metastasis.

249 Multiple molecular mechanisms involved in the formation of a premetastatic niche in bone have
250 been described (98, 137, 151, 216, 247, 248, 250, 251, 261, 271, 327, 336, 341, 371). Some of them
251 already exist in the normal bone marrow (216, 248, 250, 251), whereas others are initiated by systemic
252 signals coming from primary tumors (98, 137, 151, 327, 336, 341, 371). For example, interleukin (IL)-6
253 secreted from senescent osteoblasts promotes osteoclast-mediated bone resorption that, in turn,
254 increases tumor cell seeding and subsequent breast cancer bone metastasis formation in animals
255 (216). Similarly, in the absence of estrogen or androgen, osteoclast activity and bone resorption are
256 increased, which leads to the release of bone-derived factors from resorbed bone that shape a
257 favorable environment for tumor cells to survive and grow (248-250). Additionally, soluble factors
258 secreted from primary tumors can target stromal and/or bone cells to support future metastatic
259 colonization in the bone marrow. For example, in breast cancer models, tumor-derived IL-1 β drives
260 bone metastasis formation *in vivo* (151, 336). Blocking IL-1 β activity with the anti-IL-1 receptor
261 antagonist Anakinra or the IL-1 β specific antibody Canakinumab inhibits tumor cell dissemination from
262 the primary site into the circulation and blocks spontaneous formation of metastases to human bone
263 implants in treated mice, compared to the placebo-treated group (336). Hypoxia-induced lysyl oxidase
264 (LOX) can be secreted from primary tumors into the circulation from which LOX primes distant organs
265 for metastatic colonization, including bone (73, 98, 263, 274). The primary function of LOX is to drive
266 collagen crosslinking and extracellular matrix stiffness (7). In bone, tumor-derived LOX cooperates with
267 receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL) to accelerate osteoclastic bone
268 resorption, and the formation of premetastatic osteolytic lesions in animal models of breast or colon
269 cancer (73, 274, 335). A function-blocking antibody (AB0023) directed against LOXL2 (another member
270 of the LOX family) inhibits breast cancer bone metastasis formation in animals (10), suggesting that
271 LOXL2 could also contribute to the formation of a pre-metastatic niche in the bone marrow. Phase II
272 clinical trials with simtuzumab, a humanized anti-LOXL2 monoclonal antibody, showed however that the
273 addition of this antibody to current treatments of patients with metastatic pancreatic or colorectal cancer

274 does not improve clinical outcomes (ClinicalTrials.gov identifiers NCT01472198 and NCT01479465,
275 respectively). Conversely, the anti-IL-1 β antibody canakinumab has been shown to significantly reduce
276 the incidence of lung cancer and lung cancer mortality in patients with atherosclerosis (ClinicalTrials.gov
277 identifier NCT01327846). It remains to be established whether blocking IL-1 β , LOX or LOXL2 impedes
278 progression of bone metastasis in breast cancer patients.

279 It also appears that factors contained within cancer cell-derived exosomes can influence bone cell
280 activity before tumor cells arrive at this site. Exosomes are small extracellular vesicles (30-120 nm)
281 containing DNA, RNA [mRNA, miRNA and other noncoding RNAs], lipids and proteins that are released
282 by all types of cells and taken up by recipient cells (368). For example, exosomal amphiregulin secreted
283 by non-small cell lung carcinoma (NSCLC) cells or amphiregulin-containing exosomes released in
284 plasma of NSCLC patients promote the differentiation of human peripheral blood monocytes into
285 osteoclasts (327). In melanoma, the transfer of the MET oncoprotein from tumor-shed exosomes to
286 bone marrow progenitor cells can reprogram these cells towards a prometastatic phenotype in lungs
287 and bone *in vivo* (261). Similar findings were reported with tumor-derived exosomal miRNAs (miR-21,
288 miR-141, miR-192, and miR-940) (22, 137, 341, 371, 376). In particular, exosomal miR-141 and miR-
289 940 produced by prostate cancer cells promote osteoblast differentiation and proliferation, facilitating
290 the formation of bone metastases with an osteoblastic phenotype in mouse models (137, 376). MiRNAs
291 mainly act as negative regulators of gene expression (14). In this respect, tumor-derived exosomal miR-
292 141 promotes osteoblast differentiation by inhibiting DLC1 mRNA expression that, in turn, leads to
293 p38MAPKinase activation and increased osteoprotegerin (OPG) expression in osteoblasts (376).
294 Tumor-derived exosomal miR-940 promotes osteogenic differentiation of mesenchymal stem cells by
295 directly inhibiting ARHGAP1 (Rho GTPase Activating Protein 1) and FAM134A (Family with Sequence
296 Similarity 134 Member A) mRNA expression (137).

297 Overall, these experimental findings strongly suggest that, in addition to molecular mechanisms
298 already existing in the normal bone marrow, primary tumors can also remotely control the formation of a

299 premetastatic niche through the release of systemic factors that induce a distortion in bone remodeling.
300 Research designed to determine the mechanisms by which primary tumors promote the formation of
301 pre-metastatic niches in bone is still in its infancy and further investigations using *in vivo* model systems
302 are required to gain a more comprehensive understanding of this process. As tumor cell dissemination
303 into bone is believed to be an early process, likely to occur before the clinical detection of primary
304 tumors, the detection of these molecules in the primary tumor and/or blood may provide useful
305 biomarkers to predict future relapse in bone. Further clinical trials are needed to test this hypothesis.

306

307 The premetastatic niche: current understandings & open questions

- 308 • Preclinical and clinical studies support the existence of premetastatic tissues for future
309 metastasis.
- 310 • The general applicability of these mechanisms associated with the formation of a premetastatic
311 niche remains to be validated *in vivo* for other model systems and for other cancer types.
- 312 • Beside the observation that primary tumors can generate systemic changes that modify the
313 bone microenvironment, there is also some preclinical evidence suggesting that bone may
314 remotely control growth of primary tumors at distant sites (85, 97, 166, 257). These latter
315 observations are intriguing and clearly deserve further study.

316

317 **B. Mechanisms of Tumor Cell Extravasation and Homing to the Bone Marrow**

318 In response to pro-migratory and pro-inflammatory molecules produced by the pre-metastatic
319 niche, circulating tumor cells (CTCs) cross the endothelial cell barrier and basement membrane of blood
320 vessels (a process called extravasation) in order to home in the newly invaded parenchyma where they
321 interact with specific extracellular matrix components that facilitate their survival.

322 *1. Tumor cell extravasation*

323 In the bone marrow, the vascular endothelium that constitutes blood vessels (called sinusoids) is
324 predominantly discontinuous and fenestrated, which facilitates the traffic of hematopoietic stem cells
325 (HSCs) (241). Therefore, the sinusoids are likely to be more permissive to CTCs, suggesting there is a
326 limited requirement of extravasation mechanisms for tumor cells to invade the bone marrow (241, 273).
327 Indeed, tumor cells hijack molecular mechanisms that are used by HSCs. In particular, E-selectin
328 (Endothelial selectin) and CXCL-12 are constitutively expressed on sinusoidal endothelial cells, aiding
329 the homing of HSCs in the bone marrow (301, 316). Similarly, E-selectin- and CXCL-12-expressing
330 bone marrow endothelial cells mediate attachment of breast and prostate cancer cells through
331 interaction with E-selectin ligands and CXCR-4, respectively (267, 273). Using high-resolution real-time
332 fluorescence microscopy to track breast cancer cell migration in the calvarial bone marrow *in vivo*, Price
333 *et al.* (267) showed that 2 hours after intracardiac injection, tumor cells interacted with endothelial cells
334 in sinusoidal vascular and perisinusoidal vascular regions where expression of E-selectin and CXCL-12
335 is high. Of special interest, the preventive treatment of mice with a selective inhibitor of E-selectin,
336 before tumor cell injection, substantially blocked tumor cell interaction with E-selectin-expressing
337 endothelial cells, whereas pretreatment of animals with a small molecule inhibitor of CXCR-4
338 (AMD3100) did not inhibit breast cancer cell homing to the bone marrow *in vivo* (267). By contrast,
339 AMD3100 treatment of mice after tumor cell engraftment forced breast cancer cells residing in
340 perivascular niches to migrate from the bone marrow into the peripheral circulation. Overall, these
341 findings demonstrate that E-selectin is critical for allowing breast cancer cells to extravasate in the bone
342 marrow, whereas CXCR-4/CXCL-12 maintains tumor cells in the perivascular environment and controls
343 their exit from the bone marrow (267). The CXCR-4/CXCL-12 axis is the most well-described and
344 prominent mechanism involved in regulating tumor cell entry in the bone marrow environment (235).
345 However, it should be noted that, not all breast cancers that metastasize to bone express CXCR4 (243),
346 and other chemokines produced by the bone microenvironment (CXCL-5, CXCL-10, CXCL-13, CX3CL-
347 1, CCL-2) have also been implicated in mediating tumor cell colonization in the bone marrow (158, 159,

348 193, 213, 232, 275, 300) (Table 1). However, correlations between expression of these chemokines
349 and relapse in bone, in clinical samples, remains to be established.

350 Another factor that has been implicated in tumor cell extravasation is the cytokine IL-1 β whose
351 expression in breast cancer cell lines and primary breast carcinomas is strongly associated with bone
352 metastasis (151, 336). IL-1 β drives metastasis by inducing epithelial-to-mesenchymal transition and
353 increasing dissemination of breast cancer cells into the circulation (151, 336). Once in bone, IL-1 β
354 facilitates adhesion of CTCs to sinusoidal endothelial cells by inducing the expression of vascular cell
355 adhesion molecules [intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1
356 (VCAM-1), and E-selectin] (273). Then, IL-1 β stimulates expansion of the metastatic niche, increasing
357 proliferation of blood vessels and osteoblasts, thereby promoting tumor cell extravasation and
358 metastatic outgrowth of tumor cells that disseminated in this site (273, 336). Interestingly, IL-1 β -
359 expressing E0771 primary breast tumors spontaneously metastasize to bones in IL-1 β -knockout
360 animals and to an extent similar to that observed in normal mice, indicating that tumor-derived IL-1 β
361 rather than IL-1 β from the bone marrow microenvironment promotes bone metastasis formation (336).
362 Although these mechanisms have all been identified in mouse models it is likely that these, at least in
363 part, explain why increased IL-1 β in primary breast tumors associates with recurrence in bone in cancer
364 patients (151, 336).

365 2. *Tumor cell homing*

366 Bone extracellular matrix proteins (*e.g.*, type I collagen, tenascin C, periostin, fibronectin,
367 SIBLINGs) by binding to tumor cell surface integrins play an important role in mediating tumor cell
368 attachment to the bone matrix (17, 57, 221, 255, 260, 285, 311, 317, 333). In particular, type I collagen,
369 which is the most abundant protein among bone extracellular matrix components, mediates attachment
370 of human prostate cancer cells through binding tumor cell $\alpha 2\beta 1$ integrin (311). Fibronectin in the bone
371 marrow mediates survival of human triple negative breast cancer cells by binding to tumor cell surface

372 $\alpha 5\beta 1$ integrin (255). Tenascin C is a hexameric protein that fosters the early colonization of prostate
373 cancer cells in the bone marrow through binding tumor cell surface $\alpha 9\beta 1$ integrin (285). Like tenascin
374 C, periostin is produced by stromal cells and mediates tumor cell adhesion by binding tumor $\alpha v\beta 3$
375 integrin (221). As such, integrins have been therefore considered as attractive drug targets. For
376 example, $\alpha v\beta 3$ integrin recognizes an Arg-Gly-Asp (RGD) peptide motif expressed by extracellular
377 matrix proteins and the treatment of animals with RGD-based peptide antagonists (PSK104, S247,
378 GLPG0187) of $\alpha v\beta 3$ integrin suppresses breast and prostate cancer bone metastasis formation,
379 supporting the crucial role played by this integrin in bone metastasis formation (136, 303, 344, 388).
380 However, despite these encouraging experimental studies, ensuing clinical trials that used integrin
381 antagonists have been mainly unsuccessful (132). There may be a need to develop alternative
382 strategies that target specific integrin signaling pathways promoting tumor cell survival and drug
383 resistance (132). Integrins are also emerging as valuable cancer imaging probes to identify bone
384 metastases in clinical studies. For example, integrin $\alpha v\beta 3$ is highly expressed in osteotropic tumor cells
385 and osteoclasts and, using PET/CT imaging, this property has been used to show that an RGD peptide-
386 containing $\alpha v\beta 3$ integrin tracer (^{99m}Tc -3P-RGD₂) is superior to ^{99m}Tc -bisphosphonates to detect
387 osteolytic bone metastases in patients with advanced lung cancer (297). Similarly, PET/CT imaging with
388 a tracer targeting gastrin-releasing peptide receptor and integrin $\alpha v\beta 3$ (^{68}Ga -BBN-RGD) shows a
389 significant uptake in bone metastases from patients with advanced breast cancer (384).

390 RANK and RANKL have an established role in regulating bone remodeling (183). RANKL secreted
391 by osteoblasts and osteocytes binds to its receptor RANK on osteoclast precursors, leading to the
392 formation of mature osteoclasts and osteoclast-mediated bone resorption (183). Interestingly, tumor
393 cells can also express RANK in breast, prostate, and lung carcinomas and RANKL triggers *in vitro*
394 migration of RANK-expressing breast and prostate cancer and melanoma cells (21, 164, 325, 346). In
395 the case of breast cancer, a high RANK expression in hormone receptor-negative primary tumors is
396 associated with poor relapse-free survival and high risk of bone metastasis in patients (272, 286, 347). It

397 has been therefore suggested that RANK-expressing cancer cells may be specifically attracted to bone
398 where high local concentrations of RANKL exist (346). This contention was supported by the
399 observation that inhibition of RANK/RANKL signaling by soluble decoy receptor OPG, which binds to
400 RANKL, reduces skeletal tumor burden and bone metastasis in a melanoma model that does not
401 activate osteoclasts, whereas metastasis in other organs (ovaries, brain, adrenal glands) remain
402 unchanged (164). However, administration of OPG to a mouse model of breast cancer did not reduce
403 the number of tumor cells that disseminated in bone (249). In clinical studies, the RANKL inhibitor
404 denosumab, when given in patients with early-stage breast cancer or non-metastatic castration-resistant
405 prostate cancer (CRPC), had no effect on disease recurrence in either pre- or postmenopausal women
406 with breast cancer (63) and only modestly increased bone metastasis-free survival in CRPC patients
407 (306), thereby suggesting that RANK/RANKL does not play a major role in tumor cell colonization in
408 bone.

409 Tumor cell extravasation and homing: current understandings & open questions

- 410 • Recent studies on the bone microvasculature in mouse models have shown that there are
411 particular vessel subtypes within the same vascular bed, termed H (CD31^{hi}Endomucin^{hi}) and L
412 (CD31^{lo}Endomucin^{lo}) vessels, which have different characteristics (182). It will be of particular
413 interest to determine whether disseminated tumor cells preferentially associate with a particular
414 vessel subtype.
- 415 • Results obtained with PET/CT imaging using integrin-binding tracers to detect skeletal lesions
416 and evaluate treatment response in patients with advanced cancer and bone metastases are
417 very encouraging and deserve further investigations.

418

419 **C. Hiding in Plain Sight –Tumor Cell Dormancy and Dormant Cell Reactivation** 420 **Mechanisms in Bone Marrow Niches**

421 Entering a foreign environment, such as the bone marrow, poses tumor cells with numerous
422 challenges regarding survival and proliferation. It is hypothesized that tumor cells in the bone marrow
423 compete with HSCs for occupancy in the vascular and endosteal niches. Once in the niche, tumor cells
424 can enter a dormant state as a mechanism to help them survive until environmental conditions are
425 sufficiently permissive for proliferation and tumor outgrowth (298, 309). This hypothesis of tumor
426 dormancy is supported by an extensive body of clinical research. First, long before the development of
427 clinically detectable metastases, tumor cells disseminate to the bone marrow, only becoming active
428 following years or decades after primary tumor diagnosis (247). Second, disseminated tumor cells
429 (DTCs) are present in the bone marrow of patients with various types of cancer and are predictive of
430 future relapse, yet some of these cancer types will never develop clinically detectable bone metastases
431 (205). Lastly, for those cancer types that have a high propensity to develop overt bone metastases,
432 such as breast and prostate cancers, the rate of detection of DTCs in the bone marrow is higher than
433 the proportion of patients who subsequently develop skeletal lesions, suggesting the bone marrow
434 microenvironment influences DTC fate (32, 228). Thus, these clinical observations provide strong
435 evidence of tumor dormancy in the bone marrow. However, DTCs isolated from the bone marrow of
436 non-metastatic patients with cancer (breast, prostate, melanoma) fail to generate tumor xenografts in
437 immunodeficient mice (364). Consequently, only tumor cell lines derived from overt metastases from
438 patients with advanced cancer (breast, prostate) have been used in animal models to study tumor
439 dormancy in bone (31, 46, 117, 119, 163, 177, 267, 320, 381, 382). The use of these metastatic cell
440 lines, therefore, poses a major limitation as metastatic cancer cells and DTCs have different genotypic
441 and phenotypic traits (309). Determining the different mechanisms controlling the ability of tumor cells to
442 seed in the bone marrow and those responsible for metastatic outgrowth may require the development
443 of more clinically relevant models. Yet, tumor dormancy is an emerging research area and we believe
444 that unravelling molecular mechanisms associated with tumor dormancy using these existing preclinical
445 models may still help us better understand the earliest stages that precede the clinical development of

446 bone metastases. Below we describe our current understanding of the mechanisms involved in
447 mediating tumor cell interactions with cells from bone marrow niches (Figure 2) and then detail
448 molecular signaling mechanisms proposed to keep these tumor cells in a dormant state (Figure 3).
449 Finally, we describe how osteoclast-mediated bone resorption creates an environment that promotes
450 dormant cell reactivation.

451 1. *Tumor cells interactions with cells from bone marrow niches*

452 The vascular niche surrounds E-selectin-expressing endothelial cells that form bone marrow
453 sinusoids, and is made up of perivascular cells expressing high levels of CXCL-12 [called CXCL-12-
454 abundant reticular (CAR) cells], leptin receptor (Lepr)-expressing perivascular stromal cells, and
455 mesenchymal stem cells (MSCs) (301, 316). This vascular niche regulates HSC quiescence and the
456 supply of lineage-committed progenitors (301). Real-time *in vivo* microscopy of bone marrow sinusoids
457 in a breast cancer xenograft model has revealed opposing roles of E-selectin and CXCL-12 in tumor cell
458 trafficking (267). Whereas E-selectin interactions are critical for allowing breast cancer cell entry into the
459 bone marrow, CXCL-12/CXCR-4 interactions maintain breast cancer cells dormant in the vascular niche
460 (267). Additional mechanisms are involved in maintaining tumor cells dormant in the vascular niche. For
461 example, endothelium-derived extracellular matrix protein thrombospondin-1 (TSP-1) induces sustained
462 dormancy of breast cancer cells *in vivo* (119). Conversely, MSCs with both endothelial and pericytic cell
463 surface markers prevent the homing of breast and prostate cancer cells to the bone marrow (282). In
464 model systems, the tumor-suppressive nature of the vascular endothelium is lost when endothelial cells
465 start sprouting, which is characterized by reduced TSP-1 expression and enhanced expression of pro-
466 metastatic factors (periostin, tenascin, fibronectin) that promote tumor outgrowth (119). Interestingly,
467 immunohistochemical analysis of the bone marrow from breast cancer patients with micrometastatic
468 disease shows that dormant (Ki67-negative) breast cancer cells are preferentially localized in
469 perisinusoidal, CXCL12-rich vascular regions (267). By contrast, proliferative (Ki67-positive) breast
470 cancer cells in bone marrow biopsies from patients with macrometastatic disease are frequently

471 observed adjacent to the bone surface (267). This observation (267) is in agreement with the fact that
472 calcium levels, which are high at the endosteal mineral surface, can promote breast cancer cell
473 proliferation and bone metastasis formation in animals (354). Thus, it appears that the vascular niche
474 provides a microenvironment supportive of dormancy at least in breast cancer.

475 As the name suggests, the endosteal niche is localized at the inner surface of the bone cavity in
476 the endosteum, and is primarily made up of undifferentiated osteoblastic cells, such as spindle-shaped
477 N-cadherin⁺/CD45⁻ osteoblast (SNO) cells (138). Mature osteoblasts are short-lived cells and, as such,
478 they are unlikely to be part of the endosteal niche (76, 256). Beside SNO cells, CAR cells are also
479 present and are proposed to maintain the quiescent HSC pool through CXCL-12/CXCR-4 interactions
480 (316). The disruption of this connection using CXCR4 antagonists, in mouse models, results in
481 increased mobilization of HSCs from the bone marrow into the circulation (316). Osteoclasts are
482 dispensable for HSC maintenance in the endosteal niche and may function as negative regulators in the
483 hematopoietic system (231). With regard to the homing of tumor cells to the endosteal niche, ER-
484 negative (but not ER-positive) breast cancer cells compete with HSC to interact with SNO cells through
485 a specific Jagged-Notch2 interaction that mediates tumor cell dormancy both *in vitro* and *in vivo* (46). It
486 must be pointed out however that these particular *in vivo* experiments were conducted using intratibial
487 tumor cell inoculation, thereby bypassing the blood circulation, which impedes breast cancer cells from
488 homing to the vascular niche. Other studies, using more clinically relevant mouse models in which
489 tumor cells were disseminated into the bone via intra-arterial injection, reported that SNO cells support
490 survival of ER-positive breast cancer cells through specific N-cadherin/E-cadherin interactions and
491 connexin-43 (Cx43) gap junctions that trigger pro-survival mTOR signaling and calcium signaling
492 pathways in tumor cells, respectively, hence promoting micrometastatic progression (354, 355). In
493 addition, independently of the hormone receptor status or breast cancer subtype, CXCL-12 triggers
494 activation of a Src-dependent AKT signaling pathway by binding to CXCR-4, enhancing the survival of
495 breast cancer cells in the bone marrow (387). Of note, Werner-Klein *et al.* (364) performed single-cell

496 RNA-sequencing analysis of DTCs isolated from the bone marrow of non-metastatic breast cancer
497 patients (n=30 DTCs; 21 patients) and found that mRNA expression of the IL-6 signal transducing unit
498 gp130 (*IL6ST*) is strongly enriched in these cells, whereas the mRNA of the IL-6 binding receptor alpha
499 chain CD126 (*IL6RA*) is absent. In the absence of CD126, the IL-6 signaling pathway can be activated
500 in trans through the binding of IL-6 to the soluble form of CD126 (sIL6RA) prior to binding to gp130.
501 Both IL-6 and sIL6RA are abundant in the bone marrow, and IL-6 trans-signaling through the PI3K/AKT
502 pathway can be activated in tumor cells (364). However, the endosteal niche renders DTCs
503 unresponsive to IL-6 trans-signaling (364). Interestingly, genetic analysis of DTCs revealed that only
504 4.4% (3/68) of nonmetastatic breast cancer patients harbored mutations in the gene for PI3K (*PIK3CA*),
505 whereas 34.3% (23/67) of metastatic breast cancer patients displayed *PIK3CA* mutations, indicating
506 that DTCs may undergo further selection to become more independent from their microenvironment
507 during cancer progression. Overall, these results strongly indicate that the endosteal niche provides
508 breast cancer cells with an environment supporting their survival, outgrowth and/or enabling tumor cells
509 to acquire genetic alterations (e.g., *PIK3CA* mutation) that render them more autonomous (354, 355,
510 364, 387).

511 In prostate cancer, CXCR-4/CXCL-12 and Annexin 2 (*ANXA2*)/CXCL12 interactions also play a
512 crucial role in the recruitment of tumor cells in the endosteal niche (167, 298, 318, 357). The targeting of
513 CXCR4 in model systems results in increased numbers of prostate cancer cells in the circulation,
514 supporting the notion that these tumor cells inhabit this endosteal niche (298, 357). The current
515 hypothesis is that prostate cancer cells compete with HSCs for space in the endosteal niche (298).
516 However, as opposed to breast cancer, it seems that prostate cancer cells homing in the endosteal
517 niche may benefit from this supportive environment for maintenance of dormancy but not tumor
518 outgrowth (42, 298, 320, 357). Notably, growth arrest-specific 6 (*GAS6*) is an osteoblast-derived ligand
519 of the MER, TYRO3 and AXL tyrosine kinase receptors that has been shown to induce tumor dormancy
520 in prostate cancer (320). When prostate cancer cells bind to osteoblastic cells in the endosteal niche,

521 they increase their expression level of AXL and consequently GAS6 inhibits tumor cell proliferation by
522 binding to AXL (320). Similarly, high MER expression levels in prostate cancer cells are associated with
523 tumor dormancy in the bone marrow (42). By contrast, when TYRO3 expression levels exceed AXL
524 levels, prostate cancer cells exhibit rapid growth (320). Thus, a balance between expression levels of
525 TYRO3 and AXL/MER may regulate prostate cancer cell dormancy in the endosteal niche. A similar role
526 for AXL in promoting dormancy in models of multiple myeloma has been reported (173). Overall, the
527 relative contribution of these niches/molecules to tumour cell dormancy in these various bone metastatic
528 cancers has yet to be validated in clinical samples.

529 The bone microenvironment is also an immune privileged site, offering protection of HSCs from
530 environmental insults and the resulting immune response. High resolution *in vivo* imaging shows co-
531 localization of HSC and regulatory T cells (Treg) on endosteal surfaces in the trabecular bone marrow
532 areas in mice (111). Treg cells are known to be potent immune suppressors. In addition to vascular and
533 endosteal niches, it has been therefore proposed that Treg cells helped create an immune niche
534 supporting stem cell function whilst providing sanctuary from immune attack (111). The bone marrow
535 also contains very high numbers of myeloid-derived suppressor cells (MDSCs) (254, 370). MDSCs
536 suppress anti-cancer immune activity by inhibiting NK and CD8+ T cells (254, 370). Thus, this type of
537 protected environment would clearly also benefit resident tumor cells, preventing their elimination and
538 promoting their survival in bone. In addition, bone marrow mesenchymal stem cells also promote tumor
539 cell dormancy (247). *See section VII* for further discussion on the contribution of immune cells to tumor
540 development.

541

542 2. *Tumor cell dormancy*

543 Tumor cell dormancy is defined as the arrest in the cell cycle (also known as mitotic or cellular
544 dormancy). A second mode of dormancy refers to tumor mass dormancy of micrometastases where

545 there is a balance between cell proliferation and cell death, the latter is widely believed to be due to
546 immune surveillance and/or lack of blood supply (309). The signaling pathways through which tumor
547 mass dormancy is controlled are largely unknown, mostly because of the lack of appropriate animal
548 models that reproduce tumor dormancy in bone. Thus, although these two modes of dormancy coexist
549 in the bone marrow, we have concentrated here on molecular mechanisms that regulate tumor cell
550 dormancy in laboratory models.

551 In breast cancer, tumor cell dormancy appears to be determined by a balance between the
552 activities of activated protein kinases ERK1/2 and p38, where a switch towards ERK1/2 phosphorylation
553 favors proliferation whereas activation of p38 leads to quiescence (309). Mitogen- and stress-activated
554 kinase 1 (MSK1) is a downstream effector of the p38 and ERK1/2 signaling pathways (117). Using
555 experimental models of ER-positive human breast cancer (T47D, ZR-75) in which tumor cells form
556 latent micrometastatic bone lesions *in vivo*, Gawrzak and colleagues (117) showed that p38 depletion in
557 ER-positive human breast cancer cells decreases MSK1 expression. In turn, MSK1 depletion increases
558 the capacity of poorly metastatic ER-positive breast cancer cells to form overt metastasis in animals
559 (117). Thus, MSK1 is a dormancy enforcer and a negative regulator of metastasis initiation.

560 Another signal that regulates breast cancer dormancy in the bone marrow is leukemia inhibitory
561 factor (LIF) (163). By binding to LIF receptor (LIFR), LIF negatively regulates *STAT3* (signal transducer
562 and activator 3) in breast cancer cells. The loss of LIFR or *STAT3* enables otherwise quiescent human
563 MCF-7 breast cancer cells to proliferate and specifically metastasise to bone (163). Indeed, LIFR
564 expression levels in primary tumor of breast cancer patients who are predicted to relapse in bone are
565 significantly lower compared with those with a good prognosis (163), further supporting the observation
566 that LIFR signaling mediates tumor cell dormancy in animal models of bone metastasis.

567 In prostate cancer, bone morphogenetic protein (BMP)-7 secreted from bone marrow stromal cells
568 promotes dormancy of prostate cancer stem-like cells, and an inverse correlation between expression of
569 the BMP7 receptor BMPR2 and occurrence of bone metastasis is found in patients with prostate cancer

570 (177). By binding to BMPR2, BMP7 induces the quiescence of prostate cancer stem-like cells through
571 p38 activation and increased expression of the cell cycle inhibitor p21 (177). BMP7 also inhibits breast
572 cancer stem cell population and reduces bone metastasis formation in animals (41).

573 Bone-derived growth factors TGF β 1 and TGF β 2 exhibit competing functions on the behavior of
574 tumor cells in the bone marrow (309). TGF β 2 promotes tumor cell dormancy, whereas TGF β 1 switches
575 off dormancy, leading to rapid tumor growth *in vivo* (309). In a head and neck squamous cell carcinoma
576 model of bone metastasis, TGF β 2 (but not TGF β 1) activates p38, which up-regulates the metastasis
577 suppressor gene *DEC2* (31). In turn, DEC2 induces p27 and down-regulates cyclin-dependent kinase 4
578 (CDK4), leading to tumor cell quiescence (31). In model systems of prostate cancer bone metastasis,
579 TGF β 2 induces dormancy through p38 activation and AXL/GAS-6 expression (381, 382).

580 Due to the diversity of the molecular mechanisms that regulate tumor cell dormancy in
581 laboratory models, these processes are difficult to validate in clinical samples. However, future research
582 will establish if targeting key drivers of dormancy can be used as a method of retaining tumor cells in
583 this state indefinitely, thereby preventing metastatic outgrowth and symptomatic disease.

584 3. *Dormant cell reactivation*

585 Bone resorption likely creates an environment that promotes tumor cell reactivation. Intravital
586 imaging of the bone microenvironment in murine models of multiple myeloma has shown that tumor
587 cells colonizing endosteal niches are in a dormant state (189). However, these tumor cells are reactivated
588 and released from the endosteal niche upon treatment of tumor-bearing animals with a soluble form of
589 RANKL that stimulates osteoclast-mediated resorption (189). By contrast, sRANKL treatment has no
590 effect on tumor cells colonizing soft tissue sites (189). Androgen deprivation by orchidectomy stimulates
591 bone turnover of castrated animals bearing disseminated hormone-insensitive prostate cancer cells in
592 the bones, thereby also increasing the incidence of overt bone metastasis in these animals (251). A
593 similar effect was reported in an animal model of breast cancer, where ovariectomy-induced bone loss

594 triggered growth of disseminated hormone-insensitive breast cancer cells in bone (249,250). Thus,
595 osteoclast-mediated bone resorption plays an important role at an early stage in the establishment of
596 bone metastasis. This contention is also supported by experiments conducted in a mouse model of
597 indolent breast cancer bone metastasis, showing that VCAM-1 overexpression in tumor cells promotes
598 the recruitment of osteoclast precursors by binding to osteoclast integrin $\alpha 4\beta 1$, leading to osteoclast
599 formation and osteoclast-mediated bone resorption (214). In turn, bone-derived growth factors TGF β 1
600 released from resorbed bone switches off dormancy, leading to rapid tumor growth *in vivo* (309).
601 Furthermore, PTHrP expressed by tumor cells can act in autocrine fashion by reducing pro-dormancy
602 LIFR gene expression (163), suggesting that PTHrP also plays a role in promoting tumor cell exit from
603 dormancy. Thus, changes to the bone environment in favor of bone resorption are sufficient to trigger
604 dormant cell reactivation. This idea is supported by the fact that bisphosphonates, by decreasing bone
605 resorption, improve elimination of DTCs in the bone marrow of breast cancer patients with a minimal
606 residual disease (307), and reduce development of bone metastases when given as a neoadjuvant
607 treatment (66, 123).

608

609 Niches, tumor cell dormancy and reactivation: current understandings & open questions

- 610 • Experimental and clinical studies support the notion that vascular and endosteal niches can be
611 hijacked by arriving tumor cells to provide immediate shelter, thereby preventing their
612 elimination and promoting their survival in the bone marrow. Other existing niches, such as the
613 immune niche, may also support tumor cell survival in the bone marrow. However, many
614 aspects of the interplay between these niches and tumor cells remain elusive. The use of
615 clinically applicable imaging technologies such as PET and SPECT with niche-specific tracers
616 and single cell-omics techniques will certainly help to understand the dynamics of tumor-niche
617 interactions in the future.

618 • The diversity of the molecular mechanisms associated with tumor dormancy in the bone
619 marrow niches represent both a challenge and an opportunity for therapeutic targeting. How to
620 avoid unwanted effects on normal homeostasis while disrupting interactions that maintain tumor
621 cells in these bone marrow niches remains an open question.

622

623 IV. FITTING IN - ADAPTATION OF TUMOR CELLS TO THE BONE MARROW 624 MICROENVIRONMENT

625 During the time tumor cells are resident in the bone marrow, exiting and re-entering a dormant
626 state, they rewire their biology to meet the demands of the tissue colonized, thus modifying their primary
627 properties in order to adopt a genetic phenotype similar to bone cells that, in turn, facilitates their
628 survival in the bone microenvironment (16, 291). This process is called osteomimicry (178).

629 Immunohistochemical analysis of human clinical samples in breast and prostate carcinomas clearly
630 shows that cancer cells metastatic to the bone highly express bone proteins *in situ* (16, 55, 108, 196,
631 353). In particular, paired immunochemistry on human primary breast tumor samples and matched liver,
632 lung or bone metastases showed that only bone metastatic tumor cells express bone proteins such as
633 cathepsin K (CTSK), osteonectin, cadherin-11 (CDH-11), and Cx-43, which are normally expressed by
634 osteoblasts or osteoclasts (16, 108, 196).

635 The functions of these osteomimicry genes have been studied in animal models of bone
636 metastasis. CDH-11 mediates interactions of breast and prostate cancer cells with osteoblasts *in vitro*,
637 and its silencing in tumor cells greatly reduces bone metastasis formation *in vivo* (55, 154, 323). Breast
638 cancer cells can get calcium from the osteogenic niche through Cx43 gap junctions that facilitate
639 calcium influx from osteogenic cells to breast cancer cells and, in turn, calcium promotes tumor cell
640 proliferation (354). Similarly, Cx43 overexpression in human LNCaP prostate cancer cells enhanced
641 their capability to induce bone destruction *in vivo* following intratibial tumor cell injection, and moderately
642 augmented tumor cell proliferation *in vitro*, when tumor cells were cocultured with osteoblasts (184).

643 Another example of osteomimicry is the expression of transcription factor RUNX2 (a master regulator of
644 osteoblast differentiation) in osteotropic tumor cells. The disruption of *RUNX2* expression in breast
645 cancer cells abolishes their ability to form osteolytic lesions *in vivo* (266). *RUNX2* in osteotropic breast
646 cancer cells promotes expression of metastasis-related factors [MMP-9, MMP-13, VEGF, osteopontin,
647 bone sialoprotein (BSP), ITGA5] and bone-resorbing factors (PTH-rP, IL-8), thereby explaining why
648 *RUNX2* inhibition in tumor cells decreases skeletal tumor burden and osteolysis (200, 266). Forkhead
649 box F2 (FOXF2) is another example of master transcription factor that mediates epithelial-to-
650 osteomimicry transition, increasing the tendency for breast cancer cells to metastasise to bone. In
651 particular, the orthotopic implantation of murine 4T1 breast cancer cells overexpressing FOXF2 or the
652 intracardiac inoculation of FOXF2-overexpressing human MDA-MB-231 breast cancer cells enhances
653 the formation of osteolytic bone metastases in animals (358). Mechanistically, FOXF2 directly
654 upregulates *CTSK* that, in turn, increases breast cancer cell invasion (358). Interestingly, high
655 expression levels of transcription factors FOXF2 and RUNX2 in primary mammary carcinomas correlate
656 with bone-specific metastasis in patients with breast cancer (200, 358).

657 It is highly likely that miRNA dysregulation in tumor cells contributes to osteomimicry (36). For
658 instance, the downregulation of miR-30, miR-135, and miR-203 enhances abnormal expression of
659 osteoblast-specific genes (*CDH-11*, *RUNX2*, *SOST*, *ITGA5*, *BSP*, *OPM*), which endows tumor cells with
660 full competence for survival in the bone marrow (75, 320). Other genes associated with osteomimicry
661 (*DKK-1*), osteoclastogenesis (*IL-8*, *IL-11*) and tumour cell invasiveness (*CTGF*, *ITGA5*, *ITGB3*) are
662 direct targets for repression by miR-30 family members, these miR-30s being downregulated in
663 osteotropic breast cancer cells (75). Conversely, miR-218 is highly expressed in human MDA-MB-231
664 breast cancer cells and acts as a promoter of bone metastasis formation through stimulation of the
665 expression of metastasis-related genes (*CXCR-4*, *BSP* and *OP*) that are associated with osteomimicry
666 and production of the bone-resorbing factor PTH-rP (321).

667

668 Osteomimicry: current understandings & open questions

- 669 • *In situ* expression of bone proteins in tumor cells from human bone metastasis specimens
670 unequivocally establishes osteomimicry as a process occurring during the development of bone
671 metastases in patients with advanced breast or prostate cancer.
- 672 • Experimentally, RUNX2, FOXF2 and some miRNAs (miR-30, miR-135, miR-203, and miR-218)
673 function as master regulators of osteomimicry.
- 674 • The importance of osteotropic factors as potential biomarkers for the prediction of bone
675 metastasis risk and/or response to bone-targeted agents remains to be investigated.

676

677 **V. DISRUPTING THE BALANCE - TUMOR-INDUCED BONE DESTRUCTION**

678 The radiographic appearance of bone metastases ranges from typically destructive (osteolytic) to
679 mostly bone-forming (osteoblastic) with most tumors demonstrating a mixture of lesions (Figure 1).
680 There is always an imbalance between bone formation and bone resorption during the development of
681 bone metastases. Therefore, predominantly osteolytic lesions are associated with high osteoclast
682 activity and reduced osteoblast activity, whereas predominantly osteoblastic lesions have a high
683 osteoblast activity and variable, but also often increased, osteoclast activity (35, 67).

684 The different molecular mechanisms associated with the formation of osteolytic lesions are
685 described below (Figure 4), whereas tumor-derived factors governing the formation of osteoblastic
686 lesions are described in the next section.

687 **A. Factors Promoting Osteoclast-Mediated Bone Resorption**

688 Several factors secreted by tumor cells stimulate osteoclast activity and bone resorption (PTHrP,
689 lysophosphatidic acid, macrophage-stimulating protein, prostaglandin E2, IL-8, IL-11, MMP-1, CCN3,
690 granulocyte macrophage-colony stimulating factor) (18, 26, 126, 252, 329, 361, 362). Among them,

691 PTHrP was the first to be recognized as involved in malignant osteolysis (126, 265). Using
692 immunohistochemistry in a retrospective series of 31 human breast cancer metastasis specimens,
693 PTHrP has been shown to be expressed in 92% of bone metastases (12 out of 13 samples) and 17% of
694 metastases to non-bone sites (3 out of 18 samples) (265). Early investigations showed that preventive
695 treatment of animals with a neutralizing antibody against PTHrP reduced the development of osteolytic
696 lesions caused by human MDA-MB-231 breast cancer cells (126). PTHrP binds to the type 1
697 parathyroid hormone receptor (PTHr1), a seven-transmembrane G protein-coupled receptor expressed
698 by osteoblast, which stimulates the expression of RANKL. In turn, RANKL binds to its receptor RANK on
699 osteoclast precursors, leading to the formation of new osteoclasts and therefore enhanced bone
700 resorption (126, 329). Moreover, tumor-derived PTHrP inhibits OPG production, thus promoting bone
701 metastasis (329). The production of PTHrP by tumor cells is induced by transcription factors RUNX2
702 and Gli2. *RUNX2* is upregulated in osteotropic breast cancer cells and directly activates the Indian
703 Hedgehog (IHH) pathway characterized by the upregulation of the Gli family of zinc finger transcription
704 factors (Gli1, Gli2 and Gli3) (266). TGF β released from resorbed bone also induces Gli2 expression in
705 tumor cells (3). In turn, Gli2 (but not Gli1 and Gli3) induces PTHrP expression in bone metastatic human
706 breast cancer cells and osteolysis in tumor-bearing animals (314). As a result, the blockade of the
707 RUNX2-IHH pathway in MDA-MB-231 breast cancer cells by Runx2 short hairpin RNA inhibition
708 prevents the osteolytic disease in bone metastatic animals (266). Likewise, the transcription factor MAF
709 mediates breast cancer bone metastasis through the control of many factors including PTHrP (259).
710 Interestingly, MAF expression in primary mammary tumors has been shown to predict treatment
711 outcomes of the bisphosphonate zoledronic acid in reducing the incidence of bone metastases in early-
712 stage breast cancer (64). See section IX for further discussion.

713 Hypoxia also induces PTHrP expression and secretion by tumor cells through a HIF-dependent
714 mechanism (222). Although bone is highly vascularized, the absolute oxygen tension in the bone
715 marrow is quite low, and there is a moderate oxygen gradient between the peri-sinusoidal regions,

716 which have the lowest levels of oxygen tension (9.9 mmHg), and the endosteal region (13.5 mmHg),
717 which is perfused with small arteries (313). Thus, tumor cells experience hypoxic conditions in the bone
718 marrow. Moreover, tumor cells are also susceptible to hypoxia as they grow in the bone marrow, which
719 is caused by reduced vascular supplies of oxygen and nutrients. The role of HIF-1 α in bone metastasis
720 formation has been therefore tested experimentally (146). The extent of bone destruction and
721 vascularisation of bone metastases in animals injected with MDA-MB-231 cells overexpressing an
722 active form of HIF-1 α was significantly increased compared to mock-transfected cells (146). HIF-1 α
723 also directly regulates the expression of transcription factor *Twist* in human breast cancer cells (374),
724 and *Twist* overexpression in osteotropic breast cancer cells promotes bone metastasis formation
725 through a mechanism dependent of miR-10b, facilitating tumor cell invasion and cancer-induced bone
726 destruction (74).

727 Platelet-derived lysophosphatidic acid (LPA) supports progression of osteolytic bone metastases in
728 breast cancer (26,27). By binding to its receptor LPA1 at the tumor cell surface, LPA promotes tumor
729 cell proliferation through the stimulation of a Pi3K/ZEB1/miR-21-dependent pathway (284). LPA also
730 induces the production of interleukins IL-6 and IL-8 by human breast cancer cells, which then stimulate
731 osteoclast-mediated bone resorption (26,27). Pharmacological inhibition of LPA action on its receptor,
732 using a LPA1 antagonist, substantially reduces progression of osteolytic bone metastases caused by
733 MDA-MB-231/B02 breast cancer cells in immunodeficient animals (27). Likewise, the treatment of
734 immunocompetent animals with a LPA1 antagonist inhibits spontaneous dissemination of murine 4T1
735 breast cancer cells in distant organs (lungs, liver) with no effect on primary tumor size (225).

736 Macrophage-stimulating protein (MSP) is produced by tumor cells in breast cancer (362). It binds
737 to the RON receptor tyrosine kinase, which is expressed by osteoclasts but not osteoblasts, and
738 stimulates osteoclast survival and activity (but not osteoclast differentiation) through a RANK-
739 independent, Src phosphorylation-dependent pathway (4). The intratibial injection of MSP-expressing
740 breast cancer cells in syngeneic wild-type mice causes a profound osteolysis (4). Moreover, the

741 therapeutic targeting of RON with tyrosine kinase inhibitor BMS-777607/ASLAN002 inhibits the
742 formation of osteolytic lesions in tumor-bearing animals (4) and reduces bone resorption in
743 postmenopausal women with advanced cancer (phase-I trial; ClinicalTrials.gov identifier
744 NCT01721148).

745

746 B. Factors Suppressing Osteoblast-Mediated Bone Formation

747 Tumor cells not only stimulate osteoclast activity, but also inhibit osteoblast activity, thereby
748 worsening the imbalance between bone formation and bone resorption, and promoting bone destruction
749 (361). Main factors produced by tumor cells that have been shown to suppress osteoblast differentiation
750 include activin A, the BMP inhibitor noggin, dickkopf-1 (DKK-1), and sclerostin (SOST-1) (198, 293, 326,
751 330, 395, 396).

752 Activin A is a member of the TGF- β superfamily of growth factors. It binds to activin type IIA
753 (ActRIIA) or type IIB (ActRIIB) receptors and induces the recruitment and phosphorylation of an activin
754 type I receptor (ActRIB), which then phosphorylates Smad2 and Smad3 intracellular signaling proteins
755 (198). In multiple myeloma, it has been reported that activin A secreted by plasma cells inhibits
756 osteoblast differentiation *via* Smad2-dependent downregulation of DLX (distal-less homeobox)-5 (198).
757 In breast and prostate cancer, activin A might modulate, via Smad signaling, the expression of pro-
758 osteoclastic factors (IL-11, CTGF, MMP-1) (198). Interestingly, in animal models of breast cancer bone
759 metastasis and of multiple myeloma with osteolytic lesions, the treatment of mice with a soluble activin
760 receptor type IIA fusion protein (ActRIIA.muFc) blocks bone destruction (51). Specifically, ActRIIA.muFc
761 stimulates osteoblastogenesis and promotes bone formation in tumor-bearing animals, thereby
762 preventing cancer cell-induced suppression of bone formation (51).

763 Noggin is a BMP antagonist encoded by *NOG*. In breast cancer, *NOG* mRNA expression levels are
764 significantly upregulated in bone metastatic lesions, compared to that observed in brain, lung and liver

765 lesions (326). The silencing of *NOG* in osteotropic breast cancer cell lines substantially reduces bone
766 metastasis formation in animals (326). Similarly, *NOG* is expressed in human prostate cancer cells that
767 metastasize to bone and cause osteolytic lesions in animals (293). Tumor-derived noggin interferes with
768 physiologic bone coupling by inhibiting bone formation, which thereby prevents repair of osteolytic
769 lesions generated by an excess of osteoclast-mediated bone resorption (293).

770 DKK-1 and SOST-1 are two Wnt (Wingless/int) protein antagonists. WNT agonists promote
771 osteoblast proliferation by binding to a receptor complex consisting of a member of the Frizzled
772 transmembrane receptor family and either LRP (low-density lipoprotein receptor-related protein) 5 or
773 LRP 6 (8). Both DKK-1 and SOST-1 exhibit redundant functions by blocking LRP5/6 binding to WNTs,
774 thereby inhibiting WNT signaling (8). High circulating levels of DKK-1 were first reported in multiple
775 myeloma patients with osteolytic lesions (332). Multiple myeloma cells express DKK-1 and the blockade
776 of DKK-1 using neutralizing antibodies results in a decrease of both osteolysis and skeletal tumour
777 growth in murine models of multiple myeloma (76, 372). DKK-1 is also expressed in breast, lung and
778 prostate cancers (39, 76, 130, 131). DKK-1 knockdown in breast and prostate cancer cell lines
779 decreases bone metastasis formation, while DKK-1 overexpression increases bone metastasis and
780 bone destruction *in vivo* (330, 396). Mechanistically, tumor-derived DKK-1 promotes osteolysis in animal
781 models of multiple myeloma and breast cancer bone metastasis and decreases the formation of
782 osteoblastic lesions in a model of prostate cancer bone metastasis by silencing canonical WNT
783 signaling of osteoblasts (76, 330, 396).

784 With regard to SOST-1, this WNT inhibitor is expressed in human primary breast tumors and
785 breast cancer cell lines, especially those that are hormone unresponsive (143, 395). An anti-SOST
786 antibody was shown to decrease the extent of osteolytic lesions in mouse models of MDA-MB-231
787 breast cancer bone metastasis (143, 395). As previously reported for DKK-1 in breast cancer (396),
788 SOST-1 promotes cancer-induced bone destruction by silencing canonical WNT signaling of
789 osteoblasts (143, 395). Furthermore, a treatment with an anti-SOST antibody also protects tumor-

790 bearing animals from cancer-induced muscle weakness, which is a debilitating event that can be
791 associated with bone metastases in breast cancer patients (143). Plasma cells in multiple myeloma do
792 not express SOST-1 (227). SOST-1 is however produced by osteocytes and treatment of animals with
793 an anti-SOST antibody reduces osteolytic lesions induced by multiple myeloma, thereby preventing
794 bone destruction (227).

795

796 C. Osteocytes – Silent Partners with a Role to Play

797 Osteocytes are terminally differentiated osteoblast lineage cells that reside in lacunae within the
798 mineralized bone matrix (6, 80). Osteocytes are by far the most abundant cells of the bone. They are
799 stellate cells that communicate with their environment *via* cytoplasmic projections termed dendrites.
800 Dendrites of osteocytes form Cx43-dependent gap junctions with dendrites of neighbouring osteocytes
801 as well as osteoblasts on the bone surface and cells in the bone marrow and vascular space, which
802 results in the formation of a communication network in the bone matrix (80). Osteocytes modulate bone
803 turnover by regulating osteoblast and osteoclast functions through the secretion of RANKL, SOST and
804 DKK-1, and control calcium homeostasis through remodeling of the osteocytic perilacunar matrix (6,
805 80). They act as mechanosensors to control responses to mechanical loading of the skeleton (6, 80).
806 Moreover, osteocytes regulate phosphate homeostasis through secretion into the circulation of
807 fibroblast growth factor (FGF)-23 (80).

808 The contribution of osteocytes to bone metastasis is only beginning to be uncovered. This may be
809 explained by the fact that studying osteocytes remains very challenging due to their location within the
810 mineralized bone matrix. Methods used to isolate osteocytes from the bone matrix remain difficult and
811 the phenotype of isolated osteocytes is not necessarily maintained *in vitro* (80). For example, human
812 primary osteocytic cells in 2D culture do not express SOST-1, DKK-1 and FGF-23 (54). Thus, *in vitro*
813 methods that are used to isolate and culture osteocytes may be a limitation to the study of osteocyte

814 functions such as in bone metastasis. Despite the technical challenges, it has been shown that tumor
815 growth in bone induces pressure due to the lack of expansible space, suggesting that physical forces
816 might modulate mechanotransduction properties of osteocytes (310). Indeed, application of hydrostatic
817 pressure to cultures of MLO-Y4 osteocytic cells stimulated the secretion of factors associated with
818 enhanced survival and invasion of prostate cancer cells *in vitro* (310). Osteocyte-derived CCL-5 and
819 MMPs were among these factors promoting prostate cancer cell invasion *in vitro* (310). Whether these
820 osteocyte-derived factors promote tumor cell invasion *in vivo* remains to be determined. Tissue
821 engineered 3D bone models formed by primary human osteocytes have facilitated investigation into
822 osteocyte functions (54). For example, primary human osteocytes in a 3D-culture system produce FGF-
823 23, SOST-1, and DKK-1 as opposed to osteocytes in 2D culture (54). Using a 3D model, it has been
824 shown that primary human prostate cancer cells induce a significant increase in the expression of FGF-
825 23, RANKL and, to a lower extent, DKK-1 in primary human osteocytes, whereas SOST-1 expression is
826 drastically decreased when compared to that observed with osteocytes in the absence of tumor cells
827 (54). The authors suggested that the greater decrease in SOST-1 could favor the formation of
828 osteoblastic lesions (54). It is however unclear how SOST-1 expression in osteocytes is downregulated
829 by prostate cancer cells. These experimental findings are in contrast with the observation that high
830 circulating levels of SOST-1 are found in prostate cancer patients with osteoblastic bone metastases
831 (6). Further studies are therefore required to better understand the contribution of osteocytic-derived
832 SOST-1 in prostate cancer bone metastasis.

833 In an *in vivo* model of bone disease caused by human JJN3 multiple myeloma cells, it has been
834 shown that osteocytic dendrites were in direct contact with JJN3 cells in the bone marrow, leading to
835 increases in osteocyte apoptosis and osteocytic RANKL and SOST production (83). *In vitro* cocultures
836 between osteocyte-like MLO-A5 cells and JJN3 myeloma cells showed that cell-to-cell contact activated
837 bidirectional Notch signaling in osteocytes and multiple myeloma cells, which increased multiple
838 myeloma cell proliferation and induced osteocyte apoptosis. In turn, the induction of apoptosis promoted

839 osteocytic RANKL secretion, which then stimulated osteoclast formation (83). Thus, interactions
840 between osteocytes and multiple myeloma cells generate a microenvironment supportive of increased
841 tumor growth and bone destruction (83). These findings are in agreement with the fact that treatment of
842 animals with an anti-SOST antibody prevents bone destruction in different preclinical models of multiple
843 myeloma bone disease (5TGM1, 5T2MM, and MM1.5) (227).

844 In a breast cancer animal model, Cx43 hemichannels in osteocytes have been shown to play a
845 critical role in the suppression of bone metastasis (393). Specifically, Cx43 osteocyte-specific knockout
846 mice and osteocyte-specific Δ 130-136 transgenic mice with impaired Cx43 gap junctions and
847 hemichannels showed increased tumor growth after intra-tibial injection of Py8119 mouse mammary
848 carcinoma cells (393). Additionally, R76W transgenic mice with functional hemichannels but not gap
849 junctions in osteocytes did not display a significant difference (393). Cx43 gap junctions mediate
850 communication between adjacent cells, whereas Cx43 hemichannels serve as a portal for the exit of
851 molecules in the extracellular microenvironment (80). Zhou and colleagues (393) established a specific
852 role for osteocytic Cx43 hemichannels in suppressing breast cancer growth and bone metastasis,
853 whereas osteocytic Cx43 gap junctions did not play such a role. In agreement with this observation
854 (393), ATP is released from osteocytes through Cx43 hemichannels and exerts inhibitory effects on
855 breast cancer cell migration *in vitro* and tumor growth *in vivo* (392). However, these findings are
856 contrary to the pro-metastatic role of Cx43 gap junctions between osteoblasts and breast cancer cells,
857 which promote progression of osteolytic lesions in animals (354).

858 Overall, these findings strongly suggest that osteocytes have a role to play in the development of
859 bone metastases. However, a lot of uncertainties remain as to whether osteocytes have bone
860 metastasis suppressor or promoter activities, and whether this activity depends on the cancer cell type
861 that metastasizes to bone. Further studies are therefore warranted to investigate the contribution of
862 osteocytes in bone metastasis formation.

863

864 D. The Fertile Soil - Contribution of the Bone Matrix

865 Bone is a rich source of growth factors, including TGF β , IGFs and PDGF (platelet-derived
866 growth factor) (361). For example, while there is no difference in bone marrow TGF- β levels between
867 healthy controls and castration-resistant prostate cancer patients without bone metastases, patients
868 with bone metastases have aberrantly high levels of TGF- β (161). Indeed, when released from the
869 resorbed bone matrix, TGF β acts on tumor cells, via SMAD- and COX2-dependent signaling pathways,
870 and stimulates the expression of factors such as Gli2, PTHrP, the Notch ligand Jagged-1, IL-11 and
871 PGE2 (3, 168, 295, 361). Jagged-expressing tumor cells are capable of directly activating osteoclasts
872 by activating the Notch signaling pathway and the therapeutic targeting of Jagged-1 with a monoclonal
873 antibody inhibits bone metastasis formation in animals (295, 390). In addition, TGF β released from the
874 bone matrix during bone destruction contributes to muscle weakness by decreasing Ca²⁺-induced
875 muscle force production (359). Bone-derived IGF-I stimulates growth of breast cancer cells *via*
876 activation of the IGF type I receptor (IGF-IR)/Akt/NF κ B pathway, and IGF-IR was found to be elevated
877 in 13/15 cases of bone metastases obtained from patients with a range of tumor types, supporting a role
878 for the IGF axis in development of human disease (147). Similarly, bone-derived IGF-II stimulates
879 skeletal outgrowth of prostate cancer cells *in vivo* (174). Bone-derived PDGF activates the Akt/PKB
880 survival pathway in osteotropic breast and prostate cancer cells, as PDGF receptors in tumor cells
881 growing in bone are highly expressed compared to nonmetastatic cancer cells (89, 199). Thus,
882 evidence from both model systems and clinical samples support that bone-derived growth factors
883 contribute to bone metastasis formation by promoting skeletal tumor outgrowth. To date, therapeutic
884 targeting of growth factors has so far not resulted in patient benefit however trials including anti-growth
885 factor agents as part of combination therapy for patients with bone metastases are ongoing. For
886 example, the XENERA-1 trial (ClinicalTrials.gov Identifier: NCT03659136) aims to assess the antitumor
887 activity of xentuzamab, a monoclonal antibody that binds both IGF-I and IGF-II and inhibits the binding

888 of these ligands to IGF-R, in patients with ER+/HER2- advanced or metastatic breast cancer and bone
889 metastases.

890 Bone is a mineralized tissue, rich in calcium. We previously discussed the contribution of calcium
891 from the osteogenic niche that facilitates tumor cell proliferation through Cx43 gap junctions (354).
892 However, calcium is also released from bone during osteoclastic resorption. It binds on tumor cells
893 (breast, prostate, renal cell carcinoma) *via* a calcium-sensing receptor (CaSR) and promotes tumor cell
894 proliferation and migration (29, 110, 354). Additionally, calcium stimulates the secretion of PTHrP and
895 epiregulin by tumor cells (29, 361). PTH-rP promotes osteoclast-mediated bone resorption and
896 epiregulin decreases *OPG* expression in osteoblasts, thereby both contributing to the progression of
897 osteolytic lesions (29, 361).

898

899 Osteolysis: current understandings & open questions

- 900 • Understanding the cancer-associated mechanisms that stimulate osteoclast-mediated bone
901 resorption has led to the development of anti-resorptive pharmaceutical agents that have
902 become established as a valuable additional approach to the treatment of bone metastases in
903 patients with advanced cancer.
- 904 • The observation that tumor cells not only stimulate osteoclast activity, but also inhibit osteoblast
905 activity, suggests that stimulating osteoblastic bone formation to promote bone repair could be
906 a novel alternative approach to treat malignant skeletal lesions.
- 907 • The contribution of osteocytes to bone metastasis is only beginning to be uncovered. A better
908 understanding of the interplay between osteocytes and tumor cells will represent an opportunity
909 for therapeutic targeting.

910

911 VI. TOO MUCH OF A GOOD THING - TUMOR-DERIVED FACTORS REGULATING
912 OSTEOSCLEROSIS

913 A number of molecular mechanisms responsible for the formation of osteoblastic lesions have
914 been identified (Figure 5), described in the following sections.

915 A. Factors Promoting Osteoblast-Mediated Bone Formation

916 Tumor cells in the bone environment secrete factors that activate osteoblasts, leading to the
917 formation of skeletal lesions with extensive new bone deposition(osteosclerosis) (210, 248). Among
918 them, endothelin-1 (ET-1) was recognized as a major mediator of osteosclerosis; it stimulates
919 osteoblast proliferation and inhibits osteoclast activity and motility (2, 53, 210, 378). In this respect,
920 prostate cancer patients with bone metastases have far higher circulating levels of ET-1, compared to
921 those with localized cancer (276). Interestingly, *TMPRSS2-ERG* is the most frequent fusion gene
922 expressed in prostate cancer, it is associated with cancer progression, and its expression in PC3c-T1E4
923 prostate cancer cells has been demonstrated to promote *ET-1* expression and formation of osteoblastic
924 lesions in animals (84). Human ZR-75-1 breast cancer cells that produce ET-1 stimulate new bone
925 formation and osteoblast proliferation in organ cultures, and osteoblastic metastases in animals (378).
926 Stimulatory effects of ET-1 on osteoblasts are mediated by two receptors, ETAR and ETBR, which
927 activate similar signaling pathways and down-regulate expression of the Wnt signaling inhibitor DKK-1
928 (276). Osteoblast proliferation and bone metastasis are both inhibited by ETAR antagonists atrasentan
929 and zibotentan, as well as by the dual ETAR and ETBR antagonist bosentan, highlighting the prominent
930 role played by ET-1 in the formation of osteoblastic lesions in preclinical settings (276, 378). Despite
931 this, both atrasentan and zibotentan have failed to show benefit in CRPC patients with bone metastases
932 (47, 240).

933 Breast and prostate cancer cells can produce BMPs, such as BMP-2, BMP-4 or BMP-6, which
934 facilitate the development of osteoblastic bone metastases by stimulating tumor growth and
935 osteogenesis (77, 78, 171, 195). In this respect, prostate cancer cell-derived BMP-4 mediates

936 conversion of endothelial cells into osteoblasts, thereby promoting aberrant bone formation (204).
937 Analyses of human samples of prostate cancer bone metastases confirmed the presence of cells co-
938 expressing endothelial and osteoblastic markers (Tie-2 and osteocalcin, respectively), which together
939 with the detection of increased expression of BMP-4 in bone metastases compared to that of primary
940 prostate tumors, support the hypothesis that endothelial-to-osteoblast conversion could also take place
941 in human disease (204). Overall, these findings (77, 78, 171, 195, 204) illustrate how the dysregulation
942 of BMPs can have deleterious effects on the bone microenvironment. Furthermore, the BMP inhibitor
943 noggin is also secreted by tumor cells and it is the balance between BMPs and noggin that determines,
944 at least in part, the phenotype of breast and prostate cancer bone metastases (293).

945 Prostate cancer cells secrete multiple WNT agonists, including canonical WNTs 3A, 7B and
946 10B, which, by binding to LRP5/6, are known mediators of osteoblast differentiation and mineralization
947 (129, 202, 210, 237). However, the WNT antagonist DKK-1 is also secreted by prostate cancer cells
948 and, as aforementioned for BMPs and noggin (293), it is the relative expression levels of WNT agonists
949 and DKK-1 that determine the phenotype of skeletal lesions (130). For example, C4-2B prostate cancer
950 cells express the WNT agonists WNT7A and WNT8B, but not DKK-1, and they induce mixed
951 osteoblastic/osteolytic lesions in animals (130). DKK-1 overexpression in C4-2B cells antagonizes WNT
952 functions, which leads to the suppression of WNT signaling in osteoblasts and results in the formation of
953 highly osteolytic lesions in animals (130). Among the many proteins downstream of WNT, autocrine
954 WNTs induce BMP-4 and BMP-6 expression in prostate cancer cells that, in turn, promotes osteoblast
955 differentiation (77, 195). *WNT* expression in tumor cells is itself regulated by many factors. For example,
956 T-box family transcription factor TBX2 is overexpressed in human prostate cancer specimens and bone
957 metastases from xenograft mouse models of human prostate cancer (239). It promotes transcription of
958 *WNT3A* in prostate cancer cells and the blockade of WNT3A with neutralizing antibodies dramatically
959 reduces experimental bone metastasis formation (239). Similarly, *WNT5A* and *WNT7B* are targets for
960 the transcription factor $ERR\alpha$ ("Estrogen Receptor Related Receptor alpha") and the androgen receptor

961 (AR), respectively, which are both highly expressed in castration-resistant prostate cancer cells, and
962 they promote tumor growth and development of osteoblastic lesions in animals (108, 391). Thus, there
963 is evidence that WNT signaling is central to osteoblast-stimulatory activity of metastatic prostate cancer,
964 however therapeutic targeting of this pathway is in its infancy (237).

965 PTHrP can be actively involved in the progression of osteoblastic lesions in prostate cancer by
966 enhancing proliferation of bone marrow stromal cells and early osteoblast differentiation (203).
967 Moreover, prostate-specific antigen (PSA), a serine protease expressed by prostate cancer cells and a
968 well-known marker of cancer progression, can cleave IGF binding protein (IGFBP)-5, rendering IGF-I
969 available to bind to its receptor and stimulate osteoblast proliferation (219). PSA also enhances the
970 bioavailability of TGF- β in the bone microenvironment (379). Like PSA, production of urokinase-type
971 plasminogen activator (uPA) by prostate cancer cells can increase IGF-I and TGF- β bioavailability to the
972 bone microenvironment (113).

973 Several other osteoblast-regulatory factors expressed by tumor cells have been identified. These
974 include growth factors [PDGF BB (377), FGFs (FGF-8, FGF-9) (201, 342) and VEGF (176)],
975 adrenomedullin (299), TGF β -regulated gene PMEPA1 (107) and prostatic acid phosphatase (PAP)
976 (188). Prostate cancer cells can also secrete neuropeptides, such as substance P (124) and Sema3A
977 (112), which stimulate osteoblast differentiation.

978

979 **B. Factors Suppressing Osteoclast-Mediated Bone Resorption**

980 Tumor cells that induce osteoblastic lesions not only stimulate osteoblast activity but may
981 sometimes also inhibit osteoclast activity. Among the osteoclast inhibitors produced by cancer cells are
982 ET-1 and OPG (210). Patients with metastatic prostate cancer have high circulating levels of ET-1 and
983 OPG (90, 210, 383). Tumor-derived ET-1 directly inhibits osteoclast-mediated bone resorption by
984 binding to the surface of osteoclasts *via* membrane receptors ETA and ETB (2, 53). Tumor-derived

985 OPG inhibits osteoclast differentiation by binding to RANKL, thereby preventing its interaction with
986 RANK (183). The overexpression of OPG in human C4-2 prostate cancer cells protects these cells from
987 TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis, decreases
988 osteoclast formation and promotes the formation of osteoblastic lesions in animals (70). OPG
989 expression in prostate cancer cells is regulated by factors such as PAP, a tumor-derived acid
990 phosphatase that promotes osteoblast differentiation and bone mineralization (175, 188). PAP
991 knockdown in pro-osteoblastic VCAP prostate cancer cells decreases OPG while increasing
992 RANK/RANKL expression (175). Conversely, PAP overexpression in pro-osteolytic PC3M prostate
993 cancer cells has the inverse effect, increasing OPG while decreasing RANK/RANKL expression (175).
994 The transcription factor $ERR\alpha$ induces OPG expression in MDA-MB-231-B02 breast cancer cells,
995 thereby inhibiting osteoclast differentiation (109). Tumor-derived PSA also stimulates OPG production
996 and inhibits RANKL expression in osteoblasts (379). BMP-2 enhances Wnt/beta-catenin-dependent
997 transcriptional activation of the OPG promoter in osteoblasts (287). Thus, tumor-derived ET-1 and OPG
998 produced by both tumor cells and osteoblasts, by means of their ability to inhibit osteoclast activity,
999 contribute substantially to the formation of osteosclerotic bone metastases in model systems. When it
1000 comes to human disease, evidence that tumor-derived OPG contributes to the pathology of bone
1001 lesions is limited, hampered by the lack of bone metastases available for research. A meta-analysis
1002 found that in studies of prostate cancer, patients with bone metastases had higher levels of serum OPG
1003 compared to patients with metastases in other sites or healthy control (383). However, as this study did
1004 not include any information about the lesion types of the patients (sclerotic/lytic/mixed) it was not
1005 possible to link elevated serum OPG levels to decreased osteoclast activity and a shift in the balance
1006 towards osteoblastic bone lesions.

1007

1008 Osteosclerosis: current understandings & open questions

- 1009 • Despite elevated levels of the osteoblast stimulating factor ET-1 in patients with bone
1010 metastases and supportive data from a number of *in vitro* and *in vivo* model systems, drugs
1011 targeting ET receptors have failed to provide benefit in trials of CRPC.
- 1012 • WNT signalling regulates osteoblast differentiation and is activated in human prostate cancer,
1013 however the precise role of WNT family members in development and progression of the
1014 disease remains to be established.
- 1015 • Studies that combine detailed characterisation of bone lesions with paired measurements of
1016 factors modifying bone turnover in serum and/or bone marrow are required to provide evidence
1017 of which molecules are the most promising therapeutic targets in metastatic prostate cancer.

1018

1019 **VII. CONTRIBUTION OF BONE MARROW CELLS TO TUMOR DEVELOPMENT –** 1020 **MULTIPLE INTERACTIONS BEYOND THE VICIOUS CYCLE**

1021 In addition to the main cell types responsible for bone remodeling described above (osteoblasts,
1022 osteocytes and osteoclasts), the bone microenvironment includes a myriad of interconnected cell
1023 populations, including a rich vascular network, immune cells, adipocytes, nerve cells, and
1024 megakaryocytes. Tumor cells arriving in this environment are proposed to utilize the mechanisms that
1025 regulate normal physiological processes in order to avoid immune surveillance and establish cellular
1026 interactions that support their expansion to overt metastases. As described in earlier sections,
1027 endothelial cells contribute in tumor cell extravasation, tumor cell dormancy and formation of
1028 osteoblastic lesions (119, 204, 267, 273). The role of platelets in stimulating bone metastasis formation
1029 has also been described above (26, 27, 190, 191). With regard to megakaryocytes, the platelet-
1030 producing cells, little is known about their role in bone metastasis, with both promoting and inhibitory
1031 roles having been reported (191, 223). In the following sections we chose to cover some of the key
1032 discoveries linking immune cells, nerve cells and adipocytes to the development of bone metastases in
1033 solid tumors.

1034 A. The Immune Cells of the Bone Microenvironment

1035 It has long been clear that the immune system plays an integral part of both normal bone
1036 homeostasis, as well as in a number of pathologies associated with bone loss, mainly through the link
1037 with inflammation. Combining the bone biology and immunology research fields to increase our
1038 understanding of their close connection has resulted in the new discipline of “osteimmunology” (245).
1039 Initially focused on the bone-destructive effects of immune infiltrates through stimulation of osteoclasts
1040 by pro-inflammatory cytokines, research is expanding to other areas, including bone metastasis (253,
1041 370). Although the inflammatory response undoubtedly contributes to the extent and severity of cancer-
1042 induced bone disease, as covered in the following sections, evidence from model systems support that
1043 immune cells may also affect tumor cell colonization and progression in bone (Figure 6).

1044

1045 1. Immune cells inhibiting local tumor growth in the bone microenvironment.

1046 CD8⁺ T cells

1047 CD8⁺ T cells are central players in controlling infections and cancer, recognised as one of the most
1048 important immune cells associated with tumour destruction (253). By cross-presenting tumor antigens,
1049 dendritic cells (DCs) activate CD8⁺ T cells. In turn, tumor-specific cytotoxic CD8⁺ T cells participate in
1050 the killing of antigen-positive tumor cells (45). The anti-tumor effects of tumor-specific cytotoxic CD8⁺ T
1051 cells is dependent on their ability to produce interferon (IFN)- γ . Pioneering work by the Faccio group has
1052 demonstrated that activation of CD8⁺ T cells reduces bone metastasis formation in animals, whereas
1053 depletion of CD8⁺ T cells enhances it (386). Specifically, using phospholipase C gamma (PLC γ) 2^{-/-}
1054 mice, which have broadly compromised immune responses and are osteopetrotic due to reduced
1055 osteoclast number and functionality, Zhang and colleagues (386) reported an unexpected increased
1056 tumor growth in bone despite osteoclast dysfunction. This was found to be due to a defective anti-tumor
1057 T cell response in tumor-bearing PLC γ 2^{-/-} mice. Similar experiments were then conducted in Lyn^{-/-} mice,
1058 which have enhanced T-cell responses and decreased bone mass due to high number of osteoclasts.

1059 *Lyn*^{-/-} mice had a reduced bone tumor burden despite osteolysis (386). Importantly, injection of antigen-
1060 specific wild-type cytotoxic CD8⁺ T cells in *PLCγ2*^{-/-} mice or depletion of CD8⁺ T cells in *Lyn*^{-/-} mice
1061 normalized tumor growth in bone, regardless of osteoclast activity (386). This study is important in that it
1062 used both genetic and pharmacological approaches to demonstrate that the extent of tumor growth in
1063 bone is not only linked to the level of osteoclast activity as stipulated by the vicious cycle of cancer-
1064 induced bone destruction. In addition, these findings demonstrate that CD8⁺ T cells have the potential to
1065 act as regulators of tumor growth in bone. This contention is supported by the observation that
1066 transcription factor *ERRα* in murine 4T1 breast cancer cells inhibits the progression of bone metastases
1067 by increasing the recruitment of CD8⁺ T cells in the bone marrow (28). However, this remains to be
1068 established for human cancers where our capacity to identify T cell subsets in bone metastatic foci is
1069 limited.

1070

1071 Natural killer (NK) cells

1072 Mature NK cells represent 1% of the lymphocyte population in bone, which is the primary site of murine
1073 NK cell development (253). In contrast, human NK cells are shown to differentiate from precursors and
1074 located in the secondary lymphoid organs like spleen and lymph nodes, and single cell RNA sequencing
1075 of NK cells isolated from both blood and bone marrow of healthy donors has revealed the presence of
1076 multiple heterogenous subsets with potentially different functions (373).

1077 NK cells are involved in the nonspecific elimination of tumor cells through the production of IFN γ ,
1078 release of cytolytic granules or TRAIL/FASL-induced apoptosis (253). Pathways of IFN induction are
1079 regulated by IFN regulatory factors (IRF3, IRF5 and IRF7) and NF κ B (20). Bidwell and colleagues (20)
1080 found that *irf7* expression was suppressed in murine 4T1.2 mouse breast cancer cells isolated from
1081 bone metastases, compared to those of matched primary mammary tumors. Enforced expression of *Irf7*
1082 in bone metastatic 4T1.2 cells restored an antimetastatic immune response in immunocompetent tumor-
1083 bearing animals (20). Conversely, the inoculation of *Irf7*-overexpressing 4T1.2 cells to mice deficient in

1084 NK and CD8⁺ T cell responses led to accelerated development of bone metastases, compared to
1085 immunocompetent mice (20). Similarly, the impairment of NK-cell-mediated anti-tumour immunity with a
1086 JAK/STAT inhibitor enhanced skeletal tumor burden in preclinical models of breast cancer metastasis
1087 (25). Taken together, these data indicated that NK cells (and CD8⁺ T cells), through the production of
1088 IFN- γ , contributed to the suppression of bone metastasis and that NK cells are potential therapeutic
1089 targets in this setting. The clinical relevance of these findings was confirmed in over 800 patients in
1090 whom high expression of Irf7-regulated genes in primary tumors was associated with prolonged bone
1091 metastasis-free survival (20). A comprehensive review describing how NK cells may control metastasis
1092 points out that NK cells appear to have a particular role in reducing metastatic dissemination and
1093 speculates that this may be due to their ability to eliminate tumor cells that escape the
1094 immunosuppressive microenvironment of the primary tumor (211). It also includes an overview of
1095 clinical trials with immunotherapy agents boosting NK cell effector functions, the outcomes of which will
1096 provide important information about the impact of NK cells in metastatic disease, including in skeletal
1097 metastasis.

1098

1099 *2. Immunosuppressive cells promoting local tumor growth in the bone*
1100 *microenvironment*

1101

1102 Myeloid Derived Suppressor Cells (MDSCs)

1103 MDSCs describe a heterogeneous collective of immature progenitor populations for the myeloid cells,
1104 for which a variety of roles in tumor progression have been reported (172). Bone marrow accumulation
1105 of MDSCs is found in many cancer types, indicating pathological disruption of myeloid cell maturation
1106 (114). An important role of MDSCs in the metastatic process is their immune-suppressive functions,
1107 which include induction of oxidative stress, interference with lymphocyte trafficking and expansion of
1108 Treg cells (172). MDSCs are proposed to increase the number of Treg cells and modify tumor growth in
1109 bone independent of osteoclast activation through modification of T cell responses (45). For instance,
1110 PLC γ 2^{-/-} mice are osteopetrotic due to reduced osteoclast number and functionality (45). Interestingly,

1111 despite osteoclast dysfunction, tumor growth in bone of PLC γ 2^{-/-} mice was significantly higher than that
1112 observed in their wild-type counterparts due to an aberrant increased percentage of MDSCs in the bone
1113 marrow that, in turn, inhibited anti-tumor T cell response in tumor-bearing PLC γ 2^{-/-} mice (45, 386).

1114

1115 Few studies have investigated the potential role of MDSCs in human bone metastases, but a recent
1116 report compared polymorphonuclear (PN-) MDSC distribution in primary prostate tumours (n=90) and
1117 their corresponding lymph node metastases (n=37) to that of bone metastases (n=35) (363). PN-
1118 MDSCs were found to mainly infiltrate the stroma (rather than the epithelial areas), and that this was
1119 more prominent in the metastases compared to the primary tumour. The authors propose that this
1120 stromal location would facilitate better suppression of infiltrating T cells by the PN-MDSCs and that the
1121 high levels of CXCL5 in bone may drive MDSC infiltration and ultimately metastatic progression (363).

1122

1123 A combination of the CXCR4 antagonist AMD3465 and the IDO1 inhibitor D1MT has been shown to
1124 delay the progression of breast cancer bone metastases in mice through activation of CD8⁺ T-cells and
1125 inhibition of Treg cells and MDSCs, supporting that suppression of MDSCs could potentially reduce
1126 metastatic progression in bone (385).

1127

1128 MDSCs isolated from tumor bearing mice have been also shown to be able to differentiate into
1129 functional osteoclasts *in vitro* and *in vivo* (81, 288). Interestingly, only MDSCs from mice with confirmed
1130 tumor growth in bone had osteoclastic potential, whereas those isolated from mice with peripheral
1131 tumors or control mice did not (288). The increased understanding of the inter-connectivity between
1132 cells residing in the bone marrow has resulted in studies exploring the effects of anti-resorptive agents
1133 beyond their traditional osteoclast targets. For example, in mouse models, a single, clinically relevant
1134 dose of the osteoclast inhibitor zoledronic acid has widespread effects on a number of cell types in the
1135 bone marrow, including hematopoietic stem cells, myeloid-biased progenitor cells and lymphoid-biased

1136 cells (340). Importantly, bone marrow cells isolated from zoledronic acid treated animals, but not from
1137 control, were able to suppress tumor growth *in vivo* when co-injected with tumor cells, supporting the
1138 finding that anti-resorptive agents could support the generation of tumor-suppressing myeloid cells
1139 (340). In follow-up studies, these findings were confirmed, demonstrating that even a single dose of
1140 zoledronic acid skews myeloid progenitor cells to enter the macrophage, rather than the osteoclast
1141 lineage (339). This exemplifies the potential for unexpected (both beneficial and harmful) effects of anti-
1142 cancer therapies on bone marrow cell populations with implications for tumour progression, generally
1143 not considered when assessing the clinical benefits of cancer treatment. As the anti-resorptive
1144 bisphosphonates are increasingly used as adjuvant therapies in post-menopausal breast cancer without
1145 the precise mechanism conveying their positive effects on survival (66), it will be interesting to see if
1146 additional patient benefit could be linked to effects of these agents on a range of bone marrow cell
1147 populations.

1148

1149 Macrophages

1150 Macrophages develop from circulating monocytes within tissues and are heterogeneous and highly
1151 plastic cells, which can polarize into pro- or anti-inflammatory sub-types (M1 and M2, respectively)
1152 depending on signaling cues (45, 118). However, there are many other discrete sub-populations across
1153 the M1/M2 spectrum determined by the location and activation status of macrophages (45, 118).
1154 Macrophages are consistently found in bone metastases from patients with prostate cancer (369). In
1155 breast cancer, tumor-associated macrophages are also significantly increased in bone metastases
1156 compared to matched primary mammary tumors (394). Experimentally, tumor-associated macrophages
1157 were found to promote breast and lung cancer bone metastasis formation (102, 148). Additionally, a
1158 population of specialist osteal tissue macrophages termed 'osteomacs', whose normal function is to
1159 regulate osteoblast differentiation (50), were found to facilitate formation of osteoblastic lesions in an
1160 animal model of prostate cancer (369). Nonetheless, their specific role in bone metastasis has proven

1161 elusive, in part because of ablation techniques that remove a number of myeloid related populations,
1162 including the closely related osteoclast precursors that are established as major drivers of bone
1163 metastasis. For example, treatment of animals with clodronate-encapsulated liposomes markedly
1164 reduced the number of monocytes in peripheral blood, and the formation of bone metastasis when
1165 HARA-B lung cancer cells were injected intracardiacally to mice (148). However, in this study,
1166 clodronate-encapsulated liposomes not only reduced macrophages within tumors, but also osteoclasts
1167 in metastatic bone lesions, thereby explaining the reduction of bone destruction (148). Similarly, the
1168 use of an anti-mouse CD115 monoclonal antibody, which specifically targets monocytic cells, inhibited
1169 breast cancer bone metastasis formation in animals by blocking osteoclast activity (102). Beside the use
1170 of techniques that can directly interfere with osteoclast function (102, 148), tumor cells can recruit
1171 macrophages and osteoclasts through the same mechanism of action. For example, breast cancer-
1172 derived CCL2, which is a ligand for the chemokine receptor CCR2 expressed by myelomonocytic
1173 progenitors such as macrophages and osteoclast precursors, can stimulate through the same molecular
1174 mechanism the migration of macrophages in lung parenchyma and the differentiation of osteoclasts in
1175 the bone marrow, which ultimately aids metastasis to lungs and bone (214). Similar findings were
1176 reported with CCL2 produced by human prostate cancer cells, which promoted recruitment of
1177 macrophages within subcutaneous tumor xenografts and osteoclast-mediated bone destruction in
1178 animals bearing bone metastases (232). Thus, despite some suggestions that macrophages and
1179 osteomacs can contribute to bone metastasis (45, 369), further studies, including of samples human
1180 bone metastases, are needed to establish the precise mechanisms that could regulate these highly
1181 adaptable cells in the context of tumour growth in bone.

1182

1183

1184 Dendritic cells

1185 Dendritic cells are specialised antigen-presenting cells that are derived from hematopoietic bone
1186 marrow progenitor cells that differentiate into 2 subsets: conventional or myeloid dendritic cells (mDCs,
1187 similar to monocytes, produce IL-12) and plasmacytoid dendritic cells (pDCs, resembling plasma cells,
1188 produce IFN- α). These cells are responsible for presentation of antigens on their surface to induce T
1189 cell activation and prime CD8⁺ T cells (253). However, DCs in tumors can have limited antigen-
1190 presenting function, thereby affecting the generation of anti-tumor immune responses (218).
1191 Additionally, DCs in cancer may exhibit immunosuppressive properties under certain circumstances
1192 (218). For example, using different syngeneic breast cancer models, Sawant and colleagues (289)
1193 observed an increased number of pDCs with increased bone metastasis in animals. Conversely,
1194 depletion of pDCs following treatment of animals with PDCA1 antibody prevented breast cancer bone
1195 metastasis formation (289). Furthermore, isolated CD8⁺ T cells from pDC-depleted mice exhibited
1196 enhanced cytotoxic activity compared to those from untreated animals, indicating that in bone-
1197 metastatic disease pDCs exhibit immunosuppressive properties on CD8⁺ T cells (289). Thus, there is
1198 some evidence to support that pDCs may be critical regulators of bone metastasis, however this is one
1199 of the least investigated immune cell types in this context, with a paucity of informative clinical studies to
1200 provide solid data to allow a conclusion regarding their importance to be drawn at this point.

1201

1202 Regulatory T (Treg) cells

1203 Treg cells are potent immune suppressors, impairing CD8⁺ cell proliferation (45). The role of Treg cells
1204 in bone remodeling has not been extensively studied, with a few examples of inhibition of osteoclast
1205 maturation by Treg cells due to their production of IL-10, IL-4 and TGF β (30). In prostate cancer, Treg
1206 cells are significantly increased in the bone marrow of patients with bone metastasis compared to those
1207 without (389). Furthermore, the intravenous injection of activated Treg cells to immunodeficient
1208 NOD/SCID mice bearing human PC3 prostate cancer skeletal lesions leads to a reduction of bone
1209 destruction, due to the osteoclast-inhibitory effect of Treg cells (389).

1210 A study by Jiao *et al* (161) has shed light on why a combination of anti-CTLA-4 (ipilimumab) and anti-
1211 PD-1 (nivolumab) checkpoint inhibitors that reduce primary prostate tumour growth in patients are
1212 largely ineffective in reducing bone metastatic disease. The study compared levels of TGFβ in bone
1213 from patients with and without bone metastases and mapped T cell subsets in primary tumors and bone
1214 metastases from patients treated with ipilimumab. Results showed that the increased levels of TGFβ
1215 released during cancer-induced bone resorption caused helper T cells to polarise into Th17 CD4 cells
1216 instead of the Th1 CD4 effector cells required to trigger an anti-tumour immune response (161).
1217 Combining anti-TGFβ and anti-CTLA-4 therapy in a mouse model resulted in reduced bone metastasis,
1218 a strategy that the researchers now will take forward in clinical trials of patients with metastatic prostate
1219 cancer (161). This study (161) is an example of 'reverse translation', where a clinical observation is
1220 explored in model systems to identify mechanisms responsible for the observed effects and how they
1221 can be overcome. It also highlights that the specific immune tumor environment in bone presents a
1222 particular challenge when considering immunotherapy approaches in patients with bone metastases.

1223

1224 Immune cells and bone metastasis: current understandings & open questions

- 1225 • Depletion of CD8+ T cells results in increased bone metastasis in model systems, but direct
1226 evidence for a role of CD8+ T cells in development of human skeletal metastases is missing.
- 1227 • The anti-metastatic functions of NK cells are not yet established in human disease, but
1228 evidence from models of bone metastasis support their role in suppressing tumour growth.
- 1229 • Myeloid Derived Suppressor Cells (MDSCs) have been identified in bone metastases in model
1230 systems and in human samples, where they are proposed to increase Tregs and inhibit immune
1231 elimination of tumour cells.
- 1232 • Macrophages are highly plastic cells with context-specific functions, their role in the different
1233 stages of human bone metastasis remains to be defined.

1234 • Plasmacytoid dendritic cells (pDCs) may be critical regulators of bone metastasis in model
1235 systems, however there is a paucity of informative clinical studies to draw any conclusion at this
1236 point.

1237 • The specific immune tumour environment in bone presents a particular challenge when
1238 considering immunotherapy approaches in patients with bone metastases. Differences between
1239 murine and human immune cell populations must be considered when translating findings from
1240 in vivo model systems to human disease.

1241

1242 **B. Nerve cells**

1243 Bone is highly innervated, and a recent extensive review describes how bone homeostasis is
1244 influenced by sympathetic, parasympathetic, and sensory nerves (95). How bone remodelling is
1245 regulated by nerve cells is illustrated by the example of norepinephrine, released by sympathetic
1246 nerves, which activates β 2-adrenergic receptors expressed by osteoblasts, stimulating synthesis of
1247 RANKL that in turn modifies both osteoblast and osteoclast activity (95).

1248 As a cancer diagnosis is associated with increased levels of stress and depression, a number of
1249 studies have investigated whether stress-induced activation of the sympathetic nervous system (SNS)
1250 results in increased bone metastasis. In a model of learned helplessness (chronic immobilization
1251 stress), intracardiac injection of MDA-MB-231 breast cancer cells in mice that had undergone 2 weeks
1252 of SNS activation resulted in increased number of bone metastatic foci associated with larger lytic bone
1253 lesions, compared to control (44). Similar results were obtained when injecting MDA-MB-231 breast
1254 cancer cells in mice pre-treated for 3 weeks with isoproterenol (ISO), which is used as a surrogate of
1255 SNS activation through stimulation of the β 2-adrenergic receptor (44, 234). Importantly, these effects
1256 observed in mice under chronic stress or treated with ISO were inhibited by administration of the β 2-
1257 blocker isopropranolol (44, 234), supporting that sympathetic nerve activity mediates the 'pro-metastatic'
1258 effect of chronic stress. Furthermore, breast cancer bone metastasis in animals that are under chronic

1259 stress is inhibited by knocking down RANK in MDA-MB-231 cells (44). Mechanistically, it is proposed
1260 that stress-induced activation of the β 2-adrenergic receptor in osteoblasts stimulates RANKL production
1261 that, in turn, promotes MDA-MB-231 cell migration to bone in a RANK-dependent manner (44). ISO pre-
1262 treatment of bone marrow stromal cells also induce the expression of pro-inflammatory cytokines (IL-1 β ,
1263 IL-6) that increase the expression of E- and P-selectins by endothelial cells and the subsequent
1264 adhesion of MDA-MB-231 breast cancer cells to these cells under static and dynamic conditions *in vitro*
1265 (56). It has also been suggested that DTCs residing in endosteal niches could be affected by
1266 norepinephrine (82). Specifically, Decker *et al.* (82) found that the binding of norepinephrine to β 2-
1267 adrenergic receptors affects osteoblasts *in vitro* through downregulation of the dormancy-inducing
1268 molecule GAS6, thereby re-activating proliferation of dormant disseminated prostate cancer cells that
1269 interacted with these osteoblasts. Taken together, these experimental data (44, 56, 82) suggest that
1270 stress-induced activation of the SNS prior to tumor cells arriving in bone alters the microenvironment to
1271 become more supportive of tumor outgrowth. Whether this also applies in human disease is difficult to
1272 investigate. A study including over 100,000 women in UK did not find any evidence of increased risk of
1273 breast cancer in those who reported high levels of stress (292). The current experimental evidence for
1274 neuronal involvement therefore relates to progression of established disease, and great care should be
1275 taken not to suggest that patients are in any way responsible for their disease progression through their
1276 ability to manage the inevitable stress associated with a cancer diagnosis and treatment.

1277 Once bone metastases are established and progressing, their interaction with the nervous system
1278 is obvious. Pain is one of the most common and difficult to treat complications associated with skeletal
1279 metastases (229). Tumor cells, their associated stromal cells and osteoclasts can generate pain by
1280 releasing algogenic substances including protons (create acidosis), bradykinin, endothelins,
1281 prostaglandins, proteases, and tyrosine kinase activators such as nerve growth factor (NGF) (100).
1282 Sensory fibers in the bone marrow express acid-sensing nociceptor TRPV1 (transient receptor potential
1283 vanilloid 1) and the NGF tyrosine kinase receptor type 1 (TrkA) (23, 351). In murine models of intra-

1284 femoral or intra-tibial injection of NGF-expressing breast, prostate or Lewis lung cancer cells, tumour
1285 growth in bone is associated with induced sprouting of sensory nerve fibers and lytic bone lesions (23,
1286 162, 226, 351). SNS activation and bone pain (as judged by hyperalgesia and flinching) caused by
1287 Lewis lung cancer cells are substantially reduced in TRPV1^{-/-} mice compared to wild-type animals with
1288 comparable tumor burden (351). Similarly, systemic administration of a neutralizing anti-NGF antibody
1289 to animals bearing breast or prostate cancer bone metastasis reduces ectopic nerve fiber sprouting and
1290 attenuates nociceptive behaviors (spontaneous guarding and flinching) (23,162, 226). Overall these
1291 findings suggest that targeting NGF and/or TRPV1 are potential strategies to treat bone pain.

1292

1293 Nerve cells and bone metastasis: current understandings & open questions

- 1294 • Bone homeostasis is influenced by sympathetic, parasympathetic, and sensory nerves. These
1295 interactions are affected by numerous factors released by tumor cells.
- 1296 • In model systems, stress-induced activation of the sympathetic nervous system alters the bone
1297 microenvironment to become more supportive of tumor outgrowth. However, there is no
1298 evidence that it also applies in human disease.

1299

1300 **C. Adipocytes**

1301 The adipose content of the bone marrow increases with age, obesity levels and metabolic
1302 conditions (134, 230). A large body of research underpins the current view that bone marrow fat is a
1303 hormone-sensitive endocrine tissue with the capacity to modify bone mass, and hence could contribute
1304 to skeletal tumour growth through a range of mechanisms, including provision of energy and pro-
1305 survival factors for tumor cells (134). However, the majority of these studies are from model systems,
1306 often involving injection of large numbers of tumor cells directly into bone of immunocompromised mice
1307 fed a high fat diet. Due to suitable clinical material being difficult to obtain, the relevance of the

1308 information generated in model systems described in this section remains to be validated in studies of
1309 human samples.

1310 Adipocytes can be drivers of chronic inflammation, resulting in immune cell infiltration and release
1311 of high levels of pro-inflammatory cytokines, including CXCL-1, CXCL-2, IL-1 β , IL-6 and TNF α ,
1312 molecules known to stimulate bone resorption and bone metastasis (134, 135, 140, 151). Using an *in*
1313 *vivo* diet-induced obesity model, Herroon *et al.* (142) have demonstrated a direct link between
1314 adipocytes and prostate cancer growth in bone. Specifically, the intratibial injection of PC3 prostate
1315 cancer cells into high-fat-diet-fed mice led to larger tumors than those observed in mice on normal diet
1316 (142). *In vitro*, prostate cancer cells exposed to lipids supplied by bone marrow adipocytes displayed
1317 increased invasive and proliferative capacity compared to control, which was associated with induction
1318 of lipid chaperone FABP4 (fatty acid binding protein 4) and IL-1 β in tumor cells (142). Although FABP4
1319 is known for its expression in adipocytes, it was also expressed by PC3 cells co-cultured with
1320 adipocytes, and its inhibition with a selective inhibitor (BMS309403) blocked PC3 cell invasion *in vitro*
1321 (142). Immunohistochemical staining showed that in the small number of human prostate cancer bone
1322 metastasis samples analysed (n=5), FABP4 positivity was more pronounced compared to benign
1323 prostate lesions and primary tumor tissues (142). The authors acknowledge that further studies are
1324 however required to establish whether FABP4 acts as a mediator between adipocytes and tumor cells
1325 to stimulate tumor growth in human bone metastases.

1326 In addition to FABP4, the expression of oxidative stress enzyme HO-1 (heme oxygenase 1) was
1327 also found to be significantly upregulated in prostate cancer bone metastases from high-fat-diet-fed
1328 mice (141). *In vitro*, bone marrow adipocytes induced the upregulation of HO-1 in prostate cancer cells,
1329 whereas, *in vivo*, HO-1 overexpression in human prostate cancer cells promoted skeletal tumor growth
1330 and osteolysis (141). Importantly, a link to human prostate cancer was established by analysis of five
1331 datasets of patients with metastatic disease (n=89), showing significant upregulation of HO-1 in
1332 metastatic foci (including in bone) compared to primary tumors. HO-1 expression was identified by

1333 immunohistochemical staining in two samples from prostate cancer bone metastasis biopsies, providing
1334 some limited support that HO-1 is associated with tumor progression in bone, which requires validation
1335 in a larger sample set.

1336 Bone marrow adipocytes also promote metabolic reprogramming of prostate cancer cells in bone
1337 towards a glycolytic phenotype (87) (*see section VII for further discussion*). Additional studies utilizing
1338 these diet-induced models of increased bone marrow adiposity demonstrated that the adipose-derived
1339 chemokines CXCL1 and CXCL2 cause accelerated osteoclastogenesis *in vitro*, leading to enhanced
1340 prostate cancer-associated bone degradation *in vivo* (135) (Table 1).

1341 Adipocytes have also been linked to bone metastasis in a number of cancer types other than
1342 prostate cancer. In multiple myeloma, bone marrow adipocytes support tumor cell proliferation and
1343 migration *in vitro* through mechanisms that are, at least in part, mediated by leptin (43). Furthermore,
1344 myeloma cells promote MSC differentiation into adipocytes at the expense of osteoblasts by inhibiting
1345 expression of the ubiquitin ligase MURF1, thereby suppressing osteoblast-mediated bone formation in
1346 tumor-bearing animals and in cells from patients with myeloma (208). In a model of breast cancer bone
1347 metastasis, using conditioned medium generated from cultured adult human bone fragments, migration
1348 of MDA-MB-231 cells was found to correlate with increasing levels of the adipokine leptin and IL-1 β
1349 (328). Direct co-cultures demonstrated high numbers of breast cancer cells associated with the marrow
1350 adipose tissue within the bone fragments, supporting that tumour cells colonise areas of bone with high
1351 adiposity (328). Similar findings were reported from studies of melanoma models of bone metastasis,
1352 showing that melanoma cells were located in close proximity to adipocytes when colonising bone (52,
1353 356). Furthermore, the intra-tibial injection of B16F10 melanoma cells resulted in larger tumors and
1354 increased osteoclast numbers in mice fed a high-fat diet, compared to that observed in mice fed a
1355 normal diet (52). In agreement with these findings, melanoma cell-adipocyte co-culture experiments
1356 showed an increase in pro-inflammatory and pro-osteoclastic production of cytokines and chemokines
1357 (IL-6, IL-1 β , CXCL-1, CXCL-2, and CXCL-5) by melanoma cells (52). Wang and colleagues (356)

1358 reported that following intra-cardiac injection of B16F10 melanoma cells into mice, regardless of diet,
1359 there was a transient, highly significant increase in bone marrow adiposity and serum leptin levels
1360 compared to that of age-matched controls. *In vitro*, conditioned medium from melanoma cells promoted
1361 differentiation of adipocytes; conversely melanoma cell proliferation was stimulated by exposure to
1362 adipocyte-conditioned medium. The authors conclude that adipocytes may contribute to the lytic bone
1363 disease caused by melanoma cells (356). However, as tumours grow and induce lytic bone disease the
1364 number of adipocytes is rapidly reduced (356). This decrease in the number of adipocytes may result
1365 from a lipolysis that fuels tumor cells with adipocyte-derived fatty acids, thereby promoting tumor growth
1366 (140).

1367 Collectively, the experimental studies described above represent the increasing volume of data
1368 providing a strong link between bone marrow adipocytes and the progression of bone metastasis,
1369 however evidence from human disease to support this remains surprisingly limited. A relatively small
1370 retrospective study of 2,731 patients with early breast cancer found no significant link between body
1371 mass index and the subsequent pattern of metastasis, but in agreement with other reports, obese and
1372 overweight patients had significantly shorter survival compared to the normal weight group (375). As
1373 levels of obesity are rising, including in young people, there are concerns that we are facing an increase
1374 in almost all cancers, as well as the potential for more aggressive metastatic disease, including in bone,
1375 driven by some of the mechanisms described in this section.

1376

1377 Adipocytes and bone metastasis: current understandings & open questions

- 1378 • Bone marrow fat is a hormone-sensitive endocrine tissue with the potential to modify bone
1379 mass and to influence skeletal tumour growth through provision of energy and tumour cell
1380 survival factors, as well as through generation of a pro-inflammatory environment.

1381 • Solid evidence from clinical studies for a link between obesity and bone metastasis is lacking,
1382 this important area should be the focus of retrospective analyses of large datasets that include
1383 detailed information about patient BMI and metastatic sites.

1384

1385 VIII. FUELING EXPANSION - REPROGRAMMING ENERGY METABOLISM TO 1386 FACILITATE BONE METASTASIS PROGRESSION

1387 Metastatic tumor cells colonizing distant organs must rewire their biology in order to grow in the
1388 colonized organ (1,133, 271, 291). Most cancer cells use glycolysis (an oxygen-independent metabolic
1389 pathway) for glucose metabolism even when oxygen is sufficient (133). This phenomenon is called “the
1390 Warburg effect” or “aerobic glycolysis”. Consequently, glucose is utilized for ATP generation through
1391 lactate production and *via* the pentose phosphate pathway (PPP) for nucleotide synthesis that is
1392 essential for cell proliferation (Figure 7). Not only does the Warburg effect allow cells to maintain ATP
1393 levels but also reduce oxidative stress and generation of ROS, enabling cancer cells to survive at the
1394 metastatic site (291). The existence of these metabolic adaptation mechanisms in cancer cells has been
1395 observed *in situ* in patients with bone metastasis (262), as visualized by ¹⁸F-FDG-PET scanning of
1396 breast cancer bone metastases (Figure 8).

1397 In order to increase glucose uptake, cancer cells upregulate glucose transporters, notably glucose
1398 transporter 1 (GLUT1), phosphoglycerate kinase and lactate dehydrogenase A (133). These enzymes
1399 of the glycolytic pathway, including phosphoglycerate kinase and PPP-associated proteins, such as 6-
1400 phosphogluconolactonase, were observed to be highly expressed in osteotropic breast cancer cells and
1401 bone metastases from patients with breast cancer (48). Moreover, osteotropic MDA-MB-231 breast
1402 cancer cells produce large amounts of lactate, compared to non-osteotropic ones (197). Lactate is
1403 released from MDA-MB-231 cells by monocarboxylate transporter 4 (MCT4) and uptaken by osteoclasts
1404 through the transporter MCT1, which then fuels their oxidative metabolism and promotes osteoclast-
1405 mediated bone resorption (197) (Figure 7). These experimental findings are supported by
1406 immunohistochemical analysis of a small number of human breast cancer bone metastasis specimens

1407 (n = 4) showing a strong staining for MCT4 in tumor cells at the bone metastatic site (197). In prostate
1408 cancer bone metastasis, bone marrow adipocytes promote aerobic glycolysis in tumor cells *in vitro* and
1409 *in vivo* by up-regulating *HIF-1 α* (87). In turn, HIF-1 α stimulates the expression of proteins involved in
1410 glucose uptake, such as GLUT1, and glycolytic genes, such as phosphoglycerate kinase and lactate
1411 dehydrogenase A (87). Thus, aerobic glycolysis seems to be key in supporting skeletal tumor growth
1412 and osteolysis, at least experimentally. This metabolic adaptation system of cancer cells in bone also
1413 occurs in breast cancer patients with bone metastasis (262), as judged by FDG-PET (Figure 8).
1414 Although some clinical studies suggest that FDG has a higher sensitivity for detecting bone metastasis
1415 than primary lesions in prostate cancer, FDG remains however of limited use in this cancer type when
1416 compared to imaging agent ¹⁸F-fluorocholine (FCH), thereby suggesting prostate cancer cells also use
1417 nonglucose metabolic pathways to thrive in colonized organs (352).

1418 Beside aerobic glycolysis, cancer cells in bone can use additional sources of energy. In the
1419 previous section we have discussed the contribution of bone marrow adipocytes, which can provide free
1420 fatty acids as an energy source for tumor cell survival and growth. In addition, the cell membrane
1421 phospholipid choline can be abnormally metabolized and internalized in tumor cells overexpressing
1422 choline kinase (352). This abnormal regulation of the phospholipid metabolism has been observed *in*
1423 *situ* in cancer cells metastatic to bone (290, 348), as visualized by FCH-PET scanning of prostate
1424 cancer bone metastases (Figure 8). Autophagy could be another potential source of energy for tumor
1425 cells in bone (233). For example, the small-GTPase Rab5a and Runx2 stimulate autophagosome
1426 trafficking in human osteotropic metastatic breast cancer cells (224, 324). However, further studies are
1427 clearly needed to understand how autophagy facilitates bone metastasis formation. Of note, cancer
1428 cells transport a significant portion of glucose-derived pyruvate into mitochondria where it serves as an
1429 anaplerotic substrate to replenish tricarboxylic acid (TCA) cycle intermediates used for the biosynthesis
1430 of fatty acids and cholesterol as well as protein acetylation (1) (Figure 7). In this respect, there is
1431 experimental evidence that this mitochondrial metabolism can be used as a source of energy for

1432 prostate cancer cells in bone (331). High levels of cholesterol in human prostate cancer bone
1433 metastasis specimens were observed, compared to normal bone (331). In addition,
1434 immunohistochemistry shows intense staining of the low-density lipoprotein receptor and variable levels
1435 of the scavenger receptor class B type 1 and 3-hydroxy-3-methylglutaryl-coenzyme reductase in
1436 prostate cancer cells that are metastatic to bone, thereby indicating possibilities for influx and *de novo*
1437 synthesis of cholesterol (331).

1438 Taken together, these studies provide evidence that aerobic glycolysis and/or abnormal
1439 phospholipid and mitochondrial metabolism in tumor cells may contribute to skeletal tumor burden and
1440 bone destruction *in vivo*.

1441

1442 Reprogramming energy metabolism: facts & open questions

1443 • There is substantial evidence from model systems and human studies that energy metabolism
1444 is disrupted to favour glycolysis in breast cancer bone metastases. However, further work is
1445 required to validate the importance of aerobic glycolysis in other model systems of cancer and
1446 bone metastasis, and in patients with advanced cancer and bone metastasis other than breast
1447 cancer.

1448 • The importance of other mechanisms (autophagy, increased cholesterol synthesis) and the
1449 potential for therapeutic targeting of energy metabolism to inhibit bone metastasis needs to be
1450 established in model systems in order to determine the implications for human disease.

1451

1452 **IX. BLOCKING BONE DECONSTRUCTION - CURRENT THERAPIES FOR THE** 1453 **TREATMENT OF BONE METASTASIS**

1454 In general, the treatment of bone metastases is aimed at palliating morbidity associated with
1455 skeletal lesions. It cures only rarely (*e.g.*, in lymphoma) and treatment varies depending on the tumor
1456 type. The treatment of bone metastasis includes external beam radiotherapy, systemic therapy with

1457 cytotoxic antineoplastic drugs (chemotherapy) and endocrine agents, targeted therapies and targeted
1458 radionuclide therapy. In addition, orthopedic intervention may be necessary for impending
1459 pathological fractures. Optimal management of skeletal metastases requires a multimodality approach
1460 that involves the combined expertise of medical and radiation oncologists, interventional radiologists,
1461 nuclear medicine and orthopedic oncologists, general physicians, palliative medicine specialists and the
1462 symptom control team (69, 72). Treatment decisions depend on whether the bone disease is localized
1463 or widespread, the presence or absence of extra-skeletal metastases, and the nature of the underlying
1464 malignancy. Systemic therapy for bone metastases can be directed against the tumor cell to reduce
1465 both cell proliferation and, in consequence, the production of cytokines and growth factors influencing
1466 bone cell function. Alternatively, systemic treatment is directed toward blocking the effect of these
1467 substances on host cells. Chemotherapy, biologically targeted agents, and endocrine treatments have
1468 direct antitumor effects, whereas bone targeted agents (BTA) such as the bisphosphonates and
1469 denosumab are effective by preventing host cells (primarily osteoclasts) from reacting to tumor
1470 products.

1471 In the past two decades BTA have become established as a valuable additional approach to the
1472 range of current treatments (60, 345). Biochemical data indicate that osteoclast-mediated bone
1473 resorption is of importance not only in osteolytic bone metastases such as in breast and lung cancer but
1474 also in prostate cancer osteoblastic lesions, with values of resorption markers in the latter at least as
1475 high as those seen in breast cancer and other solid tumors (*see section X for further discussion*). As a
1476 result, the osteoclast is a key therapeutic target for skeletal metastases irrespective of the tissue of
1477 origin.

1478 BTA provide an additional treatment approach for the relief of bone pain across a range of tumor
1479 types, with effects that seem to be independent of the nature of the underlying tumor or radiographic
1480 appearance of metastases (367).

1481 Approved BTA for use in oncology include the bisphosphonates, the RANK ligand inhibitor
1482 denosumab and bone seeking radionuclides including radium-223, strontium-89 and samarium-153.

1483 As our understanding of the signaling mechanisms between bone cells and tumor cells increases,
1484 several new, targeted agents have entered clinical development. These agents include inhibitors of
1485 cathepsin K and Src kinase (both key regulators of osteoclast function), mammalian target of rapamycin
1486 (mTOR) inhibitors such as everolimus, endothelin antagonists such as atrasentan, several agents
1487 targeting TGF beta and various anabolic agents including inhibitors of the WNT signaling pathway.

1488 Below, we describe the progress and future directions of existing bone-targeted therapies and
1489 report emerging therapies that have arisen from advances in our understanding of the biology of bone
1490 metastases (Figure 9).

1491

1492 **A. Inhibiting Bone Resorption by Targeting Osteoclasts**

1493 *1. Bisphosphonates*

1494 The bisphosphonates are pyrophosphate analogs, characterized by a P-C-P-containing central
1495 structure rather than the P-O-P of pyrophosphate and a variable R' chain that determines the relative
1496 potency, adverse effects, and precise mechanism of action (58). The P-C-P backbone renders
1497 bisphosphonates resistant to phosphatase activity and promotes binding to the mineralized bone matrix.
1498 The absorption of oral bisphosphonates from the gut is poor, variable, and dramatically inhibited by food
1499 intake. After intravenous administration of a bisphosphonate, the kidney rapidly excretes approximately
1500 25% to 40% of the absorbed dose and the remainder binds avidly to exposed bone around resorbing
1501 osteoclasts, leading to high local concentrations of bisphosphonate in the resorption lacunae.

1502 During bone resorption, bisphosphonates are internalized by the osteoclast, where they cause
1503 disruption of several biochemical processes involved in osteoclast function, ultimately leading to
1504 apoptotic cell death. Nitrogen-containing bisphosphonates inhibit farnesyl pyrophosphate (FPP)
1505 synthase within the mevalonate pathway that is responsible for events that catalyse post-translational

1506 modification of a number of proteins, including the small guanosine triphosphatases such as Ras and
1507 Rho (58). Bisphosphonates that do not contain nitrogen, such as clodronate, induce osteoclast
1508 apoptosis through the generation of cytotoxic adenosine triphosphate analogs. The biological half-life of
1509 bisphosphonates is long with effects after a single dose still detectable several years later (34, 58).

1510 Based on the results of large randomized controlled trials (see below), BTA have become the
1511 standard of care for the treatment and prevention of skeletal complications associated with bone
1512 metastases in patients with solid tumors (60, 345). The primary end point of these studies was the
1513 influence of bone-targeted treatment on the number of patients experiencing SREs, as well as the time
1514 to the first SRE and the rate of SREs as determined by either a simple annual rate or more complex
1515 multiple event analysis techniques.

1516

1517 *Bisphosphonates for metastatic bone disease*

1518 The greatest experience with BTAs from the use of bisphosphonates in the management of bone
1519 metastases from breast cancer, where the value of the agents is undisputed. Placebo controlled
1520 randomized trials have shown that both the oral agents, clodronate and ibandronate, and the
1521 intravenous formulations of pamidronate, ibandronate and zoledronic acid all have useful clinical
1522 efficacy.

1523 Pamidronate was the first intravenous bisphosphonate to be systematically evaluated and has
1524 clinically important efficacy on skeletal morbidity, quality of life, pain and analgesic use in patients with
1525 breast cancer (153, 207). Zoledronic acid is the most potent bisphosphonate available and has been the
1526 bisphosphonate of choice in most clinical settings and health care systems around the world for more
1527 than a decade. In a placebo-controlled trial of zoledronic acid, the percentage of patients with at least
1528 one SRE after one year was significantly reduced from 50% in the placebo group to 30% with zoledronic
1529 acid ($P = .003$) (179). In comparison with placebo, zoledronic acid also significantly delayed the time to
1530 first SRE and reduced the overall risk of SREs by 41% (179). Zoledronic acid is somewhat more

1531 effective than pamidronate and oral ibandronate in preventing SREs (9, 278). In a multiple event
1532 analysis, zoledronic acid reduced the risk of developing skeletal complications by an additional 20%
1533 compared with pamidronate ($P = .025$) (277). In a randomized comparison of oral ibandronate and
1534 intravenous zoledronic acid, the two agents had broadly similar activity although ibandronate did not
1535 meet the strict statistical criteria for non-inferiority defined in the study protocol (9).

1536 Bisphosphonates have been shown to reduce bone pain and biochemical markers of bone
1537 resorption in patients with osteoblastic bone lesions that are associated with advanced prostate cancer.
1538 Despite somewhat disappointing preliminary results with other bisphosphonates, zoledronic acid was
1539 investigated in patients with CRPC and bone metastases and showed that the increased potency of this
1540 compound translated into improved clinical benefit (283). Treatment with zoledronic acid reduced the
1541 overall risk of skeletal complications by 36% and extended the time to first skeletal complication by
1542 more than 4 months. Bone pain was also reduced at all time points (283).

1543 The pathophysiology of bone metastases is broadly similar in all tumor types, and BTA could thus
1544 be expected to be of value in preventing skeletal morbidity across the range of tumors involving the
1545 skeleton, especially if metastatic bone disease was a patient's dominant site of disease. As part of the
1546 development program for zoledronic acid, a phase-III randomized, placebo-controlled trial was
1547 performed in patients with bone metastases from a wide range of solid tumours other than breast or
1548 prostate cancer; more than half of the persons recruited had lung cancer (279, 280). Zoledronic acid
1549 significantly reduced the proportion of patients with at least one SRE, almost doubled the time to the
1550 first SRE compared with placebo and reduced the overall risk for SRE(s) by about 30% compared with
1551 placebo.

1552 There remains uncertainty regarding the most appropriate duration and schedule of treatment.
1553 Bone targeted therapy should certainly not be stopped following the development of a first skeletal
1554 related event whilst on treatment; this should not be considered a failure of treatment, as the trials
1555 demonstrate a significant reduction in second and subsequent complications with continued treatment.

1556 Several trials have investigated the schedule of bisphosphonate treatment and suggested that
1557 the efficacy of 3 monthly and monthly administration of zoledronic acid is similar. For example, the
1558 CALGB 70604 (Alliance) trial, randomized patients with bone metastases from a range of different
1559 primary tumor types to zoledronic acid on a monthly or three-monthly schedule from the outset of
1560 treatment for two years (145). This study demonstrated non-inferiority of less frequent administration; in
1561 both arms, 29% of patients developed ≥ 1 SRE and suggests that three monthly administration of
1562 zoledronic acid is a reasonable choice for most patients (145).

1563

1564

1565 *Bisphosphonates for prevention of cancer treatment induced bone loss*

1566 There are now increasing numbers of long-term survivors from cancer who have received
1567 combination chemotherapy, radiotherapy, and hormonal cancer treatments. Many of these survivors are
1568 at increased risk of osteoporosis, largely because of the endocrine changes induced by treatment.
1569 Cancer treatment-induced bone loss (CTIBL) is a particularly important long-term problem for women
1570 with breast cancer and men receiving androgen deprivation therapy (ADT). For example, the fracture
1571 incidence in women with breast cancer on an aromatase inhibitor was found to be around 18–20% after
1572 5 years follow-up suggesting that about one in five women on an aromatase inhibitor without a bone
1573 protective agent will sustain a fracture (121).

1574 More than half of cases of prostate cancer occur in men over the age of 70 and thus many men
1575 with prostate cancer are at risk of osteoporosis, exacerbated by the ADT many will receive as treatment
1576 for their cancer. ADT reduces serum concentrations of testosterone to less than 5% of normal level and
1577 estrogen to less than 20% of normal level with consequent adverse effects on bone turnover and an
1578 increase in fracture rate, as clearly demonstrated by large retrospective epidemiological studies (296).
1579 In addition, ADT affects muscle mass, making falls more likely.

1580 The effects of both bisphosphonates and denosumab (*see next section*) on CTIBL have been

1581 studied in multiple randomized clinical trials. These studies have used dosing regimens that are similar,
1582 but not necessarily identical to those used for the treatment of age-related osteoporosis. In breast
1583 cancer, zoledronic acid is the most comprehensively studied bisphosphonate. In premenopausal
1584 women, zoledronic acid (4 mg IV every 6 months) prevented the bone loss associated with ovarian
1585 function suppression (OFS) and tamoxifen or an aromatase inhibitor whereas in the control group
1586 reductions in BMD at 3 years were around 5% and 10% with OFS plus tamoxifen and anastrozole
1587 respectively (123). In postmenopausal women, three companion trials (Z-FAST, ZO-FAST, E-ZO-FAST)
1588 compared the efficacy of a similar dosing schedule of zoledronic acid given either in conjunction with
1589 initiation of an aromatase inhibitor (immediate group), or if required due to a decline in BMD during
1590 adjuvant aromatase inhibitor therapy to a T-score < -2.0 at any site or a non-traumatic fracture (delayed
1591 group). At 5 years all three trials reported similar results with 7-9% and 4-6% differences in lumbar spine
1592 and hip BMD respectively between the two treatment arms in favor of zoledronic acid (38, 62, 209).
1593 None of these studies were designed to show a significant difference in fracture incidence between the
1594 treatment arms. Nevertheless, the BMD effects are similar to those seen in trials performed in
1595 postmenopausal osteoporosis in which bisphosphonates confer a relative risk reduction (RRR) of 45%
1596 for vertebral fractures and approximately 16% RRR for non-vertebral fractures (94).

1597 Several other randomized clinical trials have investigated the efficacy of oral bisphosphonates for
1598 preventing CTIBL in breast cancer (127). The numbers of patients included in each study is somewhat
1599 less than for the zoledronic acid studies and thus, unlike for other forms of osteoporosis, the evidence
1600 for efficacy of oral bisphosphonates in this specific setting is less robust. Indirect cross trial comparisons
1601 suggest the increases in BMD with oral regimens are somewhat less than with zoledronic acid or
1602 denosumab, especially in younger women receiving ovarian function suppression (OFS) or experiencing
1603 chemotherapy induced menopause. Again, none of the trials with oral agents were designed to assess
1604 reliably the impact of oral bisphosphonates on fracture risk.

1605 Alendronate, risedronate, pamidronate, and zoledronic acid have all been shown to prevent loss

1606 in BMD in patients with prostate cancer but the studies have been small and, while the preservation of
1607 BMD would argue for a favorable effect on fractures, the magnitude of effect cannot be reliably
1608 assessed (144).

1609 Overall, current guidelines for preventing CTIBL suggest that patients having adjuvant endocrine
1610 treatment should be managed according to risk of fracture (60, 127). Patients with a T-score of greater
1611 than -2 and no additional risk factors for fracture are advised to exercise and receive calcium and
1612 vitamin D, with risks and BMD monitored every one-two years. If the T-score is less than -2, or there are
1613 two or more risk factors for fracture, patients should receive the same advice and supplements plus a
1614 bisphosphonate or denosumab (*see next section*). Guidelines recommend continuing anti-resorptive
1615 therapy for as long as the patient is receiving endocrine treatment.

1616

1617 *Disease modifying effects of bisphosphonate treatments*

1618 The potential benefits of bone-targeted treatments on the clinical course of breast cancer in terms
1619 of prevention of recurrence and death from breast cancer have been an area of intense study over the
1620 past 20 years. In breast cancer patients with no sign of distant metastases, but having a minimal
1621 residual disease in the bone marrow, CTIBL leads to the release of bone-derived growth factors from
1622 resorbed bone that, in turn, may activate DTCs from a dormant to a proliferative state and trigger bone
1623 relapses. Of note, adjuvant zoledronic acid treatment of patients with early breast cancer improves
1624 elimination of DTCs (307). Bisphosphonates exert, at least experimentally, a variety of direct and
1625 indirect anticancer activities (58, 312). Moreover, bisphosphonates, by decreasing bone resorption, may
1626 also make the bone microenvironment less hospitable for tumor cells, thereby explaining the elimination
1627 of DTCs. These findings suggested a greater role for the use of bisphosphonates than has previously
1628 been considered. Individual trials provided varying results that suggested benefits were restricted to
1629 women who had low levels of reproductive hormones due to either natural age-related menopause or
1630 ovarian function suppression. This hypothesis was confirmed by the Early Breast Cancer Trialists'

1631 Collaborative Group (EBCTCG) meta-analysis of individual patient data from >18,000 breast cancer
1632 patients included in randomized trials of adjuvant bisphosphonates. The meta-analysis showed that
1633 adjuvant bisphosphonates (intravenous zoledronic acid, oral clodronate and oral ibandronate) only
1634 reduced breast cancer recurrences and breast cancer deaths in postmenopausal women (93). Overall,
1635 across all age and menopausal groups, despite a reduction in bone metastases, adjuvant use of a
1636 bisphosphonate had no significant effect on breast cancer recurrence (rate ratio (RR)=0.94) and the
1637 effect on breast cancer mortality, though statistically significant, was small (RR=0.91) (93). However, in
1638 postmenopausal women or those receiving ovarian suppression with goserelin, clinically important
1639 benefits were seen with statistically significant improvements in overall breast cancer recurrence
1640 (RR=0.86), distant recurrence at any site (RR=0.82), bone recurrence (RR=0.72) and breast cancer-
1641 specific mortality (RR=0.82) (123). This equates to prevention of more than 1 in 6 breast cancer deaths
1642 at 10 years. Several international guidelines now recommend the use of adjuvant bisphosphonates in
1643 postmenopausal early breast cancer, especially for those at moderate to high for recurrence (86, 128).

1644 Understanding why the benefits of adjuvant bisphosphonates appear restricted to
1645 postmenopausal women is a priority area for further research. There does not appear to be a link
1646 between bone resorption rates and treatment efficacy (33). On the other hand, more detailed evaluation
1647 on the primary tumor may help identify patients who will benefit from an adjuvant bisphosphonate. For
1648 example, a study demonstrated that patients with a MAF negative tumour (79% of all patients),
1649 evaluated using a FISH assay for the transcription factor, had significantly improved survival at 10 years
1650 and a lower relapse rate with the use of adjuvant zoledronic acid (64). On the other hand, in the 21% of
1651 women with tumors that over-express MAF no benefits were seen in this subset of patients treated with
1652 an adjuvant bisphosphonate and, in younger patients, disease outcomes were significantly worse (64).

1653 For reasons that remain unclear, the disease modifying effects of bisphosphonates in
1654 postmenopausal breast cancer have not been seen in men with prostate cancer treated with ADT. In the
1655 randomized controlled STAMPEDE trial, the addition of zoledronic acid alone or in combination with

1656 docetaxel chemotherapy to ADT for men with advanced prostate cancer did not improve survival,
1657 despite extending the time to first skeletal complication (157). By contrast, docetaxel showed evidence
1658 of survival improvement when combined to ADT (157).

1659

1660 2. *Anti-RANKL antibody: Denosumab*

1661 Therapeutic candidates to inhibit RANK/RANKL interaction were developed. Fusion proteins were
1662 initially engineered. These are recombinant proteins comprising the Fc portion of human IgG1 fused with
1663 the N-terminal ligand binding cysteine-rich domain (CRD) of OPG (OPG-Fc/AMGN-007 and Fc-OPG) or the
1664 four extracellular CRDs of RANK (RANK-Fc) (183). Both Fc-OPG and RANK-Fc potently inhibit bone
1665 resorption in preclinical models of osteoporosis and of cancer and bone metastasis (183, 312). However,
1666 following repeat dosing of human RANK-Fc in primates, autoantibodies were detected (183). This
1667 highlighted the potential risk of an immune response against endogenous RANK or OPG in patients, when
1668 using RANK-Fc or AMGN-007, respectively. An anti-RANKL antibody approach was therefore preferred,
1669 which led to the development of denosumab.

1670 Denosumab is a fully human, synthetic antibody that binds to RANKL with high affinity, thereby
1671 preventing its interaction with RANK in a way similar to that of OPG (183). Denosumab is administered
1672 by subcutaneous injection. The biological half-life of denosumab is only weeks compared to the months
1673 or years seen with bisphosphonates (34). Rebound osteolysis may occur following discontinuation of
1674 denosumab with accelerated bone loss and, in a few patients, an increased incidence of vertebral
1675 fractures 12-36 months after treatment cessation (334).

1676

1677 *Denosumab for metastatic bone disease*

1678 Denosumab has been shown to be superior to zoledronic acid for the prevention of SREs from
1679 breast cancer. 2046 patients were randomly assigned to receive four weekly subcutaneous injections of
1680 denosumab (120 mg) or intravenous zoledronic acid (4 mg), with supplements of calcium and vitamin D.

1681 Denosumab was statistically superior to zoledronic acid in delaying the first SRE (315). Overall,
1682 denosumab treatment delayed the occurrence of all types of SREs. However, no differences in survival
1683 or investigator-reported disease progression were found between the two treatment groups.

1684 Denosumab was investigated in patients with CRPC and bone metastases and showed that in a
1685 randomized trial versus zoledronic acid, this compound was statistically superior to the bisphosphonate
1686 in delaying the first SRE and the overall risk of SREs (105).

1687 Denosumab has also been studied in advanced solid tumors other than breast and prostate
1688 cancers. Non-inferiority to zoledronic acid was demonstrated with a trend to better control of skeletal
1689 morbidity (139).

1690

1691 *Denosumab for prevention of cancer treatment induced bone loss*

1692 Denosumab is the only agent to have a specific license for CTIBL following large randomised
1693 trials in postmenopausal women with breast cancer receiving an aromatase inhibitor and in men with
1694 prostate cancer receiving ADT (121, 305). In both studies, fracture incidence was the primary endpoint.
1695 The ABCSG-18 trial compared adjuvant denosumab (60 mg by subcutaneous injection given twice a
1696 year) with placebo (both with calcium and Vitamin D supplements) in 3425 postmenopausal women
1697 receiving adjuvant aromatase inhibitor treatment (121). Women treated with denosumab had a 50%
1698 (95% CI 39–65%, $p < 0.0001$) risk reduction for any clinical fracture. The fracture risk reduction appeared
1699 to be irrespective of age and baseline BMD. Furthermore, the disease-free survival (secondary
1700 endpoint) was significantly improved in the denosumab group (HR=0.82, 95% CI 0.69 – 0.98, $p = 0.026$)
1701 compared to placebo group (122). In a placebo-controlled trial of denosumab in 1468 men receiving
1702 ADT for non-metastatic prostate cancer, 36 months of denosumab treatment was associated with a
1703 significantly reduced incidence of new vertebral fractures (1.5% with denosumab *vs.* 3.9% with placebo;
1704 relative risk [RR] 0.38; 95% CI 0.19-0.78). BMD increased from baseline at all sites in the denosumab
1705 group, whereas it declined in the placebo group (305).

1706 As aforementioned for bisphosphonates, current guidelines for preventing CTIBL recommend
1707 continuing anti-resorptive therapy for as long as the patient is receiving endocrine treatment. Patients
1708 treated with denosumab may need additional bone protection with a bisphosphonate when denosumab
1709 is discontinued to prevent rebound osteolysis and the increased risk of multiple vertebral fractures
1710 associated with treatment withdrawal (334).

1711

1712 *Disease modifying effects of denosumab treatment*

1713 The disease modifying effects of denosumab have also been assessed in early breast cancer but
1714 this agent, at least when given in the intensive schedule selected in the adjuvant D-CARE study, had no
1715 effect on disease recurrence in either pre- or postmenopausal women (63). The osteoporosis schedule
1716 of denosumab has a beneficial effect on the underlying disease, as observed in the ABCSG-18 study
1717 (121,122). However, no survival benefits have been yet seen and the agent is therefore only
1718 recommended for fracture prevention (122). Of note, the presence of RANK-positive CTCs in the
1719 bloodstream of metastatic breast cancer patients (n = 20/42) is associated with a better response to
1720 denosumab therapy with respect to time to first SRE [HR=0.25 (0.1 – 10.62), P = 0.0012)], compared to
1721 metastatic patients with RANK-negative CTCs (n = 22/42) (254). It would be interesting to determine if
1722 RANK expression in CTCs could help identify high-risk early-stage breast cancer patients who might
1723 benefit from adjuvant denosumab. Similarly, a *post-hoc* analysis of the D-CARE trial will be conducted
1724 to determine if the expression of transcription factor MAF in primary tumors will help clarify the potential
1725 anticancer mechanism of denosumab (122) (*see section X-C for further discussion*).

1726 Denosumab may have some disease modifying effects in prostate cancer: 1432 men with non-
1727 metastatic CRPC who were at high risk for bone metastasis by virtue of either a PSA of ≥ 8.0 ng/mL
1728 and/or PSA doubling time ≤ 10.0 months were randomized to receive monthly denosumab, 120 mg, or
1729 placebo in addition to continuation of ADT. Denosumab significantly increased bone metastasis-free
1730 survival by a median of 4.2 months compared with placebo and delayed the time to symptomatic first

1731 bone metastases (306). However, this effect on the disease process did not translate into an
1732 improvement in overall survival and, in light of the relatively high cumulative incidence of ONJ (4% at 3
1733 years), the marginal benefits were not considered sufficient to change clinical practice.

1734 The RANK/RANKL pathway has been shown to play a crucial role in the initiation and
1735 progression of inherited breast cancer caused by mutation in the tumor-suppressor gene breast cancer
1736 1 (*BRCA1*) (269). *BRCA1* mutation carriers have a greater propensity to generate cancer stem cells,
1737 whose expansion is RANKL/RANK dependent, and denosumab inhibits the expansion of these cancer
1738 stem cells *in vitro* (269). Therefore, these findings strongly suggest that targeting the RANK/RANKL
1739 pathway could be beneficial for the prevention of breast cancer in *BRCA1* mutation carriers. Two pilot
1740 studies are currently evaluating the biological effects of denosumab on Ki67 proliferation index (primary
1741 endpoint) in normal breast and fallopian tube fimbrial tissues from *BRCA1* and *BRCA2* mutation carriers
1742 (ACTRN12614000694617 and ClinicalTrials.gov NCT03382574 studies). Furthermore, a randomized,
1743 double-blind, placebo-controlled, multi-center, international phase 3 study will determine if denosumab
1744 can prevent breast cancer development in women carrying a *BRCA1* germline mutation (ABCSG-50,
1745 EudraCT number 2017-002505-35; estimated number of subjects to be enrolled in the study: 2,918).

1746

1747 3. *Novel antiresorptive agents*

1748 *LGR4/RANKL and small-molecule RANKL inhibitors*

1749 The leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4) has recently been
1750 identified as a RANKL receptor that negatively regulates osteoclast differentiation (215). LGR4
1751 competes with RANK to bind RANKL and suppresses canonical RANK signaling during osteoclast
1752 differentiation (215). A soluble LGR4 extracellular domain (ECD), which binds to RANKL, was examined
1753 in animal models of osteoporosis. LGR4-ECD notably increased bone mass and inhibits osteoclast
1754 differentiation *in vivo* (215). Interestingly, LGR4-ECD had little physiological effect on osteoclast

1755 differentiation in normal mice, which suggests that LGR4-ECD could antagonize excessive RANKL in
1756 benign and malignant osteoclast-related diseases with few side effects.

1757 The efficacy of a small-molecule RANKL inhibitor (AS2676293) has been tested in an animal
1758 model of bone metastasis (238). Oral administration of AS2676293 to animals inhibits formation of
1759 osteolytic lesions caused by MDA-MB-231 breast cancer cells. *In vitro*, AS2676293 inhibits
1760 osteoclastogenesis.

1761 These antiresorptive agents are still at a preclinical stage of development.

1762 *Cathepsin K inhibitors*

1763 Cathepsin K is a lysosomal cysteine protease highly expressed in osteoclasts, which degrades
1764 collagen during bone resorption. Cathepsin K inhibitors (AFG-495, L-235) reduce bone destruction and
1765 skeletal tumor burden in animal models of breast cancer bone metastasis (92, 196), providing a direct
1766 proof for causal role of cathepsin K in bone metastasis formation. Additionally, metastatic tumor cells in
1767 bone express cathepsin K and AFG-495 dose-dependently inhibits breast cancer cell invasion *in vitro*,
1768 but not tumor growth *in vivo*. These results suggest that cathepsin K inhibitors in the treatment of bone
1769 metastasis could potentially have a dual effect, inhibiting both osteoclast-mediated bone resorption and,
1770 to a less extent, tumor burden (196). Interestingly, a phase II trial in women with breast cancer and bone
1771 metastases showed that cathepsin K inhibitor odanacatib (which is structurally related to L-235)
1772 successfully reduced circulating levels of bone resorption markers after 4 weeks of treatment (160).
1773 Similarly, a phase-II trial in postmenopausal women with osteoporosis showed that odanacatib therapy
1774 was effective at inhibiting bone resorption and increasing bone mineral density (186). A large phase III
1775 trial, Long-Term Odanacatib Fracture Trial (LOFT), enrolling 16,713 participants with osteoporosis from
1776 387 centres was therefore conducted (24). However, development of the agent has been discontinued
1777 due to possible cardiovascular adverse events. A phase III trial assessing the efficacy of odanacatib in

1778 reducing the risk of bone metastasis in women with breast cancer (ClinicalTrials.gov identifier
1779 NCT00692458) has also been withdrawn for undisclosed commercial reasons.

1780

1781 *Mammalian target of rapamycin (mTOR) inhibitors*

1782 In bone physiology, RANKL and M-CSF promote osteoclast survival by signalling through
1783 mTOR (19). Rapamycin and everolimus (a rapamycin analog) are both mTOR inhibitors that block
1784 osteoclast differentiation *in vitro* and suppress bone resorption in an animal model of bone loss caused
1785 by ovariectomy (19, 36). In addition to their antiresorptive effects, mTOR inhibitors exhibit anti-cancer
1786 effects. Everolimus and temsirolimus were the first mTOR inhibitors to be approved in the treatment of
1787 advanced breast and renal cell cancers. Interestingly, everolimus and temsirolimus inhibit skeletal tumor
1788 burden and osteolysis in animal models of bone metastasis caused by breast and renal cell carcinoma,
1789 respectively (36, 343). Everolimus combined with aromatase inhibitor exemestane has been approved
1790 for patients with advanced hormone receptor-positive/HER2-negative breast cancer who progress on
1791 prior nonsteroidal aromatase inhibitor therapy with either letrozole or anastrozole (BOLERO-2 study). In
1792 this study, the progression-free survival was significantly improved in the everolimus plus exemestane
1793 arm, compared to the placebo plus exemestane arm (11). Because exemestane is known to increase
1794 bone turnover, an exploratory analysis in the BOLERO-2 study has been conducted in patients with or
1795 without bone-only disease (120, 152). Compared to placebo plus exemestane, everolimus plus
1796 exemestane increased the median progression-free survival (5.3 and 12.9 months, respectively),
1797 regardless of bisphosphonate use and presence of bone metastases at baseline, indicating a 67%
1798 reduction in the risk of progression (120, 152). In addition, bone marker levels increased with
1799 exemestane monotherapy, but decreased when used in combination therapy with everolimus (120).
1800 Overall, these results suggest that everolimus plus exemestane decreases disease progression in the
1801 bone, probably by suppressing increased bone turnover observed with exemestane monotherapy in
1802 addition to the greater antitumor effects of the combination.

1803 *Src non-receptor tyrosine kinase inhibitors*

1804 Src is one of eleven members of a family of non-receptor tyrosine kinases that interact with
1805 several protein-tyrosine kinase receptors, G-protein-coupled receptors and integrins, which are
1806 expressed at the plasma membrane (281). c-Src plays multiple roles in regulating cell proliferation,
1807 survival, adhesion, migration, invasion, metastasis, and angiogenesis (281). Although Src is
1808 ubiquitously expressed in vertebrate cells, much higher protein levels are found in osteoclasts, platelets
1809 and neurons than most other cells. Of note, the most noticeable phenotype of Src knock-out mice is
1810 osteopetrosis (enhanced bone mass) as a result of osteoclast dysfunction (281). In fact, following
1811 integrin $\alpha v \beta 3$ activation, Src phosphorylation regulates the organization of osteoclast's actin
1812 cytoskeleton, which enables the osteoclast to attach and spread to bone and optimally resorb bone.
1813 Additionally, following RANK/RANKL interaction, Src activation triggers signaling through the
1814 PI3K/AKT/mTOR pathway and promotes osteoclast survival (312). As exemplified in experimental bone
1815 metastasis, the injection of cancer cells in Src knock-out mice shows that these animals are protected
1816 from tumor-associated bone destruction because Src-defective osteoclasts do not resorb bone (361).
1817 Thus, Src plays a central role in osteoclast function. In addition, elevated expression and activity of c-
1818 Src have been reported in a variety of cancers. Three Src inhibitors (dasatinib, saracatinib and
1819 bosutinib) underwent clinical studies in patients with cancer and (bone) metastases (312). To date
1820 clinical development has yielded disappointing results in the setting of solid tumors and bone
1821 metastases.

1822 *RON receptor tyrosine kinase inhibitor.*

1823 RON is a receptor tyrosine kinase receptor expressed by osteoclasts (4). Tumor-derived MSP
1824 promotes the formation of osteolytic lesions in an animal model of breast cancer bone metastasis,
1825 whose extent is inhibited upon treatment of metastatic animals with RON tyrosine kinase inhibitor BMS-
1826 777607/ASLAN002 (4). A phase-I trial in postmenopausal women with advanced cancer shows that

1827 BMS-777607/ASLAN002 reduced bone resorption after 4 weeks of treatment (ClinicalTrials.gov
1828 identifier NCT01721148).

1829 *Dual c-MET and VEGFR2 receptor tyrosine kinase inhibitor (cabozantinib)*

1830 Receptor tyrosine kinases c-MET and vascular endothelial growth factor (VEGF) receptor 2
1831 (VEGFR2) and their respective ligands, hepatocyte growth factor (HGF) and VEGF, are expressed by
1832 both osteoblasts and osteoclasts, enabling the activation of autocrine and paracrine HGF/c-MET and
1833 VEGF/VEGFR2 signaling pathways for the regulation of osteoblast and osteoclast activities (194).
1834 Additionally, c-MET and VEGFR signaling facilitates tumor progression through the activation of multiple
1835 pathways (PI3K/AKT/mTOR, SRC, MAPKinases) (194). Dual tyrosine kinase inhibitors that target both
1836 VEGFR2 and c-MET (cabozantinib and TAS-115) were therefore developed and investigated in pre-
1837 clinical and clinical settings (194).

1838 Cabozantinib inhibits human osteoclast differentiation and osteoclast-mediated bone resorption
1839 and increases OPG production by human osteoblasts *in vitro* (104). In animal models of prostate
1840 cancer, cabozantinib decreased skeletal tumor burden and formation of osteoblastic lesions, indicating
1841 that this compound suppresses bone metastasis formation, at least in part, through inhibition of bone
1842 remodeling (79, 192). Similarly, TAS-115 inhibits human PC-3 prostate cancer bone metastasis
1843 formation and suppresses bone destruction (360). The effect of cabozantinib was therefore investigated
1844 in the treatment of prostate cancer patients with bone metastasis.

1845 In phase-II studies, cabozantinib treatment of metastatic CRPC patients with bone metastases
1846 showed a remarkable 68% rate of normalization of bone scans and suggested disease benefits with
1847 prolongation of progression-free survival (194). However in the phase-III trial COMET-1, comparing
1848 cabozantinib with prednisone in patients with metastatic CRPC and bone metastases following prior
1849 treatment with docetaxel and either abiraterone or enzalutamide, although cabozantinib improved
1850 progression-free survival and time to first SRE, it failed to improve overall survival, the primary end point
1851 of the trial (304). In the light of these data, further development of cabozantinib in prostate cancer has

1852 been halted and a second trial (COMET-2) comparing cabozantinib with mitoxantrone plus prednisone
1853 in a similar patient population as COMET-1 closed prematurely. It is likely that the dramatic effects on
1854 bone scan appearances seen in the initial studies reflected the direct effects of cabozantinib on bone
1855 cell function and skeletal blood flow rather than effects on the underlying malignant disease.

1856 Cabozantinib is approved for the treatment of patients with advanced renal cell carcinoma after
1857 previous antiangiogenic therapy on the basis of significant improvements in progression-free survival
1858 and overall survival when compared with everolimus (phase-3 METEOR trial). Pre-specified analyses of
1859 progression-free survival and overall survival were conducted in a sub-group of patients with bone
1860 metastasis from the METEOR trial (99). Compared to everolimus, cabozantinib treatment was
1861 associated with a significant improvement of progression-free survival and overall survival in patients
1862 with bone metastases, indicating it is a good treatment option for these patients.

1863 *miR-34a mimic (MRX34)*

1864 MiR-34a is a critical suppressor of osteoclastogenesis and bone resorption by directly targeting
1865 the pro-osteoclastic factor Tgif2 (transforming growth factor- β -induced factor 2) (181). Its expression is
1866 therefore downregulated during osteoclast differentiation. The pharmacological administration of a
1867 miR-34a mimic delivered in nanoparticles (whose aim is to replenish the lost miRNA expression) can
1868 attenuate bone metastases in animals bearing breast or skin tumours (181). A phase I, open-label,
1869 multicenter, dose-escalation study investigated the safety, pharmacokinetics and pharmacodynamics
1870 of a miR-34a mimic (MRX34) encapsulated in lipid nanoparticles, in patients with unresectable primary
1871 liver cancer or advanced or metastatic cancer with or without liver involvement or hematologic
1872 malignancies (15). However, the clinical trial was terminated prematurely due to cases of immune-
1873 related serious adverse events (ClinicalTrials.gov identifier NCT01829971).

1874 *BET inhibitor*

1875 The bromodomain and extraterminal (BET) protein family (BRD2, BRD3, BRD4 and BRDT) is an
1876 important class of chromatin readers, regulating chromatin accessibility to transcription factors and RNA
1877 polymerase. JQ1, a thienotriazolo-1,4-diazapine that binds selectively to BET bromodomain proteins,
1878 inhibits osteoclast differentiation by interfering with BRD4-dependent RANKL activation of *NFATC1*
1879 transcription (185). Moreover, JQ1 inhibits bone resorption in experimental models of malignant
1880 osteolytic lesions and osteoporosis (12, 185). JQ1 is still at a preclinical stage of development.

1881 *Dock5 inhibitor*

1882 Dock5 (Dedicator of cytokinesis 5), a guanine nucleotide exchange factor for the small GTPase
1883 Rac, participates to the formation of the sealing zone in osteoclasts. C21, a chemical inhibitor of Dock5,
1884 reduces osteoclast-mediated bone resorption *in vitro* and blocks bone destruction in a melanoma model
1885 of bone metastasis *in vivo* (349). C21 is still at a preclinical stage of development.

1886 *Jagged/Notch inhibitor*

1887 In bone metastasis, tumor-derived Jagged1 activate Notch signaling in osteoclast precursors,
1888 promoting osteoclast differentiation and bone resorption (295). Tumor-derived Jagged1 also engages
1889 Notch signaling in osteoblasts, stimulating IL-6 production. In turn, IL-6 secreted from osteoblasts
1890 stimulates tumor growth (295). Therefore, a fully human monoclonal antibody (15D11) against Jagged-1
1891 has been developed. 15D11 inhibits bone metastasis formation in animals and sensitizes bone
1892 metastases to chemotherapy (390). 15D11 is still at a preclinical stage of development.

1893

1894 **B. Promoting Bone Formation by Targeting Osteoblasts**

1895 *1. Agents blocking WNT inhibitors*

1896 Anti-DKK1 (BHQ880 and DKN-01) and anti-SOST (blosozumab, BPS804 and romosozumab)
1897 antibodies have been developed to block the inhibitory effect of Wnt antagonists DKK-1 and SOST on
1898 osteoblast-mediated bone formation (71, 170, 227, 395). BHQ880 and DKN-01 are in phase I/II clinical

1899 trials for patients with multiple myeloma and other solid tumors, such as cholangiocarcinoma,
1900 esophageal cancer and gastric cancer, whereas romosozumab, blososumab and BPS804 are in phase
1901 II/III clinical trials for osteoporosis (170). In a phase III trial romosozumab decreased the risk of vertebral
1902 fractures in postmenopausal women with osteoporosis (36% lower risk with romosozumab than
1903 placebo) (71). Experimentally, an anti-SOST antibody decreased the extent of osteolytic lesions in a
1904 mouse model of breast cancer bone metastasis and multiple myeloma (227, 395). However, up to know,
1905 there is no clinical study investigating the effect of anti-SOST antibodies in cancer-induced bone
1906 diseases.

1907 Of note, the inhibition of one of these two WNT inhibitors (DKK1 and SOST) may engender a
1908 compensatory response in order to return to a steady state (106). By contrast, a bispecific antibody
1909 targeting SOST and DKK-1 (Hetero-DS) leads to synergistic bone formation in rodents and non-human
1910 primates (106). Thus, Hetero-DS could have a valuable role in increasing bone mass and improving
1911 healing of lytic bone lesions associated with bone metastasis.

1912 2. *Endothelin-1 receptor inhibitors*

1913 Preclinical studies have uncovered a prominent role for ET-1 in the formation of osteosclerotic
1914 lesions (276, 378). However, phase 3 trials of the inhibitors, atrasentan and zibotentan in combination
1915 with docetaxel failed to improve overall survival in patients with metastatic castration-resistant prostate
1916 cancer compared with docetaxel alone and this treatment approach seems unlikely to reach the clinic
1917 (47, 240).

1918 3. *Androgen Inhibitors*

1919 Abiraterone acetate is an orally administered selective androgen biosynthesis inhibitor derived from
1920 the structure of pregnenolone. It is an irreversible inhibitor of cytochrome CYP17A, resulting in virtually
1921 undetectable serum and intratumoral androgen levels (244). Abiraterone was evaluated in
1922 chemotherapy-naïve and chemotherapy-treated men with metastatic castration-resistant prostate
1923 cancer (COU-AA-301 and COU-AA-302 trials). The data from these two phase-III trials showed that

1924 abiraterone treatment significantly improves overall survival and skeletal outcomes (delay of
1925 symptomatic progression and reduction of time to first SRE) (116). These benefits of abiraterone
1926 treatment on metastatic bone disease may not only be related to a systemic control of the disease but
1927 also associated with a direct effect in the bone. Indeed, abiraterone exhibits direct bone anabolic and
1928 anti-resorptive effects (156).

1929 Another promising inhibitor is the androgen receptor antagonist enzalutamide. Enzalutamide was
1930 evaluated in chemotherapy-naïve and chemotherapy-treated men with metastatic castration-resistant
1931 prostate cancer patients (13, 116). In these two phase-III trials (AFFIRM and PREVAIL), enzalutamide
1932 significantly decreased the risk of death and improved skeletal outcomes (time to first SREs and
1933 radiographic progression, respectively).

1934 However, despite approval of abiraterone and enzalutamide in metastatic castration-resistant
1935 prostate cancer, virtually all patients eventually acquire secondary resistance. One plausible explanation
1936 for resistance may involve the presence of the androgen-receptor isoform encoded by splice variant 7
1937 (AR.V7), which lacks the ligand-binding domain, but remains constitutively active as a transcription
1938 factor (5).

1939 4. *Activin A inhibitors*

1940 Activin A binds to activin type IIA (ActRIIA) or type IIB (ActRIIB) receptors and induces the
1941 recruitment and phosphorylation of an activin type I receptor (ActRIB), which then phosphorylates
1942 Smad2 and Smad3 intracellular signaling proteins (312). The treatment of non-human primates with a
1943 soluble chimeric protein composed of the extracellular domain of ActRIIA fused to human IgG-Fc
1944 (sotatercept, formerly called ACE-011) increased bone volume by decreasing bone resorption and
1945 increasing bone formation (212). In animal models of multiple myeloma with osteolytic lesions, the
1946 treatment of mice with a soluble activin receptor type IIA fusion protein (ActRIIA.muFc) blocked bone
1947 destruction (51). Specifically, ActRIIA.muFc treatment significantly stimulated osteoblastogenesis,
1948 prevented myeloma-induced suppression of bone formation, blocked the development of osteolytic

1949 bone lesions and increased survival (51). In the clinic, sotalercept improved bone mineral density and
1950 bone formation in multiple myeloma patients (312). These pre-clinical and clinical findings suggest that
1951 stimulating osteoblastic bone formation to facilitate bone repair might be an alternative or additional
1952 therapeutic approach to the use of antiresorptive agents to treat osteolytic lesions. Although higher
1953 serum levels of activin A were reported in breast or prostate cancer patients with bone metastases,
1954 compared with those of patients without bone metastases, there are currently no ongoing trials in breast
1955 or prostate cancer with bone metastases (312).

1956

1957 C. Targeting the Bone Matrix and the Microenvironment

1958 1. *Bone targeted radiopharmaceuticals*

1959 The therapeutic use of radioactive-labeled tracer molecules is currently an area of considerable
1960 interest and research. Targeted radiotherapy has potential advantages over external beam radiotherapy
1961 in that the radiation dose may be delivered more specifically to the tumor and normal tissues may
1962 partially be spared unnecessary irradiation (242). Theoretically, it should also be possible to administer
1963 high doses of radiation to the tumor on a recurrent basis if necessary.

1964 The α -emitting radiopharmaceutical 223 radium dichloride (radium-223, a calcium-mimetic
1965 radioisotope) and the β -emitting radiopharmaceuticals 89 strontium (strontium-89, a calcium-mimetic
1966 radioisotope) and ethylene diamine tetramethylene phosphonate- 153 samarium (samarium-153, a
1967 bisphosphonate-conjugated radioisotope) bind to bone mineral and preferentially to newly formed bone
1968 matrix, such as areas of osteosclerotic bone metastatic lesions (59, 312). These radiopharmaceuticals
1969 emit radiation causing DNA damage and cell death. Radium-223 almost exclusively produces alpha
1970 particles that produce a high-linear energy transfer (LET) with ultra-short penetration ($< 100 \mu\text{m}$; 2-10
1971 cell diameters) resulting in a highly localized antitumor effect on adjacent bone metastases while limiting
1972 damage to the surrounding normal tissue (242). In contrast to radium-223, strontium-89 and samarium-

1973 153 have a low-LET with a penetration range of 3 to 8 millimetres, which results in considerably more
1974 dose to normal tissues, notably the bone marrow and this limits the use of these agents and the ability
1975 to combine with other treatments (59, 312).

1976 Strontium-89 and samarium-153 are approved for palliation of bone pain (103), but only
1977 occasionally used and it is the bone-seeking, alpha particle emitting, radium-223 that is of most
1978 relevance to current practice. Radium-223 is now approved for the treatment of bone metastases from
1979 CRPC following a placebo-controlled randomized phase-III trial (ALSYMPCA) in which radium-223
1980 increased the survival of patients by 3.6 months and further reduced skeletal morbidity over and above
1981 a bisphosphonate (258). Treatment was well tolerated and improved quality of life with no significant
1982 long-term toxicities identified (258, 348). Radium-223 has subsequently been studied earlier in the
1983 course of metastatic prostate cancer and in combination with other therapies. However, a double-
1984 blinded, placebo-controlled randomized phase-III trial (ERA 223; NCT02043678) investigating the
1985 efficacy and safety of radium-223 and abiraterone *versus* abiraterone alone in chemotherapy-naïve
1986 CRPC patients with bone metastases showed that the combination of radium-223 and abiraterone did
1987 not improve either disease or skeletal outcomes compared with abiraterone alone. Furthermore, more
1988 bone fractures were observed in the combined treatment arm, particularly in patients not receiving
1989 concomitant antiresorptive agents (zoledronic acid, denosumab). In breast cancer, experimental
1990 findings showed that radium-223 inhibits skeletal tumor burden and bone destruction in a mouse model
1991 of breast cancer bone metastasis (319). A phase IIa study was conducted in breast cancer patients with
1992 bone-dominant disease. Radium-223 induced metabolic changes, as judged by a 25% decrease of ¹⁸F-
1993 fluorodeoxyglucose uptake in osteosclerotic lesions using positron emission tomography and
1994 computed tomography (59).

1995

1996 2. *Agents targeting nerve- or bone-derived growth factors.*

1997 The release of algogenic factors by cancer/stromal cells and osteoclasts can induce sensitization
1998 and activation of sensory fibers that innervate the bone. Bisphosphonates and denosumab have been

1999 approved for the treatment of bone pain (100). A phase II study evaluated the safety and efficacy of the
2000 anti-NGF antibody tanezumab in patients with painful bone metastases taking daily opioids (308). The
2001 data are encouraging and suggest that tanezumab treatment results in sustained analgesic
2002 improvements.

2003 The TGF β signaling pathway plays a critical and dual role in cancer progression. Several inhibitors
2004 of TGF β signaling, such as neutralizing antibodies, antisense oligonucleotides, and receptor kinase
2005 inhibitors, have been developed and shown to have inhibitory effects on bone metastases in animal
2006 models (40, 101).

2007

2008 X. THE VALUE OF BONE TURNOVER BIOMARKERS IN BONE METASTASIS

2009

2010 As previously discussed in *section I-C*, clinical presentations of bone metastases are highly
2011 diverse, and many locations remain asymptomatic. Nowadays, plain radiography is insufficient to
2012 correctly identify bone metastases since more than 50% of an affected bone is required to be detected.
2013 As a consequence, bone metastases are often diagnosed at the time symptoms occur, increasing the
2014 risk of developing SREs, which significantly impair patients' quality of life (64).

2015 In adults, the bone mass is maintained by a continuous bone remodeling, which is a balance
2016 between osteoclast-mediated bone resorption and osteoblast-mediated bone formation (76). The
2017 turnover between resorption and formation leads to the release of bone-derived molecules that are
2018 amenable to measurement in blood and urine (350). Some of these molecules have been used as
2019 biochemical biomarkers of bone turnover, reflecting ongoing rates of bone formation or bone resorption
2020 (Table 2). Since cancer cells cause a distortion of bone turnover, several clinical studies examined
2021 whether variations in the expression levels of these bone biomarkers in blood or urine were associated
2022 with malignant bone disease progression (61, 90). However, at present, the high inter- and intra-
2023 individual variability represents a limitation to the routine use of these biomarkers (90). Clinical

2024 applications of these biomarkers for the detection and monitoring of bone metastasis and response to
2025 antiresorptive therapies have recently been reviewed (90) and are outlined in the following sections.

2026

2027 **A. Bone Formation Markers**

2028 Biochemical markers of bone formation include bone alkaline phosphatase (BALP), which is an
2029 enzyme localized at the plasma membrane of osteoblasts, and procollagen I carboxyl-terminal and
2030 amino-terminal propeptides (PICP and PINP, respectively), which are cleaved during the processing of
2031 type I collagen (Table 2) (350). Their clinical applications in the management of malignant bone
2032 diseases are summarized below.

2033 *Diagnosis of bone metastasis*

2034 In breast and prostate cancer, serum concentrations of PINP were found significantly increased in
2035 patients with bone metastases (90). In a retrospective analysis, PINP was measured in the serum of
2036 prostate cancer patients with different clinical outcomes (N0/M0: no metastases, N1/M0: lymph node
2037 metastases only, and M1: bone metastases) (180). Increased PINP levels in the M1 group were
2038 detectable 8 months before the first positive bone scintigraph (180). PICP and BALP were also
2039 investigated in the prostate cancer setting. In particular, serum BALP concentrations significantly
2040 correlated with the extent of bone involvement (90). Furthermore, a meta-analysis including 19 trials and
2041 3,628 patients with solid tumors showed that serum BALP levels in patients with bone metastases were
2042 2.9-fold higher ($P < 0.05$) than in patients without bone lesions (91). Another meta-analysis in lung
2043 cancer including 16 trials and 1,720 patients with or without bone metastases showed that high
2044 concentrations of BALP were also associated with bone metastasis (155).

2045 *Prognosis of bone metastasis*

2046 BALP levels were assessed in patients with bone metastases from breast cancer (n = 1,648),
2047 castration-resistant prostate cancer (n = 643) and lung cancer and other solid tumors (n = 773) treated
2048 with a bisphosphonate (zoledronic acid or pamidronate). High serum levels of BALP at baseline and on-
2049 study were associated with increased risks of SREs, disease progression and death in patients who did
2050 not receive bisphosphonate therapy (61, 90). Similar findings were reported in a retrospective analysis
2051 involving 5,543 patients who received zoledronic acid or denosumab for bone metastasis treatment
2052 (206). Furthermore, after 3 months of treatment with either denosumab or zoledronic acid, patients with
2053 serum BALP levels \geq median at month 3 had significantly reduced overall survival compared with those
2054 who had serum BALP levels $<$ median (HR = 2.44; $P < 0.0001$) (206).

2055 The prognosis value of PINP for bone metastasis was investigated in breast cancer (33).
2056 Specifically, PINP was measured at baseline in the serum from 872 patients from a large randomized
2057 trial of adjuvant zoledronic acid (AZURE) in early breast cancer (33). High baseline PINP was
2058 prognostic for future bone recurrence at any time ($P < 0.006$), but was not predictive for distant
2059 metastasis taken as a whole, demonstrating the bone metastasis specificity of PINP (33).

2060 *Response to bone-targeted therapies*

2061 The predictive value of BALP and PICP has been evaluated in castration-resistant prostate cancer
2062 patients with bone metastases (n = 778) treated on a placebo-controlled phase III trial of docetaxel with
2063 or without atrasentan (SWOG S0421) (187). High baseline serum levels of BALP and PICP were
2064 associated with poor overall survival (HR = 1.23; $P < 0.001$ and HR = 1.38; $P < 0.001$, respectively).
2065 Increasing BALP and PICP levels by week 9 of therapy were associated with a significantly increased
2066 risk of death (HR = 1.28; $P < 0.001$ and HR = 1.35; $P < 0.001$, respectively). For patients with the
2067 highest biomarker levels (upper 25th percentile), improved survival was observed in the atrasentan arm
2068 compared with placebo arm (HR = 0.65; $P < 0.04$ and HR = 0.61; $P < 0.02$ for BALP and PICP,
2069 respectively).

2070

2071 B. Bone Resorption Markers

2072 Biochemical markers of bone resorption include (1) the carboxyterminal telopeptide of type I
2073 collagen (ICTP), which is a degradation product of mature type I collagen cleaved by MMP, (2) C- and
2074 N-telopeptides (CTX and NTX, respectively), which are proteolytic fragments generated by cathepsin K
2075 cleavage of type I collagen, (3) pyridinoline (PYD) and deoxypyridinoline (DPD), which are nonreducible
2076 pyridinium cross-links present in the mature form of type I collagen, and (4) tartrate resistant acid
2077 phosphatase 5b (TRACP), which is an osteoclast-derived enzyme (Table 2) (350). Additional potential
2078 bone resorption markers are RANKL and OPG, the RANK-L/OPG ratio being used to estimate the
2079 osteolysis rate, and miRNAs (Table 2), the latter being significantly upregulated in the serum of patients
2080 with osteoporotic fractures and breast cancer patients with osteolytic bone metastases (96, 294). BSP
2081 and osteopontin, which are osteoblast-derived bone matrix components, have been also investigated as
2082 potential bone markers associated with osteolysis (165). ICTP, TRACP, serum CTX, and urinary NTX
2083 are the most common resorption markers used in clinical practice. Clinical applications for these bone
2084 resorption markers in the management of malignant bone diseases are summarized below.

2085 *Diagnosis of bone metastasis*

2086 Serum concentrations of TRACP were found increased in patients with bone metastases from
2087 breast cancer, but not lung cancer (90, 155). In prostate cancer, high NTX and CTX levels were
2088 associated with bone metastases (35). In lung cancer, a meta-analysis including 16 trials and 1,720
2089 patients with or without bone metastasis showed that high concentrations of NTX and ICTP (but not
2090 CTX) were associated with bone metastasis (155).

2091 Increased serum levels of BSP and OPN have been associated with bone metastases from breast,
2092 lung and prostate cancer (165). However, these proteins are also expressed by tumor cells, suggesting
2093 they can be considered tumor markers rather than bone biomarkers (165).

2094 Increased serum levels of RANKL and/or OPG have been associated with bone metastases from
2095 prostate cancer (165). Similarly, the RANKL/OPG ratio is increased in severe osteolysis associated with
2096 primary bone tumors and bone metastasis from lung, renal and breast cancer (125). However, the low
2097 sensitivity of the assays to measure circulating levels of RANKL and OPG and the observation that
2098 RANK, RANKL and OPG are expressed in a wide variety of different cell types including tumors cells
2099 have so far limited the routine measurement of these molecules (165, 183).

2100 Circulating miRNAs originating from tissues, being remarkably stable in blood, may be able to
2101 serve as biomarkers. For example, Seeliger *et al.* (294) identified 5 miRNAs (miR-21, miR-23a, miR-25,
2102 miR-100 and miR-125b) that were upregulated in both the serum and the bone tissue of osteoporotic
2103 patients with bone fractures. Although these miRNAs are not bone tissue-specific, they have been
2104 reported to play a role in bone remodeling when they are expressed by osteoblasts (294). Similarly, Eil
2105 *et al.* (96) identified a series of 4 miRNAs (miR-16, miR-211, miR-378 and Let-7a) that were specifically
2106 upregulated during osteoclast differentiation. The authors then thought to investigate this series of
2107 miRNAs as potential biomarkers for osteolytic bone metastases. They found that miR-16 and miR-378
2108 were consistently increased in the serum from breast cancer patients with bone metastases (n = 38),
2109 compared to healthy female donors (n = 21) (96).

2110 *Prognosis of bone metastasis*

2111 NTX levels were assessed in patients with bone metastases from breast cancer (n = 1,648),
2112 castration-resistant prostate cancer (n = 643) and lung cancer and other solid tumors (n = 773) treated
2113 with a bisphosphonate (zoledronic acid or pamidronate). High serum levels of NTX at baseline and on-
2114 study were associated with increased risks of SREs, disease progression and death in patients who did
2115 not receive bisphosphonate therapy (61, 90). Similar findings were reported in a retrospective analysis
2116 involving 5,543 patients who received zoledronic acid or anti-RANKL antibody denosumab for bone
2117 metastasis treatment (206). Furthermore, after 3 months of treatment with either denosumab or

2118 zoledronic acid, patients with urinary NTX levels \geq median at month 3 had significantly reduced overall
2119 survival compared with those who had urinary NTX levels $<$ median (HR = 1.85; $P < 0.0001$) (206).

2120 The prognosis value of CTX and ICTP for bone metastasis was investigated in breast cancer (33).
2121 These bone resorption markers were measured at baseline in the serum from 872 patients from a large
2122 randomized trial of adjuvant zoledronic acid (AZURE) in early breast cancer (33). High baseline CTX or
2123 ICTP was prognostic for future bone recurrence at any time ($P < 0.009$ and 0.008 , respectively), but
2124 were not predictive for overall distant recurrence, demonstrating the bone metastasis specificity of CTX
2125 and ICTP (33).

2126 *Response to bone-targeted therapies*

2127 The value of measuring NTX levels to assess response to bisphosphonate therapy was
2128 investigated by exploring databases from phase III trials of zoledronic acid in solid tumors and multiple
2129 myeloma. The analysis revealed that patients with high NTX levels at baseline that normalize during
2130 zoledronic acid treatment have improved survival as compared to patients with persistent elevated NTX
2131 levels (61).

2132 The predictive value of NTX and PYD has been evaluated in castration-resistant prostate cancer
2133 patients with bone metastases ($n = 778$) treated on a placebo-controlled phase III trial of docetaxel with
2134 or without atrasentan (SWOG S0421) (187). As for bone formation markers BALP and PICP, high
2135 baseline levels of NTX and PYD were associated with poor overall survival (HR = 1.40; $P < 0.001$ and
2136 HR = 1.52; $P < 0.001$, respectively). Increasing bone resorption marker levels by week 9 of therapy
2137 were associated with a significantly increased risk of death (HR = 1.36; $P = 0.002$ and HR = 1.36; $P =$
2138 0.002 for NTX and PYD, respectively). In contrast to what was observed for patients with the highest
2139 bone formation marker levels, there was however no survival benefit from atrasentan when using NTX
2140 or PYD in the upper 25th percentile. Nonetheless, when combining all four biomarkers in the highest
2141 quartile, there was clear evidence that patients had a survival benefit from atrasentan (HR = 0.33;
2142 median survival = 13 [atrasentan] vs 5 months [placebo]; $P = .005$) (187).

2143

2144 C. Insights from markers not associated with bone turnover

2145 Westbrook and colleagues (365) have identified two proteins [macrophage-capping protein (CAPG)
2146 and PDZ domain-containing protein (GIPC1)] from proteomic analysis of osteotropic human breast
2147 cancer cell lines whose expression in tumor cells was subsequently validated by immunohistochemistry
2148 using tumor tissue microarrays (TMAs) from breast cancer patients (n = 364) of the AZURE trial. Clinical
2149 validation of these two markers showed that patients who did not receive a bisphosphonate therapy
2150 were more likely to develop a first distant recurrence in bone (HR = 4.5; $P < 0.001$) and die (HR = 1.8; P
2151 = 0.045) if CAPG and GIPC1 were highly expressed in the primary tumor. Moreover, patients with high
2152 expression of CAPG and GIPC1 had a 10-fold increase in treatment benefit, compared with patients on
2153 standard therapy (365).

2154 Dedicator of cytokinesis protein 4 (DOCK4) is another protein specifically expressed in osteotropic
2155 tumor cells (366). DOCK4 expression in primary tumors was validated by immunohistochemistry, using
2156 TMAs from breast cancer patients (n = 345) of the AZURE trial (366). Adjusted Cox regression analyses
2157 showed that patients who did not receive a bisphosphonate therapy were more likely to develop a first
2158 distant recurrence in bone (HR = 2.13; $P = 0.034$) if DOCK4 was highly expressed in the primary tumor
2159 (366).

2160 MAF is a transcription factor of the AP-1 family shown to mediate breast cancer bone metastasis
2161 (260). The value of MAF expression in primary tumors to predict the treatment outcomes of adjuvant
2162 zoledronic acid in breast cancer patients from the AZURE trial (n = 1,739) has been investigated (64). In
2163 patients with MAF-negative tumors (79% of all patients), there was a lower relapse rate with the use of
2164 zoledronic acid (HR = 0.74), but not in patients who had MAF-positive tumors (64). Additionally, MAF
2165 positivity was associated with increased extraskeletal recurrence in the zoledronic acid group (HR =
2166 6.92) (64). Data from ABCSG-18 trial are also being used in a *post-hoc* analysis addressing MAF to
2167 help clarify the anticancer mechanism of denosumab (122).

2168

2169 **XI. CONCLUSION**

2170 Bone is one of the most common sites for metastasis, especially from breast, prostate and lung
2171 cancer. These skeletal metastases contribute substantially to morbidity and mortality in patients with
2172 advanced cancer. It is therefore essential to better understand the pathophysiology of bone metastasis
2173 in order to improve therapies for the treatment and prevention of bone metastasis and predict the risk of
2174 disease relapse. In this review, we described the importance of the systemic effect of primary tumors in
2175 preparing a pre-metastatic niche to facilitate the arrival of tumor cells in the bone marrow and we
2176 highlighted the prominence of metastatic niches in mediating dormancy of tumor cells. We also
2177 discussed the key role of the environment for reactivation of dormant tumor cells, which then undergo
2178 further selection to acquire a full complement of metastasis-colonization functions that dormant tumor
2179 cells did not express before. We also explained how, at a later stage, tumor cells induce osteolytic or
2180 osteoblastic lesions. These findings provide the rationale for the use of bone-targeted agents such as
2181 the bisphosphonates, the RANK ligand inhibitor denosumab and bone seeking radiopharmaceuticals.
2182 However, as our understanding of the signaling mechanisms between tumor cells and cells in the bone
2183 marrow microenvironment increases, several new, targeted agents have entered clinical development.
2184 They could be used in combination with anti-resorptive agents to efficiently block the development of
2185 skeletal lesions. Another attractive avenue of research would be to reconstruct bone lesions by restoring
2186 osteoblast anabolic functions. Finally, we showed that bone markers potentially provide important
2187 insight for predicting the risk of disease relapse in patients with cancer and evaluating a patient's risk of
2188 worsening skeletal health.

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2194

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2202

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- 3614
3615

3616 **Figure legends:**

3617 **Figure 1:** Patterns of bone metastases from solid tumors ranging from mostly destructive (osteolytic) to
3618 mostly bone-forming (osteoblastic). Representative radiographs and histology of bone metastases with
3619 osteolytic (white arrow) or osteoblastic (black arrow) lesions are shown. For bone tumor sections,
3620 mineralized bone is stained green, whereas bone marrow and tumor cells (*) are stained red. Of note, in
3621 osteoblastic lesions, extensive new woven bone (stained dark red) can be observed, leading to the
3622 formation of new trabecular bone that fills the bone marrow cavity (white arrow).

3623

3624 **Figure 2:** Bone colonization by tumor cells is a stepwise sequence of events that include (i) the
3625 formation of a pre-metastatic niche to attract circulating tumor cells (CTCs) in bone, (ii) the
3626 extravasation and homing of CTCs within the pre-metastatic niche where they bind to bone extracellular
3627 matrix proteins, and (iii) the maintenance of tumor cells in the vascular niche and the osteoblastic niche
3628 where tumor cells become quiescent through specific adhesive interactions with host cells. ANXA2:
3629 annexin A2; AXL: tyrosine kinase receptor; CAR cells: CXCL-12-abundant reticular cells; CDH:
3630 cadherin; Cx43: connexin 43; CXCL: chemokine; CXCR: chemokine receptor; ECM: extracellular matrix;
3631 GAS-6: growth arrest-specific 6; IL-1: interleukin-1; IL-6: interleukin-6; LOX: lysyl oxidase; RANK-L:
3632 receptor activator of nuclear factor kappa-B (RANK) ligand; SNO: spindle-shaped N-cadherin+
3633 osteoblast; TSP-1: thrombospondin-1.

3634

3635 **Figure 3:** The fate of bone-resident tumor cells is determined by a balance between activities of multiple
3636 activated protein kinases (MAPK) ERK1/2 and p38, where a switch towards ERK1/2 phosphorylation
3637 favors proliferation whereas activation of p38 leads to quiescence. This balance between ERK1/2 and
3638 p38 activities is governed by several factors that either promote dormancy (boxes in green), helping
3639 tumor cells to survive in the vascular and osteoblastic niches, or enhance tumor cell reactivation and
3640 proliferation (boxes in red). However, proliferative tumor cells become vulnerable to immune

3641 surveillance, leading to tumor cell killing by CD8⁺ T cells and NK cells. The bone microenvironment also
3642 contains immunosuppressive cells (MDSCs, Treg, pDC) that help tumor cells to escape from adaptive
3643 immunity. AXL: tyrosine kinase receptor; BMP-7: bone morphogenetic protein-7; C CAR cells: CXCL-
3644 12-abundant reticular cells; CXCL-12: chemokine;; GAS-6: growth arrest-specific 6; IFN- γ : interferon
3645 γ ; LIF: leukemia inhibitory factor; MDSCs: myeloid-derived suppressor cells; MSK1: mitogen- and
3646 stress-activated kinase 1; NK cell: natural killer cell; POSTN: periostin; pDC: plasmacytoid dendritic cell;
3647 PTHrP: parathyroid hormone-related peptide; RANK-L: receptor activator of nuclear factor kappa-B
3648 (RANK) ligand; SNO: spindle-shaped N-cadherin+ osteoblast; TGF β : transforming growth factor beta;
3649 Treg: regulatory T cells; TSP-1: thrombospondin-1; TYRO3: tyrosine kinase receptor; VCAM-1: vascular
3650 cell adhesion protein 1; VEGF: vascular endothelial growth factor.

3651

3652 **Figure 4:** Mechanisms governing the formation of osteolytic bone metastases. Several factors secreted
3653 by tumor cells enhance osteoclast-mediated bone resorption, either directly (*e.g.*, IL-8) or indirectly
3654 (*e.g.*, PTHrP, IL-6) *via* stimulation of RANK-L secretion and inhibition of OPG production by osteoblasts.
3655 In turn, the binding of RANK-L to RANK on osteoclast precursors leads to the formation of new
3656 osteoclasts. LPA by binding to its receptor LPA1 at the tumor cell surface promotes tumor cell
3657 proliferation and the production of IL-6 and IL-8, further enhancing osteoclast-mediated bone resorption.
3658 In addition, tumor-derived LOX and IL-1 β accelerate RANKL-induced osteoclastogenesis.
3659 Consequently, growth factors (TGF β , IGFs, PDGF) and calcium are released from the resorbed bone
3660 matrix. TGF β acts on tumor cells and stimulates the expression of factors such as PTHrP and Notch
3661 ligand Jagged-1. In turn, Jagged/Notch signaling promotes osteoclast differentiation. IGFs and calcium
3662 promote tumor cell proliferation. Calcium also stimulates the secretion of PTHrP and epiregulin by tumor
3663 cells. Tumor-derived epiregulin decreases *OPG* expression in osteoblasts. Thus, there is a vicious cycle
3664 where tumor cells stimulate bone destruction and factors released from resorbed bone stimulate tumor
3665 growth. This cycle is enhanced by the secretion of tumor-derived factors (DKK-1, SOST-1, noggin,

3666 activin A) that inhibit osteoblast activity, thereby worsening the imbalance between bone formation and
3667 bone resorption, and promoting bone destruction.

3668 DKK-1: dickkopf-1; IGF: insulin-like growth factor; IL-6: interleukin-6; LOX: lysyl oxidase; LPA:
3669 lysophosphatidic acid; OPG: osteoprotegerin; PDGF: platelet-derived growth factor; PTHrP: parathyroid
3670 hormone-related peptide; RANK-L: receptor activator of nuclear factor kappa-B (RANK) ligand; SOST-1:
3671 sclerostin; TGF β : transforming growth factor beta.

3672

3673 **Figure 5:** Mechanisms governing the formation of osteoblastic bone metastases. Several factors
3674 secreted by tumor cells directly enhance osteoblast differentiation (ET-1, BMP-2, BMP-6, Wnts). BMP-4
3675 mediates conversion of endothelial cells into osteoblasts. The stimulation of osteoblast differentiation is
3676 associated with increased OPG production, whereas RANK-L secretion is decreased. Tumor cells also
3677 produce OPG. Tumor-derived ET-1 directly acts onto mature osteoclasts to inhibit osteoclast activity.
3678 Therefore, there is a strong imbalance between bone formation and bone resorption, leading to aberrant
3679 bone formation. In addition, tumor-derived PSA and uPA increase the bioavailability of tumor growth-
3680 promoting factors to the bone microenvironment, such as IGF-I and TGF- β .

3681 BMP: bone morphogenetic protein; ET-1: endothelin-1; IGF: insulin-like growth factor; OPG:
3682 osteoprotegerin; PSA: prostate specific antigen; TGF β : transforming growth factor beta; uPA:
3683 urokinase.

3684

3685 **Figure 6:** Contribution of immune cells to bone metastasis formation. The innate and adaptive immune
3686 cells in the bone tissue microenvironment harbor both tumor-promoting and tumor-suppressing
3687 activities. CD8⁺ T cells and natural killer (NK) cells eliminate tumor cells through the production of
3688 interferon (IFN)- γ or TRAIL/FASL-induced apoptosis. However, these tumor cells may escape to the
3689 cytotoxic activity of immune cells (*e.g.*, CD8⁺ T cells), by inducing the recruitment of myeloid derived
3690 suppressor cells (MDSC), plasmacytoid dendritic cells (pDC) and regulatory T cells (Treg) that induce

3691 an immunosuppressive state within the bone tissue microenvironment. Beside tumor-suppressing
3692 activities, MDSCs can differentiate into functional osteoclasts. Furthermore, tumor-associated
3693 macrophages and a population of specialist osteal tissue macrophages termed osteomacs facilitate
3694 bone metastasis formation. RANK-L: receptor activator of nuclear factor kappa-B (RANK) ligand.

3695

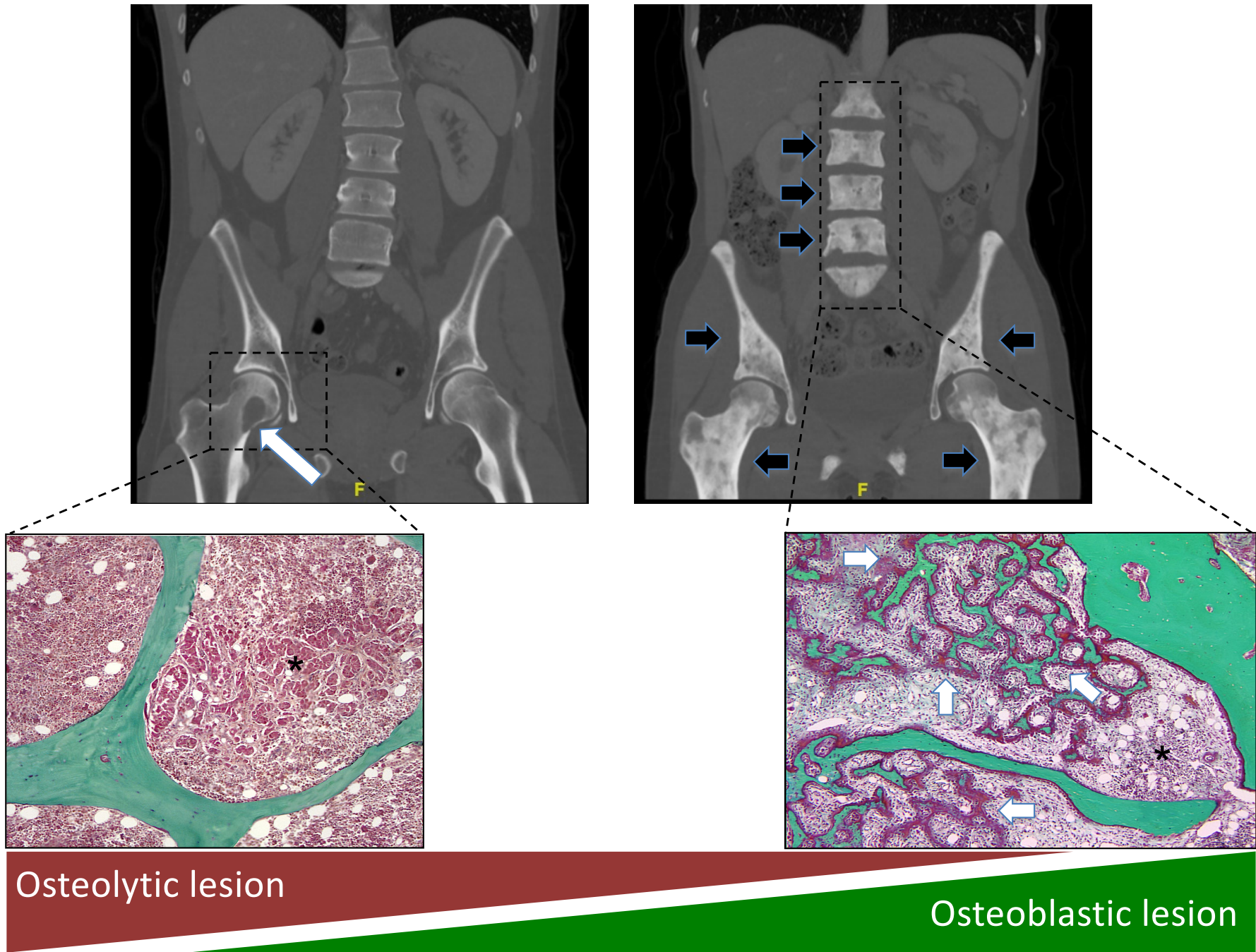
3696 **Figure 7:** Metabolic pathways associated with bone metastasis progression. In order to increase
3697 glucose uptake, cancer cells up-regulate glucose transporters, notably glucose transporter 1 (GLUT1).
3698 Glucose is then utilized for ATP generation through lactate production (aerobic glycolysis), *via* glucose-
3699 6-phosphate (G6P) and the pentose phosphate pathway (PPP) for nucleotide synthesis and through the
3700 tricarboxylic acid (TCA) cycle for lipid biosynthesis and protein acetylation. Lactate is released from
3701 tumor cells by monocarboxylate transporter 4 (MCT4) and then uptaken by osteoclasts through the
3702 transporter MCT1. Lactate stimulates osteoclast-mediated bone resorption, whereas fatty acids,
3703 cholesterol and nucleotides stimulate tumor cell proliferation.

3704

3705 **Figure 8: (A)** Clinical presentation of a solitary asymptomatic bone metastasis in a 54-year-old patient
3706 with breast cancer. A hot spot localized on L1 left pedicle (black arrow) was initially detected using
3707 technetium-99m (99m Tc)-bisphosphonate (BP) planar whole-body scintigraphy. Further analysis was
3708 conducted using fluorodeoxyglucose (FDG) positron emission tomography (PET) imaging. FDG/PET
3709 imaging displayed a hypermetabolic focus (white arrow) congruent to 99mTc-BP scintigraphy. **(B)**
3710 Clinical presentation of a solitary asymptomatic bone metastasis in a 67-year-old patient with prostate
3711 cancer. A hot spot localized on L3 vertebral body (black arrow) was initially detected using 99mTc-BP
3712 planar whole-body scintigraphy. Further analysis was conducted using fluorocholine (FCH)/PET
3713 imaging, displaying a hypermetabolic focus (white arrow) on L3, which was congruent to 99mTc-BP
3714 scintigraphy.

3715

3716 **Figure 9:** Current and emerging bone-targeted therapies. Summary of cellular and molecular targets
3717 and corresponding bone-targeted agents that are approved by the FDA and EMA for use in oncology or
3718 evaluated in phase trials. NCT: ClinicalTrials.gov identifier (ID) number.

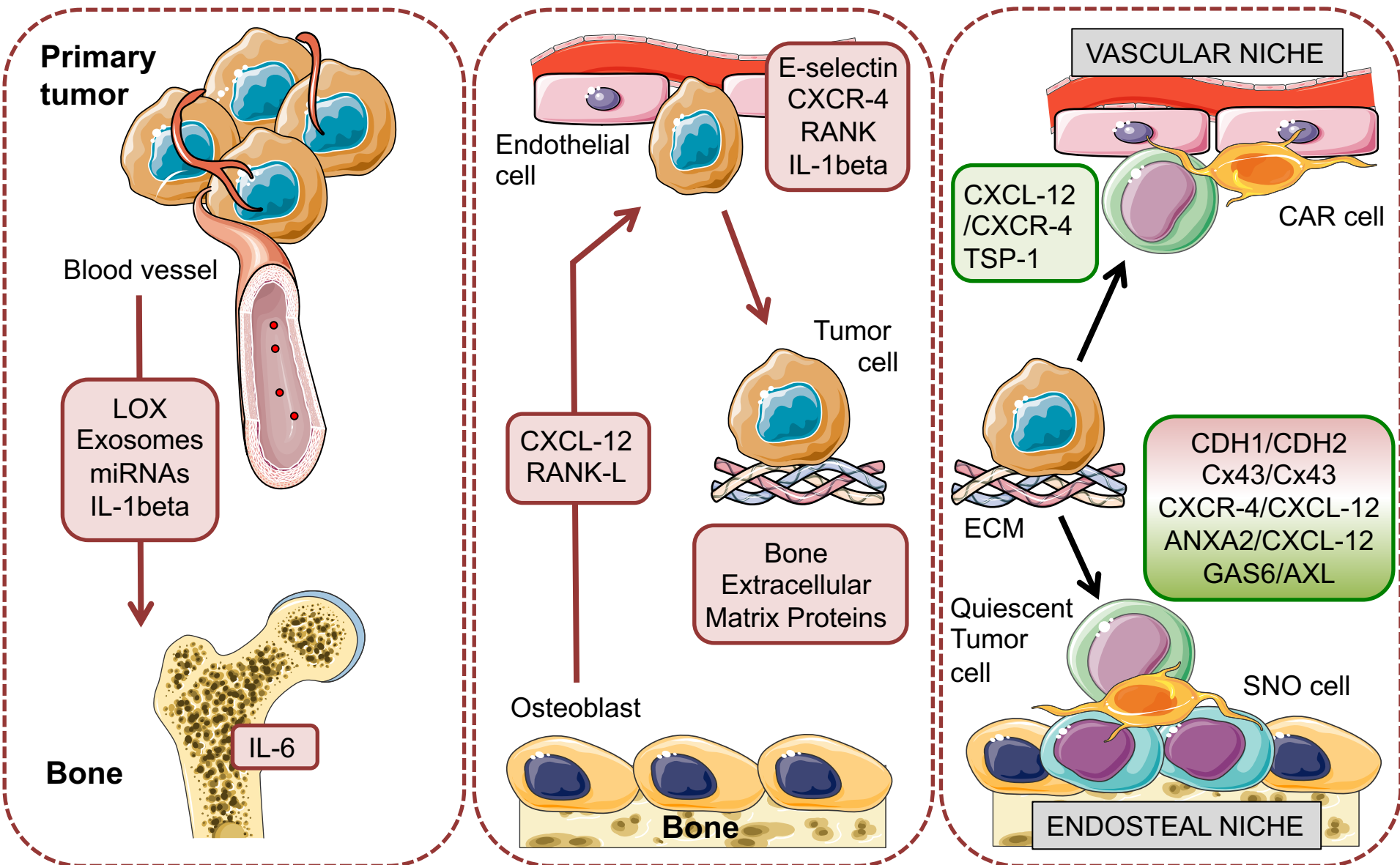
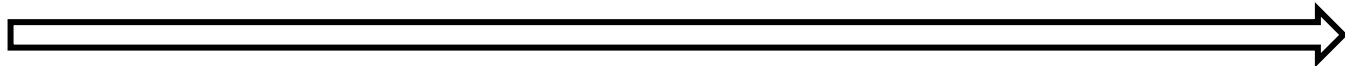


Osteolytic lesion

Osteoblastic lesion

Figure 1

PROGRESSION OF BONE COLONIZATION



Primary tumor

Blood vessel

LOX
Exosomes
miRNAs
IL-1beta

Bone

IL-6

Premetastatic niche

Endothelial cell

E-selectin
CXCR-4
RANK
IL-1beta

Tumor cell

CXCL-12
RANK-L

Bone
Extracellular
Matrix
Proteins

Osteoblast

Bone

Tumor cell extravasation and homing

VASCULAR NICHE

CXCL-12
/CXCR-4
TSP-1

CAR cell

ECM

Quiescent
Tumor
cell

CDH1/CDH2
Cx43/Cx43
CXCR-4/CXCL-12
ANXA2/CXCL-12
GAS6/AXL

SNO cell

ENDOSTEAL NICHE

Maintenance within the bone marrow niches

Figure 2

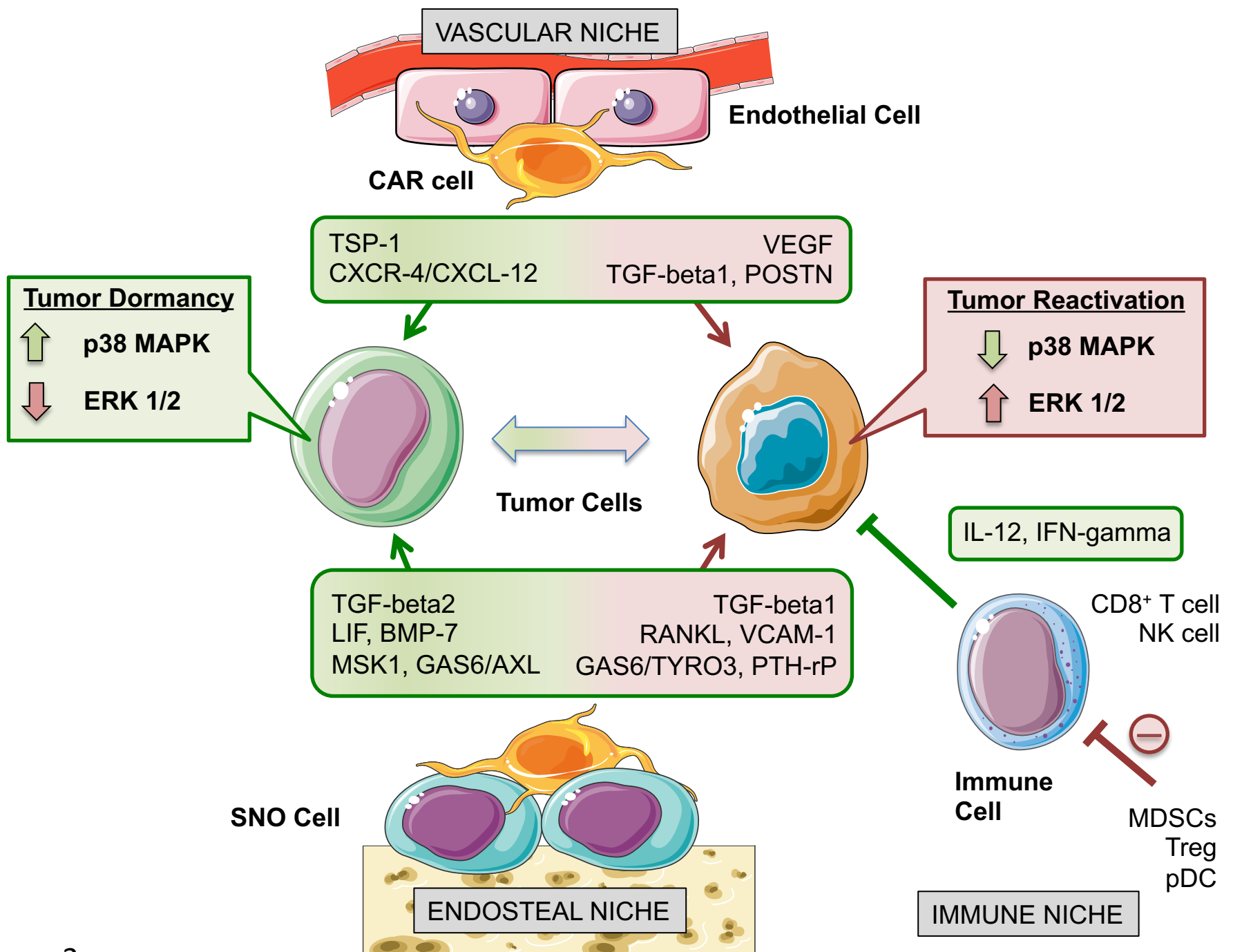


Figure 3

Figure 4

PROGRESSION OF OSTEOLYTIC BONE METASTASES

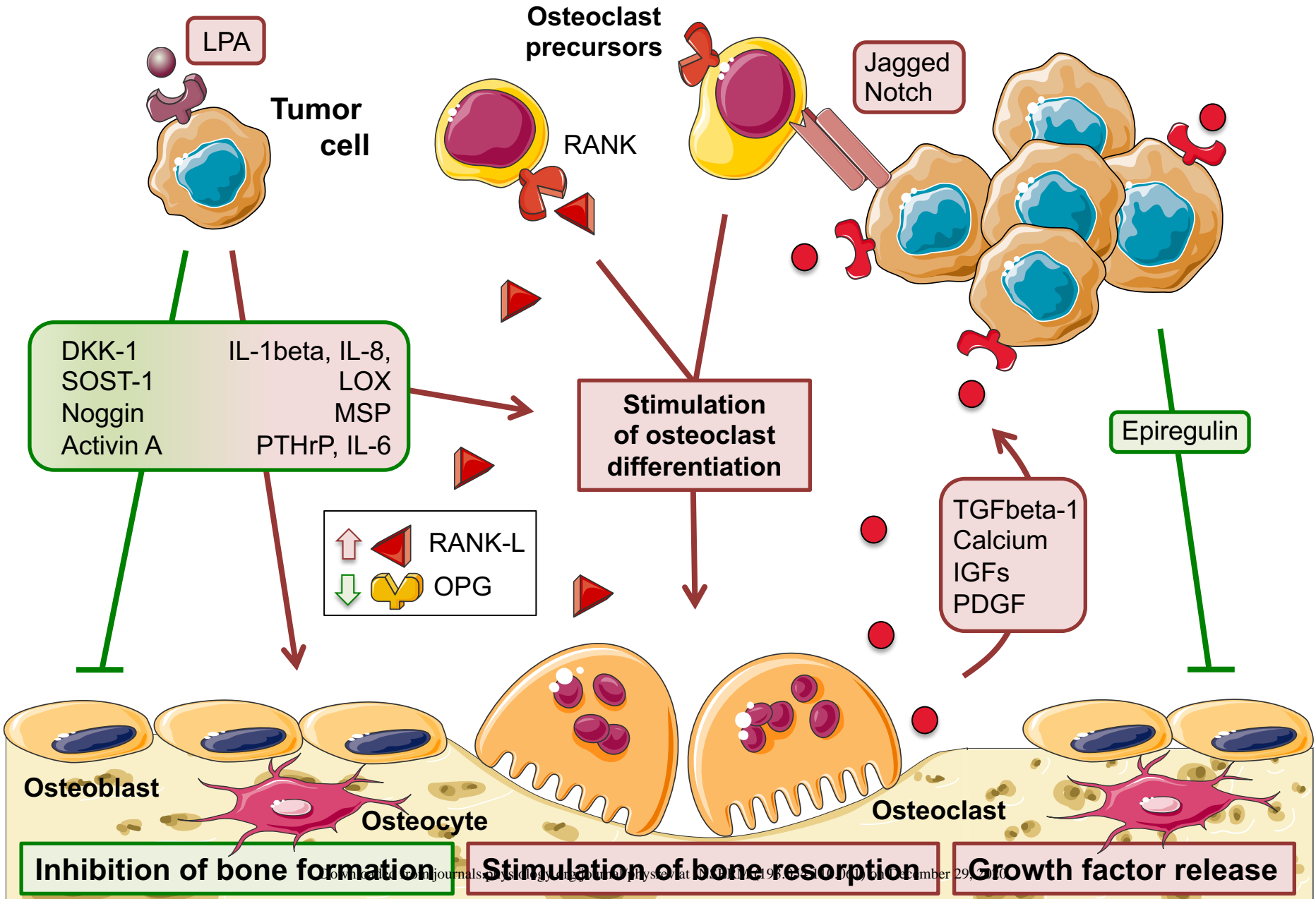
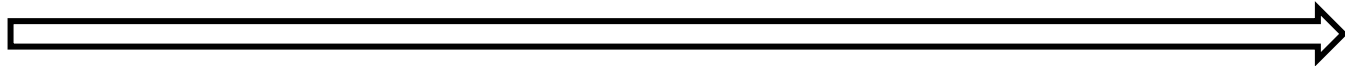
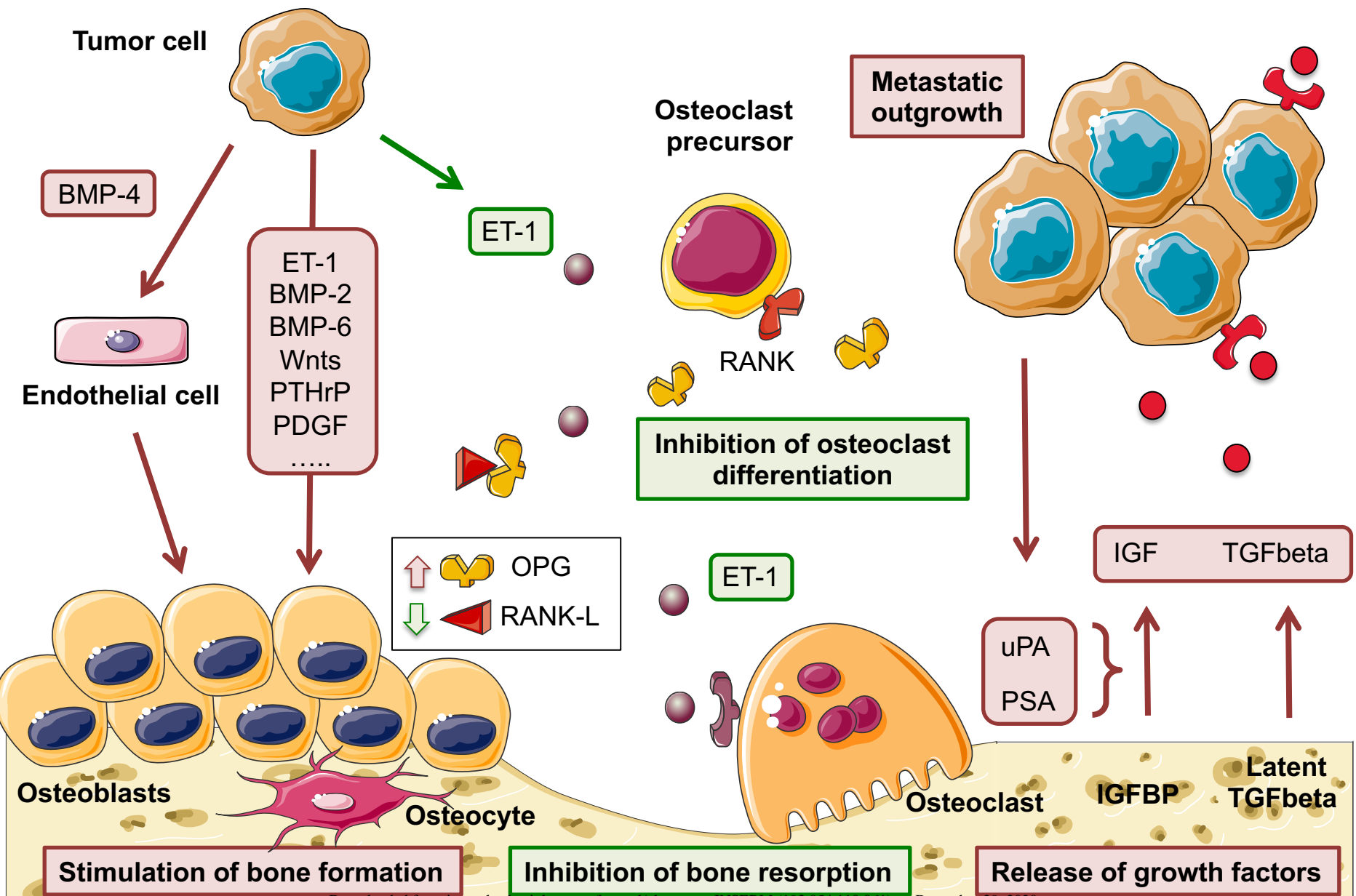
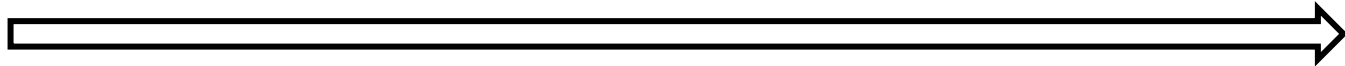
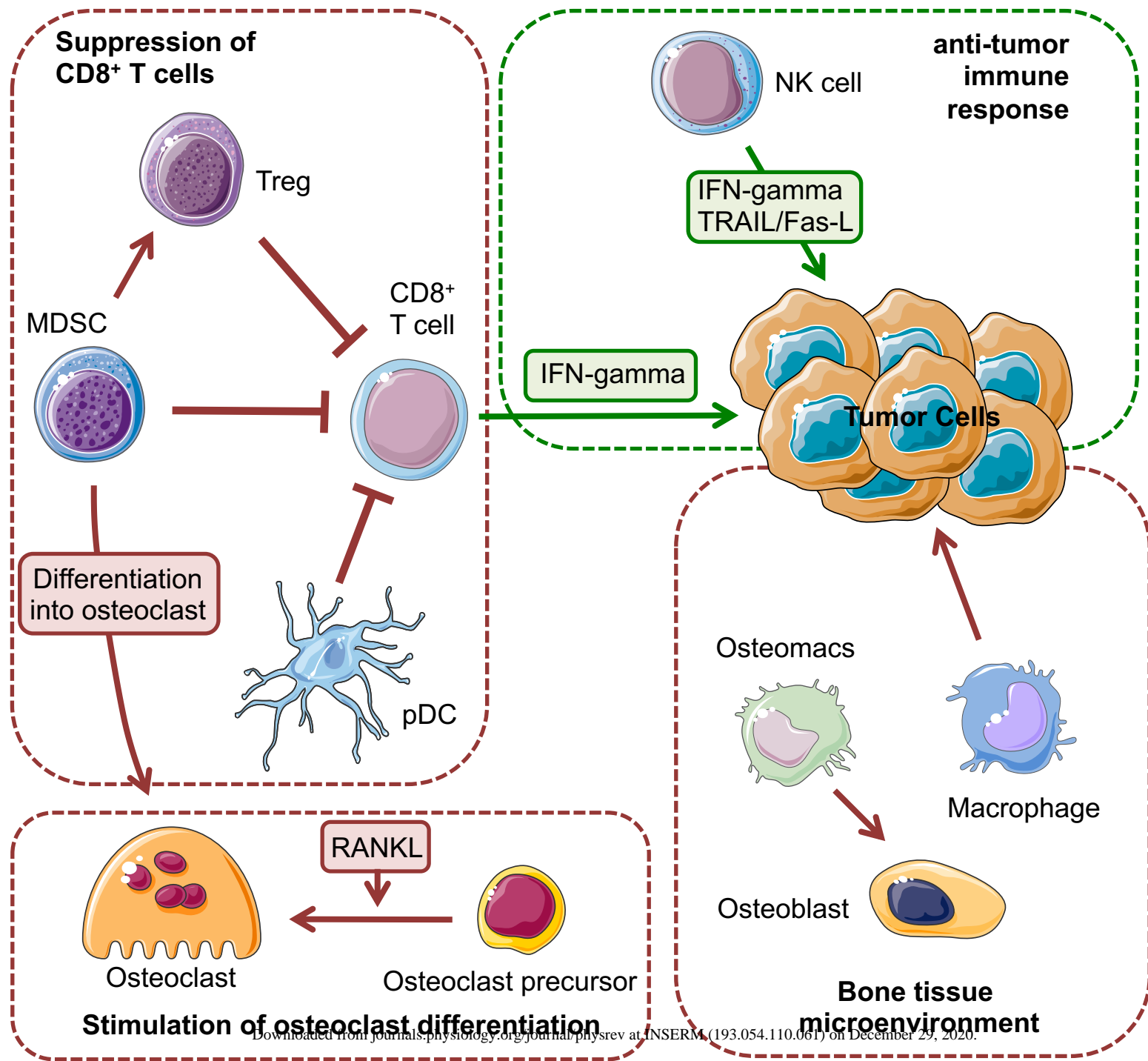


Figure 5

PROGRESSION OF OSTEOBLASTIC BONE METASTASES





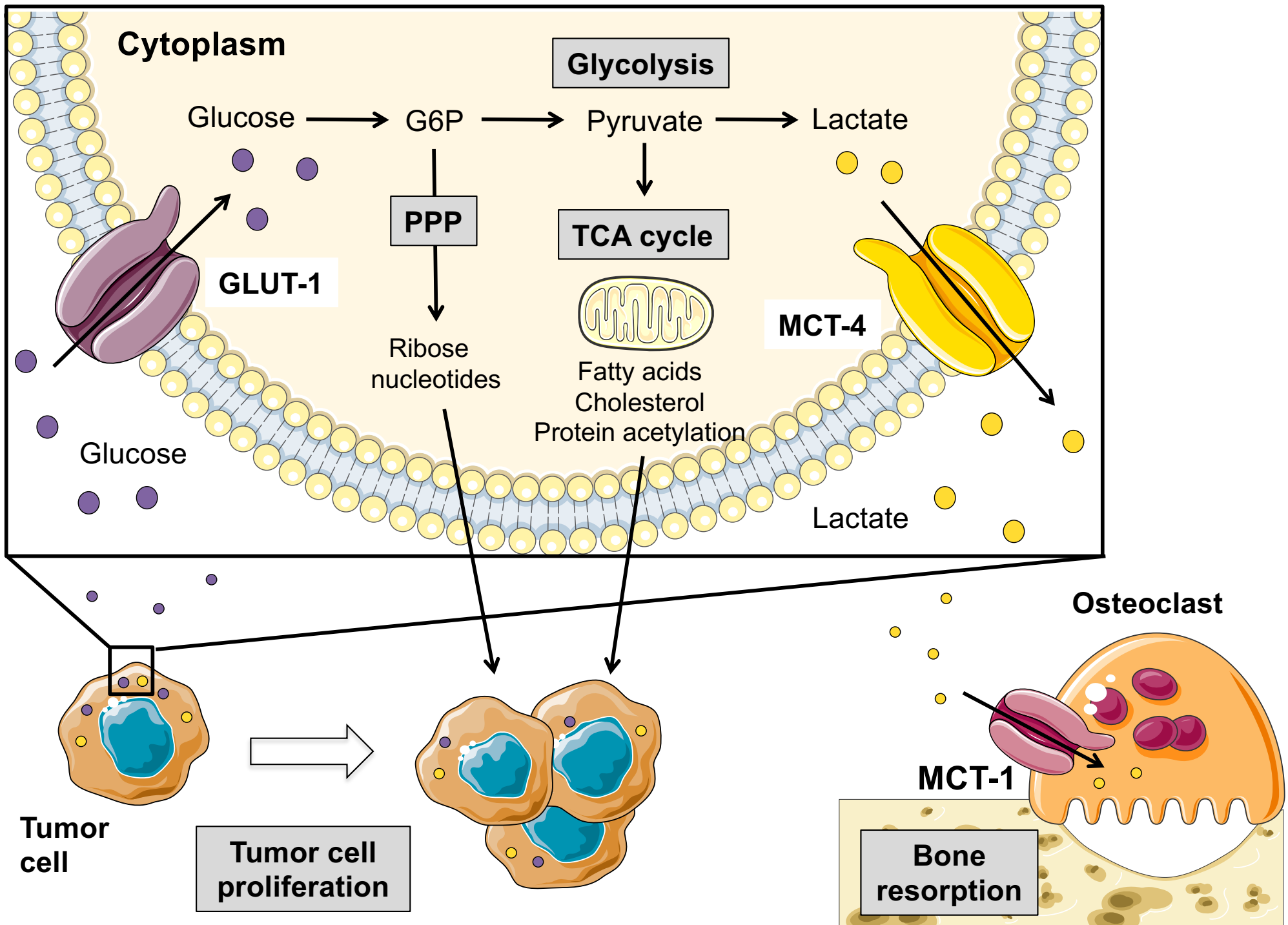
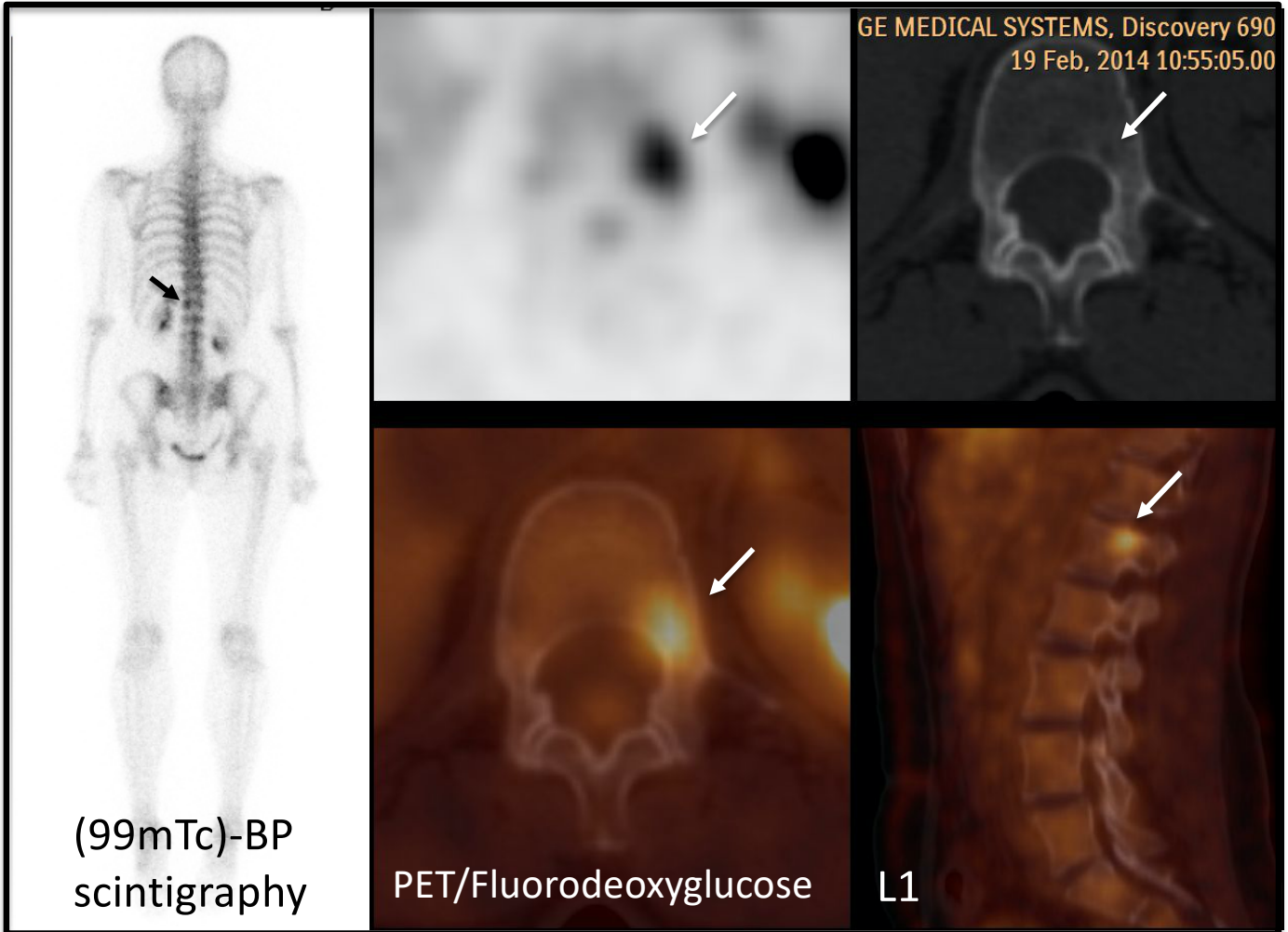
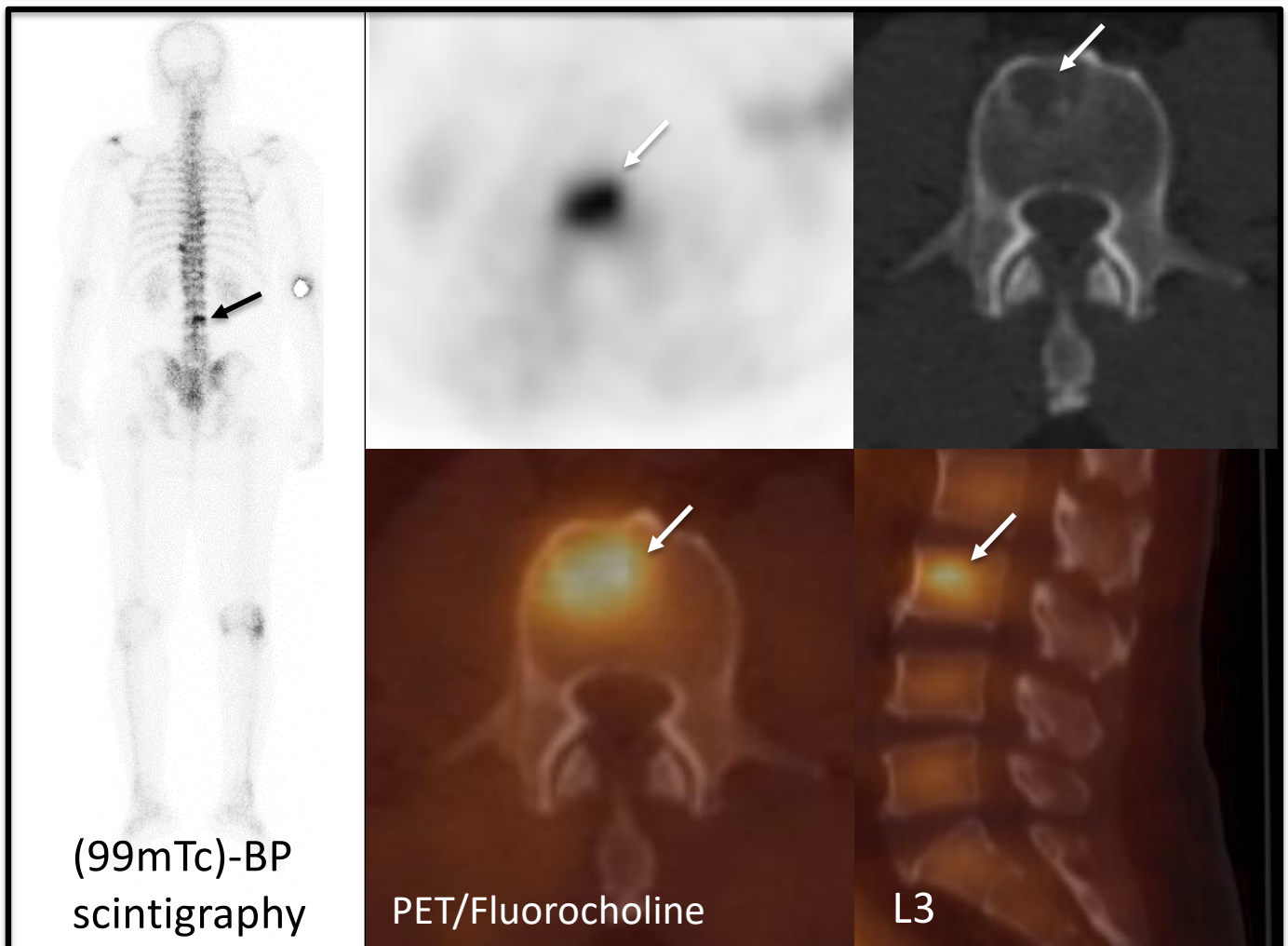


Figure 7

A**BREAST CANCER****B****PROSTATE CANCER**

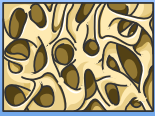



Matrix, Cell	Molecular target	Drug	Phase / Trial ID (NCT)
 Bone matrix	Calcium	Bisphosphonates, Radium-223, Samarium-223, Strontium-89	Approved
	TGFβ	Galinusertib	II / NCT02452008
 Osteoclast	FPPS	Bisphosphonates	Approved
	RANKL	Denosumab	Approved
	mTOR	Everolimus	Approved
	VEGFR, c-MET	Cabozantinib	Approved
	RON	BMS777607/ASLAN002	I / NCT01721148, NCT00605618
 Osteoblast	Androgen receptor	Enzalutamide	Approved
	CYP17A	Abiraterone	Approved
	Activin A	Sotatercept	II / NCT01562405, NCT00747123
	DKK-1	BHQ880	II / NCT01302886, NCT01337752, NCT00741377
 Nerve cell	NGF	Tanezumab	III / NCT00545129, NCT02609828

Table 1. Chemokines and their receptors involved *in the formation of bone metastasis*.

Chemokine receptor	Chemokine Ligand	Tumor type	Function in bone metastasis	Reference
CXCR-2	CXCL-1, CXCL-2	Prostate	Marrow adipocyte-derived CXCL-1 and CXCL-2 contribute to osteolysis in metastatic prostate cancer	135
CXCR-2	CXCL-5	Breast	The CXCR-2/CXCL-5 axis promotes tumor cell colonization in the bone marrow	275
CXCR-2	CXCL-8 (IL8)	Breast	Tumor-derived IL8 contributes to osteoclastic bone destruction in bone metastasis	18, 26, 27, 361
CXCR-3	CXCL-10	Breast, melanoma	CXCL-10 facilitates trafficking of CXCR-3-expressing cancer cells to bone	193
CXCR-4	CXCL-12	Breast, prostate	The CXCR-4/CXCL-12 axis regulates tumor cell entry in the bone marrow	235, 267, 273
CXCR-5	CXCL-13	Prostate	CXCL-13 mediates attachment of CXCR-5-expressing tumor cells to bone marrow endothelial cells <i>in vitro</i>	300
CX3CR-1	CX3CL-1	Breast, prostate	CX3CR-1 and CX3CL-1 interactions promote tumor cell extravasation in the bone marrow	158, 159
CCR-2	CCL-2	Breast, prostate	CCL2-expressing tumor cells engage CCR-2-expressing macrophages and pre-osteoclasts to facilitate colonization in bone	213, 232

Table 2. *Potential clinical utility of bone turnover biomarkers.*

Biomarker	Abbreviation	Clinical application	Reference
Bone formation marker			
Bone alkaline phosphatase	BALP	Diagnosis of bone metastasis in solid tumors. Prognosis of bone metastasis in solid tumors. Risk of skeletal related events. Prognosis during anti-resorptive therapy. Prediction of response to treatment.	61, 67, 90, 91, 155, 187, 206
procollagen I carboxyl-terminal propeptide	PICP	Diagnosis of bone metastasis in prostate cancer. Prediction of response to atrasentan in prostate cancer.	187
Procollagen I amino-terminal propeptide	PINP	Diagnosis of bone metastasis in breast and prostate cancer.	35, 90, 180
Bone resorption marker			
C-telopeptide	CTX	Diagnosis of bone metastasis in prostate cancer. Prognosis of bone metastasis in breast cancer.	33, 35
N-telopeptide	NTX	Diagnosis of bone metastasis in prostate and lung cancer. Prognosis of bone metastasis in solid tumors. Risk of skeletal related events. Prognosis during anti-resorptive therapy. Prediction	35, 61, 67, 90, 155, 187, 206

of response to treatment.

carboxyterminal telopeptide of type I collagen	ICTP	Diagnosis of bone metastasis in lung cancer. Prognosis of bone metastasis in breast cancer.	33, 155
Tartrate resistant acid phosphatase 5b	TRACP	Diagnosis of bone metastasis in breast cancer.	90, 155
pyridinoline	PYD	Prediction of response to atrasentan in prostate cancer.	186
Receptor activator of nuclear factor κ B-ligand/osteoprotegerin	RANKL/OPG	Diagnosis of bone metastasis in solid tumors	125, 165, 183
microRNAs	miRNAs	Diagnosis of bone metastasis in breast cancer.	96, 294

