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Review

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How the "seed" prepares the "soil": the bone/bone marrow pre-metastatic niche

Antonio Maurizi, Marco Ponzetti, Nadia Rucci

Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila 67100, Italy.

Correspondence to: Dr. Nadia Rucci, Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Via Vetoio-Coppito 2, L'Aquila 67100, Italy. E-mail: rucci@univaq.it

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Abstract

Cancer is one of the leading causes of death in women and men worldwide. The fatal outcome usually occurs after metastatic dissemination, and bone is by far the most common site of metastasis for breast and prostate cancer, the highest incidence neoplasia in women and men, respectively. However, while this is clear, the mechanisms through which the metastatic preference is established is not. An emerging concept in this regard is the pre-metastatic niche (PMN) establishment, i.e., the process through which tumors can influence the bone microenvironment from the primary site and make it permissive for their engraftment, before they migrate to the blood flow and metastasize. In this review, we discuss key microenvironmental players in the bone/bone marrow PMN, including osteoblasts, osteoclasts, and bone marrow adipocytes. We also describe the known PMN-educating factors, as well as the role of extracellular vesicles as emerging players in the bone/bone marrow PMN. An overview of current therapeutic developments aimed at targeting the bone PMN is also provided.

Keywords: Bone metastasis, pre-metastatic niche, bone microenvironment, extracellular vesicles

INTRODUCTION

Cancer is one of the most common classes of non-transmittable diseases and one of the leading causes of death in women and men worldwide[1]. The fatal outcome usually occurs after metastatic dissemination of cancer cells from the primary tumor. In fact, even though considerable effort has been put into cancer

research in the last century, metastatic cancer is still incurable[2]. The occurrence of metastasis is a stepwise process, in which cancer cells invade locally, enter the blood flow, and extravasate in a distal site[3]. For this process to happen, not only tumor cells need to acquire a specific set of "skills", but also the tissue that will be metastasized must change to allow tumor cell engraftment and growth. The fact that several types of cancer show a predilection to a specific distal tissue has always been a key issue in cancer research, from the days of the "seed and soil" theory of Dr. Paget[4] to our time. The latest development in the field is the conceptualization of the pre-metastatic niche (PMN). This is the phenomenon through which cancer cells prepare the "soil" for their dissemination by secreting soluble mediators and exosomes to make it suitable for their engraftment. Perhaps the most remarkable aspect of this process is that it could happen years before overt metastases appear. Hence, the reprogramming of the microenvironment appears to be operated by the tumor directly from the primary site before metastatic dissemination.

In cell biology, a niche is a specific area of a tissue, usually composed by few cells in close contact with each other, working in tandem to achieve a specific function, together with the surrounding extracellular matrix[5]. Niches are very dynamic structures, at both the cellular and molecular levels, and they are able to quickly integrate and adapt to external stimuli, providing an additional layer of regulation to especially important processes, such as hematopoietic stem cells' proliferation *vs*. quiescence. In fact, a deregulation in such process may lead to severe consequences, including microenvironment-induced leukemogenesis or stem cell exhaustion[6]. The bone marrow microenvironment is the prototypical tissue where most of the known niches have been discovered, and this should not come as a surprise, given that it presents much more flexibility and degrees of freedom for resident and circulating cells to rearrange themselves in small, specific groups compared to solid organs. Of note, this microenvironment is also a frequent site of metastasis for the most common human cancers, including breast, prostate, ovarian, colon, liver, and lung[7]. In fact, the bone marrow is the site where the metastatic niche is most commonly established[8]. The mechanisms underlying the bone marrow PMN are very complex[9], and this is due to the fact that cellular and molecular players may differ from one tumor to another, and that no spontaneous bone metastasis models are currently available. The fact that the PMN is established early during tumor growth poses an important challenge, as well as an opportunity: on the one hand, it means that circulating tumor cells (CTCs) may be able to prime the microenvironment and engraft during the first stages of the disease, thus making metastasis prevention a challenge. On the other hand, understanding the mechanisms inducing the PMN formation may provide crucial molecular players which may be targeted to prevent homing and metastatic growth in the bone marrow.

In this review, we describe the known cellular and molecular players in the bone/bone marrow PMN, as well as the factors produced by both tumor and resident cells that allow CTCs homing into the bone/bone marrow. Finally, we discuss possible therapeutic implications of the PMN in term of prevention of the metastatic dissemination.

THE HALLMARKS OF THE BONE/BONE MARROW PRE-METASTATIC NICHE

Liu and Cao[10] proposed six characteristics of the PMN influencing the fate of the CTCs upon arrival in the secondary site. These are represented by immunosuppression, inflammation, angiogenesis, vascular permeability, lymphangiogenesis, organotropism, and reprogramming. Their elegant description of this phenomenon is generalizable to all PMNs; however, there are specific hallmarks that could be more important than others in the bone/bone marrow PMN. Establishing an immunosuppressive niche is a central strategy for tumor cells to overcome the immunosurveillance of the host. This process includes the recruitment of immune and regulatory cells such as T cells Treg, macrophages, and myeloid-derived suppressor cells in the PMN[11-13].

It is widely demonstrated that chronic inflammatory processes play a pivotal role in tumor progression and metastasis[14,15], and that they are related to physiological phenomena such as aging[16,17]. In the context of the bone/bone marrow PMN, inflammation plays at least a two-faced role: on the one hand, it promotes osteoclast differentiation[18], which fuels bone loss and tumor cells homing and engraftment, while, on the other hand, it increases vascular leakiness, favoring tumor extravasation[19]. The increase of vascular permeability and angiogenesis is a crucial event for bone metastasis development. Key molecules involved in this event include vascular endothelial growth factor (VEGF), angiopoietin-2, mesenchymal epithelial transition factor (c-MET), and matrix metalloproteinases[10,20-24]. Although not focused on the bone/bone marrow pre-metastatic niche *per se*, a recent study demonstrated that, in breast cancer-bearing mice, bone marrow-derived suppressor cells can migrate to the lungs through C5a signaling and cause an angiogenic switch and vascular leakiness in the lungs, favoring the establishment of a PMN before tumor arrival[25]. Moreover, recent cutting-edge studies revealed that extracellular vesicles released by tumor cells in the primary site influence the vascular permeability in the PMN to promote the extravasation of the CTCs, as discussed in further detail in the following paragraphs.

BONE/BONE MARROW CELLS AND TUMOR-DERIVED FACTORS SHAPING THE BONE PRE-METASTATIC NICHE

Tumor cells are able to recruit and reprogram normal resident cells to promote tumor dissemination[10]. Therefore, different cell types in the bone/bone marrow microenvironment, including osteoblasts, osteoclasts, osteocytes, endothelial cells, bone marrow macrophages, and adipocytes [Figure 1 and Table 1], have a role to play in the establishment of the PMN, as discussed in the following paragraphs.

Osteoblasts and osteoclasts

Two of the most crucial players in the establishment of overt bone metastases are osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells. Their role, albeit less well-described, seems to also be pivotal in the establishment of the PMN in the bone/bone marrow compartment. Osteoblasts have been shown to respond to primary tumor-secreted factors such as PTHrP, which activates their RANKL production and increases osteoclast differentiation, thus starting a classic cancer-bone "vicious cycle" at a distance[8]. This activation of osteoclastic bone resorption triggers the release of factors such as insulin-like growth factor-1, bone morphogenic proteins, and platelet-derived growth factor from the bone matrix, which may help establish a pre-metastatic microenvironment. Another important action of the PTHrP secreted by the primary tumor site is the induction of the (C-C motif) ligand 2 chemokine expression by osteoblasts, which in turns recruits and activates M2 macrophages. The latter may partake in the PMN and foment tumor growth[26,27] once they migrate to the bone microenvironment. Other primary tumor-secreted factors that act similarly to PTHrP are heparanase (HPSE), its derived soluble factor Syndecan-1, and IL-8. HPSE is a matrix-remodeling enzyme that cleaves heparan sulfate, but it also has signaling roles in multiple pathways that are important for cell growth, including proto-oncogene tyrosine-protein kinase Src, extracellular signal-regulated kinase (ERK), hepatocyte/insulin-like/epidermal-growth factors (HGF, IGF, and EGF, respectively), signal transducer and activator of transcription (STAT), and protein kinase B (PKB)[24]. HPSE has been shown to promote bone resorption in breast cancer-inoculated mice, in absence of detectable bone metastases[25]. This is accomplished through the shedding of syndecan-1 and IL-8 from the primary site, which activate osteoclastogenesis directly[26], and possibly through osteoblastic RANKL upregulation, at least in multiple myeloma[27]. HPSE is considered an oncogene, since it is also able to promote primary tumor growth and angiogenesis, but, intriguingly, a close homolog of HPSE, called heparanase-2 (Hpa2) but lacking any heparanase activity, has been shown to have oncosuppressor activity[24], indicating that the catalytic activity is crucial for the oncogenic function and that this pathway is worthy of more investigation as a therapeutic target, despite clinical trials so far having been

Table 1. Cell types and factors involved in the bone PMN formation

	Cell type	Molecules	Target(s)	Function
Primary tumour	Tumour cells	PTHrP	Osteoblasts	Acts on Osteoblasts inducing the RANKL and CCL2 expression
		MINDIN	Osteoblasts	Increase osteoclastogenesis and tumour cell adhesion on osteoblasts and bone
		LOX	Osteoblasts	Induce bone matrix remodelling establishing a permissive environment for tumour cels
		HPSE	Osteoblasts	Acts on Osteoblast inducing the RANKL expression
		IL8 Syndecan1	Osteoclast precursors	Activate osteoclastogenesis
		SAA1/3 TLR4 TNFα Il6	Immune cells and osteoclasts	Recruitment of immune and regulatory cells in the PMN, as well as enhance osteoclastogenesis
		Integrin αvβ3	Bone matrix	Enhances the homing of tumour cells into bone
		CXCR4	Tumour cells	Promotes bone homing and colonization
Bone/Bone marrow microenvironment	Osteoblasts	RANKL	Osteoclasts precursors	Activate osteoclastogenesis
		CCL2	M2 Macrophages	Recruits and activates M2 macrophages
		SDF1 DKK1	Tumour cells	Enhances the homing of tumour cells into bone
	Osteoclasts (Activity)	IGF-1 BMPs PDGF TGFβ	Tumour cells and bone/bone marrow cells	Growth factors stimulating tumour growth and microenvironmental changes in bone/bone marrow
	Osteocytes	CCL12	Tumour cells	Enhances the homing of tumour cells into bone
		IL6 HFG HIF-1	Tumour cells	Affect the homing and the cancer cells proliferation in bone/bone marrow microenvironment
		GDF15	Tumour cells	Enhances cancer cells invasion and growth through the growth response 1 (EGR1) transcription factor-mediated mechanisms
		Notch	Tumour cells	Mediate cell-cell communication
		VEGF BMP7 Sclerostin	Endothelial cells	Increase vascular permeability
		RANKL	Endothelial cells and osteoclasts	Increase vascular permeability and osteoclastogenesis
	Bone marrow adipocytes (BMAs)	Inteleukines (6,1β) TNFα ANGPTL2/4 Visfatin SDF1	Endothelial and tumour cells	Induce vascular leakiness
	Endothelial cells	VCAM-1 Integrin α4β1	Tumour cells	Enhances the vascular adhesion and the homing of tumour cells into bone
	Immune cells	RANKL IL17F	Osteoclasts	Activate osteoclastogenesis

PTHrP: Parathyroid hormone-related peptide; LOX: lysil oxidase; HPSE: heparanase; SAA1: serum amyloid A1; SAA3: serum amyloid A3; TLR4: toll-like receptor 4; TNF: tumor necrosis factor; RANKL: receptor-activator of nuclear factor κB ligand; CCL2: CC-motif chemochine ligand 2; SDF1: stromal cell-derived factor 1; DKK1: Dikkopf-related protein 1; IGF-1: insulin-like growth factor-1; BMPs: bone morphogenic proteins; PDGF: platelet-derived growth factor; HIF-1: hypoxia inducible factor 1; GDF15: growth-derived factor 15; VEGF: vascular endothelial growth factor; SDF1: stromal cell-derived factor 1; VCAM-1: vascular cell adhesion molecule 1.

unsuccessful[28]. Therefore, PTHrP, HPSE, and its derivatives IL-8 and Syndecan-1 can start the vicious cycle directly from the primary site and prime the bone microenvironment to metastasis by increasing the osteoclast differentiation and bone resorption.

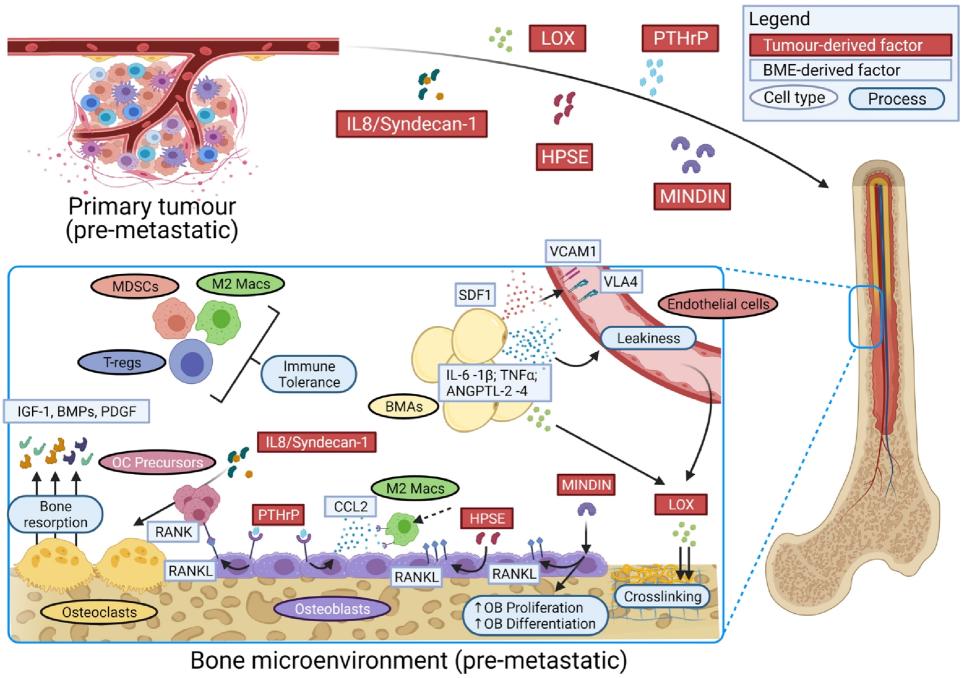

Figure 1. Primary tumor-secreted soluble factors in the establishment of the bone/bone marrow (BM) PMN. Lysil oxidase (LOX) crosslinks collagen and elastin in the bone/bone marrow microenvironment (BME), making the extracellular matrix stiffer and more susceptible to CTCs engraftment. Tumor cells also induce LOX secretion in bone marrow adipocytes (BMAs) as well as Interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)α, angiopoietin-like 2 (ANGPTL2), and angiopoietin-like 2 (ANGPTL4), which concur to determine vascular leakiness. Moreover, BMA-derived stromal cell-derived factor 1 (SDF1) induces vascular cell adhesion molecule 1 (VCAM-1) and very large antigen 4 (VLA4) expression in endothelial cells. Prostate cancer-secreted MINDIN is able to target osteoblasts, increase their proliferation and differentiation, and increase their production of receptor-activator of nuclear factor κB ligand (RANKL), which activates osteoclastogenesis, leading to bone resorption. This causes the release of growth factors such as insulin-like growth factor 1 (IGF-1), bone morphogenic proteins (BMPs), and platelet-derived growth factor (PDGF) from the bone matrix. Primary breast tumors have been reported to secrete syndecan-1, IL-8, parathyroid hormone-related peptide (PTHrP), and heparanase (HPSE), which are all able to activate osteoclastogenesis either directly or by activating the RANK-RANKL pathway through osteoblasts. PTHrP is also able to induce CC-motif chemochine ligand 2 (CCL2) expression in osteoblasts, which summons anti-inflammatory M2 Macrophages (M2 Macs). M2 Macs, together with myeloid-derived suppressor cells (MDSCs) and t-regulatory cells (T-regs), help establish immune tolerance in the BME. Image created with BioRender.com.

Osteoblasts are responsible for deposing bone matrix, which tumor cells are able to modify, even from a distance, to promote their future engraftment. In this regard, breast cancer cells growing in the primary site produce lysyl oxidase (LOX), a soluble enzyme that increases the tensile strength and stiffness of the extracellular matrix (ECM) by regulating the crosslinking between collagen and elastin[28,29]. Tumor-secreted LOX can reach the bone microenvironment through the blood flow and effectively enact a bone matrix remodeling that establishes a permissive environment that allows the engraftment and dissemination of tumor cells derived from colon[30] and breast cancers[31]. Recently, Ardura *et al.*[32] demonstrated that prostate cancer secretes the ECM protein MINDIN, which may affect bone cells in the PMN, inducing changes in the bone microenvironment eventually promoting prostate cancer bone metastasis[32]. In particular, MINDIN is able to increase osteoclastogenesis *in vivo*, which is particularly important in prostate cancer. In fact, although prostate cancer more frequently produces osteosclerotic metastases, it is now well known that osteoclasts also play a crucial role in these kinds of metastases, increasing bone deposition by releasing growth factors from the bone matrix, which increase osteoblast activity and tumor growth, thus fueling the osteosclerotic vicious cycle that leads to the development of overt metastases[33,34]. Current knowledge suggests that osteoclast activation is actually triggered before osteoblastic lesions can be detected, hence MINDIN could be a strong candidate as a tumor-secreted PMN initiator in bone. Furthermore, through *ex*

vivo and *in vitro* assays, Ardura *et al.*[32] showed that MINDIN was able to increase prostate cancer cells adhesion to bone and osteoblasts, thus strengthening the idea that this could be an important factor in the PMN establishment and prostate cancer cells homing.

The expression of several matrix proteins produced by osteoblasts have also proved important in homing to bone, and thus could be considered PMN factors. However, direct proof of this is currently lacking. An emerging topic in PMN establishment is represented by tumor-derived extracellular vesicles (EVs), which we and others found to be influencing the bone microenvironment in a way that favors tumor growth, affecting osteoblasts, osteoclasts, and endothelial cells[35,36]. The role of EVs is discussed in detail in the following paragraphs.

Osteocytes

Osteocytes represent 90%-95% of bone-resident cells[37-40]. They arise from osteoblasts, which depose matrix around themselves and remain trapped in the mineralized matrix, eventually differentiating into mature osteocytes[41]. These cells regulate bone physiology acting on both osteoblast and osteoclast activities[42]. It has in fact been demonstrated that they negatively regulate osteoblast differentiation and function by releasing DKK1 and sclerostin[42-44]. These molecules act as antagonist of WNT pathway by preventing the binding of WNT ligands to the Frizzled or LRP 5 and 6 receptors. Osteocytes are also the main source of RANKL, the most important osteoclast differentiation- and survival-inducing factor[45-49]. In addition to RANKL, osteocytes produce other factors involved in osteoclasts differentiation, such as OPG, MCSF, tumor necrosis factor (TNF), IL-6 and IL-11[50,51]. Moreover, in specific circumstances, osteocytes can even depose or resorb their neighboring bone matrix to respond to the needs of the organism[52]. Osteocytes also regulate other cell types within the bone/bone marrow niche. Indeed, osteocyte-derived VEGF stimulates angiogenesis by activating the MAPK-ERK pathway in endothelial cells[53]. Other angiogenetic factors produced by osteocytes are sclerostin, BMP7, and RANKL[47,54]. Osteocytes also regulate skeletal muscle by secreting myogenic factors such as sclerostin, prostaglandin E2 (PGE2), and Wnt3[55-58]. Moreover, recent observations demonstrated that connexin 43, a key component of gap junctions that are abundant in osteocytic processes, may also play a role in the bone-skeletal muscle crosstalk affecting muscle formation and function[59]. Finally, osteocytes are also endocrine cells, able to regulate phosphate homeostasis by fibroblast growth factor 23 (FGF23) secretion. This is a *bona fide* hormone, able to target renal proximal tubular cells and reduce their expression of the sodium-phosphate transporters type IIa, thus increasing phosphate excretion. FGF23 also reduces renal production of $1,25(OH)_2D$, which in turn reduces gut and bone absorption of phosphate[60].

As with other bone cells, osteocytes also play an important role in the development of bone metastases[61]. Indeed, they produce several factors involved in homing and proliferation of cancer cells in the bone microenvironment. These include CCL12, IL-6, hepatocyte growth factor (HGF), and hypoxia inducible factor 1 (HIF-1)[61]. Recent studies demonstrated that osteocyte-derived conditioned medium increases proliferation of human breast and prostate cancer cell lines[62]. Moreover, skeletal loading and changes in the mechanical forces in bone microenvironment affect cancer cells behavior through direct or indirect osteocytes stimulation[63-66].

Looking at the other side of the coin, tumor cells are able to impact osteocytes too, enhancing their pro-tumoral behavior. Indeed, it has been reported that prostate cancer cells stimulate osteocytes to produce growth-derived factor 15 (GDF15), which in turn enhances prostate cancer cells invasion and growth through an early growth response 1 (EGR1) transcription factor-mediated mechanism[67]. Finally, osteocytes are also able to establish a bidirectional communication with myeloma cells by cell-cell interaction,

mediated by the Notch pathway. This leads to the disruption of the bone remodeling compartment thus inducing osteolytic lesions[68,69].

Bone marrow adipocytes and vascular leakiness

It is well-established that bone marrow adipocytes (BMAs) increase in number with age, as well as in response to lifestyle changes and other external factors[70-72], and it is becoming clear that their number is inversely correlated with bone mineral density and directly correlated with osteoclast activation. This makes BMAs crucial in bone metastasis development and growth, but it is not the only function exerted by these cells in the tumoral context. In fact, BMAs seem to play a key role in establishing vascular leakiness, which allows tumor cells to escape circulation and engraft in the bone marrow. This action is exerted through several factors, including the inflammatory cytokines IL-6, IL-1β, and TNFα and novel players such as angiopoietin-like 2 (ANGPTL2) and ANGPTL4, visfatin, and stromal cell-derived factor 1 (SDF1). This, together with the fact that bone marrow sinusoids are by nature permissive for intra- and extravasation of CTCs, can create an ideal condition for tumor engraftment in the bone marrow[73]. Furthermore, SDF1 is one of the most widely recognized homing factors for CTCs, as described above. Another important action BMAs exert in the context of the PMN establishment is the secretion and promotion of expression of LOX[74]. As stated above, this protein creates a stiffer matrix that more readily receives circulating tumor cells[74]. Finally, the SDF1/CXCR4 pathway increases adhesion molecules expression in bone marrow endothelial cells, key examples being vascular cell adhesion molecule-1 (VCAM-1) and integrins α4β1, which enhance multiple myeloma, prostate, and lung cancer homing to bone[75,76].

Immune cells

The bone/bone marrow PMN needs to be an immune-privileged area since cancer cells need to escape immune surveillance to engraft and survive in such an immune cells-rich environment. This process is achieved by the recruitment of immune-suppressive cells such as Treg, macrophages, and myeloid-derived suppressor cells[11,12,77,78] in the PMN. Consistent with this idea, increasing number of the immunosuppressive Treg in the bone marrow has been associated with metastatic dissemination of different neoplasias[79,80]. Conversely, CD8+ T-cells, NK cells, and other immune cells expressing specific markers such as caveolin-1 and IL-1β have been reported to prevent the formation of the PMN or to keep the cancer cells in a dormant state[81-83]. Recently, Monteran *et al.*[84] demonstrated that 4T1 mouse breast cancer cells induce systemic immune suppression, associated with an increase of granulocytes and myeloid cells along with a decrease of NK cells and lymphocytes in the early steps of bone marrow pre-metastatic niche formation[84]. Intriguingly, Monteiro and Bonomo[85] also recently found that cancer cells can exploit immune cells in a completely different way, which still results in the priming of the bone/bone marrow microenvironment for metastasis. In fact, after coming in contact with the primary tumor, primed CD4+ T-cells can migrate to the bone marrow and activate osteoclastogenesis by means of RANKL and IL-17F, which results in bone loss, before the tumor can be detected in bone. This bone loss of course primes the microenvironment for metastatic engraftment. Intriguingly, in this case, dendritic cells are used as osteoclast precursors. Finally, primary tumor-derived molecules such as S100A8/9, serum amyloid A1, serum amyloid A3, toll-like receptor 4, IL-6, and TNFα have been linked with the recruitment of immune and regulatory cells in the PMN, as well as osteoclastogenesis, thus inducing a microenvironmental remodeling that creates a PMN and may promote metastasis[73,86,87].

TAKING ADVANTAGE OF THE PRE-METASTATIC NICHE: HOW CANCER CELLS HOME TO BONE

After the establishment of the PMN, the bone/bone marrow microenvironment becomes a better "soil" for the cancerous "seed". The next step for the CTCs is to extravasate into the bone/bone marrow milieu and

survive in the new microenvironment until they can develop into an overt metastasis. This process is defined as "homing" and is regulated by very complex relationships among cancer cells, resident cells, and the local ECM. Although we do not yet have a complete picture of how this process takes place, several important cellular and molecular players have been identified over the years on both the CTCs and the bone/bone marrow sides. Interestingly, to improve their ability to home to the bone/bone marrow, CTCs can disguise themselves through complex molecular contrivances, so that resident cells consider them part of the tissue, rather than a non-self element. This ability to "camouflage" is defined as "mimicry", and the two most studied cancer mimicry scenario are the so-called osteo-mimicry, where cancer cells "pretend" to be bone cells, and the hematopoietic stem cell (HSC)-mimicry, where CTCs express typical HSC molecules to home into their niches. Both osteo- and HSC-mimicry are important in CTCs homing. A key osteomimetic molecule involved in CTCs bone homing is RANK, the most important receptor in osteoclast differentiation and survival. In fact, osteoblast/osteocyte derived RANK-ligand (RANKL) creates a gradient through which RANK-expressing tumors such as breast, prostate, and melanoma can migrate towards bone. Another key osteomimetic homing factor is the αvβ3 integrin, which is well studied in osteoclasts, being a key mediator of their adhesion to the bone matrix during the bone resorption process[88-90]. This molecule can promiscuously bind to ECM components, including fibronectin, vitronectin, fibrinogen, and osteopontin (OPN)[91]. The latter is a key component of the bone matrix and is well-recognized as a microenvironmental homing factor for bone metastasizing cancer cells[92]. The αvβ3 integrin works in concert with another important ECM receptor expressed by a subset of CTCs: CD44. The latter has been proposed as a stemness marker in breast cancer cells, and it is also able to bind OPN, along with other ECM components such as hyaluronan, thus co-mediating the bone homing of this subset of cancer cells. This is in agreement with the concept that, once in the bone marrow microenvironment, CTCs may assume a stem-cell-like phenotype and hibernate into a dormant state for months or even several years, before currently unknown factors mediate their reactivation. This is achieved by means of HSC-mimicry. HSCs have tightly regulated homing and quiescence-inducing mechanisms, which can be replicated and exploited by CTCs to their advantage. Central for the homing mechanism is the SDF1-CXCR4 axis[93-95]. Indeed, SDF1 is highly expressed in bone, and this promotes the colonization of CXCR4-positive tumors[96-100]. In line with this, preventing the interaction between SDF1 and CXCR4 reduces bone metastasis[100]. Annexin II is another factor that can bind SDF1 and is used by HSCs to localize in the niche. In this case as well, cancer cells can mimic HSCs and express this protein, thus occupying their place in the niche[101]. In addition to the "mimicry" molecules, other homing factors have been identified that at least partially mediate this phenomenon. Key examples are microenvironment-derived CCL12 and CCL22, binding CCR7- and CCR4-positive cancer cells, respectively[96,102,103]. Another interesting example is Dikkopf-related protein 1 (DKK1), a Wnt pathway inhibitor that, according to a recent report, seems to determine bone *vs.* lung metastasis[104]. In fact, tumors with high expression of DKK1 are prone to metastasize to the bone, while DKK1-low tumors metastasize preferentially to lungs. Although the authors did not focus specifically on dissecting the role of DKK1 in homing, they observed a reduction in osteoblastic bone deposition in DKK1-low tumors, which can be considered a microenvironmental reprogramming. However, whether this happens before or after metastasis is still an open topic, which may provide valuable information for metastasis treatment. Another important molecule in the tumor colonization of the bone is transforming growth factor β (TGFβ), which is abundantly present in the bone matrix. TGFβ has a proliferative effect on both bone cells (osteoblasts) and cancer cells. It has been demonstrated that the TGFβ released upon tumor-induced bone resorption is able to increase invasion, chemotaxis, and angiogenesis[105-107]. Moreover, TGFβ stimulates tumor cells to produce osteolytic factors that enhance osteoclast activity, eventually leading to increased bone resorption[8,18]. This further increases the release of TGFβ from the bone matrix, fueling the so-called "vicious cycle" in the bone microenvironment. Recently, Waning *et al.*[108] demonstrated that TGFβ released from bone also contribute to muscle weakness in an *in vivo* model of bone metastases. In line with this, inhibition of TGFβ and its signaling pathway leads to increased bone mass[109], thus paving the way for possible therapeutic

applications[110,111].

Finally, other integrins, such as very late antigen 4 and VLA5, are important homing factors, being able to bind endothelial VCAM-1 and bone matrix fibronectin, thus partaking in cancer cell homing to bone, at least in multiple myeloma[112].

Another class of adhesion molecules, i.e., the cadherins, which mediate homophilic interactions between cells, can also mediate homing to bone. In particular, cadherin 11, (also referred to as osteoblast cadherin) can also be expressed by prostate cancer cells and promote their homing to the endosteal niche. Consistently, cadherin 11 is not expressed by normal prostate tissue, but its expression emerges when the normal tissue becomes cancerous[113]. This molecule may also be considered an example of osteo-mimicry. Similar findings were also observed in breast cancer cells[114], and, after homing, cadherin 11 overexpressing cells were able to increase osteoclastogenesis, thus propelling the osteolytic vicious cycle.

EXTRACELLULAR VESICLES: AN IMPORTANT TOOL TO SHUTTLE THE PRO-METASTATIC MESSAGE

The role of extracellular vesicles (EVs) as crucial mediators of intercellular communication is well established. EVs are structures surrounded by a lipidic bilayer, naturally released by non-apoptotic cells in both physiological and pathological conditions[115]. According to their origin and size, the scientific community has classically distinguished two major populations of EVs: microvesicles with a diameter of 100-1000 nm, which are directly shed by the plasma membrane, and exosomes, which 30-150 nm vesicles originating from the endo-lysosomal compartment after fusion of multivesicular bodies with the plasma membrane[116,117]. Although this classification is falling out of use[118,119], for the purpose of this review, it is adopted to describe the works published using this nomenclature.

EVs can be found in all biological fluids, such as blood, urine, saliva, and cerebrospinal and seminal fluid, and they often mirror the molecular setup of the cell of origin, which makes them very attractive as diagnostic tools[117]. EVs serve as cargo for a wide range of molecules, including DNA, proteins, lipids, and different types of RNA (mRNAs and miRNAs)[120]. Based on these characteristics, EVs represent an optimal means for tumor cells' colonization of distant organs. Tumor cells release significantly higher levels of EVs compared to normal cells, and this is also true for tumor cells that preferentially metastasize the bone, such as prostate[121,122] and breast cancer[123].

EVs released by primary tumor cells actively participate in the initiation of the PMN[73]. As a paradigm of this role, a few years ago, Hoshino *et al.*[124] identified a specific cluster of integrins expressed on the surface of tumor-derived exosomes, which allows their adhesion to a specific cell type or extracellular matrix molecule present in a distant organ, thus driving the organotropism of tumor cells. In particular, they found that exosomes expressing integrin αvβ5 specifically bind to Kupffer cells, promoting liver tropism, while exosomal integrins α6β4 and α6β1 bind to lung-resident fibroblasts, thus mediating lung metastases. Therefore, the authors proposed the exosomal integrin expression profile as a potential predictor of patient organ-specific metastasis[124]. Consistent with these results, in a colon cancer *in vivo* model, Ji *et al.*[125] demonstrated that the primary tumor releases integrin beta-like 1 (ITGBL1)-enriched EVs to convert lung fibroblasts and hepatic stellate cells to a cancer-activated phenotype, which in turn promotes PMN establishment by secreting proinflammatory cytokines. Moreover, the EVs enrichment in ITGBL1 is driven by the RUNX2 transcription factor. Similarly, Costa-Silva *et al.*[126] found that pancreatic cancer-derived EVs contribute to the PMN formation in the liver by shuttling macrophage migration inhibitory factor.

In bone metastases, osteoblasts and osteoclasts are reprogrammed by the tumor cells directly from the primary site, through the release of EVs containing an osteotropic cargo. With regards to prostate cancer, first studies from Itoh *et al.*[127] showed that EVs derived from different prostate cancer cell lines stimulate osteoblast differentiation of MC3T3 cells by releasing v-ets erythroblastosis virus E26 oncogene homolog 1, which is an osteoblast differentiation-related transcriptional factor. Similarly, it has been demonstrated that different prostate cancer cell line-derived exosomes shuttle phospholipase D2, which targets the osteoblasts, stimulating their proliferation and differentiation by activating the ERK1/2 pathway[128]. This effect is lost when prostate cancer-derived exosomes are generated in the presence of a PLD pan inhibitor[128]. Interestingly, a bidirectional cross-talk between prostate cancer and osteoblast is conceivable, as demonstrated some years ago by Millimaggi *et al.*[129], who found that osteoblast-derived conditioned media stimulated membrane vesicle shedding in prostate cancer cells.

microRNAs are typical cargo of EVs and several reports showed their crucial role in PMN formation. Their shuttling in the bone microenvironment by means of EVs can drive bone metastasis development towards an osteolytic or an osteosclerotic phenotype. Indeed, Probert *et al.*[130] recently demonstrated the delivery of a set of RNAs by prostate cancer-derived EVs to osteoblasts, which positively regulates osteoblast behavior. This mRNA cargo is enriched in genes related to cell surface signaling, cell-cell interaction, and protein translation. Among the non-coding RNA, the authors also found an enrichment in miRNA-21, which has already been linked to ovarian cancer exosome-mediated development[131] and could be considered a prognostic factor in the urine and plasma of prostate cancer patients[132,133]. Consistently, it has been demonstrated that hsa-miR-940 contained in prostate cancer-derived EVs promotes the *in vitro* osteogenic differentiation of human mesenchymal stromal cells by targeting ARHGAP1 and FAM134A. This in turn induces extensive osteoblastic lesions in the bone metastatic microenvironment *in vivo*[121].

With regards to breast cancer, we recently demonstrated by *in vivo* studies the ability of breast cancer-derived EVs to reach the bone microenvironment and be integrated by osteoblasts and osteoclasts[35]. Moreover, these EVs impaired osteoblast viability and differentiation, while enhancing the expression of pro-osteoclastogenic and inflammatory cytokines. In line with this profile, breast cancer EVs increase osteoclastogenesis, thus indicating a direct and indirect effect of EVs on osteoclast formation through the osteoblasts. To complete this picture, the effect of breast cancer cell-EVs on *in vitro* and *in vivo* angiogenesis was also investigated, finding a stimulatory role[35]. Following this paper, Yuan *et al.*[134] confirmed the crucial role of breast cancer secreted exosomes in establishing a PMN in the bone by stimulating osteoclast differentiation and function, while, from a mechanistic point of view, they identified the involvement of exosomal miR-21, which decreases the expression of programmed cell death 4, a factor known to have a suppressive function on c-Fos transactivation[135]. The authors also found significantly higher levels of miR-21 in the serum from breast cancer patients with bone-metastasis compared to that from breast cancer patients with non-bone metastases, thus giving a prognostic and therapeutic meaning to miR-21 shuttled by EVs[134].

Among other miRNAs driving organotropism of breast cancer cells, there is miR-940. When the human breast cancer cell line MDA-MB-231, known to induce osteolytic bone metastases, was forced to overexpress miR-940, it led to the switch towards an osteosclerotic phenotype of bone metastases[121]. A similar study, performed by Ye *et al.*[122], demonstrated that miR-141-3p shuttled by prostate cancer exosomes favors osteoblast differentiation and osteosclerotic bone metastasis development *in vivo*.

Multiple myeloma is quite frequently associated with a severe impairment of bone integrity, characterized by the development of extensive osteolytic lesions, caused by an exacerbated osteoclast activity which,

however, cannot be strictly considered as bone metastases. It has been shown that multiple myeloma-derived exosomes, as well as exosomes isolated from multiple myeloma patients, are internalized by osteoclast precursors, eventually leading to an enhancement of pre-osteoclast migration and osteoclast differentiation and functions, thus greatly contributing to the development of osteolytic lesions[136]. Later on, a mechanism of action was proposed by Raimondo *et al.*[137], who found an enrichment of amphiregulin (AREG) content, a molecule overexpressed in several types of cancers, in multiple myeloma-derived exosomes, which in turn activated the EGF pathway, eventually leading to an increase of osteoclast formation and activity. Similarly, Taverna *et al.*[138] showed that exosomes from non-small cell lung cancer carry AREG, which again activated osteoclast differentiation by a RANKL-dependent mechanism through the EGF pathway, eventually leading to bone metastases development.

Among other bone-related malignancies, we should also mention melanoma, which rarely metastasizes to bone; however, when this happens, both prognosis and quality of life of patients dramatically worsen. One of the first studies on this topic revealed that melanoma cells are able to educate bone marrow progenitor cells towards a prometastatic phenotype by upregulating the oncoprotein MET[139]. Consistently, recent studies point out a crucial role for EVs in influencing the migration and invasiveness of melanoma cells towards the bone[140]. Melanoma-derived EVs induce immune suppression and, in turn, defective dendritic cell functions[140]. They also promote PMN to distant organs by delivering a cargo of factors that increase the release of immunosuppressive cytokines, as well as angiogenic factors and matrix metalloproteinases[141]. With regards to the specific role of melanoma EVs on osteotropism, Mannavola *et al.*[142] found that bone conditioned medium significantly increased *CXCR4*, *CXCR7* and *PTHrP* expression in osteotropic melanoma cells, while their exosomes were able to reprogram non-osteotropic melanoma cells by reverting their original poor bone tropism towards an osteotropic phenotype. This effect is accomplished through the upregulation of membrane *CXCR7* by melanoma cells, which is required to promote their chemotaxis toward SDF1 gradient, eventually leading to bone colonization. A summary of the main proposed roles of EVs in the PMN establishment is presented in Figure 2.

CLINICAL RELEVANCE OF PRE-METASTATIC NICHE-TARGETING IN THE BONE METASTASES

The concept of the PMN represents a paradigm shift in the way we look at metastasis prevention and therapy. Indeed, new therapeutic approaches have been developed to target not only cancer cells but also the microenvironment, to prevent the tumor from homing to bone and/or developing into overt metastases.

With specific regards to the bone PMN, it could be targeted at different levels: (1) inhibiting the production of tumor-derived factors that allow the establishment of PMN; (2) blocking the interaction of the CTCs with the reprogrammed stromal cells or counteracting their reprogramming; and (3) reverting the immunosuppressive niche[10]. While the former option would probably be a very effective approach, thus far, no attempt has been made in clinical trials to the best of our knowledge. LOX would be an attractive therapeutic target in this regard, especially considering that a recent report[143] showed that LOX inhibition is able to overcome chemotherapy resistance in triple negative breast cancer (TNBC) subtypes. This would allow preventing PMN formation, while making the primary tumor easier to treat, even in its most aggressive form, i.e., chemotherapy resistant TNBC. An oral pan-LOX inhibitor has been developed by Pharmaxis for the treatment of myelofibrosis, which recently passed a phase I clinical trial and seemed to be well tolerated. Therefore, we have the tools and the knowledge to exploit this pathway. As for the latter point, i.e., blocking the interaction of CTCs with the reprogrammed stromal cells, or counteracting their reprogramming, this is surely the issue where most of the efforts have been put thus far. In fact, anti-resorptive drugs such as bisphosphonates (BPs) and denosumab have been tested in clinical trials as

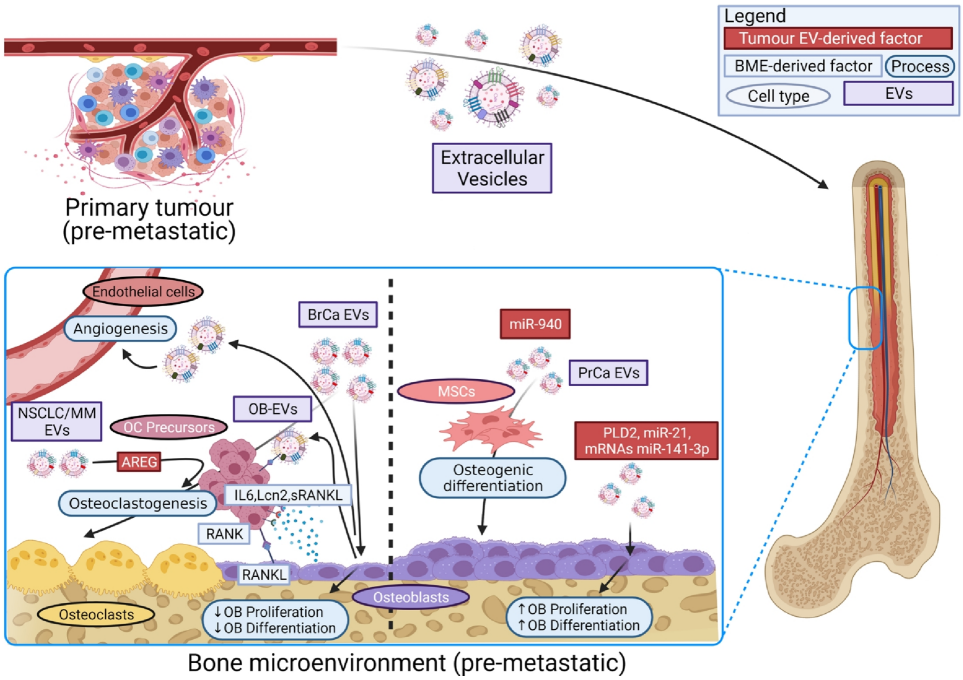

Figure 2. Proposed effects of tumor-derived extracellular vesicles (EVs) in the establishment of the bone/bone marrow PMN. Tumors secrete extracellular vesicles (EVs) that reach the bone/bone marrow microenvironment (BME). Prostate cancer (PrCa) can induce osteoblast (OB) proliferation and differentiation through their EVs, by shuttling phospholipase D2 (PLD2), as well as miR-21 and miR-141-3p. They can also induce mesenchymal stromal cells (MSCs) osteogenic differentiation through miR-940. This induces extensive osteoblastic lesions. Non-small cell lung cancer (NSCLC) and multiple myeloma (MM) EVs induce osteoclastogenesis through amphiregulin (AREG), which activates the receptor-activator of nuclear factor κB (RANK)-RANK ligand (RANKL) pathway. Breast cancer (BrCa) EVs induce osteoclast formation from precursors directly and through osteoblastic production of RANKL (membrane bound and soluble sRANKL), interleukin-6 (IL-6), and Lipocalin-2. BrCa can also induce the release of "educated" OB-EVs, which express RANKL on their surface, thus further increasing osteoclast formation and bone resorption. Educated OB-EVs also increase *in vitro* and *in vivo* angiogenesis. Image created with BioRender.com.

adjuvant therapy[144]. Bisphosphonates have been studied in several clinical trials for both the prevention of cancer treatment-induced bone loss (CTIBL) and the reduction of incidence and severity of bone metastases. The large body of evidence supporting the use of BPs in an adjuvant setting led to a consensus paper published in 2016 and authored by the leaders in the field of bone metastases treatment[145]. In this consensus, the panel recommends the use of adjuvant BPs in conjunction with anti-estrogenic therapy be considered as standard, given the compelling evidence of positive effects on preventing CTIBL and the amelioration of bone metastases severity and incidence, at least in post-menopausal women, since evidence of efficacy in pre-menopausal women is lacking. As for denosumab, clinical trials are currently in progress (ABCSG-18 study) and already provided promising results in an adjuvant setting to prevent skeletal-related events in conjunction with an aromatase inhibitor. Interestingly, this treatment regimen was also able to increase overall survival by about 2% over the follow-up period[144]. However, despite the positive effects of denosumab on bone health and promising preclinical data, a very recent international, multicenter, randomized, controlled, phase 3 trial investigating denosumab in the adjuvant setting (D-CARE) demonstrated that it is not effective in preventing bone metastases in the adjuvant setting, in women with histologically confirmed stage II or III breast cancer[146]. Additionally, and at variance with BPs, there were no subgroups that benefitted from adjuvant denosumab.

Another important effort in the field is represented by radionuclide therapy, mainly the bone-targeting α-emitter Radium-233. This agent has proved effective in castration resistant prostate cancer with two or more

bone metastases, although its adoption is hindered by low cost-effectiveness compared to other similar therapies[147]. However, where Radium-233 could really shine is the adjuvant setting: by accumulating in the bone tissue before bone metastases are detected, radium could target micrometastases, especially dormant tumor cells, more effectively than any other known agent, since they do not need actively cycling cells to perform their cytotoxic activity[148]. This could address one of the most significant issues in cancer nowadays, and several clinical trials are in progress with radium-233, alone or in combination with other drugs such as nivolumab, both in overtly metastatic breast and in prostate cancer, as well as oligometastatic or non-metastatic prostate cancer (www.clincaltrials.gov; keywords: prostate cancer, radium).

As for Point (3), conventional chemotherapy, alone or in combination with anti-angiogenetic molecules such as anti-ANG2, subverts the immunosuppressing niche, inducing an anti-tumor immune response along with a reduction of the inflammatory and angiogenetic processes in the secondary site[149]. Moreover, anti-angiogenic agents such as the TSU68 have been demonstrated to be a valid approach to target both the primary tumor and the PMN[150].

Extracellular vesicles could also represent attractive therapeutic targets to prevent PMN establishment, but to date there is no therapy available that would allow the targeting of these particles without significant side effects for the host. However, an early feasibility study aimed at removing EVs from the host's blood to prevent the establishment of EV-induced immune-suppressive PMNs in squamous cell carcinoma of the head and neck is in progress (NCT04453046). This trial features treatment with pembrolizumab (anti-PD-1 mAb) and treatment of patients' blood with a Hemopurifier, an extracorporeal circulation device that uses a lectin affinity matrix cartridge, originally designed to clear viruses from the bloodstream. This device is also able to clear cancer-derived EVs, which have a similar glycosylation status and size to those viruses. This approach could potentially be applied to all cancers, including those that metastasize to bone, thus this is a trial worth following. The field is young, and many exosome-release inhibitors are being developed, targeting the Ras-related proteins RAB27A/RAB27B or sphingomyelinase, as well as indirect inhibitors such as cannabidiol, ketotifene, and, interestingly, proton pump inhibitors (PPIs)[151]. The latter mechanism of action is based largely on the fact that cancer cells secrete more EVs when their microenvironment is acidic, which is mainly achieved through tumoral and microenvironmental V-ATPases. As we recently demonstrated[152], omeprazole is able to reduce bone pain and, at least at later stages, osteolysis. Therefore, this could be an interesting treatment which could inhibit osteoclastic acidification, hence influencing PMN formation, reduce acidity-induced pain, and inhibit tumor EVs secretion. PPIs are used worldwide and have few side effects; therefore, although we cannot realistically imagine a single-agent adjuvant therapy based on PPIs for cancer, these could be an interesting addition to a standard therapy which would be worth exploring. In addition to this, efforts are in place to exploit EVs as diagnostic tools that would on the one hand help diagnose primary tumors of unknown origin, and on the other hand monitor treatment response, onset of drug resistance, and propensity to metastasize[153].

CONCLUSIONS

In this review, we discuss the specific hallmarks of the bone/bone marrow PMN, focusing on key microenvironmental players (i.e., osteoblasts, osteoclasts, osteocytes, bone marrow adipocytes, endothelial cells, and immune cells), as well as the molecular pathways involved in its establishment. We then give an overview of the homing process, which is the next step in metastasis, where CTCs becoming DTCs in the bone microenvironment, using specific molecular cues and proteins to their advantage. This process includes proteins that can fall into the concepts of osteo- or HSC-mimicry, where cancer cells can express bone microenvironmental factors to persuade, at the molecular level, that they belong in that milieu. Extracellular vesicles are also very important in PMN establishment. These nano-scale molecular carriers

secreted by cancer cells are potentially able to reprogram the entire bone microenvironment, including at least osteoblasts, osteoclasts, and endothelial cells. However, what is of the utmost importance is translating these important basic science concepts to the clinic. There has been significant attention given to how the bone microenvironment can be targeted to reduce PMN formation, and many clinical trials have explored the use of bone-targeting agents to prevent metastasis, with promising results. In addition, EVs hold great promise for therapy, with many potential candidates in preclinical phase. However, since the field is very young, there is still a long way to go to get to an EV-targeting anti-PMN therapy. Nonetheless, EVs are incredibly powerful diagnostic and therapy-monitoring tools, as well as potential drug delivery systems, which hold much potential for the future.

DECLARATIONS

Authors' contributions

Collected the data, wrote the manuscript and prepared figures and tables: Maurizi A, Ponzetti M
Coordinated the work, wrote and reviewed the manuscript: Rucci N
Revised and approved the final version of the manuscript: Maurizi A, Ponzetti M, Rucci N

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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