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RESEARCH PAPER

Changes in the peripheral blood and bone marrow from untreated advanced breast cancer patients that are associated with the establishment of bone metastases

Leandro Marcelo Martinez · Valeria Beatriz Fernández Vallone · Vivian Labovsky · Hosoon Choi · Erica Leonor Hofer · Leonardo Feldman · Raúl Horacio Bordenave · Emilio Batagelj · Federico Dimase · Ana Rodriguez Villafañe · Norma Alejandra Chasseing

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Abstract Bone metastasis is an incurable complication of breast cancer affecting 70–80 % of advanced patients. It is a multistep process that includes tumour cell mobilisation, intravasation, survival in the circulation, extravasation, migration and proliferation in the bone marrow/bone. Although novel findings demonstrate the bone marrow microenvironment significance in bone metastatic progression, a majority of studies have focused on end-stage disease and little is known about how the pre-metastatic niche arises in the bone marrow/bone tissues. We demonstrated a significant increase in patients' peripheral blood plasma ability to induce transendothelial migration of MCF-7 cells compared with healthy volunteers. Moreover, high RANKL, MIF and OPG levels in patients' peripheral blood could play a role in the intravasation, angiogenesis, survival and epithelial–mesenchymal transition of circulating tumour cells. Also, we observed a significant increase in patients' bone marrow plasma capacity to induce transendothelial migration of MDA-MB231 and MCF-7 cells compared with healthy volunteers. Furthermore, patients' bone marrow mesenchymal stem cells could control the recruitment of tumour cells, modifying the MCF-7 and MDA-MB231 cell migration. In addition, we found a significantly higher MDA-MB231 cell proliferation when we used patients' bone marrow plasma compared with healthy volunteers. Interestingly, PDGF-AB, ICAM-1 and VCAM-1 levels in patients' bone marrow were significantly higher than the values of healthy volunteers, suggesting that they could be involved in the cancer cell extravasation, bone resorption and cancer cell proliferation. We believe that these results can reveal new

Leandro Marcelo Martinez and Valeria Beatriz Fernández Vallone have contributed equally to this study.

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L. M. Martinez · V. B. F. Vallone · V. Labovsky · E. L. Hofer · N. A. Chasseing (✉)
Immunohematology Laboratory, Experimental Biology and Medicine Institute (IBYME), National Council of Scientific and Technical Research (CONICET), 2490 Vuelta de Obligado, 1428 Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina
e-mail: achasseing@ibyme.conicet.gov.ar; alejachase@gmail.com

H. Choi
Texas A&M Health Science Center, College of Medicine, Institute for Regenerative Medicine at Scott & White, 5701 Airport Road, Module C., Temple, TX 76502, USA

L. Feldman
Department of Bone Marrow Transplantation, Favaloro Fundation, 443 Solis, 1428 Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina

R. H. Bordenave
Department of Oncology, Iriarte Hospital, 770 Alison Bell, 1878 Quilmes, Buenos Aires, Argentina

E. Batagelj
Department of Oncology, Central Militar Hospital, 726 Luis María Campos, 1426 Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina

F. Dimase
Department of Hemotherapy, Central Militar Hospital, 726 Luis María Campos, 1426 Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina

A. R. Villafañe
Department of Diagnosis and Treatment, Central Militar Hospital, 726 Luis María Campos, 1426 Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina

information about what alterations happen in the bone marrow of advanced breast cancer patients before bone colonisation, changes that create optimal soil for the metastatic cascade progression.

Keywords Breast cancer · Bone marrow · Pre-metastatic niche · Mesenchymal stem cells · Bone metastasis

Introduction

The ability to invade and metastasise allows cancer cells to leave the sites of primary tumour formation and recolonise in new tissues. Bone metastasis is an important clinical entity in patients with malignant tumours, and blood circulation emerges as a vector for tumour cells to spread and establish metastatic lesions. The bone marrow (BM)/bone microenvironment per se seems to play an important role, facilitating colonisation, establishment and growth of tumour cells [1].

The metastatic process involves a series of distinct steps that together comprise the invasion–metastasis cascade. As the first step, cancer cells in the primary tumour acquire the ability to invade the surrounding tissues; in carcinomas, this requires breaching the basement membrane that confines the epithelial compartment. Tumour cells then must gain access to lymphatic and blood vessels, enter into the lumina of these vessels (intravasation), survive transport through these vessels and exit from the vasculatures (extravasation). Small cell clumps or singly disseminated tumour cells (micrometastasis) must acquire the ability to survive and proliferate in the microenvironment of a foreign tissue to form macroscopic metastasis [2]. Finally, it is well known that local growth factors' release by active bone basic multicellular units (osteoblasts and osteoclasts) in close proximity to a BM micrometastasis (colonised BM) may evoke the growth and invasive potential of the breast cancer (BC) cells (BCCs), ultimately leading to the development of an overt bone metastatic cascade [3].

Although the stepwise progression of the metastatic cascade is relatively well described from the point of view of the intrinsic mechanisms of tumour cells, greater emphasis needs to be placed on the study of systemic factors that influence 'pre-metastatic niche' formation and metastatic progression [4].

Over 100 years ago, Paget [5] enunciated 'the seed and soil' hypothesis, stating that seeds of metastatic BCCs preferentially settle on the soil of the bone matrix. Tumour expansion in bone could be achieved by two mechanisms: osteoclastic bone resorption and extracellular matrix (ECM) degradation [6, 7]. The colonisation and growth of BC metastasis in the BM/bone depend on the cooperative interaction of the cancer cells with the host cells in the BM/bone microenvironment. It includes the resident osteoblasts, osteoclasts, mesenchymal stem cells (MSC), progenitor stromal cells, fibroblasts, adipocytes, endothelial cells, haematopoietic stem cells (HSC) and progenitors, bone lining cells and transient cells, such as macrophages, lymphocytes, neutrophils and other blood cells. Whilst cell–cell contact is established between cancer cells and BM/bone cells via adhesion molecules, a wider network of communications occurs through secreted cytokines and growth factors. These soluble molecules play a critical role in the normal haematopoietic and bone-remodelling processes, as well as in the cancer cell colonisation of the BM [8].

Accumulating the findings of the receptor activator of nuclear factor kappa B ligand (RANKL)/RANK (RANKL receptor)/osteoprotegerin (OPG) molecular triad, the key regulators of bone remodelling, opened a new era of bone research [9, 10]. Although RANK is an essential receptor for osteoclast formation, activation and survival, functional RANK expression has been recently identified upon the migration and bone-specific metastatic behaviour of BCCs [11–13]. However, it has been appreciated that macrophage migration inhibitory factor (MIF) constitutes an important link between chronic inflammation and cancer. MIF levels are markedly elevated in numerous tumour entities such as BC [14]. Inflammatory leucocyte recruitment is dependent on MIF-CXC chemokine receptor-2 (CXCR-2) and MIF-CXCR-4 interactions [15]. Moreover, Verjans et al. found that the expression of MIF receptor CD74 was elevated in MDA-MB231 cells. Autocrine MIF promoted tumour cell proliferation, as indicated by the blockade of MIF or CD74 in MDA-MB231 and MDA-MB468 cells, and MDA-MB231 invasiveness was enhanced by exogenous MIF, demonstrating that MIF functions as a chemoattractant [16]. Moreover, our group found CXCR-4 expression in BCCs of MCF-7 and MDA-MB231 lines, as other authors did [17]. Therefore, the interaction between MIF/RANKL and their receptors in BCCs favours its invasive and migratory capacity, to be precise the egress of metastatic cells from the primary tumour. Moreover, MIF and OPG are not only pro-angiogenic factors [18, 19] but also have an anti-apoptotic effect over BCCs favouring circulating BCC survival [20, 21]. Also, it is known that MIF induces the production of proinflammatory factors such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in stromal cells [22], factors that induce the epithelial-to-mesenchymal transition (EMT) of BCCs facilitating the process of invasion and intravasation [23, 24]. Furthermore, CXCR-4 receptor and its ligand stromal cell-derived factor-1 (SDF-1) are specifically used by BCCs to arrest and migrate into secondary organs, in particular BM/bone [25].

After survival and arrest in the circulation, tumour cells must escape out of the blood and lymphatic vessels in a process known as extravasation [26]. The role of CAMs, such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular endothelial cell adhesion molecule-1 (VCAM-1), has been studied extensively in the process of inflammation. Some circulating cancer cells have been shown to extravasate to a secondary site using a process similar to inflammatory cells [27]. Although tumour cells mimic mechanisms used by leucocytes, the adhesion molecules and ligands involved in tumour cell extravasation are sometimes different, suggesting additional or alternative non-leucocyte-like mechanisms [28]. Increasing evidence suggests that ICAM-1 plays an important role in the adhesion of BCCs to endothelial cell monolayers and in subsequent transendothelial migrations [29, 30]. Significantly high serum concentrations of ICAM-1 were found in patients with metastatic BC with liver and/or bone metastasis. The patients who did not respond to chemo/endocrine therapy showed significantly higher soluble ICAM-1 levels compared with those who responded to therapy. Moreover, high ICAM-1 levels predicted a significantly poorer overall survival in both univariate and multivariate analyses [31]. Another finding highlights the importance of VCAM-1 interactions in metastatic bone BC [30, 32]. The elevation in VCAM-1 has prognostic significance in patients with breast carcinoma and was associated with the presence of circulating cancer cells [33, 34].

Additionally, there is a role for vascular endothelial growth factor (VEGF) in endothelial retraction [26]; this last factor is induced by MIF [16]. In parallel, MIF induces the recruitment and migration of endothelial progenitor cells [35]; it has been proposed that the expressions of ICAM, VCAM, E-selectin and CC chemokine ligand (CCL)-2 in endothelial cells are MIF dependent [36]. A number of putative angiogenic factors, including platelet-derived growth factor (PDGF) and the same MIF, have been shown to upregulate VEGF expression in different types of cells (stromal cells, tumour cells, etc.) [37]. Moreover, it is known that PDGF and PDGF-receptor α and β are expressed in the BCCs that grow in the bones of nude mice. The interaction between PDGF and its receptors on the BCCs is coincident with decreased apoptosis, increased proliferation and higher microvessel density in the tumours. Interfering with PDGF-receptor signalling may be an approach to control the progressive growth of BCCs and thus to reduce bone lysis in a mouse model [38]. Another anti-apoptotic factor of BCCs is the stanniocalcin-1 (STC-1) [39].

On the other hand, Neville-Webbe et al. proposed that BM stromal cells may increase the survival of the BCCs protecting them from tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [40]. The role of TRAIL is of particular interest in BCC survival, as this molecule selectively induces apoptosis in BCCs, but not in normal cells [41]. TRAIL can bind to TRAIL-R1 (death receptor 4, DR4), TRAIL-R2 (DR5), TRAIL-R3 (decoy receptor, DcR1), TRAIL-R4 (DcR2) and OPG, a soluble factor that is also a receptor of RANKL. DR4 and DR5 induce apoptosis, whereas DcR1, DcR2 and OPG are unable to induce such signalling due to the lack of an intracellular death domain or due to their status as secreted molecules [42] that are expressed and/or released, for example, by BCCs, BM stroma and osteoclasts [43]. Recently, Lee et al. showed that infusing mice weekly with human MSC preactivated with TNF-α inhibited the progression of metastasis formed from MDA-MB231 cells. In co-culture, preactivated MSC induced apoptosis through the expression of TRAIL in MDA-MB231 cells and several other TRAIL-sensitive cancer cell lines [44]. These authors also found that cell-to-cell contact was required, since the human MSC had no effect in transwell co-cultures and an increase in soluble TRAIL was not detected in conditioned media (CM) from the cells, suggesting that the high amount of TRAIL expressed by MSC was transmembrane [44].

Finally, various studies have provided strong evidence that BM/bone active cytokines are potent EMT effectors that have been shown to be released from the BM-ECM and/or the extracellular bone matrix upon osteoclastic bone resorption and/or secreted by the BM stromal cells [45, 46]. An EMT is currently defined as a biological process that allows a polarised epithelial cell to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components [47, 48]. Recent findings have shown that RANKL, PDGF and MIF promote EMT phenotype in cancer cells [24, 49, 50].

Therefore, although the genetic and phenotypic makeup of a tumour determines its metastatic efficiency, a receptive BM microenvironment is a prerequisite for the arrival and localisation of BCCs in this 'soil'. For all these previously described observations, the main aim of this work was to study if the BM microenvironment of untreated advanced BCP (clinical–pathological stage III-B, without BM/bone metastasis) represents an optimal 'pre-metastatic niche' hospitable for BCC metastatic colonisation, establishment and growth. It was also important to study whether the peripheral blood (PB) plasma of these patients could favour one of the early stages of the metastatic cascade such as the intravasation process. For these reasons, we evaluated the effect of PB and BM plasma from these BCP and healthy volunteers (HV), as well as the BM–CM of colony-forming unit fibroblastic (1 CFU-F = 1 MSC) cultures over the transendothelial migration, invasion, migration, proliferation and apoptosis of 2 human BCC lines, MCF-7 and

MDA-MB231. Moreover, this study investigates the levels of OPG, RANKL, TRAIL, SDF-1, PDGF-AB, STC-1, MIF, ICAM-1 and VCAM-1 in the PB and BM plasma and BM–CM of CFU-F cultures from these BCPs without bone metastasis and HV. Finally, the expressions of RANK and CXCR-4 receptors were studied in BCCs of the primary breast tumours from these patients to know whether they could respond to the respective ligands present in PB plasma of these patients.

Materials and methods

Patients

This study included 15 patients with breast infiltrative ductal carcinoma and a clinical stage of III-B and 12 HV as controls. The International Union against Cancer TNM classification was used. All of samples studied in this work (PB, BM aspirate and tumour biopsy) were harvested from advanced BCPs before primary tumour surgery and adjuvant treatment (no hormonal-, radio- or chemotherapy), and these patients did not have BM/bone metastases or osteoporosis. The BCPs were menopausal with an age range between 50 and 65 years. Patients were free of metabolic bone disease, such as vitamin D deficiency, thyroid disease, parathyroid disease or kidney damage. The HV were donors for allogeneic BM transplantation with an age range of 40–67 years old and were matched for menopausal status with patients.

All individuals gave consent prior to participating in this study. The investigations were approved by the IBYME Ethical Committee and performed in accordance with the principles of the Helsinki Declaration.

Bone and BM metastasis

The absence of bone metastases was confirmed by X-ray and bone scintigraphy. The absence of BM infiltration with neoplastic cells was confirmed by an immunocytochemistry system (K0673, Dako) and cell morphology analysis was performed by the Pappenheim technique. BM aspirates were stained with monoclonal antibodies (mAbs) against epithelial membrane antigen (EMA, Dako) and cytokeratins AE1–AE3 (IR053, Dako). Patients' BMs were considered positive for metastasis only if cells expressed EMA and cytokeratins AE1–AE3 and if cells were morphologically malignant.

Collection of PB plasma

PB plasma from 15 BCP and 12 HV was obtained from 10 ml of PB collected over heparin without preservatives (25 units/ml, Gibco). The samples were centrifuged at $1{,}125\times g$ for 45 min at 4 °C. The plasma obtained was aliquoted and stored at −20 °C until use.

Collection of BM plasma and mononuclear cells

BM samples from 15 BCP and 12 HV were collected from the posterior iliac crest into heparinised saline. A portion of the aspirates was centrifuged at $1{,}125\times g$ for 45 min at 4 °C. The plasma obtained was aliquoted and stored at −20 °C until use. Another portion was half diluted with phosphate-buffered saline (PBS) and layered onto Ficoll-Hypaque density gradient medium (density = 1,075 gr/cm^3, Sigma). Mononuclear cells (MNC) were harvested from the interface and resuspended in α-MEM containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B and 2 mM L-glutamine (basal α-MEM) (Gibco). The cellular suspension was counted with 3 % acetic acid solution (Sigma), and cell viability was determined by 0.04 % trypan blue (Sigma) dye exclusion.

CFU-F assay

From 15 BCP and 12 HV, $2\text{x}10^6$ MNC were placed in 25 cm^2 tissue culture flasks (Corning Glass Works) containing 10 ml of basal α-MEM plus 20 % foetal bovine serum (FBS, 16,000-044, Gibco). Cells were incubated in a 37 °C, 5 % CO_2-humidified environment for 7 days. After this period, non-adherent cells were removed and the medium was renewed in the flask. The cultures were incubated for an additional 7 days. At the end, the CM was harvested (CM, day 14), centrifuged at $250\times g$ for 10 min and frozen at −20 °C until use.

The fibroblastic nature of the CFU-F cultures was demonstrated by immunocytochemistry staining using a mAb against the human β-subunit of prolyl-4-hydroxylase (M0877, Dako), CD45 (M0701, Dako) and CD34 (M7165, Dako). Negative controls were performed using isotype control mAb (IgG1, X0931, Dako). Each sample was examined in duplicate. In parallel, we studied the presence of tumour cells in these BCP cultures through immunocytochemistry staining as previously described.

Cell culture of BC lines

We used two human BCC lines: MCF-7 and MDA-MB231. They were obtained from the American Type Culture Collection, USA. The cells were routinely maintained in DMEM/F12 culture medium with phenol red (Gibco) containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B and 2 mM L-glutamine (basal DMEM/F12). In the case of MCF-7, cells were also cultured with 2 μg/ml humanised pig insulin (Beta Laboratory, Argentina). All the cultures were incubated with 10 %

FBS (NATOCOR). The cell density used was 4×10^4 viable cells/cm^2, and the medium was renewed every 3 days. Adherent cells were incubated at 37 °C in a 5 % CO_2-humidified environment until confluence. After this period, the cancer cells were washed with PBS and isolated with trypsin–EDTA solution. The number of viable cells was determined by 0.04 % trypan blue dye exclusion. Aliquots of each suspension cell line were frozen under nitrogen until the respective assay to maintain the lines. For the transendothelial migration, invasion, migration, proliferation and apoptotic assays, we used cells of MCF-7 and MDA-MB 231 lines until the fourth subculture.

Preparation of CM from BCCs

Next, 5×10^4 BCCs/cm^2 were incubated in 24-well plates (Axigen Scientific) or a glass Lab-Tek chamber slide (Nunc Co) in basal DMEM/F12 without phenol red with 10 % FBS at 37 °C and 5 % CO_2 and humidity (adherence). 24 h later, the cultures were washed in PBS and incubated for 48 h in basal DMEM/F12 without phenol red (arrest). Finally, the cells were washed in PBS and incubated over 24 h with basal DMEM/F12 with 5 % FBS at 37 °C, 5 % CO_2 and humidity. The CM were harvested, centrifuged at $250 \times g$ for 10 min and frozen at −20 °C until use.

Cell culture of endothelial cell line

We used human microvascular endothelial cells (HMEC-1) provided by Marcela F. Bolontrade PhD (Laboratory of Molecular and Cellular Therapy, Fundación Instituto Leloir, Buenos Aires, Argentina). The cells were routinely maintained in basal α-MEM plus 10 % FBS (16,000-044, Gibco). The cell density used was 4×10^4 viable cells/cm^2, and the medium was renewed every three days. Adherent cells were incubated at 37 °C in a 5 % CO_2-humidified environment until confluence. After this period, the cells were washed with PBS and isolated with trypsin–EDTA solution. The number of viable cells was determined by 0.04 % trypan blue dye exclusion. Aliquots of the cell suspension were frozen under nitrogen until the respective assay to maintain the line. For the transendothelial migration assay, we used cells of HMEC-1 line until the fourth subculture.

Evaluation of the OPG, RANKL, TRAIL, SDF-1, PDGF-AB, STC-1, MIF, ICAM-1 and VCAM-1 in PB and BM plasma and BM–CM of CFU-F by ELISA assay

The OPG, RANKL, SDF-1, PDGF-AB, STC-1, MIF, ICAM-1 and VCAM-1 levels were measured using an ELISA kit from R&D Systems (DY805), Antigenix America (RHF740CK), R&D Systems (DY350), R&D Systems (DHD00), R&D Systems (DY2958), R&D Systems (DY289), R&D Systems (BBEAB) and R&D Systems (DY809), respectively, according to the manufacturers. The TRAIL ELISA kit was developed in our laboratory: 96-well plates (Nunc) were coated with 8 μg/ml in PBS-capture Ab (mouse monoclonal anti-human TRAIL, MAB3751, R&D System). The standard curve of recombinant human (rh)TRAIL was prepared in reagent diluent with a concentration range between 31.25 and 2,000 pg/ml. The detection Ab was biotinylated goat anti-human TRAIL (BAF375, R&D System), which was used at 0.4 μg/ml. Detection was performed using streptavidin-peroxidase from *Streptomyces avidinii* (S5512, Sigma) and then adding a substrate solution [H_2O_2-tetramethylbenzidine (T8767, Sigma)]. The optical density (OD) of each well was determined using a microplate reader set at 450 nm. All samples and standards were analysed in triplicate.

Evaluation of the OPG and TRAIL in BCC-CM by ELISA assay

ELISA kits were used as previously described.

Transendothelial migration assay

The transendothelial migration assays were performed using a modified Boyden chamber, which was constructed from a 24-well plate in which cellulose membranes (Transwells, PI8P01250, Millipore) were placed. Briefly, HMEC-1 cells (2×10^5) were seeded in the upper chamber of the transwell unit and cultured in basal α-MEM plus 10 % FBS for 1 day prior to the assay. During this period, the endothelial cells had formed a monolayer. Then, 10 % of the PB plasma or BM plasma from 6 BCP and 6 HV in basal DMEM/F12 or 100 % of the BM–CM of CFU-F cultures from 6 BCP and 6 HV were placed in the lower chamber and 4×10^4 calcein-labelled MCF-7 or MDA-MB231 cells [previously marked with fluorogenic dye calcein acetoxymethyl ester (calcein AM) according to the manufacturer (Vybrant Cell Adhesion Assay Kit, V-13181, Molecular Probes)] were seeded into basal DMEM/F12 in the upper chamber of the transwell unit, after washing the endothelial monolayer with PBS. In addition, we analyse the BCC transendothelial migration to (i) basal DMEM/F12 and (ii) basal α-MEM with 20 % FBS, which was pre-incubated for 7 days at 37 °C, 5 % CO_2 and humidity (CFU-F culture media) (basal transendothelial migration controls). The systems were incubated for 24 h at 37 °C in a 5 % CO_2- humidified atmosphere. After that, the cells on the upper side of the membrane were removed and the cells attached to the lower side of the membrane were fixed in 2 % formaldehyde. Because only the tumour cells were labelled, these assays allow discrimination of tumour cells

from migrated endothelial cells. The transendothelial migrated BCCs were counted using fluorescent field microscopy and a 20× objective lens: The images captured in five representative visual fields were analysed using Image J software and the mean number of cells/field was calculated. Data were expressed as a transendothelial migration percentage increase of BCCs over the basal transendothelial migration control. Each sample was assayed in duplicate.

Immunohistochemical (IHC) assay

IHC studies were carry out on paraffin blocks from five tumour tissue biopsies of these BCPs and five non-malignant breast tissues of five women. The control 'non-malignant' tissues were breast biopsies from women who had negative results of BC (non-neoplastic breast tissue). Also, these women were menopausal and did not present compromise in their BM and bone metabolism. These tissues were fixed in 10 % neutral buffered formalin, and the tumour blocks were sectioned at 5 μm thickness. The sections were deparaffinised and hydrated to passages in xylene and 100, 96 and 70 % ethanol and then incubated in 20 μg/ml Proteinase K (Fermentas, EO0491) for 30 min. Endogenous peroxidase in the tissue section was blocked by incubating it for 5 min in 3 % hydrogen peroxide. After this, we proceeded to block proteins using 1 % bovine serum albumin in PBS for 1 h. Cytokeratin AE1–AE3, RANK and CXCR-4 mAbs (as previously described, MAB683, R&D Systems and MAB172, R&D Systems, respectively) and isotype controls [MAB002, R&D Systems (for AE1–AE3 and RANK mAbs) and 08-6599, ZYMED (for CXCR-4 mAb)] were incubated overnight at 4 °C. Then, LSAB+ System-HRP (K0690, Dako) and 3-3′-diaminobenzidine (Liquid DAB+ Substrate Chromogen System, K3468, Dako) were used according to the manufacturer. Haematoxylin was used for counterstaining, followed by mounting with Canada Balsam resin (Canadax, Biopur).

Immunostained slides were evaluated by light microscopy and the immunohistochemistry signal was scored based on the Allred score by comparison with cytokeratin AE1–AE3 expression [51, 52]. Briefly, a quantitative score was assigned representing the percentage of breast epithelial cells with positive stain [0–10 % (score 0); 10–30 % (score 1); 30–60 % (score 2); 60–90 % (score 3); or 90–100 % (score 4)]. Intensity of staining in positive cells was assigned an intensity score [absent (score 0); low (score 1); moderate (score 2); or intense (score 3)]. Quantitative score and intensity score were added to obtain a total score that ranged from 0 to 7. Each sample was assayed in quadruplicate.

Transwell migration and Matrigel invasion assays

The migration and invasion assays were performed using a modified Boyden chamber, which was conducted from a 24-well plate in which cellulose membranes (Transwells, PI8P01250, Millipore) were placed. To assess the BCC migration to 10 % of the BM plasma from 6 BCP and 6 HV in basal DMEM/F12 or to 100 % of the BM–CM of CFU-F from 6 BCP and 6 HV, 2×10^4 tumour cells were seeded into basal DMEM/F12 in the upper chamber compartment and the systems were incubated for 14 h at 37 °C in a 5 % CO_2-humidified atmosphere. In contrast, for the matrigel invasion assay, each cellulose membrane was previously incubated with 20 μg/cm^3 of ECM gel (E1270, Sigma) according to the manufacturer and 6×10^4 BCCs were seeded into basal DMEM/F12 in the upper chamber compartment and incubated during 24 h. In both cases, the basal migration or invasion controls used were as follows: (i) basal DMEM/F12 and (ii) basal α-MEM with 20 % FBS, which was pre-incubated for 7 days at 37 °C, 5 % CO_2 and humidity. When the cell migration or invasion had finished, the BCCs were stained for over 10 min using 0.05 % crystal violet prepared with methanol. The non-migrated cells on the top side of the surface were removed. The cells on the bottom side of the membrane were counted in 10 standardised fields at 100× using an optic microscope. Data were expressed as a migration or invasion percentage increase of BCC over the basal migration or invasion control. Each sample was assayed in duplicate.

Proliferation assay

Next, 5×10^3 BCCs/well were incubated in adherence and post-arrest culture conditions in 96-well plates (Orange Scientific). After this period, the cells were incubated for 48 h with the following samples: (i) 10 % of the BM plasma from 8 BCP and 8 HV plus 90 % basal DMEM/F12, (ii) 10 % of the BM plasma from 8 BCP and 8 HV plus 88.75 % basal DMEM/F12 plus 1.25 % FBS, (iii) 100 % of the BM–CM of CFU-F from 12 BCP and 10 HV, (iv) 50 % of the BM–CM of CFU-F from 12 BCP and 10 HV plus 50 % basal DMEM/F12, and (v) 50 % of the BM–CM of CFU-F from 12 BCP and 10 HV plus 50 % basal DMEM/F12 containing 2.5 % FBS; controls: (i) 100 % basal DMEM/F12 (culture media for BCCs), (ii) 100 % basal DMEM/F12 with 1.25 % FBS, (iii) 100 % basal α-MEM with 20 % FBS, which was pre-incubated 7 days at 37 °C, 5 % CO_2 and humidity (culture media for CFU-F assay), (iv) 50 % basal α-MEM with 20 % FBS pre-incubated plus 50 % basal DMEM/F12, and (vi) 50 % basal α-MEM with 20 % FBS pre-incubated plus 50 % basal DMEM/F12 containing 2.5 % FBS. The cell proliferation was

evaluated by the Cell Titer 96 AQueous One Solution Cell Proliferation Assay commercial kit (G3580, Promega) according to the manufacturer. The OD was performed at 490 nm using a microplate reader. The value of each sample under study was obtained subtracting the control OD from its respective sample value, and the values obtained were compared with the positive controls. Furthermore, the differences between BCP and HV were evaluated. All experiments on the samples were carried out in triplicate.

Apoptosis assay

Additionally, 5×10^4 BCCs/cm^2 were incubated in adherence and post-arrest culture conditions in 24-well plates (Annexin V assay) or glass Lab-Tek slides [TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay]. After this period, the cells were incubated for 24 h with the following: (i) 50 % basal α-MEM with 20 % FBS pre-incubated 7 days at 37 °C, 5 % CO_2 and humidity plus 50 % basal DMEM/F12 containing 10 % FBS, with and without 50 ng/ml rhTRAIL (375-TL, R&D Systems) and (ii) 50 % of the BM–CM of CFU-F from 7 BCP and 7 HV plus 50 % basal DMEM/F12 containing 10 % FBS, with and without 50 ng/ml rhTRAIL. The percentage of cells under early, late and total apoptosis in each sample was obtained subtracting the control cell percentage from its respective sample value and the results were compared with the positive control values (rhTRAIL effect). Furthermore, the differences between BCP and HV were evaluated. Each sample was assayed in duplicate.

Annexin V-FICT/propidium iodide apoptotic assay

The apoptotic cells were evaluated with an Annexin V-FITC Apoptosis Detection commercial kit I (556547, BD Pharmingen) according to the manufacturer. Each sample was analysed by flow cytometry at 488 nm for Annexin-FITC and at 633 nm for propidium iodide (PI) within 1 h.

TUNEL assay

The apoptotic cells were evaluated using a DeadEnd™ Fluorometric TUNEL System commercial kit (G3250, Promega) according to the manufacturer. We used 1 μg/ml PI for 15 min at room temperature in the dark as a nuclear stain. Immediately, the samples were analysed under a confocal microscope to view the green and red fluorescence at 520 ± 20 nm and >620 nm, respectively.

Statistics

Statistical analysis was performed using parametric and non-parametric tests as needed (Graph Pad Prism 4 Software). Differences were considered statistically significant when $p < 0.05$.

Results

BM metastasis

Routine diagnostic tests for metastasis study were negative for all of BCPs. Non-evidence of BM infiltration with neoplastic cells was observed in all of the BM aspirates from BCP. Moreover, neoplastic cells were not found in any of CFU-F cultures which indicated that all the soluble factors were released by mesenchymal stromal cells in the CM of these cultures (data not shown).

Morphology and phenotypical characterisation of cells from CFU-F assay

The majority of stromal cells from CFU-F cultures of BCP ($n = 15$) and HV ($n = 12$) consisted of spindle shapes typical of fibroblastic morphology, which showed positive staining for ß-subunit of prolyl-4-hidroxylase and negative for CD45 and CD34, confirming their fibroblastic nature (data not shown).

Evaluation of the OPG, RANKL, TRAIL, SDF-1, PDGF-AB, STC-1, MIF, ICAM-1 and VCAM-1 in PB and BM plasma and BM–CM of CFU-F by ELISA assay

Considering that there is a wider network of communication through cytokines and growth factors to allow the intravasation process, survival in blood vessels and colonisation of BM, establishment and growth of tumour cells from advanced BCP, we investigated how the levels of the same cytokines present in PB plasma, BM plasma and BM–CM of CFU-F cultures change in untreated advanced BCP without bone metastatic development compared with HV.

The data showed that OPG, RANKL and MIF levels in BCP-PB plasma were significantly higher than HV values (Table 1). However, SDF-1 levels in BCP-PB plasma were significantly lower than HV values (Table 1). No significant difference in PB plasma VCAM-1 levels between both groups was found, but the BCP group had a tendency to have higher values (Table 1). Moreover, PDGF-AB, ICAM-1 and VCAM-1 levels in BCP–BM plasma were significantly higher than HV values (Table 1). No

Table 1 Levels of OPG, RANKL, TRAIL, SDF-1, PDGF-AB, STC-1, MIF, ICAM-1 and VCAM-1 in peripheral blood (PB) and bone marrow (BM) plasma, and BM-conditioned media (CM) of colony-forming unit fibroblastic (CFU-F) by ELISA assay

Levels (pg/ml)	PB plasma		BM plasma		CFU-F-CM	
	BCP vs HV	*p*	BCP vs HV	*p*	BCP vs HV	*p*
OPG	2,005.00 ± 195.90 ($n = 10$) vs 1,100.00 ± 124.10 ($n = 10$)	$p = 0.001$*	2,112.00 ± 341.40 ($n = 11$) vs 1,328.00 ± 203.60 ($n = 8$)	NS	888.20 ± 270.60 ($n = 15$) vs 1,422.00 ± 264.00 ($n = 12$)	$p = 0.0482$**
RANKL	130.20 ± 23.63 ($n = 12$) vs 31.29 ± 0.03 ($n = 12$)	$p < 0.0001$**	51.42 ± 7.68 ($n = 12$) vs 56.72 ± 8.70 ($n = 8$)	NS	<LLD	NA
TRAIL	<LLD	NA	<LLD	NA	<LLD	NA
SDF-1	117.00 ± 25.00 ($n = 13$) vs 254.00 ± 28.00 ($n = 12$)	$p < 0.05$*	905.00 ± 172.00 ($n = 9$) vs 846.00 ± 170.00 ($n = 10$)	NS	91.15 ± 18.28 ($n = 14$) vs 263.40 ± 70.69 ($n = 12$)	$p = 0.0334$*
PDGF-AB	<LLD	NA	4,468.00 ± 746.00 ($n = 8$) vs 2,528.00 ± 421.00 ($n = 6$)	$p < 0.05$*	<LLD	NA
STC-1	1,036.00 ± 172.60 ($n = 9$) vs 1,466.00 ± 222.80 ($n = 7$)	NS	946.30 ± 286.90 ($n = 5$) vs 1,368.00 ± 395.80 ($n = 4$)	NS	<LLD	NA
MIF	4,564.00 ± 591.00 ($n = 7$) vs 2,265.00 ± 402.00 ($n = 9$)	$p < 0.05$*	4,170.00 ± 440.00 ($n = 5$) vs 3,520.00 ± 320.00 ($n = 5$)	NS	4,550.00 ± 380.30 ($n = 10$) vs 4,299.00 ± 660.30 ($n = 10$)	NS
ICAM-1	–	–	251,818.00 ± 27,405.00 ($n = 7$) vs 165,409.00 ± 18,180.00 ($n = 7$)	$p = 0.0161$*	<LLD	NA
VCAM-1	107.10 ± 21.55 ($n = 4$) vs 57.98 ± 33.54 ($n = 4$)	NS	133.80 ± 1.93 ($n = 4$) vs 103.30 ± 9.03 ($n = 5$)	$p = 0.0317$**	<LLD	NA

Values expressed as mean±SE. Statistical analysis between breast cancer patients (BCP) and healthy volunteers (HV): *Unpaired *t*-test and **Mann–Whitney *U*-test. LLD: lower limit of detection of ELISA

NA not applicable, *NS* not significant, (−) not measured

significant difference was found in the BM plasma OPG and MIF levels between both groups, but the BCP group had a tendency to have higher values (Table 1). Furthermore, OPG and SDF-1 levels in CFU-F-CM from BCP were significantly lower than in HV (Table 1). Finally, all other soluble factor values were less than the ELISA detection limit, or the data showed similar levels between BCP and HV, independent of the type of samples (Table 1).

Evaluation of the OPG and TRAIL in BCC-CM by ELISA assay

In reference to factors measured in CM of MCF-7 and MDA-MB231 cells, OPG and TRAIL values were less than 31.25 pg/ml in these cultures.

Transendothelial migration of BCCs

To investigate the process of BCC intravasation to blood vasculature and extravasation to BM in untreated advanced BCP compared with HV, we evaluated the in vitro capacity of PB plasma, as well as BM plasma and CFU-F-CM from BCP and HV to induce transendothelial migration of BCCs.

The results showed that PB plasma from BCP induced a higher transendothelial migration increase of MCF-7 and MDA-MB231 cells compared with HV plasma; however, this observation was significant only when we used MCF-7 cells (Table 2; Fig. 1a, b). Moreover, BM plasma from BCP induced a significantly higher transendothelial migration increase of MCF-7 and MDA-MB231 cells compared with HV plasma (Table 2; Fig. 2a, b). Furthermore, data showed that BM–CM of CFU-F from BCP and HV had transendothelial migratory effects over both types of BCCs, but no significant difference was found between the groups (Table 2).

Additionally, we found that PB plasma from BCP induced a significantly higher transendothelial migration of MCF-7 cells than MDA-MB231 cells (data are expressed as mean of number of counted cells/field ± SE; Unpaired *t*-test): 57.47 ± 4.47 and 26.01 ± 5.70, respectively, $p = 0.0025$. In contrast, BM plasma from BCP induced a significantly higher transendothelial migration of MDA-MB231 cells than MCF-7 cells (data are expressed as mean of number of counted cells/field ± SE; Unpaired *t*-test): 27.33 ± 3.76 and 11.27 ± 3.66, respectively, $p = 0.0120$. However, the in vitro transendothelial migration capacity of BCCs to BM–CM of CFU-F from BCP did not show a significant difference between both types of lines (data not shown).

RANK and CXCR-4 expression in BC tissues

To evaluate the immunoexpression of key tumour cell receptors involved in the intravasation process of BCCs,

Table 2 Effects of peripheral blood (PB) and bone marrow (BM) plasma as well as BM-conditioned media (CM) of colony-forming unit fibroblastic (CFU-F) from breast cancer patients (BCP) and healthy volunteers (HV) over transendothelial migration, transwell migration, matrigel invasion and proliferation of MCF-7 and MDA-MB231 cells

Effect	PB plasma (BCP vs. HV)		BM plasma (BCP vs. HV)		CFU-F-CM (BCP vs. HV)	
	MCF-7	MDA-MB231	MCF-7	MDA-MB231	MCF-7	MDA-MB231
Trans-endothelial migration	1,513.00 ± 116.10 vs 933.30 ± 138.60 ($p = 0.0123$*)	464.40 ± 97.23 vs 352.40 ± 8.45	190.80 ± 50.68 vs 32.92 ± 14.52 ($p = 0.0303$**)	1,952.00 ± 261.90 vs 581.00 ± 96.80 ($p = 0.0027$**)	358.50 ± 112.70 vs 716.00 ± 147.80	207.80 ± 68.83 vs 259.40 ± 56.42
Transwell migration	NA	NA	167.10 ± 79.09 vs 360.50 ± 107.60	157.40 ± 82.13 vs 302.70 ± 149.30	585.00 ± 209.10 vs 41.05 ± 12.27 ($p = 0.0484$**)	374.00 ± 83.54 vs 127.10 ± 53.23 ($p = 0.0319$*)
Matrigel invasion	NA	NA	343.40 ± 100.20 vs 445.60 ± 75.21	21.00 ± 9.79 vs 8.95 ± 4.00	4,512.00 ± 412.80 vs 3,917.00 ± 768.80	8,027.00 ± 678.20 vs 9,032.00 ± 374.50
Proliferation	NA	NA	1.21 ± 0.04 vs 1.10 ± 0.07	1.56 ± 0.05 vs 1.33 ± 0.08 ($p = 0.0434$*)	NE	NE

Transendothelial migration values are expressed as mean of transendothelial migration percentage increase of breast cancer cells (BCCs)±SE. Transwell migration values are expressed as mean of migration percentage increase of BCCs±SE. Matrigel invasion values are expressed as mean of invasion percentage increase of BCCs±SE. Proliferation assay value of each sample under study was obtained subtracting the control optical density from its respective sample value, and the values are expressed as the mean±SE. Statistical analysis between BCP and HV: *Unpaired *t*-test and **Unpaired *t*-test with Welch's correction. All these experiments were carried out with six samples from each group (PB and BM plasma as well as BM–CM of CFU-F). In the case of proliferation assay, we used 8 BM plasma from each group as well as 12 and 10 BM–CM of CFU-F from BCP and HV, respectively

NA not applicable, *NE* non effect

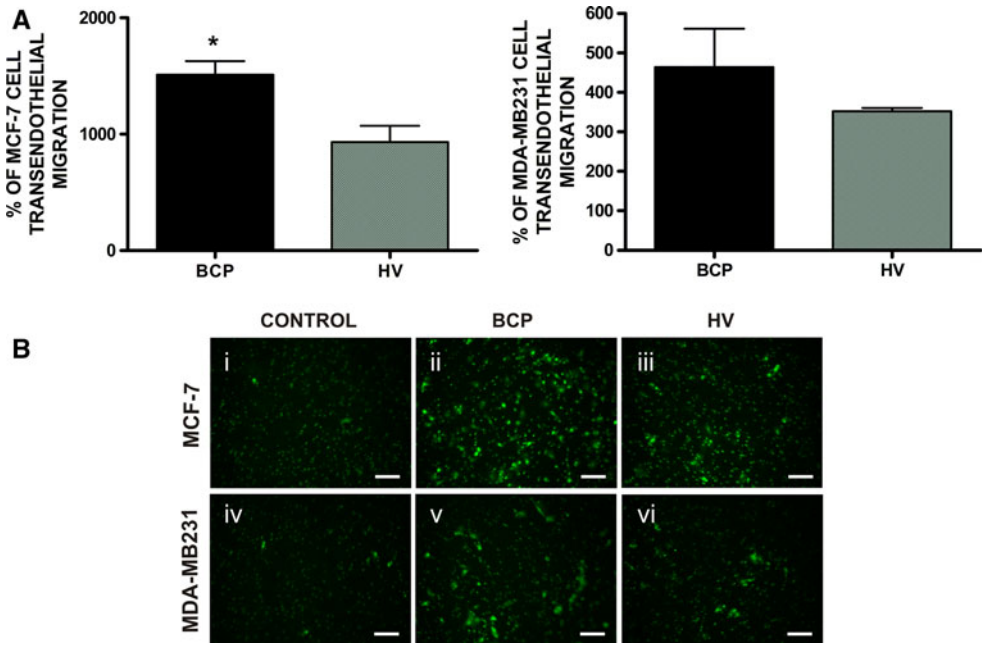

Fig. 1 **a** Capacity of peripheral blood (PB) plasma from breast cancer patients (BCP) and healthy volunteers (HV) to induce transendothelial migration of MCF-7 and MDA-MB231 cells. The data are expressed as transendothelial migration percentage increases of BC cells over the basal transendothelial migration control. The graphics show values as the mean ± SE. Statistical analysis: *asterisk* (*) indicates a significant difference $p = 0.0123$ (Unpaired *t*-test) between BCP ($n = 6$) and HV ($n = 6$) in the MCF-7 cells. **b** This figure is a representative example of the in vitro capacity of PB plasma from a BCP and a HV to induce transendothelial migration of MCF-7 and MDA-MB231 cells. Calcein-labelled MCF-7 or MDA-MB231 cells were seeded in basal DMEM/F12 on a monolayer of endothelial cells of HMEC-1 line in the upper chamber of the transwell unit to basal DMEM/F12 (basal transendothelial migration control) (*i* and *iv*, respectively) or 10 % of the PB plasma from BCP and HV in basal DMEM/F12 *(ii–iii* and *v–vi,* respectively) during 24 h. Migrated BC cells were counted using fluorescent field microscopy and a 20× objective lens: the images captured in five representative visual fields were analysed using Image J software and the mean number of cells/field was calculated. The *scale bar* represents 100 μm

we performed the IHC assays using patients' primary tumour tissues and non-malignant breast tissues. In this manner, the RANK and CXCR-4 expression (total score) in the primary tissue BCCs was higher than in the epithelial cells of non-malignant breast tissues [median, (interquartile range)]: 6 (5–7) vs. 5 (4.5–5) and 5.5 (3.5–7) vs. 2 (0–3), respectively; however, this observation was significant only when we analysed the expression of CXCR-4 between both groups ($p = 0.0317$, Mann–Whitney *U*-test) (Fig. 3a, b).

Transwell migration and Matrigel invasion of BCCs

Next, we examined whether BM microenvironment from untreated advanced BCP could control the recruitment of newly arriving cancer cells, modifying the migration and invasion of BCCs compared with HV. For this purpose, we tested in vitro BCC migratory and invasive capacity to BM plasma and BM–CM of CFU-F cultures from BCP and HV.

Data showed that CFU-F-CM from BCP induced a significantly higher transwell migration increase of MCF-7 and MDA-MB231 cells compared with HV–CM (Table 2; Fig. 4a, b). In contrast, BM plasma from BCP and HV had transwell migratory effect over both BCCs, but no significant difference was found between the groups (Table 2). In addition, in vitro migration capacity of BCCs (measured as mean of number of counted cells/field) to BM plasma and BM–CM of CFU-F from BCP did not show significant difference between both types of lines (data not shown).

In reference to matrigel invasion, BM plasma and BM–CM of CFU-F cultures from BCP and HV induce the invasion of both BCCs, but no significant difference was found between the groups (Table 2). Additionally, we found that BM plasma from BCP induced a significantly higher invasion of MCF-7 cells than MDA-MB231 cells (data are expressed as mean of number of counted cells/field ± SE; Unpaired *t*-test with Welch's correction): 151.90 ± 50.44 and 17.43 ± 11.76, respectively, $p = 0.0484$. In contrast, in vitro invasion capacity of BCCs to BM–CM of CFU-F from BCP did not show significant difference between both types of lines (data not shown).

Proliferation of BCCs

To evaluate whether the BM microenvironment induces a favourable soil for the BCCs when they arrive at the BM of

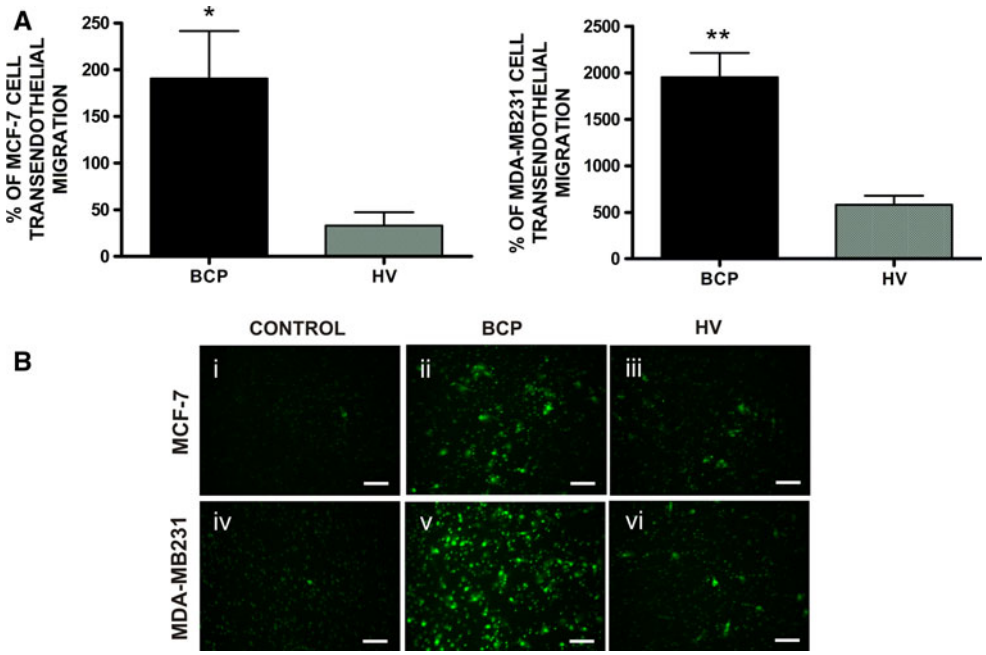

Fig. 2 **a** Capacity of bone marrow (BM) plasma from breast cancer patients (BCP) and healthy volunteers (HV) to induce transendothelial migration of MCF-7 and MDA-MB231 cells. The data are expressed as transendothelial migration percentage increases of BC cells over the basal transendothelial migration control. The graphics show values as the mean ± SE. Statistical analysis: *asterisks* (* and **) indicate a significant difference $p = 0.0303$ (*) and $p = 0.0027$ (**) (Unpaired *t*-test with Welch's correction) between BCP ($n = 6$) and HV ($n = 6$) in the MCF-7 and MDA-MB231 cell lines, respectively. **b** This figure is a representative example of the in vitro capacity of BM plasma from a BCP and a HV to induce transendothelial migration of MCF-7 and MDA-MB231 cells. Calcein-labelled MCF-7 or MDA-MB231 cells were seeded in basal DMEM/F12 on a monolayer of endothelial cells of HMEC-1 line in the upper chamber of the transwell unit to basal DMEM/F12 (basal transendothelial migration control) (*i* and *iv*, respectively) or 10 % of the BM plasma from BCP and HV in basal DMEM/F12 *(ii–iii* and *v–vi,* respectively) during 24 h. Migrated BC cells were counted using fluorescent field microscopy and a 20× objective lens: the images captured in five representative visual fields were analysed using Image J software and the mean number of cells/field was calculated. The *scale bar* represents 100 μm

untreated advanced BCP, we investigated the proliferative capacity of BM plasma and BM–CM of CFU-F cultures from BCP over MCF-7 and MDA-MB231 cells compared with HV.

We found that 10 % of BCP–BM plasma had a greater capacity to increase the proliferation of both types of BCCs compared with the effect of HV plasma; however, this observation was significant only when we used MDA-MB231 cells (Table 2; Fig. 5). In the case we added 1.25 % FBS to these cultures, no proliferative effect over BCCs was observed.

In contrast, there was no stimulant effect by CFU-F-CM from BCP and HV over the proliferation of both BCCs independent of their use at either 50 or 100 %, with or without 1.25 % FBS. Also, it is important to say that the control culture medium for CFU-F assay (basal α-MEM with 20 % FBS), after its incubation in a 37 °C, 5 % CO_2-humidified environment for 7 days, had non-detectable levels of the some of the classical cell growth and inhibitor factors like the following: Dkk-1, SDF-1, IL-1β, IL-6, M-CSF, TGF-β1, PDGF-AB, GM-CSF, basic FGF, soluble receptors of TNF-α, EGF, IL-17, RANKL, MIF, ICAM-1, VCAM-1, STC-1, TRAIL, PGE2, IL-4, TNF-α, INF-α and γ, IL-2 and IL-10 (by ELISA methodology, data not shown). So, this last comment shows that after 7 days of incubation, the effect of the soluble factors present in 20 % of serum is at a minimum, and all of the factors present in the studied CM were from the stromal cells of the CFU-F.

Apoptosis of BCCs

It is known that BM stromal cells may increase the survival of the BCCs protecting them from TRAIL-induced apoptosis. Thus, we next decided to evaluate whether BM–CM of CFU-F cultures from untreated advanced BCP (without bone metastasis) protect the MCF-7 and MDA-MB231 apoptosis induced by rhTRAIL compared to HV–CM.

Annexin V-FICT/PI apoptotic assay

Non-apoptotic effect by CM of CFU-F cultures from both groups was observed over both BCCs. However, when MDA-MB231 cells were treated with CFU-F-CM from BCP and HV in the presence of rhTRAIL (50 ng/ml), the

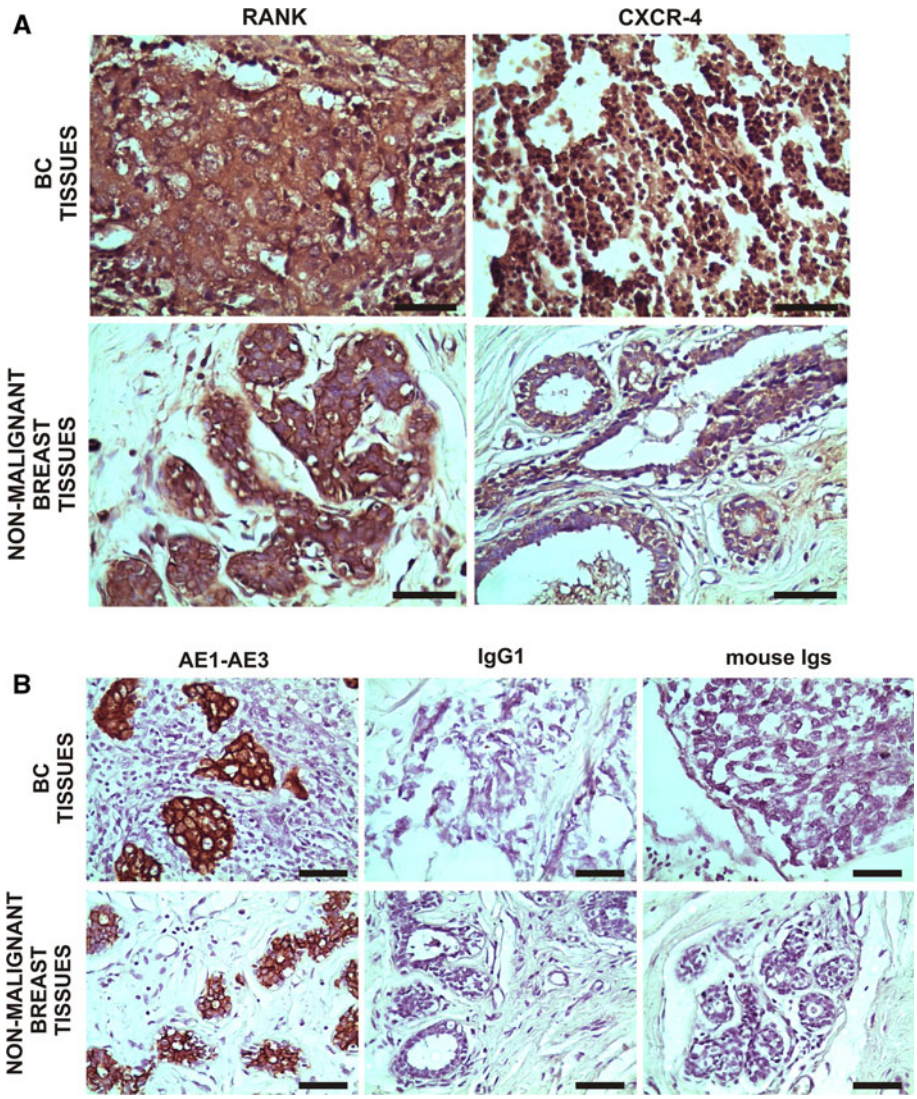

Fig. 3 **a** Expression of RANK and CXCR-4 in primary tissue of tumour biopsies from a breast cancer (BC) patient and non-malignant breast tissue from a woman by immunohistochemistry assay. **b** Positive controls were performed in both types of tissues incubating with Cytokeratin AE1–AE3 monoclonal antibody (mAb) (a pan-cytokeratin epithelial marker). No staining was observed in both types of tissues when we incubated them with irrelevant IgG1 (for AE1–AE3 and RANK mAbs) and mouse immunoglobulins (Igs, for CXCR-4 mAb) as negative isotype controls. Nuclei were counterstained with haematoxylin (*purple*). Immunohistochemistry studies were performed on paraffin blocks fixed in 10 % neutral buffered formalin and sectioned at 5 μm. Data are expressed based on Allred score by comparison with Cytokeratins AE1–AE3. The *scale bar* represents 50 μm. (Color figure online)

values of the percentage of cells under early and late apoptosis were significantly decreased compared with the values induced by rhTRAIL alone (Table 3). Furthermore, CFU-F-CM from BCP had a lower capacity to reduce the rhTRAIL apoptotic effect compared with CFU-F-CM from HV; this observation was related to the significantly lower reduction of the early apoptosis when we used BCP–CM compared with HV–CM [$p < 0.05$, One-way analysis of variance (ANOVA) followed by a Bonferroni's Multiple Comparison test] (Fig. 6 and Supplementary Figure). No change in the percentage of viable MCF-7 cells was observed when we used CFU-F-CM from BCP and HV with or without rhTRAIL (data not shown).

TUNEL apoptotic assay

The data did not show apoptosis by CM of CFU-F cultures from BCP and HV over both types of BCCs. In the presence of rhTRAIL, CFU-F-CM from BCP and HV reduced the total apoptosis of MDA-MB231 cells compared with the cultures treated with rhTRAIL alone, with similar values in both groups (Fig. 7a, b). In relation to the MCF-7 line, no change in the percentage of viable MCF-7 cells was observed when we used CFU-F-CM from BCP and HV with or without rhTRAIL (data not shown).

Discussion

BC frequently metastasises to the skeleton. BM and bone provide a unique microenvironment for tumour growth, including niches occupied by HSC and MSC. Recent data suggest that circulating tumour cells usurp these niches and compete with the normal stem cell occupants [53]. Bone colonisation is supposed to be preceded by changes in the target tissue (BM/bone) to create a permissive

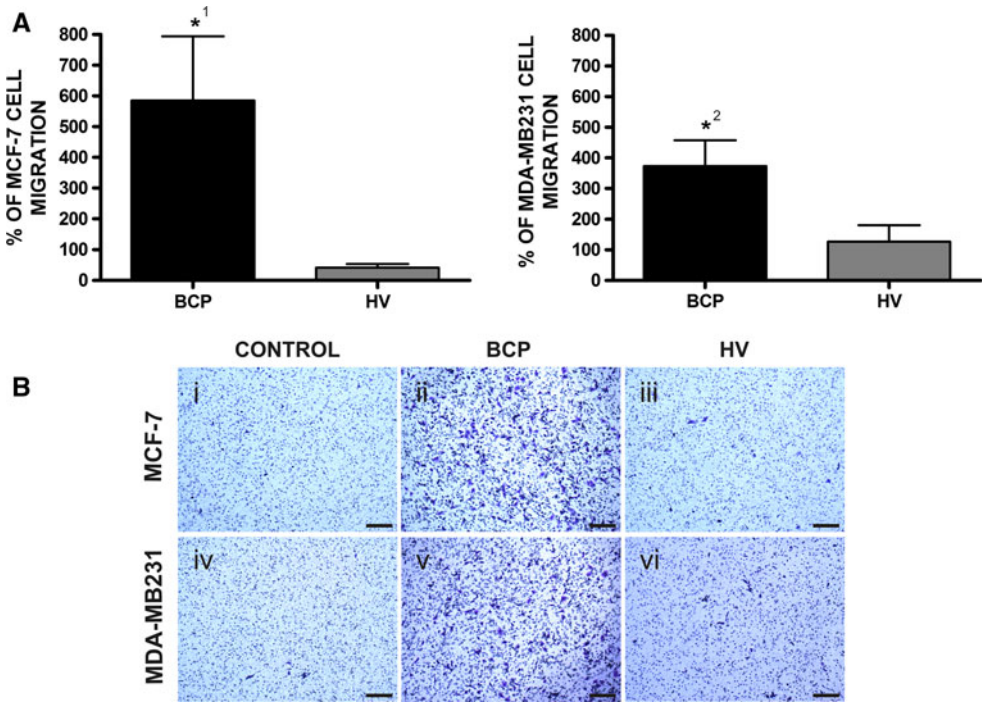

Fig. 4 **a** Transwell migration effect of bone marrow (BM)-conditioned media (CM) of colony-forming unit fibroblastic (CFU-F) from breast cancer patients (BCP) and healthy volunteers (HV) over MCF-7 and MDA-MB231 cells. The data are expressed as migration percentage increases of BC cells (BCCs) over the basal migration control. The graphics show values as the mean ± SE. Statistical analysis: *asterisks* ($*^1$ and $*^2$) indicate a significant difference $p = 0.0484$ ($*^1$, unpaired *t*-test with Welch's correction) and $p = 0.0319$ ($*^2$, unpaired *t*-test) between BCP ($n = 6$) and HV ($n = 6$) in the MCF-7 and MDA-MB231 cell lines, respectively. **b** This figure is a representative example of the migration effect of BM–CM of CFU-F from a BCP and a HV over MCF-7 and MDA-MB231 cells. MCF-7 and MDA-MB231 cells were seeded in basal DMEM/F12 into the upper compartment to basal α-MEM with 20 % FBS, which was pre-incubated for 7 days at 37 °C with 5 % CO_2 and humidity (basal migration controls) (*i* and *iv*, respectively) and 100 % of the BM–CM of CFU-F from BCP and HV *(ii–iii* and *v–vi, respectively*). After 14 h, the cell migration was finished, and the BCCs were stained with 0.05 % crystal violet prepared with methanol. The migrated cells on the bottom side of the membrane were counted in 10 standardised fields at 100× using an optic microscope. The *scale bar* represents 200 μm

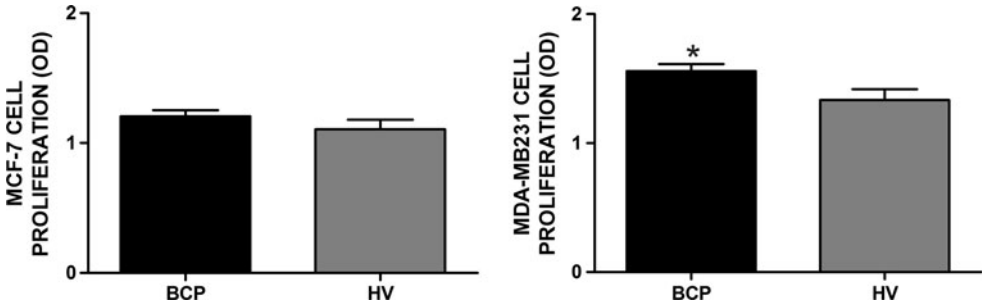

Fig. 5 Proliferative effect of bone marrow (BM) plasma from breast cancer patients (BCP) and healthy volunteers (HV) over MCF-7 and MDA-MB231 cells. In this case, after establishing the adherence and post-arrest culture conditions, the BC cells were incubated for 48 h with 10 % of the BCP and HV-BM plasma in the basal DMEM/F12 (without FBS). The value of each sample under study was obtained subtracting the control optical density (basal DMEM/F12) from its respective sample value, and the values are expressed as the mean ± SE. Statistical analysis: unpaired *t*-test. *Asterisks* indicate a significant difference ($p = 0.0434$) between BCP ($n = 8$) and HV ($n = 8$) in the MDA-MB231 cell line

microenvironment ('soil'), the pre-metastatic niche, for the establishment of the metastatic foci. Yet, how the pre-metastatic niche arises in the BM/bone tissues remains unclear.

First of all, our results showed that neoplastic cells were not found in any of the BCP–BM aspirates or CFU-F cultures, indicating that we isolated BM samples from BCP without BM metastasis. Additionally, the phenotypical

Table 3 Effect of colony-forming unit fibroblastic (CFU-F)-conditioned media (CM) from breast cancer patients (BCP) and healthy volunteers (HV) over the apoptosis of MDA-MB231 cells induced with TRAIL

Treatments	% of MDA-MB231 cells	
	Early apoptosis	Late apoptosis
rhTRAIL	15.73 ± 2.17 (*a, b*)	28.24 ± 4.39 (*c, d*)
BCP–CM + rhTRAIL	6.51 ± 0.66 (*a*)	8.77 ± 2.47 (*c*)
HV–CM + rhTRAIL	0.59 ± 0.87 (*b*)	5.54 ± 3.81 (*d*)

Study of the effect of CFU-F-CM from 7 BCP and 7 HV over the % of MDA-MB231 cells under early and late apoptosis induced with 50 ng/ml of recombinant human (rh)TRAIL by the Annexin V-FICT/propidium iodide apoptotic assay. Values expressed as mean ± SE. Statistical analysis: (*a*) and (*b*) $p < 0.001$; (*c*) and (*d*) $p < 0.01$. One-way analysis of variance (ANOVA) followed by a Bonferroni's Multiple Comparison test

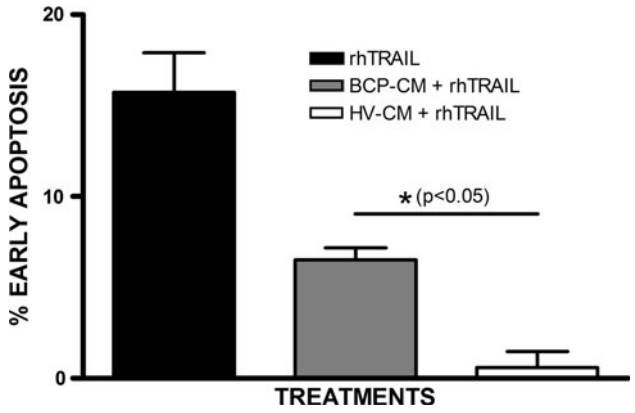

Fig. 6 Antagonistic effect of colony-forming unit fibroblastic (CFU-F)-conditioned media (CM) from breast cancer patients (BCP) and healthy volunteers (HV) over the % of MDA-MB231 cells under early apoptosis induced with 50 ng/ml of recombinant human (rh)TRAIL by the Annexin V-FICT/propidium iodide apoptotic assay. The percentage of cells under early apoptosis in each sample was obtained subtracting the control cell percentage (without rhTRAIL) from its respective sample value, and the values are expressed as the mean ± SE. Statistical analysis: One-way analysis of variance (ANOVA) followed by a Bonferroni's Multiple Comparison test. *Asterisks* indicate a significant difference ($p < 0.05$) between 7 BCP–CM + rhTRAIL and 7 HV–CM + rhTRAIL

characterisation of CFU-F indicated that these cells had specific mesenchymal markers [54]. Remember that each CFU-F was derived from a MSC, and these stromal stem cells play a fundamental role in the pathogenesis of bone metastasis and they have been shown to facilitate the entry of BCCs in the BM [55]. Also, micrometastatic foci within the BM might give rise to distant metastases, and MSC might facilitate this phenomenon.

The metastatic process itself is believed to be highly inefficient, since only a small fraction of BCCs entering the circulation will successfully generate secondary tumours. This low metastatic efficiency is considered to be the result of tumour cell death in the blood circulation [56]. Our results indicated an increase in the capacity of BCP-PB plasma to induce the transendothelial migration of BCCs from both types of BCC lines compared with HV. However, this increase was only significant when using the MCF-7 cells. In this regard, taking into account what has been said in the introduction, it is possible that the high levels of RANKL and MIF found in PB plasma from our BCP could play a role in the intravasation of BCCs into the blood vasculature after the binding of each ligand to the specific receptors (RANK and CXCR-4 or CD74, respectively) present in the cancer cells of the primary tumours of these patients. In agreement with this last result, in previous work, we found that hormone-dependent and -independent human metastatic breast carcinoma epithelial cell lines (MCF-7 and MDA-MB231) expressed RANK and CXCR-4 [17]. On the other hand, if we compare the effect of BCP-PB plasma on transendothelial migration of both BCC lines studied, the results showed that a greater number of MCF-7 cells responded. Finally, RANKL and MIF can induce, in a direct or indirect way, BCC-EMT phenotype, and as described this 'transition state EM' further promotes the process of invasion and intravasation of BCCs and their survival [22–24, 49].

Another factor significantly elevated in the BCP-PB plasma was OPG, and this factor and the MIF are not only pro-angiogenic but also favour the survival of circulating BCCs [20, 21]. Furthermore, as we mentioned in the introduction, the high VCAM-1 levels measured in BCP-PB plasma may indicate the presence of circulating cancer cells in our studied patients [33, 34]. In contrast, low levels of SDF-1 were found in BCP-PB plasma as compared to HV values. Significant decrease that suggests that this factor would not be the most important for inducing the intravasation and BCC survival in this clinical stage of tumour development (III-B).

On the other hand, the fact that the overexpression of MIF causes high-turnover osteoporosis in a mouse model provides evidence of the role played by MIF in bone remodelling and balance [22]. Furthermore, IL-1, IL-6 and TNF-α that are induced by MIF favour the bone resorption process, and IL-1 and TNF-α activate microvascular endothelial cells that could promote the transendothelial migration of circulating preosteoclasts capable of differentiating into bone-resorbing osteoclast [57]. Moreover, both RANKL and the same MIF stimulate the in vitro migration of preosteoclasts across bone endothelial cells [11]. Therefore, high RANKL and MIF levels in PB plasma of our BCP may induce the differentiation of PB monocytes and/or tumour macrophages, promoting preosteoclast formation in PB or primary breast tumours. These events could induce, in a near future, an increase in BM/bone mature osteoclasts and the bone resorption process, finally

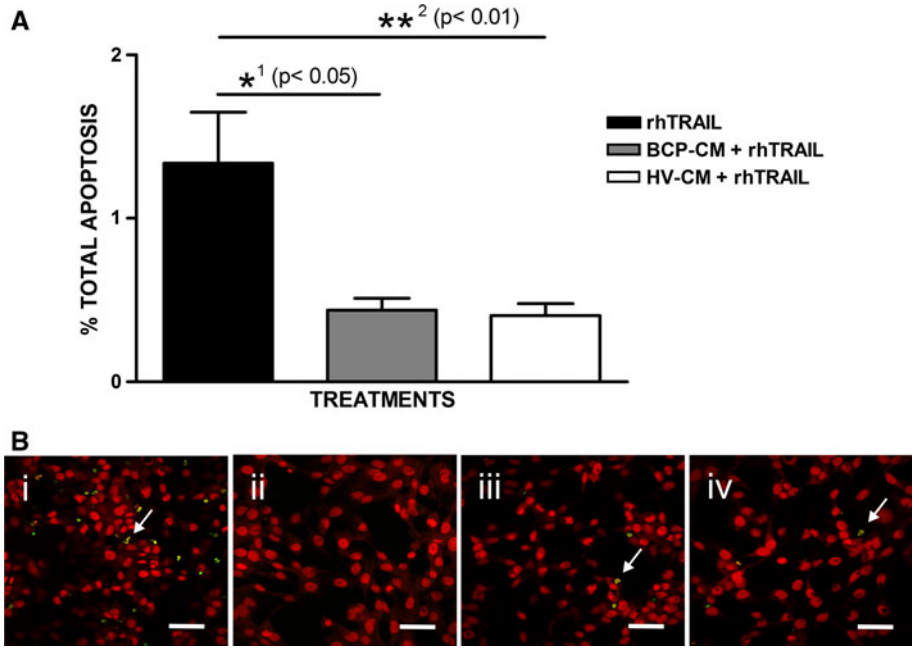

Fig. 7 **a** Antagonistic effect of colony-forming unit fibroblastic (CFU-F)-conditioned media (CM) from breast cancer patients (BCP) and healthy volunteers (HV) over the % of MDA-MB231 cells under total apoptosis induced with 50 ng/ml of recombinant human (rh)TRAIL by TUNEL apoptotic assay. The percentage of cells under total apoptosis in each sample was obtained subtracting the control cell percentage (without rhTRAIL) from its respective sample value, and the values are expressed as the mean ± SE. Statistical analysis: Kruskal–Wallis test followed by a Dunn's Multiple Comparison test. *Asterisks* ($*^1$ and $**^2$) indicate a significant difference $p < 0.05$ ($*^1$) and $p < 0.01$ ($**^2$) between 7 BCP–CM + rhTRAIL vs. rhTRAIL and 7 HV–CM + rhTRAIL vs. rhTRAIL, respectively. **b** This figure is an example of the effect of CFU-F-CM from a BCP and a HV over MDA-MB231 cells. After establishing the adherence and post-arrest culture conditions, the BC cells were treated for 24 h with the following: (*i*) 50 % basal α-MEM plus 50 % basal DMEM/F12 containing 10 % FBS, with 50 ng/ml rhTRAIL, (*ii*) 50 % basal α-MEM plus 50 % basal DMEM/F12 containing 10 % FBS, without rhTRAIL, (*iii*) 50 % of the CM of CFU-F from a BCP plus 50 % basal DMEM/F12 containing 10 % FBS, with 50 ng/ml rhTRAIL, and (*iv*) 50 % of the CM of CFU-F from a HV plus 50 % basal DMEM/F12 containing 10 % FBS, with 50 ng/ml rhTRAIL. The cells were fixed with 1 % paraformaldehyde, permeabilised with ethanol-acetic acid, incubated with recombinant terminal deoxynucleotidyl transferase (rTdT)-incubation buffer (which includes: equilibrium buffer, a nucleotide mix with fluorescein-12-dUTP and rTdT enzyme), stained with propidium iodide and analysed under a confocal microscope (400×). The arrows show an example of apoptotic cells in green (fluorescein-12-dUTP at 520 ± 20 nm). The *scale bar* represents 50 μm

leading to the release of tumour survival factors embedded in the bone matrix. Recently, Monteiro et al. [58] found that tumour-specific T cells induce osteolytic bone disease before bone colonisation in a mouse model of metastatic BC. This T cell pro-metastatic activity correlates with a pro-osteoclastogenic cytokine profile, including RANKL, a master regulator of osteoclastogenesis.

The evidence presented in our data is the first in vitro study which showed that BCP–BM plasma induced a significantly higher transendothelial migration increase of MCF-7 and MDA-MB231 cells compared with HV-BM plasma. Also, BCP–BM plasma induced a significantly higher transendothelial migration of MDA-MB231 cells than MCF-7 cells, the opposite result to the effect described before for BCP-PB plasma. This last observation could be related to the fact that BM extravasation is a complex event where MDA-MB231 cells are more invasive and metastatic, exhibiting advantage over the MCF-7 cells. Future studies are needed to understand more about the mechanisms involved in transendothelial migration of both types of BCCs. Also, the results showed that BM–CM of CFU-F from BCP and HV had transendothelial migratory effects over both types of BCCs, but no significant difference was found between the groups. However, in vitro transendothelial migration capacity of BCCs to BM–CM of CFU-F from BCP did not show significant difference between both types of line cells. This latter observation could be changed if we use, in the future, BM-MSC instead of CM of CFU-F from BCP–BM for do the transendothelial migration assays.

It is well known, as we mentioned in the introduction, that cytokines and CAMs in the BM/bone microenvironment are critical components for bone-remodelling and haematopoietic processes. The presence of cancer cells changes the normal levels of these cytokines and CAMs, which in turn disrupts the homoeostatic balance in the

bone. Whether these changes are limited to the immediate location of the cancer cells or are the result of a systemic effect has yet to be determined [8]. In this work, we demonstrated how the levels of the same BM microenvironment cytokines and CAMs change in untreated advanced BCP without metastatic development in BM/bone compared with HV.

Based on the observations previously described in the introduction, we found that soluble ICAM-1, VCAM-1 and PDGF-AB levels in BM plasma from BCP were significantly higher than HV, suggesting that they could be involved in the BCC escape out of the blood vessels into the BM (extravasation step). Furthermore, Paduch et al. demonstrated that reactive oxygen species (ROS) promote transendothelial migration [59]. In a previous study, our data showed that the oxygen radical production in PB neutrophils from advanced BCP is higher than in HV. Moreover, we reported elevated levels of TBARS (2-thiobabituric acid-reactive substances) in PB and BM plasma from these BCP compared with HV, suggesting a significant increase in lipid oxidation and ROS production in the PB and BM of untreated advanced BCP [60]. These previous results support further evidence that the BM microenvironment of these patients favours BCC intravasation as well as extravasation. In addition to ICAM-1, the fact that the BM plasma of these BCP had a tendency to present higher levels of MIF and OPG in comparison to HV may also be relevant to the regulation of angiogenesis and survival of BCCs, which create a favourable BM microenvironment to seed circulating tumour cells [18–21, 61, 62].

Although in vitro studies have shown a facilitating role for MSC in the migration of BCCs to bone, a definitive role for MSC has yet to be determined. In the present work, we examined whether BM-MSC from untreated advanced BCP could potentially control the recruitment and establishment of newly arriving cancer cells, modifying the migration of BCCs as another possible interaction that favours tumour development. Our results demonstrate that the CM of CFU-F cultures (1CFU-F = 1MSC) from BCP induced a higher transwell migration increase of MCF-7 and MDA-MB231 cells compared with HV cultures. Unfortunately, none of the studied chemoattractants in the CFU-F-CM appear to be involved in this process. Future studies need to be performed to investigate possible factors involved in this process. Furthermore, if we compare the effect of BCP–CM of CFU-F on transwell migration of both BCC lines studied, the results showed that a equal number of MCF-7 and MDA-MB231 cells responded. Results suggest that BM pre-conditioning adapts BCCs to utilise BM-MSC-derived signals to increase their migration. In contrast, other authors found no significant in vitro transwell migration of MCF-7 cells with CM of BM-MSC from HV or co-culture [63]. Meanwhile, BM plasma from BCP and HV had a transwell migratory effect over both types of BCCs, but no significant difference was found between the groups.

In reference to matrigel invasion of BCCs, BM plasma and BM–CM of CFU-F cultures from BCP and HV induce the invasion of both BCCs, but no significant difference was found between the groups. Additionally, if we compare the effect of BCP–CM of CFU-F on both types of BCC lines studied, the results showed that a higher number of MCF-7 cells responded.

To evaluate whether the BM microenvironment induces a favourable soil for the BCCs when they arrive at the BM of untreated advanced BCP, we investigated whether the BM plasma and MSC present in BCP–BM provide metastatic BCCs with an advantageous environment for proliferation. Thus, BCP and HV-BM plasma had proliferative effects over both BCCs. Furthermore, the BCP–BM plasma significantly increased the proliferation of MDA-MB231 cells compared with the effects of HV plasma. Several growth factors and cytokines may contribute to the proliferation of BCCs. One example is PDGF, which is composed of multifunctional cytokines involved in the growth, survival and differentiation of connective tissues [64, 65]. Recently, Yokoyama et al. [66] reported that PDGF-BB tended to induce the nuclear translocation of β-catenin and cell proliferation in MDA-MB231 cells, whilst these results were not found in MCF-7 cells. In addition to its angiogenic capacity [67, 68], we think that PDGF-AB, amongst another molecules, could induce a favourable BM microenvironment for the proliferation of BCCs when they enter into the BM at an early time period of the metastatic cascade. However, data have shown that the CM of CFU-F did not modify the proliferation of the BCCs, independent of the type of group. Moreover, our studies recently revealed no proliferation of BCCs in co-cultures with BM mesenchymal stromal cells (data not shown). Thus, it may be that BCCs require the complexity of the haematopoietic microenvironment to modify their behaviour.

Another interesting fact is that PDGF induces osteoblast proliferation, inhibits osteoblast differentiation [69] and induces these immature osteoblasts to release osteoclastogenic factors in the BM/bone (IL-6, RANKL, etc.) contributing to the osteoclastogenic process and bone resorption. Additionally, this last event can be induced by the direct action of PDGF on osteoclasts [69–71]. In previous work, we found an inefficient cloning and osteogenic differentiation capacity for the BM-MSC of these BCPs (clinical stage III-B, without bone metastasis) [54, 72]. Simultaneously, these BCPs showed spontaneous osteoclastogenesis in BM [Fernandez Vallone VB, Choi H, Martinez LM, Labovsky V, Batagelj E, Dimase F, Feldman L, Bordenave RH, Chasseing NA (2010) Osteoclastogenesis process in bone marrow of

untreated advanced breast cancer patients. Book of Program and Proceedings of MRS-AACR Conference: Metastasis and the Tumour Microenvironment, Philadelphia. Abst B34, page 105]. Therefore, the increase of PDGF levels in BM plasma contributed to create an optimal microenvironment, the pre-metastatic niche, for the establishment of the BCCs.

It is also reported that soluble VCAM-1 is likely to be a key factor for the recruitment of monocytic osteoclast progenitors to nascent bone micrometastasis [73]. This migration to the bone is a consequence of the interaction between α4β1 and VCAM-1. Not only do BM endothelial and mesenchymal stromal cells release VCAM-1 but BCCs also do. Therefore, it is possible that these types of cells, by upregulating VCAM-1, could locally retain a high density of monocytes as the source of osteoclast differentiation or induce the maturation of haematopoietic osteoclast progenitors [73]. Moreover, the interaction between VCAM-1 and α4β1 or αvβ3 can induce the osteoclastogenic process [32, 74]. Thus, soluble VCAM-1 could promote the transition from indolent micrometastasis to over-metastasis. Therefore, VCAM-1 represents a promising therapeutic target to restrict the progression of micrometastasis in BM/bone of our BCP. Furthermore, the increase of soluble ICAM-1 in BM plasma of BCP vs. HV can produce osteoclastogenesis through the interaction with leucocyte function-associated antigen-1 (LFA-1) present on osteoclast precursors [75].

In addition, Fernandes et al. [76] reported that soluble ICAMs have a dual effect on bone homoeostasis, increasing osteoclast activity, whilst lowering osteoblast anabolic activity. Also, CAMs could stimulate invasive and proliferative capacities of BCCs in the BM/bone environment as other authors found [74, 77, 78]. Therefore, the BM of our BCP showed a haematopoietic microenvironment suitable not only for the extravasation, invasion, migration and proliferation of BCCs but also for the induction of the transendothelial migration of circulating preosteoclasts that can develop by RANKL and other osteoclastogenic factors (PDGF, MIF, VCAM-1 and ICAM-1) into functional osteoclasts. Such data could lead, in the near future, to a 'vicious cycle' of bone destruction and BCC proliferation that favours bone metastatic progression.

In contrast, our data did not show apoptotic effects from any of the BM-CM, but we did observe an inhibition of rhTRAIL-induced apoptosis of MDA-MB231 cells when we used the CFU-F-CM from BCP and HV. Moreover, the BCP–CM had a significantly lower reduction of the early apoptosis compared with HV–CM. To identify the molecule responsible for the inhibition of the rhTRAIL effect, we measured the OPG levels in BCC and BM-CM. Several reports support the role of OPG in tumour cell survival in addition to its role in bone turnover [10]. The ability of OPG to bind to TRAIL and inhibit its apoptotic effect on tumour cells can thereby provide cells with a survival advantage [79]. The major OPG cellular sources in the BM have been shown to be released by human BM-MSC [40] and some BCCs [80]. Our results showed that the OPG value was <31.25 pg/ml in the CM from both BCC cultures. Surprisingly, the significantly lower OPG level in the BM–CM of CFU-F cultures from BCP than HV could be related to the significantly lower decrease of the rhTRAIL apoptotic effect in MDA-MB231 cells when we used these BCP–CM. These data suggest that BCP-MSC cannot protect BCCs from TRAIL-induced apoptosis as do HV cells.

Finally, high levels of PB-RANKL and MIF as well as BM-PDGF from untreated advanced BCP (without bone metastasis) may favour the EMT phenotype of circulating and disseminating tumour cells into the BM/bone, a key event that greatly enhances their malignant phenotype and activates quiescent tumour cells. Thus, if a majority of the circulating tumour cells have an EMT phenotype and are cancer stem cells, particularly at the beginning of the formation of 'pre-metastatic niches', the significant increases in ICAM-1 and VCAM-1 levels in the BM plasma of these BCP can favour the extravasation of these tumour cells in particular, as well as their invasive and proliferative capacities discussed above.

In summary, we propose that PB plasma from untreated advanced BCP favours intravasation of BCCs in the circulation, and the BM microenvironment creates the permissive soil, the pre-metastatic niche, for the establishment of BCCs. These findings suggest that early therapeutic intervention may be required to oppose the tumour-induced changes to the PB and haematopoietic microenvironment, and thus tumour progression. Therefore, further investigations aimed at deciphering the intricacies of the microenvironment should be performed to optimise therapeutic development.

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Ethical standards All individuals gave consent prior to participating in these studies. The investigations were approved by the IBYME Ethical Committee and performed in accordance with the principles of the Helsinki Declaration.

Disclosure The authors declare no conflicts of interest.

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