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RANK in cancer-associated fibroblasts: A valuable prognostic determinant for metastasis in early-stage breast cancer patients

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Abstract.
BACKGROUND: The molecular system of receptor activator of nuclear factor kappa-β (RANK) and its ligand (RANKL) plays a role in a variety of physiological and pathological processes. These encompass the regulation of bone metabolism, mammary gland development, immune function, as well as their involvement and tumorigenesis. Nevertheless, limited knowledge exists regarding their function within the tumor microenvironment.
METHODS AND RESULTS: We explored the significance of RANK expression in cancer-associated fibroblasts (CAFs) as a prognostic biomarker in early breast cancer patients (BCPs) by immunohistochemistry. Results reveal a significant correlation between high RANK expression in CAFs and an increased risk of metastasis ($p = 0.006$), shorter metastasis-free survival (MFS) [$p = 0.007$, OR (95%CI) = 2.290 (1.259–4.156)], and lower overall survival (OS) [$p = 0.004$, OR (95%CI) = 2.469 (1.343–4.541)]. Upon analyzing the phenotype of CD34(-) CAFs isolated from primary tumors in BCPs, we observed co-expression of RANK with CD105 marker by immunofluorescence and flow cytometry, characteristic of mesenchymal stem/stromal cells (MSCs), suggesting the possible cellular origin. Also RANKL-RANK system increase the OCT-4, SOX-2 and DKK-1 (dickkopf 1) gene expression in CD34(-) CAFs by RT-PCR. Moreover, this system plays a crucial role in the migration of these CD34(-) CAFs.
CONCLUSIONS: These results support the clinical relevance of RANK in CAFs and propose its potential as a future therapeutic target in the treatment of early BCPs.

Keywords: Cancer-associated fibroblasts, RANK/RANKL, breast cancer, prognostic factor, metastatic occurrence

[1]These authors share the same position.

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1. Introduction

Breast cancer is a complex system composed of tumor cells and their stromal microenvironment, where CAFs play a significant role [1,2]. These activated fibroblasts are a heterogeneous population of spindle-shaped stromal cells that do not express CD34 and CD31 but could exhibit positivity for smooth muscle actin α, fibroblast-specific protein 1 (FSP) and fibroblas activation proteinα (FAP), among other markers [3, 4]. Regards to their origin, CAFs derive from multiple sources, including MSCs [4,5,6]. It has been observed that MSCs from the bone marrow can migrate to the primary breast tumor in the early stages of the disease and contribute to tumor development, either as CAFs or in undifferentiated form [7,8,9]. These MSCs/CAFs interact with tumor cells, providing signals that promote tumor aggressiveness. In breast cancer progression and metastasis, cytokines and chemokines as well as their receptors appear to have a significant impact on numerous cellular pathways that affect the outcome [4, 10]. One key molecular system is RANKL and its receptor, RANK [11,12,13]. Studies have highlighted the proliferative effect induced by the interaction between RANKL and RANK in breast epithelial cells, suggesting involvement in the initiation and progression of breast cancer [14,15,16,17,18,19]. Also, RANKL has the capacity to induce the migration of breast cancer cells [20]. Consequently, this system could play a prominent and crucial role in facilitating the specific breast metastasis Therefore, targeting the interactions between RANKL-RANK presents a promising therapeutic opportunity for disrupting the progression of tumors [15]. In our previous investigation, we observed that the expression of RANKL and RANK was significantly higher in spindle shaped stromal cells, not associated with vasculature, in invasive ductal primary tumors of early BCPs compared to these types of stromal cells in non-neoplastic breast tissues [10]. Furthermore, we found a significant positive association between RANKL expression in spindle shaped stromal cells, not associated with vasculature, and the expression of RANK in both breast tumor cells and these stromal cells [10]. These results suggest a reciprocal communication between both cell types and an autocrine and paracrine regulation of RANK, particularly in these stromal cells. These findings, along with discoveries made by other researchers, could suggest that these type of fibroblast, through the action of RANKL, have the potential to influence the proliferation, survival, migration, and intravasation of breast tumor cells during the early stages [21,22,23,24,25]. The system RANKLRANK is also express in bone marrow MSCs [26]. Previous studies conducted by other researchers have demonstrated that bone marrow MSCs lacking RANKL exhibited deficient osteogenic differentiation and reduced self-renewal capability [27,28].

RANK functional activities have been clearly established by studying the phenotype of RANK knockout mice, which exhibit severe osteopetrosis characterized by a lack of mature osteoclasts and an absence of lymph node development, leading to impairment in B- and T-cell maturation [29,30]. RANK is thus recognized as the second key player in "osteoimmunology" [31]. Furthermore, studies have demonstrated that RANK is upregulated in osteosarcoma, a mesenchymal tumor of osteoblastic origin. Additionally, this receptor is present in normal osteoblasts and plays a crucial role in controlling their migration inside the bone, which is essential for bone modeling and remodeling [32,33,34]. Despite advancements in understanding the RANKL-RANK system, there is still a lack of knowledge and contradictions regarding its functions in CAFs and MSCs within the primary breast tumor microenvironment. Given the background of this research field and acknowledging the limitations of classic prognostic parameters in accurately predicting outcomes in early-stage breast cancer, it becomes imperative to evaluate the potential of RANK as a prognostic biomarker. Additionally, investigate the role of RANK in the self-renewal, proliferation, and migration processes of CAFs is of vital importance.

2. Materials and methods

2.1. Retrospective analysis

2.1.1. Selection and characterization of breast cancer patients

A retrospective study was conducted, including 155 consecutive patients who underwent surgical treatment for breast cancer at the Hospital Italiano in Buenos Aires, Argentina. These patients had a confirmed histological diagnosis of early-stage invasive ductal carcinoma (stage I/II) based on the TNM classification system of the International Union Against Cancer [35]. A minimum follow-up period of 10 years after surgery was ensured. Patients who received neoadjuvant therapies, those with insufficient tissue samples, and those with previous primary tumors were excluded. After surgery, all patients received appropriate treatment, including hormonal therapy, radiother-

apy, and/or chemotherapy. The treatment plan was determined based on the clinical and histopathological characteristics of each patient and the guidelines recommended by the European Society for Medical Oncology [36,37]. This study was approved by the Ethics Committees of the Institute of Biology and Experimental Medicine (IBYME) and the Hospital Italiano. Informed consent was obtained from the patients or their family members (IBYME approval: CE 050 and Hospital Italiano approval: No. 5009). The research was conducted in accordance with the principles set out in the Declaration of Helsinki. To protect the privacy of the patients, medical records were anonymized using a numerical code.

The clinical characteristics of the patients, considered classical prognostic markers, were categorized according to the cutoff values specified in the Hospital Italiano protocols [38]. These characteristics included: a) Age (< 50 or $\geqslant 50$ years), b) Tumor size ($\leqslant 2$ or > 2 cm), c) Histological grade according to the Scarff-Bloom-Richardson grading system [39], categorized as well-differentiated (G1), moderately differentiated (G2), or poorly differentiated (G3), d) Expression of estrogen receptors (ER), progesterone receptors (PR), and HER2/neu status, classified as negative or positive according to Wernicke M et al. [27], and e) Presence of regional lymph node metastasis, recorded as negative (no involvement in axillary dissection or sentinel lymph node) or positive (including micro-metastasis). Outcome data included local relapse, the occurrence of metastasis, the occurrence of bone metastasis, the occurrence of visceral metastasis, the occurrence of mixed metastasis (bone and visceral), local relapse-free survival (RFS), MFS, bone metastasis-free survival (BMFS), visceral metastasis-free survival (VMFS), mix metastasis-free survival (MMFS), and OS. MFS, BMFS, VMFS, and mix-MFS were defined as the time interval from the date of surgery to the first observation of tumor appearance (metastatic event and/or local relapse) or the last follow-up. Patients included in the mixed metastasis group were those who, at the time of follow-up, had both bone and visceral metastases, without differentiation of which event occurred first. OS was defined as the interval from the date of surgery to death or the last follow-up [40]. Supplementary Table 1 provides detailed information on the specific clinical characteristics of the patients and their corresponding outcome data. The site of breast cancer metastasis is described in Supplementary Table 2. Additionally, data on the presence of single or multiple foci of metastasis within the same organ were documented.

2.1.2. Analysis of RANK expression

Breast tissue samples were processed following the methodology outlined by Labovsky et al. [10]. In order to assess the levels of RANK expression in spindle-shaped stromal cells not associated with vasculature, we employed an immunohistochemical protocol as described in previous studies [10]. The immunohistochemical signal was evaluated using the Allred scoring system [41]. Cells with membranous staining, nuclear counterstaining, and displaying characteristic fibroblastic morphology (spindle shape), not associated with the vasculature, were counted within the intratumoral stroma. Positive cell percentages were assigned scores according to the following categories: 0 ($< 10\%$), 1 (10–30%), 2 (31–60%), 3 (61–90%), and 4 ($> 90\%$). Staining intensity was scored on a scale of 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong), based on the relative intensity of low molecular weight cytokeratin AE1-AE3 expression [10]. The final staining score, ranging from 0 to 7, was calculated by combining the percentage of positive cells and the staining intensity score. Enumeration was performed in five representative optical fields per tissue section at a magnification of 400x. The evaluation was independently conducted by two pathologists, with an 88.4% agreement in the immunohistochemical assessment between observers (Kappa value = 0.867) (Fig. 1).

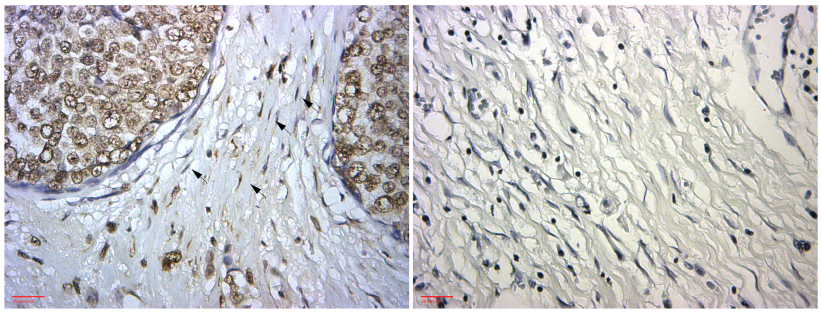
2.1.3. Intra-tumoral stromal characteristics

The histological characteristics of the tumor stroma, such as the percentage (%) of intratumoral stroma, the quantity of fibroblasts, collagen deposition, lymphocytic infiltration, myxoid changes, and blood and lymphatic vascularization, were assessed through hematoxylin and eosin staining. Intratumoral stroma was assessed as a percentage and categorized as low ($< 50\%$) or high ($\geqslant 50\%$) in quantity. Pathologists scored the presence of fibroblasts, collagen deposition, lymphocytic infiltration, myxoid changes, blood and lymphatic vascularization using a scale of absent (0%, score 0), scanty ($< 30\%$, score 1), moderate (30–50%, score 2), or abundant ($\geqslant 50\%$, score 3). The degree of desmoplasia was also documented based on information obtained from the patients' medical records, categorized as low/moderate or severe.

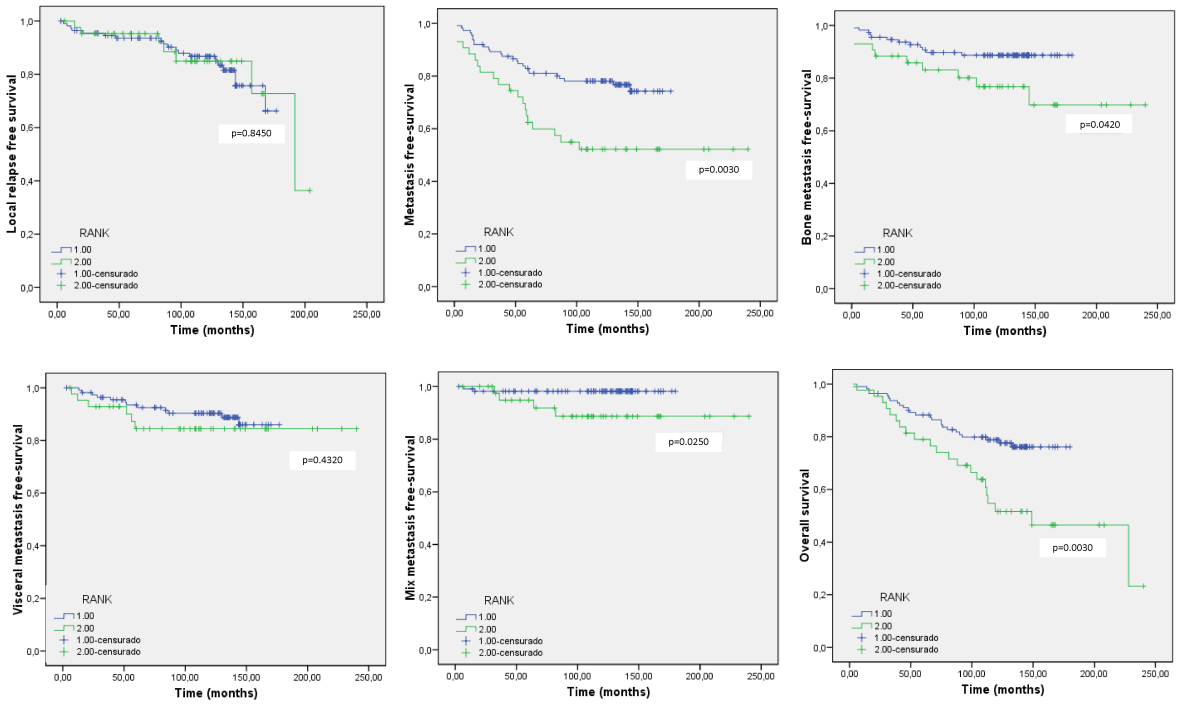
2.1.4. Statistical analysis

The statistical analysis of RANK expression and its correlation with clinical-pathological characteristics was conducted following the methodology described by Labovsky et al. [10]. To determine the optimal cutoff

A.



B.



C.

RANK	*RFS*		*MFS*		*BMFS*		*VMFS*		*MMFS*		*OS*	
	Time (months)	p	Time (months)	p	Time (months)	p	Time (months)	p	Time (months)	p	Time (months)	p
Low expression	173.02 ± 9.71	0.8450	179.04 ± 7.59	**0.0030**	187.33 ± 14.49	**0.0420**	214.45 ± 5.66	0.4320	219.07 ± 9.88	**0.0250**	180.45 ± 7.43	**0.0030**
High expression	156.06 ± 4.45		145.19 ± 5.60		163.85 ± 4.44		208.24 ± 11.96		176.95 ± 2.13		150.13 ± 13.76	

Fig. 1. Expression of RANK in CAFs from the primary tumor of BCPs. Left panel: representative example of RANK immunostaining (brown chromogen) in stromal cells assessed in the primary tumor tissue of a BCP. Right panel: isotype control. Nuclei were stained with hematoxylin (purple color). Original magnification: $\times$ 400. Scale bars represent 25 μm. B. Association of RANK expression with local relapse-free survival (RFS), metastasis-free survival (MFS), bone metastasis-free survival (BMFS), visceral metastasis-free survival (VMFS), mix metastasis-free survival (MMFS), and overall survival (OS) in early invasive ductal BCPs. Kaplan-Meier curves (univariate analysis) marked in green represent data from samples with high RANK expression, while blue curves represent samples with negative/low RANK expression. The Log Rank (Mantel-Cox) test was used to assess the Kaplan-Meier curves. $^{*}p$-value < 0.050. C. Details of RFS, MFS, BMFS, VMFS, MMFS and OS for the negative/low and high RANK expression groups.

value for receptor expression, we used the values of the first quartile (Q1), median, and third quartile (Q3) for sample classification. Associations between categorized RANK expression and patient OS were evaluated through univariable analysis. The cutoff value that provided the lowest p-value was selected. The optimal cutoff values for receptor expression were as follows: RANK = 1 (Median), quantity of fibroblasts =

2 (Q1), collagen deposition = 1 (Q1), lymphocytic infiltration = 1 (Q3), myxoid changes = 0 (Q1), blood and lymphatic vascularization = 0 (Q1). The Fisher exact test was used to assess the association between RANK expression and classical prognostic markers, as well as the occurrence of metastasis, bone metastasis, visceral metastasis, mixed metastasis, and local occurrence. Survival analyses, including RFS, MFS, BMFS, VMFS, mix-MFS, and OS, were performed using the Kaplan-Meier method, and differences were assessed with the log-rank (Mantel-Cox) test [37]. Multivariate survival analysis was conducted using the Cox proportional hazards model with backward stepwise selection (likelihood ratio). Only significant variables identified in the univariable analysis were considered. A significance level of less than 0.0500 was established for all analyses. The statistical analysis was carried out by an experienced statistician using SPSS software (version 18.00, Chicago, Illinois).

2.2. Prospective analysis

2.2.1. Patient selection

A prospective study was conducted using tumor-associated fibroblasts obtained from tumor tissue during surgery from 9 patients with early invasive ductal breast carcinoma (stages I/II, luminal type). Patients who had received neoadjuvant therapy, had a history of previous tumors, or had insufficient sample size ($<$ 1 cm) were excluded. The samples were provided by the Breast Pathology Service of the Hospital Italiano, CABA. This study was approved by the Ethics Committees of the Institute of Biology and Experimental Medicine (IBYME) and the Hospital Italiano. Informed consent was obtained from the patients or their family members (IBYME approval: CE 050 and Hospital Italiano approval: No. 5009). The research was conducted in accordance with the principles outlined in the Declaration of Helsinki. To protect the privacy of the patients, medical records were anonymized using a numerical code.

2.2.2. Isolation and expansion of CAFs from primary beast cancer tissue

Immediately after mastectomy, the tissues were placed in DMEM-F12 (#12500-062, Gibco). They were subsequently washed with the same medium supplemented with an antibiotic-antimycotic solution (ATB/ATM) (Gibco, Cat.15240), which contained a final concentration of 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 25 μg/ml of amphotericin B, and 2 mM of L-glutamine (hereinafter referred to as *supplemented medium*). The tissue was dissected with a scalpel onto a tissue culture dish and treated with 0.1% type III collagenase/hyaluronidase (Sigma-Aldrich, St. Louis, MO) overnight at 37°C with gentle agitation. After this period, the sample was centrifuged at 40g for 2 minutes. The resulting pellet, rich in organelles, as well as the undispersed tissue, was discarded. The supernatant obtained was transferred and centrifuged again at 100g for 2 minutes. The pellet obtained at this step is enriched in epithelial cells, while the supernatant is enriched in fibroblasts, the cell fraction of interest. A final centrifugation of the supernatant was performed at 200g for 5 minutes. The resulting pellet, rich in fibroblasts, was resuspended in *supplemented α medium*. Cell counting was performed using a 3% acetic acid solution in water, and fibroblast viability was determined using the trypan blue exclusion test with 0.04% trypan blue in PBS. For primary cultures, 375,000 cells were incubated per 25 cm^2 culture flask in 10 ml of supplemented α medium with 20% fetal bovine serum (FBS) (Natocor). After 24 hours, the medium was changed to remove non-adherent cells. Incubations were carried out at 37°C, 5% CO2, and humidity. The medium was renewed every 7 days, and when the culture reached 70–80% confluence, adherent cells were treated with a trypsin-EDTA solution (0.05%–0.02% in PBS; Gibco, Cat. 15400) to detach them at 37°C, 5% CO2 humidified environment for 10 minutes. The cells obtained in this first subculture are mesenchymal cells mostly composed of differentiated stromal cells, particularly CAFs. For simplicity, they will be referred to as CAFs from now on. To reduce cell density, CAFs from the first subculture were split into two 25 cm^2 culture flasks and incubated in supplemented α medium with 20% FBS. The culture medium was renewed every 7 days until they reached 70–80% confluence again. At that point, CAFs from the second subculture were trypsinized, and the CD34(-) fibroblast population was separated using a magnetic separation column (Anti-PE Multisort Kit: #130-091-271, MACS Miltenyibiotec, Ab anti CD34-PE: #130-098-140, MACS Miltenyibiotec). These step is important to remove the endothelial progenitors and endothelial cells. Subsequently, the CD34(-) CAFs fraction was incubated at a concentration of 240 viable cells/cm^2. Low cell density promotes the self-renewal of MSCs and, therefore, fibroblasts derived from them. The third subcultures were incubated with supplemented α medium with 20% FBS and the culture medium was renewed every 7 days until they reached 70–80% confluence again. Then the cells were trypsinized and to increase

cell yield, CD34(-) fibroblasts were seeded at 3,000 viable cells/cm2 and maintained by changing the medium every 7 days until they reached 70–80% confluence (fourth subculture). This cell fraction was used for the remaining assays.

2.2.3. Phenotypic characterization of CD34(-) fibroblast populations

A total of 3×10^5 cells from 4th subculture was centrifuged at 1,100 rpm for 5 minutes and re-suspended in 50 μl of PBS with 1% bovine serum albumin (BSA; Santa Cruz, Cat. sc-2323). Subsequently, the cells were incubated with specific monoclonal antibodies (Abs) conjugated with different fluorochromes targeting the following human antigens for 30 minutes at room temperature: RANK, CD105, CD34, CD90, CD73, FAP, CD19, and CD14. To analyze the co-expression of CD105, RANK, and CD34, a triple labeling was performed. The details of each Ab, along with their respective isotype controls, are summarized in Supplementary Table 3. The concentrations recommended by the manufacturers for each Ab were used in the flow cytometry analysis. Controls were simultaneously evaluated using the same protein concentration as the corresponding primary Abs. After incubation, the cells in each tube were washed twice with 1% PBS-BSA and centrifuged at 1,100 rpm for 4 minutes in each wash. Finally, the cells were re-suspended in 100 μl of PBS, and 10,000 events were analyzed in each case using flow cytometry (FACScalibur, BD Biosciences). FlowJo software was used to analyze the data, with isotype controls used to properly position the analysis quadrants and obtain relative fluorescence indices (RFI: specific surface molecule fluorescence index/specific isotype control fluorescence index). Each sample was performed in duplicate.

2.2.4. Evaluation of RANK and CD105 co-expression in CAFs isolated from fresh breast tissue and paraffin samples by immunofluorescence

CAFs isolated from breast tumor tissue were seeded onto slides at a density of 350,000 cells/ml (from 4th subculture). They were then fixed with methanol for 15 minutes and subsequently hydrated with 0.1% TBS-TW20 for 10 minutes. To perform double-label immunofluorescence, firstly, they were incubated overnight with the primary anti-RANK Ab (#MAB683, RyD Systems). On the second day, extensive washes were carried out with 1X PBS, followed by incubation with a secondary anti-mouse Ab labeled with Alexa 488 (715545150, Jackson-immunoresearch). Then, overnight incubation was performed with the primary anti-CD105 Ab (#AF1097, RyD Systems). On the third day, two washes with 1X PBS with 0.1% Tween 20 were done, followed by incubation with a secondary anti-goat Ab labeled with Alexa-647 (705605147, Jackson Immunoresearch). Subsequently, counterstaining was done using DAPI, followed by a wash with distilled water, and finally, the samples were mounted using Vectashield. CAFs expressing RANK (+) and CD105 (+) were visualized using a confocal microscope (40X). To perform immunofluorescence on breast tumor tissue embedded in paraffin, the same procedure was followed, with the difference that the initial processing of the sample was carried out as previously mentioned in the retrospective study [10]. Each sample was performed in duplicate.

2.2.5. Quantitative RT-PCR

CD34(-) stromal cells (CAF-like) for the 4th subculture was again seeded at 3,000 cells/cm^2 to obtain enough cells density for the RNA isolation. The culture conditions were as follow: i) 10 ml of supplemented α-medium with 5% FBS, and ii) 10 ml of supplemented α-medium with 5% FBS and 25 ng/ml hrRANKL. The culture was maintained until 70–80% of confluence at 37°C and 5% CO2 humidified environment. Subsequently, total RNA was extracted from 3×10^5 cells using EasyPure RNA kit (#ER101-01, Transgen biotech, Beijing, Chinese). A total of 1 μg of RNA was reverse-transcribed into cDNA using random primers (#4368814, High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). Samples were analyzed using FS UNIVERSAL SYBR GREEN MASTER ROX master mix (cat. 04913850001, ROCHE, Mannheim, Germany) on a CFX96TM TOUCH REAL-TIME PCR system (Bio-Rad, Hercules, CA, USA) following standard cycling conditions and a subsequent melting curve analysis. The threshold cycle (Ct) values were normalized to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the data are presented as the fold change in gene expression of OCT4, SOX-2 and DKK-1. Primer sequences are provided in Supplementary Table 4. Each sample was assayed in duplicate.

2.2.6. Fibroblastic colony-forming unit (CFU-F) assay

Isolated CD34(-) stromal cells (CAFs-like) were plated at a density of 100 cells of 4th subculture/cm^2 in 25 cm^2 culture flasks containing: i) 10 ml of supplemented α-medium with 5% FBS and ii) 10 ml of supplemented α-medium with 5% FBS and 25 ng/ml hrRANKL and iii) 10 ml of supplemented α-medium

with 20% FBS (standard positive control). Cells were incubated in a humidified environment at 37°C with 5% CO2 for 7 days. After this period, the culture medium was refreshed with/without hrRANKL. After another 7 days, stromal cells were washed twice with PBS, fixed with 100% methanol (Merck, Darmstadt, Germany) for 15 minutes, and finally stained with pure Giemsa (cat. 48900 Sigma, Biopure, St. Louis, MA, USA) for 5 minutes at room temperature. Colonies containing more than 50 spindle-shaped cells were identified under a microscope and recorded as CFU-F. The frequency of CFU-F was represented as the colony-forming efficiency, calculated as the number of CFU-F obtained for every 2,500 seeded CAFs. Each sample was performed in duplicate. The number of CAFs per CFU-F field (referred to as stromal cell density) was calculated by capturing ten images of different CFUF culture fields and processing them with FIJI software [42]. Morphological changes in CFU-F cultures were also evaluated. Analysis of area, ellipse longitudinal, and horizontal axis values were carried out using three pictures obtained from three typical regions (three optical fields, 200X) of each CFU-F culture, evaluating 10 cells per photo and analyzed by FIJI Software [43].

2.2.7. Proliferation assay

Viable CD34(-) fibroblasts were seeded at a density of 5×10^3 cells per well and cultured in 96-well plates (cat. 4430100, Orange Scientific, Belgium) with 200 μl of supplemented α-MEM with 20% FBS for 24 hours. Subsequently, the cultures were washed with PBS and incubated for 48 hours in α-MEM supplemented without phenol red and FBS (α-MEM without RF, cat. 41061029, Gibco, Grand Island, NY, USA). Finally, the cells were washed with PBS and incubated for an additional 48 hours at 37°C, 5% CO2, and humidity in: i) α-MEM without RF supplemented with 5% FBS, ii) α-MEM without RF supplemented with 10% FBS (*i and ii positive control*), iii) α-MEM without RF supplemented with 25 ng/ml hrRANKL and iv) α-MEM without RF and FBS (*negative control*). Cell proliferation was assessed using the Non-Radioactive CellTiter 96 AQueous Cell Proliferation Assay (cat. G5421, Promega, Madison, WI, USA) following the manufacturer's instructions. Optical density (OD) was measured at 490 nm using a microplate reader. The value for each sample was calculated by subtracting the OD of the negative control from its respective value. The data was analyzed and plotted as percentage increase relative to baseline (negative control) All experiments were performed in triplicate for each sample.

2.2.8. Migration assay

Experiments were conducted using transwell membranes with an 8 μm pore size (PI8P01250, Millipore) in 24-well plates. Each well was filled with i) supplemented α-medium with 10% FBS (positive control), ii) supplemented α-medium without FBS (basal control), iii) supplemented α-medium with 50 ng/ml hrRANKL (# GF091, RyD Systems), iv) supplemented α-medium with 50 ng/ml hrRANKL and 3.3 μg/ml anti-RANKL antibody (#MAB626, RyD Systems) or v) supplemented α-medium with 50 ng/ml hrRANKL and 5 μg/ml anti-RANK antibody (#MAB683, RyD Systems). In this particular assay, we use 50 ng/ml instead 25 ng/ml because we did not observe effect with this last dose. Subsequently, transwells were seeded with 4×10^4 CD34(-) fibroblastic cells. The assay was stopped at 14 hours by fixing the porous membranes in pure methanol for 10 minutes at room temperature. Then, the membranes were washed with water, and the remaining non-migrated cells on the membrane surface were removed with a wet cotton swab. The membranes were allowed to dry, stained with 0.05% crystal violet for 10 minutes, and subsequently observed under an inverted fluorescence microscope, where photos of 5 defined fields were taken at a magnification of 200X. The migrated cells were counted using Image J software. Each sample was performed in duplicate.

2.2.9. Statistical analysis

Results were presented as the mean $\pm$ standard error (SE). Data normality was assessed using the Shapiro-Wilk test. Parametric data were analyzed using an unpaired t-test with Welch correction to determine differences between groups. All statistical tests were two-tailed. Statistical analysis was performed using GraphPad 8 Prism software (GraphPad Prism version 8.01, GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as $p < 0.0500$.

3. Results

3.1. Retrospective study

3.1.1. Expression of RANK in spindle-shaped stromal cells, not associated with vasculature, and its association with the clinical-pathological characteristics of breast cancer patients

Out of a total of 155 BCPs diagnosed with invasive ductal breast cancer (stage I/II), 43 (27.75%) samples were found to have high RANK expression while 112

Table 1
Association of RANK expression in spindle shape stromal cells, not associated to the vasculature, with clinical-pathological characteristics (classic prognostic markers), local relapse, metastasis occurrence, bone metastasis occurrence, visceral metastasis occurrence, and mix metastasis occurrence in 155 patients with early invasive ductal breast cancer. Fisher's exact test was used for the association between variables

Clinicopathological characteristics		RANK			
		n	High expression		p
			n	%	
Age (years)	< 50	42	12	28.57%	1
	$\geqslant 50$	113	31	27.43%	
Tumor size (cm)	$\leqslant 2$	111	27	24.32%	0.1640
	> 2	44	16	36.36%	
ER	Negative	19	4	21.05%	0.5930
	Positive	136	39	28.68%	
PR	Negative	31	7	22.58%	0.6540
	Positive	124	36	29.03%	
Her2/neu	Negative	137	40	29.20%	0.1570
	Positive	18	2	11.11%	
Histological grade	G1	12	4	33.33%	0.6700
	G2	89	26	29.21%	
	G3	54	13	24.07%	
Regional lymph nodes	Negative	106	30	28.30%	0.8500
	Positive	49	13	26.53%	
Local relapse	Negative	129	36	27.91%	1
	Positive	26	7	26.92%	
Metastatic occurrence	Negative	109	23	21.10%	**0.0060**
	Positive	46	20	43.48%	
Bone metastatic occurrence	Negative	133	33	24.81%	0.0690
	Positive	22	10	45.45%	
Visceral metastatic occurrence	Negative	137	37	27.01%	0.5820
	Positive	18	6	33.33%	
Mix-metastatic occurrence	Negative	149	39	26.17%	0.0500
	Positive	6	4	66.67%	

*p-value < 0.050. ER: estrogen receptor; PR: progesterone receptor.

(72.25%) samples were found to have low RANK expression. Within the high expression group of RANK, the average percentage of fibroblasts expressing RANK was $46.90 \pm 5.12\%$ with an intensity of 1.89 ± 0.12. Whereas in the low expression group, the average percentage of RANK expression was $1.95 \pm 0.28\%$ with an average intensity of 0.38 ± 0.05.

There were no significant differences found regarding the association between RANK expression in spindle-shaped stromal cells, not associated with vasculature, "fibroblasts-like", and clinical parameters such as ER, PR, Her2/neu status, tumor size, age, histological grade, and regional lymph node status (Table 1). However, it was found that RANK expression in these stromal cells was related to the occurrence of metastasis in BCPs at early stages (I/II). High RANK expression was significantly associated with a higher risk of developing metastasis ($p = 0.006$, Table 1). Although no significant association was found between RANK expression and the occurrence of metastasis at specific sites, there was a tendency of association with bone metastasis events. RANK expression was also associated with the number of metastatic foci per organ ($p = 0.005$) (Table 2). Patients with high RANK expression had multiple foci within the same organ (Table 2). RANK expression was significantly related to MFS, BMFS, MMFS, and OS ($p = 0.033$, $p = 0.003$, $p = 0.042$, and $p = 0.025$, respectively) (Fig. 1). Patients with high RANK expression had shorter times for MFS (169.59 ± 9.55 months), BMFS (186.65 ± 10.53 months), MMFS (176.95 ± 2.13 months), and OS (178.03 ± 8.99 months) compared to the group of patients with low RANK expression (205.05 ± 5.39, 261.28 ± 1.5, 219.07 ± 9.88, and 226.67 ± 6.58 months, respectively) (Fig. 1).

3.1.2. Expression of RANK in spindle-shaped stromal cells, not associated with vasculature, and its association with intratumoral stromal characteristics

The expression of RANK was associated with the percentage of intratumoral stroma, as well as with blood and lymphatic vascularization ($p = 0.029$, $p = 0.013$, and $p = 0.006$, respectively, Table 3). Among patients with high RANK expression, 58.14% had a high per-

Table 2
Association of RANK expression in spindle shape stromal cells, not associated to the vasculature, with the number (#) of metastatic foci per organ in general. Fisher's exact test was used for the association between variables

Characteristics of the metastatic focus	n	*# metastatic foci*	
		>1 focus (n)	p
RANK			
Low expression	112	14	**0.0050**
High expression	43	15	

$^{*}p$-value < 0.0500.

centage of intratumoral stroma (Table 3). Regarding vascularization, high RANK expression was associated with a greater amount of blood and lymphatic vascularization (Table 3).

3.1.3. Association between classical prognostic markers and tumor progression

The ER status, PR status, tumor size, and histological grade were significantly associated with a worse prognosis in BCPs. Patients with ER (-) had lower MFS, BMFS, VMFS, and OS ($p = 0.0001$, $p = 0.001$, $p = 0.001$, $p = 0.0001$, respectively). Additionally, patients with PR (-) had lower MFS, BMFS, and OS ($p = 0.003$, $p = 0.013$, and $p = 0.001$, respectively). Those patients with a tumor size > 2 cm had lower MFS, BMFS, VMFS, and OS ($p = 0.0001$, $p = 0.003$, $p = 0.001$, and $p = 0.0001$, respectively). Furthermore, BCPs with a high histological differentiation grade (G3) had lower MFS and OS ($p = 0.002$ and $p = 0.006$, respectively) (*Data not shown*).

3.1.4. Multivariate analysis

RANK expression was an independent prognostic factor for MFS and OS in our BCPs ($p = 0.007$ and 0.004, respectively) (Fig. 2).

3.2. Prospective study

3.2.1. Phenotypic characterization of CD34(-) spindle-shaped stromal cells isolated from BCPs

Phenotypic analysis conducted through flow cytometry revealed that the studied CD34(-) spindle-shaped stromal cells exhibited the expression of markers associated with both CAFs and MSCs. We observed that our cell population had an expression of $97.19 \pm 1.13\%$ for CD90, $95.85 \pm 0.95\%$ for CD73, $20.42 \pm 3.18\%$ for CD105, and $66.91 \pm 6.89\%$ for FAP (Fig. 3). Regarding the RANK marker, we found that it was expressed in $37.14 \pm 8.36\%$ (Fig. 3). As for the markers CD34, CD14, and CD19, we found a low percentage of expression, considered as negative ($1.51 \pm 0.26\%$, $2.25 \pm 0.62\%$, and $2.68 \pm 0.44\%$, respectively) (Fig. 3). Furthermore, it was identified that $1516 \pm 3.29\%$ of the stromal cells isolated from the primary breast tumor co-expressed both RANK and CD105 markers (Fig. 3, panels B and C). This same co-expression was evident in breast cancer tissue embedded in paraffin (Fig. 3, panel D).

3.2.2. Gene expression of self-renewal and multipotency

We observed that CD34 (-) fibroblasts treated with 25 ng/ml hrRANKL had increased expression of genes related to cell multipotentiality as well as self-renewal, such as OCT4, SOX-2 and DKK-1, compared to CD34(-) fibroblasts that did not receive treatment (Fig. 4, panel A).

3.2.3. Capacity to generate fibroblastic colony-forming units

We did not find differences in the capacity to form CFU-F between CD34(-) spindle-shaped stromal cells treated with αMEM + 5% FBS + 25 ng/ml hrRANKL and those without RANKL treatment (αMEM + 5% FBS). (Fig. 4, panel B and G). Additionally, no differences were observed in the number of stromal cells per optical field of CFU-F (stromal cell density), cell area, or ellipse longitudinal, and horizontal axis (Fig. 4, panel C, D, E and F). Thus, in our experimental conditions in the presence of 5% of FBS, hrRANKL does not promote CD34(-) spindle-shaped stromal cells self-renewal and proliferation inside the CFU-F.

3.2.4. Proliferation capacity

Regarding the proliferation analysis performed using the MTS assay, we observed an increase in CD34(-) spindle-shaped stromal cells proliferation when we cultured these stromal cells with 25ng/ml hrRANKL alone. However, the stimulating effect of hrRANKL was lower than the values obtained with FBS (5% and 10%) (Fig. 4, panel H).

Table 3
Association between stromal histological characteristics and RANK in spindle shape stromal cells, not associated to the vasculature, expression in a cohort of 155 early breast cancer patients. Fisher's exact test was used for the association between variables

Characteristics of the breast tumor stroma		RANK		
		Total	High expression	p
% Intratumoral Stroma	$\leqslant 50$	88	18	**0.0290**
	> 50	67	25	
% Fibroblast	non-large amount	140	38	0.5620
	Large amount	15	5	
Collagen deposition	non-large amount	70	16	0.2800
	Large amount	85	27	
Lymphatic infiltration	non-large amount	135	35	0.1920
	Large amount	20	8	
Desmoplasia	non-large amount	60	10	0.0850
	Large amount	95	27	
Mixoid changes	non-large amount	59	13	0.3520
	Large amount	96	30	
Blood vascularization	non-large amount	109	1	**0.0130**
	Large amount	46	18	
Lymphatic vascularization	non-large amount	111	1	**0.0006**
	Large amount	44	18	
Total		155	43	

*p-value < 0.0500.

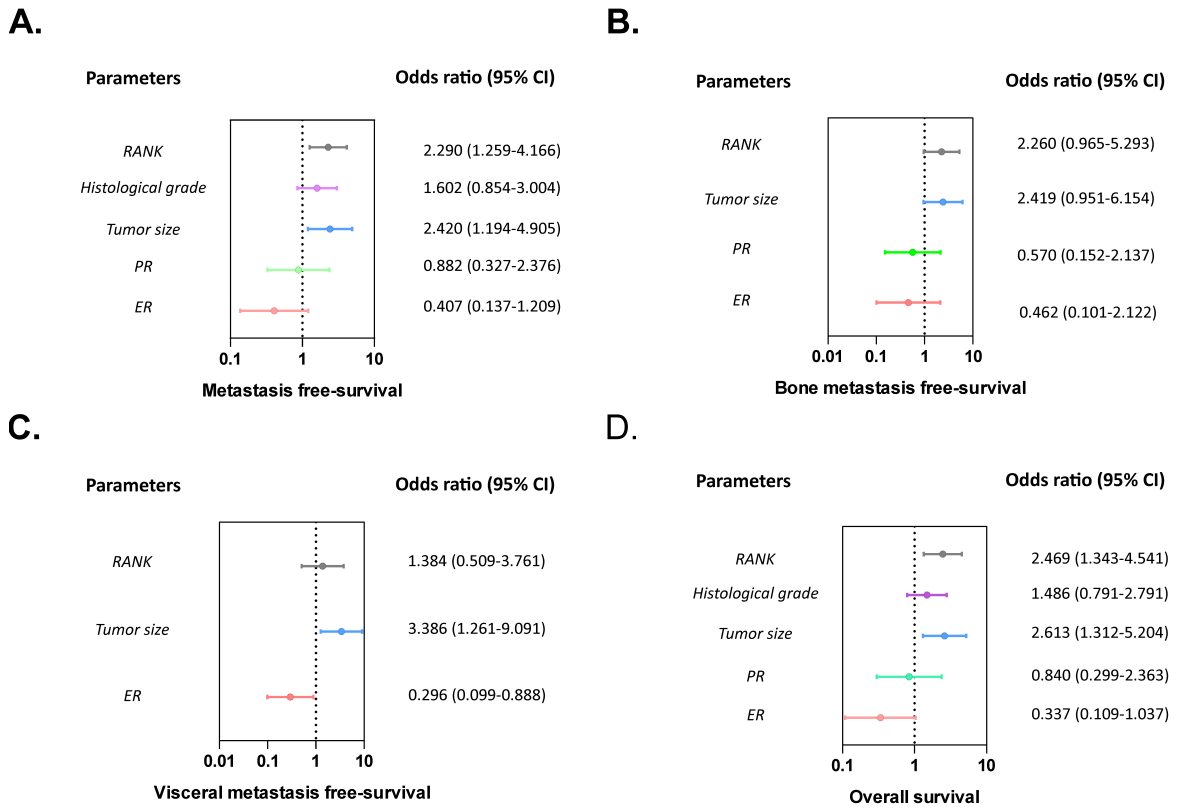


Fig. 2. Forest plot showed odds ratios for the multivariate association between classical prognosis factors and RANK, and metastasis-free survival (A), bone metastasis-free survival (B), visceral metastasis-free survival (C), and overall survival (D) in early invasive ductal BCPs.

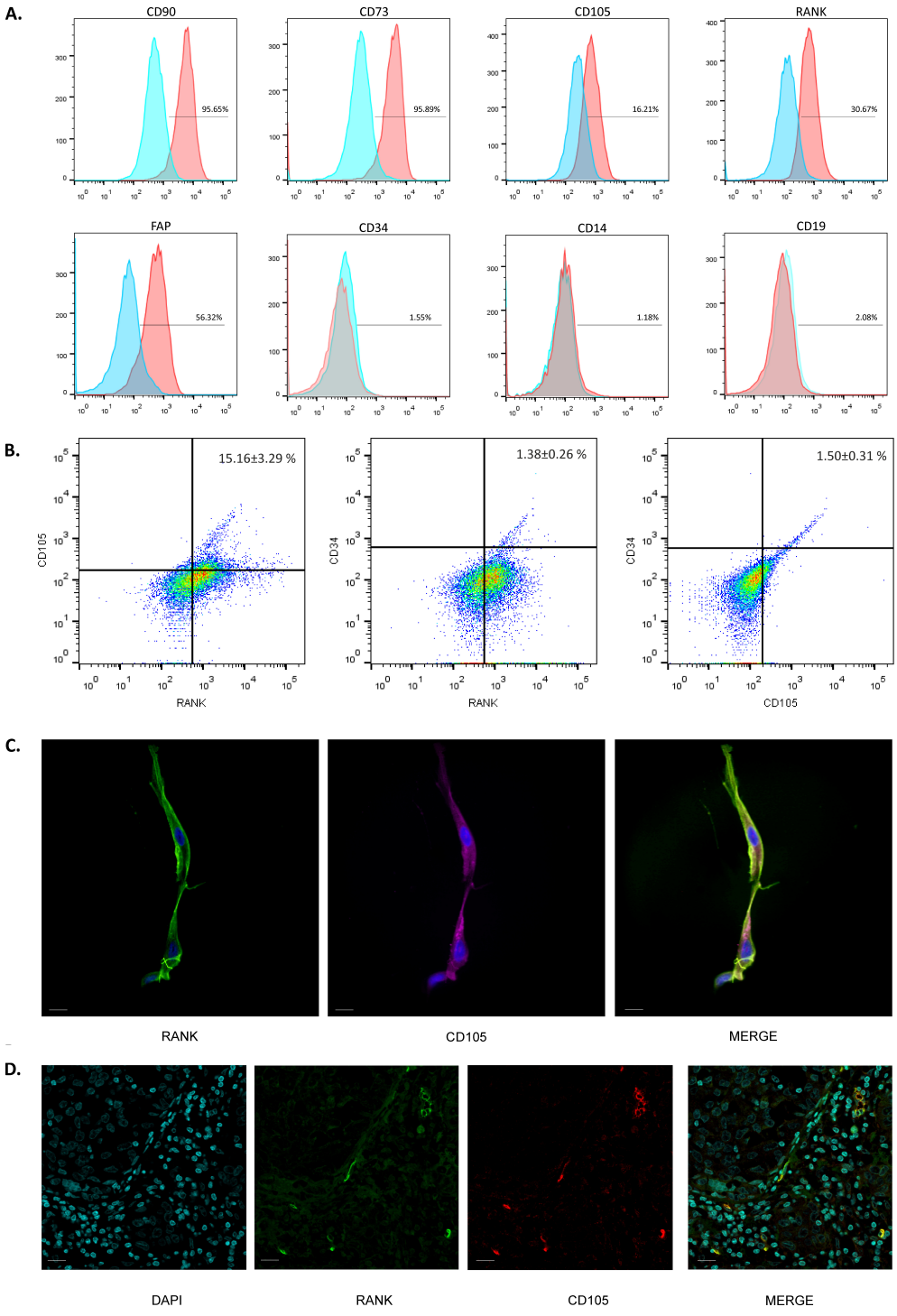


Fig. 3. Phenotypic characterization of CD34(-) CAFs. A. Representative flow cytometry surface antigen histograms of CAFs from a representative BCP. Isotype control (). B. Co-expression of RANK and CD105 in CAFs of breast tumors. A representative dot plot of RANK-CD105 co-expression, CD34-CD105, and CD34-RANK in fibroblasts isolated from the primary tumors of BCPs (I/II). C. Dual staining of RANK (green) and CD105 (red) by immunofluorescence in CD34(-) fibroblasts isolated from the primary tumors of BCPs. Counterstained with DAPI. Magnification 400X. The scale corresponds to 50 μm. D. Dual staining of RANK (green) and CD105 (red) by immunofluorescence in paraffin-embedded breast tissue from BCPs. Magnification 200X. The scale corresponds to 200 μm.

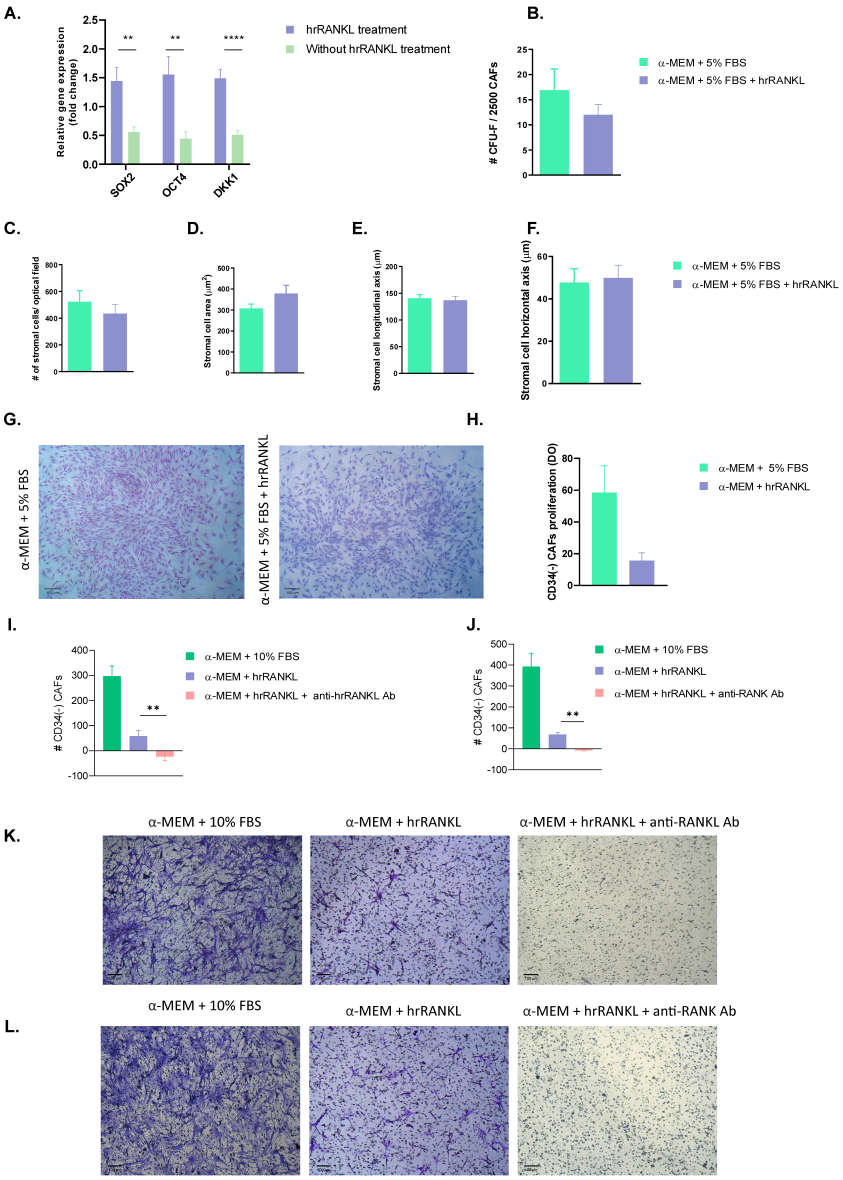


Fig. 4. Effect of RANKL-RANK system on the self-renewal, proliferation abilities, expression of pluripotency factors and migration of CD34(-) CAFs in BCPs. A. Gene expression of self–renewal and pluripotency factors in CAFs from BCPs treatment with and without RANKL. Expression of OCT-4, SOX-2 and DKK-1 by quantitative real-time polymerase chain reaction (RT-PCR). All the results were normalized against a set of reference genes. B. CFU-F Assay: The self-renewal capacity of CD34(-) CAFs from BCPs was assessed in αMEM basal medium supplemented with 5% fetal bovine serum (FBS) and in the presence of hrRANKL (25 ng/ml). The CFU-F assay was also conducted in the presence of 20% FBS as a standard positive control (#CFU-F/2500 CAFs $= 29.22 \pm 5.11$). C. Stromal cell density per optical microscope field in each CFU-F. D. Area of stromal cells in typical regions of each CFU-F culture. E. Length of stromal cells in typical regions of CFU-F cultures. F. Width of stromal cells in typical regions of CFU-F cultures. G. CFU-F size observed for a representative BCP in supplemented αMEM added with 5% FBS and in supplemented α MEM added with 5% SBF + 25 ng/ml hrRANKL. Giemsa staining (40X). H. Proliferation of CD34(-) CAFs in BCPs was evaluated in supplemented αMEM added with 5% and 10% SBF without hrRANKL, and in the presence of 25 ng/ml hrRANKL. Percentage increase relative to baseline (negative control) is plotted. All values are expressed as mean $\pm$ SE. Unpaired t-test with Welch's correction was used for statistical analysis. Asterisks indicate a significant difference ($^{*}p < 0.0500$). I and K. Migration of CD34(-) CAFs in BCPs was assessed in supplemented basal medium (α MEM) with I) 10% FBS (positive control), II) 50 ng/ml of hrRANKL and III) 50 ng/ml of hrRANKL and 3.3 μg/ml of anti-RANKL Ab. Values are expressed as mean $\pm$ standard error (SE). Unpaired t-test with Welch's correction was used for statistical analysis, ($^{**}p = 0.0089$). J and L. Migration of CD34(-) CAFs in BCP was assessed in supplemented basal medium (αMEM) with I) 10% FBS (positive control), II) 50 ng/ml of hrRANKL and III) 50 ng/ml of hrRANKL and 5 μg/ml of anti-RANK Ab. Values are expressed as mean $\pm$ standard error (SE). Unpaired t-test with Welch's correction was used for statistical analysis, ($^{**}p = 0.0049$).

3.2.5. Migration capacity

The CD34(-) spindle-shape stromal cells had migratory capacity when stimulated with 50 ng/ml of hrRANKL. Furthermore, this migratory capacity was inhibited when adding an anti-RANKL Ab to the assay (Fig. 4, panel I and K). Similarly, when the study was conducted with the blocking of the RANKL receptor, RANK, using an anti-RANK Ab, a similar reduction in migration was observed (Fig. 4, panel J and L). Thus, in our experimental conditions, hrRANKL promoted CD34(-) spindle-shaped stromal cells migration.

4. Discussion

There is substantial evidence supporting the presence of RANK in various functional organs and cell types, including osteoclasts, MSCs, osteoblasts, endothelial cells, mammary and immune system cells, and certain cancer cells, such as breast and prostate cancer [28,29, 30,44]. Although limited studies have assessed RANK expression in primary tumors as a prognostic factor, it is worth mentioning that functional RANK expression has been reported in human breast cancer cell lines [15]. Additionally, Santini et al. found that the expression of RANK in the primary tumor of BCPs, particularly those with invasive ductal breast carcinoma, is associated with the occurrence of bone metastases and the time to this type of metastasis [45]. These findings suggest that the RANK status in breast cancer cells plays a crucial role in their tendency to metastasize to bone, particularly when RANKL is abundantly expressed [15, 45]. Our study focused on evaluated RANK expression in spindle-shape stromal cells, not associated to the vasculature, (*fibroblast-like*). We investigated its correlation with clinical-pathological characteristics, to gain valuable insights into the role of these cells in breast cancer progression and prognosis. Our findings revealed a clear association between RANK expression in these stromal cells and the occurrence of metastasis. Specifically, high RANK expression was significantly associated with an increased risk of developing metastases. While no significant association was established between RANK expression and the occurrence of site-specific metastasis, the trend toward an association with bone metastasis suggests a potential role in this particular type of metastatic event. Furthermore, the observed correlation between RANK expression and the number of metastatic foci per organ added implies that RANK could serve as a valuable marker for assessing bone response in metastatic patients. This could aid in the early detection of imbalances in bone homeostasis [46]. Our results showed that the significant association between RANK expression in spindle- shape stromal cells, not associated to the vasculature, and MFS, BMFS, MMFS, and OS highlights the potential role of RANK as a prognostic indicator for early BCPs (stage I/II). Patients with high RANK expression in these type of stromal cells had a shorter MFS, BMFS, and MMFS, as well as lower OS compared to those with low RANK expression. This proposes that RANK expression could serve as a valuable prognostic biomarker for predicting disease progression and survival in early BCPs. From the study of RANK expression in relation to stromal characteristics, significant associations emerged with intratumoral stromal percentage and blood and lymphatic vascularization. Patients with high RANK expression had a higher abundance of intratumoral stroma, as well as increased blood and lymphatic vascularization. These results suggest a possible role of RANK(+) CAFs in influencing the composition of the microenvironment and vascularization patterns, factors that may play a critical role in breast cancer progression and metastasis. It is known that abundance of intratumoral stroma, as well as increased blood and lymphatic vascularization are powerful prognostic factors of poorer survival in BCPs [47,48,49]. RANK expression emerged as an independent prognostic factor for MFS and OS in BCPs, reinforcing its potential as a prognostic indicator, which can provide additional information beyond classical prognostic markers.

When performing the phenotypic characterization of CD34(-) CAFs isolated from the primary tumor of luminal BCPs, we discovered that these stromal cells express RANK in $37.14 \pm 8.36\%$ of cases. Furthermore, they exhibit the expression of typical markers of both CAFs (FAP) and MSCs (CD90, CD73, CD105). This initially suggests that they have an activated fibroblast phenotype and that these cells may have originated from MSCs that could have migrated from the bone marrow during the early stages of breast tumor development [8, 50].

Previous studies demonstrate the involvement of the RANKL-RANK system in cell self-renewal and proliferation processes [27,51]. However, in our experimental model, our results showed no significant differences in CFU-F and proliferation capacity when comparing CD34(-) fibroblasts with and without hrRANKL treatment. Nevertheless, hrRANKL treatment resulted in an upregulation of genes associated with self-renewal and multipotency. The basal expression level of OCT-4 is critical for preserving the stemness and differentiation

potential of stem cells, like MSCs, by modulating the expression of SOX-2 and NANOG [52]. In this study, we observed that treatment with hrRANKL increased the expression of OCT-4 and SOX-2. These findings suggest that the RANKL-RANK system may promote MSCs-like properties in these CAFs. Furthermore, a major concern in the field of cell therapy is the potential conversion of stem cells into malignant forms, like cancer stem cells. As tumor stem cells dedifferentiate, they often reactivate specific stem cell markers, with OCT-4 levels frequently increasing in cancer stem cells [53,54]. So the examination of RANK-RANKL expression as well as OCT-4 gene expression in stem cells such as MSCs or the CAFs that originated from them could serve as a valuable tool for predicting their potential for malignant transformation in tumor stromal microenvironment of early BCPs Also, Hiroaki K. et al. described that elevated expression of SOX2 in the stromal tissue of colorectal cancer patients is linked to increased invasiveness and a less favorable prognosis in terms of recurrence-free survival [55]. These previous observations suggest that the RANKL-RANK system, by promoting the expression of SOX-2, may induce the pro-tumoral activity of these stromal cells in early BCPs. Our results also showed that treatment with hrRANKL increased the gene expression of DKK-1. In this regard, Gregory CA. et al. discovered that human bone marrow MSCs at clonal densities, initiate the production of the Wnt inhibitor, DKK-1. This mechanism enables cells to re-enter the cell cycle by suppressing the canonical Wnt/β-catenin signaling pathway [56]. Therefore, this leads us to think that the expression of DKK-1 in fibroblasts could favor their entry into the cell cycle. Furthermore, DKK-1, which is secreted by perichondrium MSCs, plays a crucial role in regulating the Wnt/β-catenin signaling pathway in breast cancer, thereby enhancing neovascularization during tumor development [57]. So, the upregulation of these critical genes emphasizes the need for further exploration of the specific mechanisms through which hrRANKL influences these stromal cellular traits and its potential implications for breast cancer therapies.

Metastasis are a common and significant complication in many cancer patients, contributing significantly to morbidity and mortality [58]. Although breast cancer have a marked tendency to metastasize to bones [59], the underlying reasons for this osteotropic behavior remain poorly understood. Given the high expression of RANKL in the bone marrow/bone environment, the hypothesis arises that RANKL could function as a chemoattractant for breast tumor cells [9]. In particular, our study on the migratory capacity of CD34(-) CAFs observed in response to stimulation with hrRANKL, as well as the inhibition of this migration after the addition of an anti-RANKL or anti-RANK Ab, suggests that this response to RANKL is primarily mediated by the RANK receptor. Previous results of our group, suggest a reciprocal communication between RANKL expression in breast tumor cells and spindle-shaped stromal cells, not associated to the vasculature, and an autocrine and paracrine regulation of RANK, in particular in this type of CAFs [10]. Taking into account this background information and our results from the present study, it is conceivable that the RANKL-RANK system could play a significant role in the migration of CAFs within the breast tumor microenvironment, as well as in their extravasation to future pre-metastatic niches, such as the bone. Studies of other authors, showed that CAFs can enhance the survival and establishment of cancer cells in distant parts of the body by spreading through the bloodstream as either circulating CAFs or CAF clusters, sometimes in the presence of cancer cells and sometimes independently [60,61]. Circulating CAFs and clusters of CAFs have been detected in the peripheral blood of breast cancer patients with metastatic diseases [61].

All of these results together contribute in part to a better understanding of the role of RANK(+) CAFs in the tumor microenvironment and open avenues for further research on therapies targeting RANKL-RANK signaling in the context of breast cancer. Hence, blocking RANKL and RANK with Denosumab (RANKL Ab) or RANK Ab, respectively, could serve as preventive strategies to decrease the incidence of breast cancer initiation and metastasis, targeting not only the breast tumor cell but also the RANK (+) CAFs.

5. Conclusion

The results of both retrospective and prospective studies provide a comprehensive understanding of the influence of RANK expression in the tumor microenvironment and its impact on the progression and prognosis of early BCPs. RANK expression in CAFs emerges as a clinically relevant marker, associated with the occurrence of metastasis, the formation of multiple metastatic foci, and a poorer prognosis in terms of MFS and OS. Additionally, its involvement in the composition of the intratumoral stroma and its influence on vascularization are evident, highlighting its role in regulating these stromal cells as a potential prognostic

marker and therapeutic target in the management of breast cancer. This provides a solid base for future research and clinical developments aimed at manipulating the RANKLRANK signaling pathway in this context. On the other hand, migratory and proliferative response to hrRANKL as well as the upregulation of the expression of OCT-4, SOX-2 and DKK-1 genes in CAFs indicate a complex and regulated interaction with the microenvironment. These findings expand our understanding of the versatility and function of these stromal cells in breast cancer, contributing to potential therapeutic approaches in the future.

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Conflict of interest

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Abbreviations

RANK receptor activator of nuclear factor kappa-β.
RANKL receptor activator of nuclear factor kappa-β ligand.
CAFs cancer-associated fibroblast.
MSCs mesenchymal stem/stromal cells.
BCPs breast cancer patients.
DKK-1 Dickkopf 1.
MFS metastasis-free survival.
BMFS bone metastasis-free survival.
VMFS visceral metastasis-free survival.
RFS local relapse-free survival.
MMFS mix metastasis-free survival.
OS overall survival.
ER estrogen receptor.
PR progesterone receptor.
FBS fetal bovine serum.
CFU-F fibroblastic colony-forming unit.

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