

Hematogenic Dissemination of Triple-negative *Versus* Hormonal Receptor-positive Breast Cancer Cells

MARIA JOÃO CARVALHO^{1,2,3}, ANA MARGARIDA ABRANTES^{2,3,5},
MAFALDA LARANJO^{2,3,5}, BÁRBARA PAIVA⁶, ISABEL TORGAL¹, ANTÓNIO SILVÉRIO CABRITA⁴,
FILOMENA BOTELHO^{2,3,5} and CARLOS FREIRE DE OLIVEIRA³

¹Gynecology A Service, Coimbra Hospital and University Center, Coimbra, Portugal;

²Biophysics Unit – Institute for Biomedical Imaging and Life Sciences,

³Centre of Investigation on Environment, Genetics and Oncobiology, ⁴Experimental Pathology Institute,

⁵Neurosciences Institute, Institute for Biomedical Imaging and Life Sciences, and

⁶Laboratory of Biostatistics and Medical Informatics, Faculty of Medicine of Coimbra University, Coimbra, Portugal

Abstract. *The aim of this study was to characterize the hematogeneous spread, in vivo, of breast cancer (BC) cell lines that express hormonal receptors (HR) comparing with triple-negative (TN) BC, particularly considering the lung and liver. Female Balb/c nu nu mice (n=30) were injected with two breast cancer cell lines (MCF7 and HCC1806). Nuclear medicine imaging with Technetium (^{99m}Tc)-hydroxymethylene diphosphonate (^{99m}Tc-HMDP) and ^{99m}Tc-Hexakis 2-methoxy-2-methylpropylisonitrile (MIBI) were performed between the 7th and 8th weeks after injection. The histological metastatic foci were analyzed by morphometric and immunohistochemistry studies regarding estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (ERBB2) and cytokeratin (CK)-5/6. The mean area of lung metastasis in MCF7 cases was significantly higher (p=0.023), although the number of liver foci was higher in the HCC1806 group (p=0.006). Logistic regression revealed a potentiating model for liver metastasis with HCC1806 cells (odds ratio=16; p=0.03). The number and area of lung-metastatic foci were not predictive of liver dissemination. Lung metastasis study showed ER positivity in 57.1% of the MCF7 group, compared to 80% of the HCC1806 group. PR was positive in 42.9% of MCF7 cases and negative in 60% of HCC1806 cases. HR-positive cells developed massive lung metastatization. TN cells seem to potentiate liver metastasis. ER, PR, ERBB2 and basal-like CK expression in metastases was not uniformly correlated with that of primary tumor cells.*

Correspondence to: Maria João Carvalho, Azinhaga de Santa Comba, Celas, 3000-548 Coimbra, Portugal. E-mail: mariaj.carvalho@sapo.pt

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Breast cancer represents a heterogeneous group of tumors. In clinical practice, tumors are stratified into three main groups: hormonal receptor (HR)-positive, namely for estrogen (ER) and progesterone (PR) receptors, which respond to therapeutics targeting ER and to chemotherapy; ERBB2-positive tumors, candidate for treatment with the monoclonal antibody trastuzumab; and triple-negative tumors (TN), HR- and ERBB2-negative, for which the only available systemic therapy is conventional chemotherapy.

Therapeutic strategies in metastatic breast cancer depend on tumor biology. About 30-40% of cases of metastatic breast cancers respond to hormone therapy, but there are other fractions that are stable under adjuvant chemotherapy (1, 2). Hormone therapy has less toxicity and a lasting suppressive effect, but despite this, classic chemotherapy has a better response rate (50-60%) (3). The systematic evaluation of the expression of HRs (ER and PR) has great advantage in selecting patients that benefit from adjuvant hormone therapy and those who benefit from other strategies.

In metastatic disease, response depends on the maintenance of these receptors in relation to the primitive tumor. Previous studies have demonstrated that 10% to 20% of primary tumors that express ER are negative for this receptor in their metastases (4). The response rate in these cases was 12% compared to 74% when the expression of ER was positive in both metastases and primary tumor. The intensity of ER positivity also correlates with hormonal response and is potentiated by the presence of PR (1, 5). Patients with breast cancer without ER benefit from cytostatic therapy, with a risk reduction of 35% compared to only 20% in those with ER-positive tumors (6).

Tumors that overexpress ERBB2 have an aggressive biological behavior and are associated with a higher rate of recurrent disease and lower survival than ERBB2-negative

tumors (7). The expression of ERBB2 in the primary tumor and in axillary metastases is not concordant; previous studies describe a concordance rate of 77% in tumors overexpressing ERBB2 and of 95% in ERBB2-negative tumors (8). Expression in metastases may be concordant (98%) but the number of cases is less representative and refers to different locations (9).

TN breast cancer represents 10% to 17% of all breast cancer (10,11). These tumors present aggressive clinical behavior and confer worse prognosis despite response to conventional chemotherapy (12-15). The interest in TN arose due to the lack of targeted therapies and a similarity with basal-like tumors of the molecular classification of breast cancer (16-18). Another issue is the short interval to first metastasis compares with other sub-types of breast cancer (11). The preferential metastatic spread of these tumors is hematogeneous and distant metastases develop more frequently in the brain and lungs (19).

The present study aimed to evaluate the *in vivo* hematogeneous dissemination of TN (HCC1806) compared with HR-positive (MCF7) breast cancer cells. The main objective was the evaluation of metastasis after injection of these cells in the mouse tail vein, using nuclear imaging methods, histological analyses and immunohistochemistry. We aimed to evaluate the phenotype of secondary lesions compared with the initially injected cells regarding ER, PR, ERBB2 and basal cytokeratins (CK5/6).

Materials and Methods

Cell culture. The human breast cancer cell lines MCF-7, which expresses ER and PR, and HCC1806, which does not express ER, PR or ERBB2, were obtained from the American Type Culture Collection (ATCC, Middlesex, UK). The cell lines were maintained and expanded in accordance with ATCC recommendations with Dulbecco's modified Eagle's medium (DMEM), for MCF-7 cells and RPMI-1840, for HCC1806 (Sigma, Missouri, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) at 37°C in 95% air and 5% CO₂ atmosphere, in a humidified incubator.

Immediately prior to *in vivo* studies, cells were detached using a solution of 0.25% trypsin-EDTA (Gibco, Paisley, UK) and cell suspensions with 1.5×10^6 cells were prepared. The cells were labeled with ^{99m}Tc-Hexamethylpropyleneamine Oxime (HMPAO) according to Bassa *et al.* (20).

Animal studies. Four to 6-week-old female athymic nude mice (Balb/c *nu/nu*), with 18-22 g weight, were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA) and housed under conditions in accordance with the Institution of Animal Care of the University of Coimbra and European Community. The study was approved by the Ethics Committee (reference: IBB/58/08, approved in 10 July 2008).

Three experimental groups were considered: group 1 consisted of 17 animals injected in the tail vein with MCF7 cells; group 2 consisted of 13 animals injected into the tail vein with HCC1806 cells; and group 3 consisted of 10 animals as a control group.

For cell administration and for the acquisition of images, the mice were anesthetized with a solution of ketamine (77%) and chlorpromazine (23%), administered subcutaneously.

Administration of the labeled cells was performed with the animals placed in prone position over the detector of a gamma camera (GE 400AC; Milwaukee, Wisconsin, USA) coupled with a low-energy and high-resolution parallel hole collimator. During this procedure, a dynamic sequence of 60 images of 10 sec each for a matrix of 128×128 pixels resolution was acquired allowing recording the trajectory of the cells to the locations of embolization. Image processing was performed in a Xeleris workstation (GE, Buckinghamshire, United Kingdom).

Nuclear imaging studies. Seven to eight weeks after cell injection, and in order to evaluate and characterize the existence of metastases images with ^{99m}Tc- Hexakis 2-methoxy-2-methylpropylisonitrile (MIBI) (MIBI-Cardiolite®; Bristol-Myers and Squibb, New York City, New York, USA) and ^{99m}Tc- hydroxymethylene diphosphonate (^{99m}Tc-HMDP) (Medronate II Agent; GE Healthcare, Buckinghamshire, United Kingdom) were performed. ^{99m}Tc-MIBI is uptaken by mitochondria and used to perform cardiac and tumor acquisitions. ^{99m}Tc-HMDP is a tracer whose accumulation is achieved by binding phosphonate groups with the calcium of hydroxyapatite crystals, hence it is used to perform bone scintigraphy (21).

Labeling of ^{99m}Tc-MIBI was performed according to the manufacturer's instructions by adding approximately 1850 MBq of ^{99m}TcO⁻ in 4 ml of 0.9% NaCl to the pharmaceutical formulation. Immediately after dissolving, the solution was heated at 100°C for 10 minutes. To obtain ^{99m}Tc-HMDP about 11.840 MBq of ^{99m}TcO⁻ in 8 ml of 0.9% NaCl were added to the pharmaceutical formulation and incubated during 20 min according to the manufacturer's instructions. Radiochemical purity for both radiopharmaceuticals was accessed through ascendant thin-layer microchromatography.

^{99m}Tc-HMDP was injected into the dorsal vein of the tail and dynamic images were acquired immediately as described before. Static image of 256×256 pixels, zoom 2, with individual duration of 2 minutes were obtained 30, 60, 90 and 120 min after administration of the radiopharmaceutical. Processing of images was performed on a Xeleris workstation and consisted in drawing regions of interest (ROIs) on tumor projections to obtain activity-time curves.

Seven days after this acquisition, we proceeded to injection 17.6 ± 13.3 MBq of ^{99m}Tc- MIBI in the dorsal vein of the tail and used the same acquisition protocol.

Ex vivo studies. Eight weeks after tumor cell injection, animals were killed by cervical dislocation. Necropsy was performed with harvesting of the lung, liver, brain, kidneys, spleen, uterus and ovaries, as well as all locations macroscopically suspected of metastasis. After tissue collection, each organ was measured and weighed. Concerning the lungs, the different lobes were separated, considering for the right lung a sample of cranial, middle, accessory and caudal lobes and for the left lung a sample of lower and average lobes. The liver was also divided into the left lateral, middle left, middle right and right lateral lobes. The brain was divided into anterior and posterior parts. The samples were immediately preserved in buffered formalin.

Histology and morphometry. Each sample was fixed, embedded in paraffin, cut into sectors and placed on slides randomly, and stained with hematoxylin and eosin (H&E). Each section of pulmonary and

hepatic samples was evaluated for the presence of metastases. All slides with images suggestive of secondary lesions were photographed. Subsequently the images were analyzed by drawing ROIs to obtain the total area of pixels.

Immunohistochemistry. Nine cases were selected from the MCF7 group and eight cases from the HCC1806 group, according to the presentation of metastases. The presence of ER (Dako® N1575, Glostrup, Denmark), PR (Neomarkers® MS-RP-192-TO, Fremont, California, USA), ERBB2/HER-2/neu (Thermo® RB-9040-R7, Waltham, Massachusetts, USA) and CK5/6 (Dako® IR780, Glostrup, Denmark) was evaluated.

For ER, PR and CK5/6 a semiquantitative score based on the intensity and the proportion of labeled tumor cells was assigned 0, +, ++, +++. The cut-off value for positivity was 10% (+), about 50% of cells moderate (++), intense and diffuse (+++).

For ERBB2, Food and Drug Administration criteria were used considering unmarked (0); weak marking in fewer than 30% of cells (+); complete marking of the membrane in fewer than 10% of cells (++); and intense and uniform labeling membrane by at least 30% of the cells (+++). Marking of (++) was considered for overexpression of ERBB2 (22).

Statistical analysis. To study the statistical significance of histology and morphometric data, we compared the mean values obtained for each parameter by ANOVA with multiple comparisons by *post-hoc* Tukey test whenever needed. We used logistic regression analysis to iterate, by blocks, and logistic regression analysis by the enter method to identify possible predictors of liver metastasis choosing the best fit method *via* the Hosmer-Lemeshow test. The results were confirmed using principal components categorical analysis.

In the data analysis concerning marking by immunohistochemistry, McNemar test was used.

All results were evaluated at a significance level of 0.05, and obtained with SPSS, version 17.0, Armonk, New York, USA).

Results

Image analysis. After injection of cells labeled with ^{99m}Tc -HMPAO, the dynamic images revealed an embolization profile predominantly in pulmonary localization, as shown in Figure 1.

The metastatic foci were evaluated with nuclear imaging using the ^{99m}Tc -MIBI and ^{99m}Tc -HMDP radiotracers, 7 to 8 weeks after tumor cell injection. The images of target organs such as liver, lungs and brain were not significantly different considering the animals injected with tumor cells and the control group. Despite this, the use of these radiotracers detected possible metastatic foci. One of the cases (a mouse from the MCF7 group) showed an increased uptake of ^{99m}Tc -HMDP that represented a bone metastasis (Figure 2A). A second case corresponded to a pelvic metastasis detected by hyperfixation with ^{99m}Tc -MIBI, also in an animal from the MCF7 group (Figure 2B). These findings were confirmed by histological *ex vivo* studies.

Ex vivo studies. The mass, nose-tail length and nose-anus length were homogeneous for the animals of each group.

In the MCF7 group, two cases were found to have macrometastases apart from those in the lung and liver. In one of the cases, there was a neoformation in the left femur, with round, imprecise limits and white coloration, of size 15×16×14 mm, infiltrating the adjacent tissue. The other case was a pelvic mass, adherent to the retroperitoneum and deviating the uterus to the left, 10 mm in largest diameter. The mass presented an oval shape, irregular surface and yellow color. Sectioning revealed macroscopic central necrosis.

Lung metastasis. All animals injected with cell lines had foci of lung metastasis (n=30). The mass of the right lung was not significantly different considering the cell line injected, but there was a significant trend ($p=0.058$) for an increase in the mass of left lung, which was superior in the MCF7 group (0.10 ± 0.01 g) compared to the control group (0.05 ± 0.01 g).

The number of pulmonary foci was similar in mice injected with MCF7 and HCC1806 cells, but the mean area of each focus in each pulmonary lobe was significantly higher in the MCF7 group ($p=0.023$). The medial region of left lung had the highest area considering this cell line ($p=0.04$) (Table I). The histology is represented in Figure 3, showing a greater extent of pulmonary lesions in the MCF7 group compared with the HCC1806 group (Figure 3).

Liver metastasis. In general, liver metastases were detected in 14 cases (47%). Considering the group injected with MCF7 cells, liver metastases were found in 29% (n=5). On the other hand, the animals injected with HCC1806 cells developed this secondary lesion in 69% (n=9). The number of metastatic foci was significantly higher ($p=0.006$) comparing HCC1806 with MCF7 cells. There was no significant difference between the groups considering the different liver lobes (Table II). Histological images (Figure 4) illustrate a greater number of liver foci in the HCC1806 group than in the MCF7 group. The mean area of liver metastasis in each lobe was not significantly different by group ($p>0.05$).

The logistic regression showed that HCC1806 cells potentiated the development of liver metastasis (odds ratio=16; $p=0.030$); this predictive model had a sensitivity of 88.9%, specificity of 66.7% and an accuracy of 77.8%. The number, area and sum of areas of lung metastases were not predictive of liver metastasis. A second model of prediction was found, with an inferior accuracy (70%). It was found that the presence of MCF7 cells was associated with a decreased risk and did not potentiate liver metastasis. The estimated decreased risk of developing liver metastasis with MCF7 cells was approximately 5.4-fold higher ($p>0.05$). This model had a sensitivity of 75% and specificity

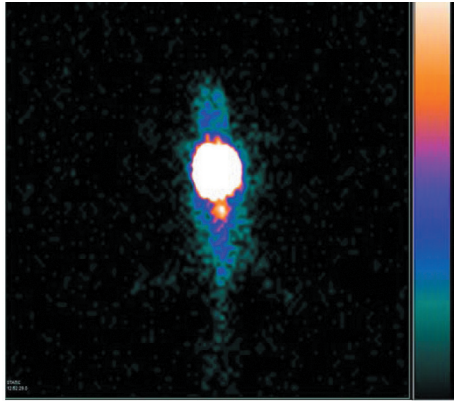


Figure 1. Pulmonary embolization of breast cancer tumor cells labelled with ^{99m}Tc - Hexamethylpropyleneamine Oxime (HMPAO).

of 64.3%. Considering Figure 5, liver metastasis was independent of the number of lung foci. On the other hand, liver metastasis was related to the presence of HCC1806 cells and absence of MCF7 cells, despite the first association being stronger. There was an association between liver metastasis and a smaller mean area of lung foci and the sum of areas in each lung lobe, but these variables were not identified as being predictive of liver metastasis in the logistic regression.

Immunohistochemistry. Immunohistochemistry was performed on lung samples for ER, PR, ERBB2 and CK5/6 (Table III). The alteration of ER, PR in HCC1806 group was identical to that of the MCF7 group ($p>0.05$). The change in ERBB2 expression in HCC1806 cells was not significantly

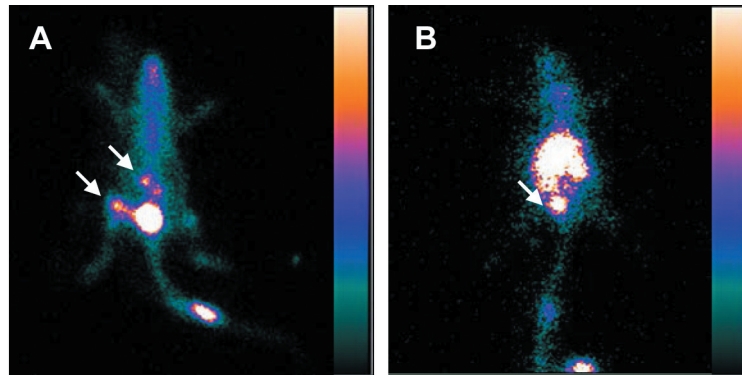


Figure 2. A: Static image obtained after ^{99m}Tc -hydroxymethylene diphosphonate (^{99m}Tc -HMDP) administration; arrows: hyperfixation representing bone metastasis. B: Static image obtained after ^{99m}Tc -Hexakis 2-methoxy-2-methylpropylisonitrile (^{99m}Tc -MIBI) administration; arrow: hyperfixation representing pelvic metastasis.

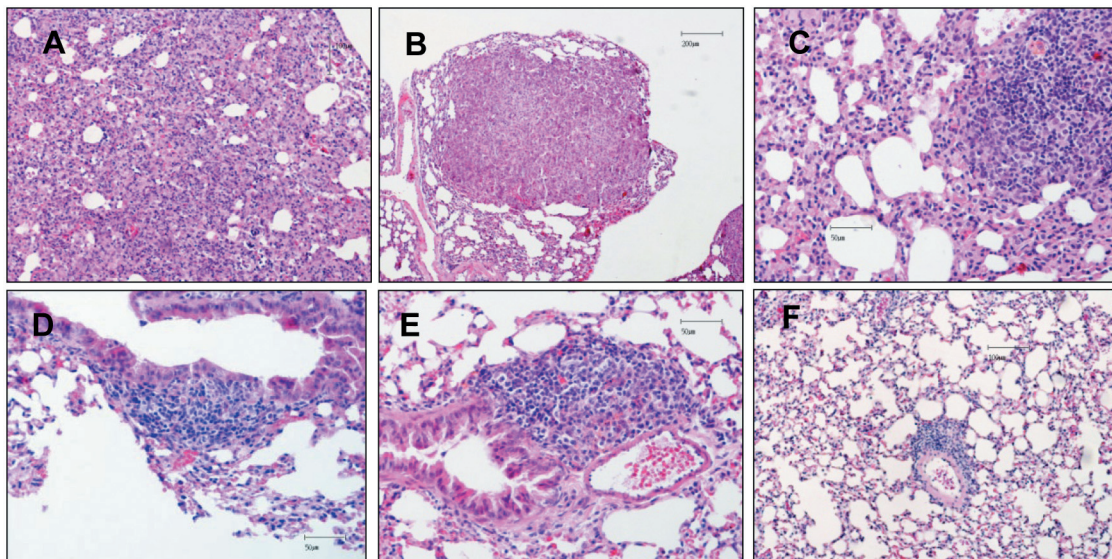


Figure 3. Histology (hematoxylin and eosin staining) of lung metastases. A, B and C: MCF7; D, E and F: HCC1806 (Magnification: A and C, 400 \times ; B, D, E, F, 100 \times).

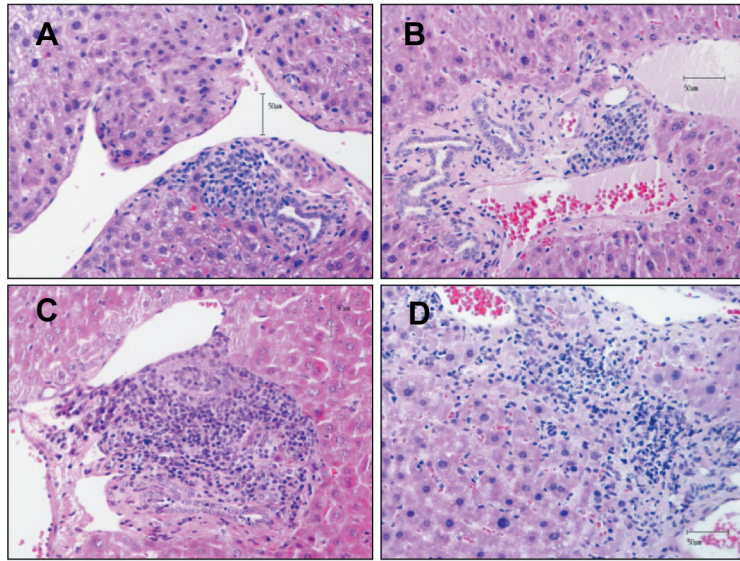


Figure 4. Histology (hematoxylin and eosin staining) of liver metastases. A and B: MCF7; C and D: HCC1806 (magnification: 100 \times).

different comparing with MCF7 cells, although a case with ERBB2 overexpression was found in the HCC1806 group. CK5/6 expression was positive in only one case of the HCC1806 group.

In the group injected with MCF7 cells, the phenotype was similar to that of the original cells in three cases (42.8%). In the HCC1806 group, the TN group, the maintenance of cell phenotype was not described in any case.

Discussion

Metastasis is still one of the main issues in breast cancer. Despite surgery, systemic therapy and radiotherapy, metastasis occurs in a large proportion of patients and about one-third already have metastases at diagnosis (23).

The development of a secondary lesion depends on several factors, including the characteristics of original tumor, cell survival in the circulation and growth in a distant organ (24). Metastasis is an inefficient process, as only a small proportion of cells complete all steps (25).

In the present study, we used an animal model to study the metastasis and growth of human breast cancer cells, a simple procedure that allows for genetic and pharmacological manipulation of tumor cells. Despite the introduction of tumor cells into the circulation not being considered a true process of metastasis (26), this methodology does allow the evaluation of circulatory dissemination according to tumor cell phenotype. The procedure starts from the presence of disseminated cells and the subsequent steps that lead to a secondary lesion. This type of dissemination is preferential

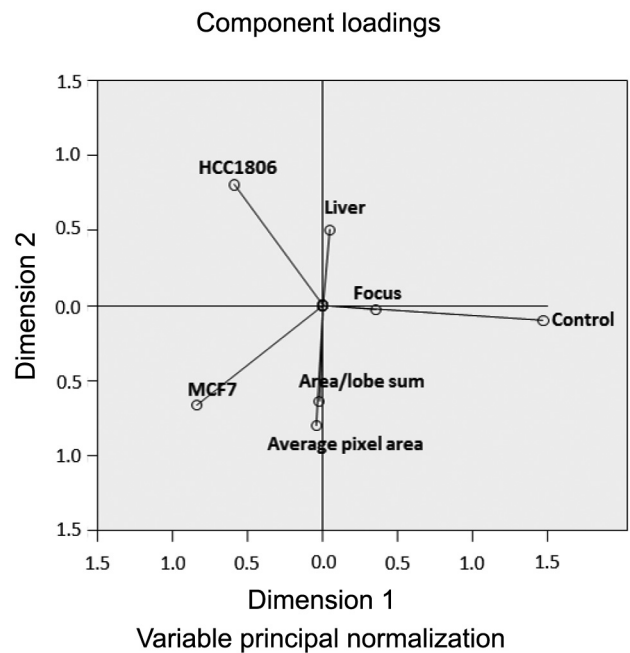


Figure 5. Graphic representation of the logistic regression model for the factors predictive of liver metastasis.

for visceral metastasis. Spontaneous metastasis from primary tumor may occur in orthotopic animal models, however, it does not happen frequently and takes a long time after cell inoculation. Nevertheless, there exist factors that should be

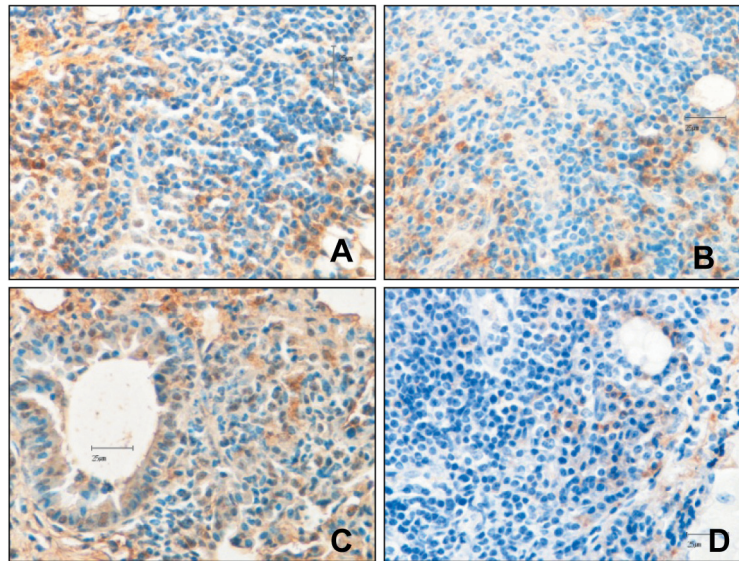


Figure 6. Immunohistochemistry of lung metastasis. A: Estrogen receptor positivity in the MCF7 group; B: progesterone receptors positivity in the VHCC1806 group; C: human epidermal growth factor receptor 2 (ERBB2) positivity in the MCF7 group; D: cytokeratin 5/6 staining in the HCC1806 group (magnification: 100 \times).

Table I. Mean area (pixels) of metastatic foci of each pulmonary lobe.

	Cell line	N	Mean area (pixels)	Standard deviation	Minimum	Maximum
Foci/lobe	MCF7	17	58192.1	49387.2	10839.5	19916.3
($p=0.023$)	HCC1806	13	24263.5	12369.9	8816.4	56895.8
	Total	30	43489.7	41248.5	8816.4	199163.3
Foci/lobe	MCF7	16	53983.6	64046.9	8854	254841
Right cranial	HCC1806	12	26646.2	22526.2	6163	74619
($p=0.171$)	Total	28	42267.5	51724.5	6163	254841
Foci/lobe	MCF7	16	37088.2	25456.9	8449	109706
Right medial	HCC1806	11	33553.6	24624.7	8898	90849
($p=0.723$)	Total	27	35648.2	24702.9	8449	109706
Foci/lobe	MCF7	13	69315.3	78741.2	5726	260222
Right caudal	HCC1806	11	21373.9	12188.4	6912	45599
($p=0.059$)	Total	24	47342.2	62408.9	5726	260222
Foci/lobe	MCF7	13	97271.2	152167.4	7564	569825
Right accessory	HCC1806	12	22935.8	15516.4	6955	59555
($p=0.106$)	Total	25	61590.2	114562.2	6955	569825
Foci/lobe	MCF7	15	50386.8	41533.7	11015	136705
Left medium	HCC1806	11	22213.8	11655.1	7462	40996
($p=0.040$)	Total	26	38467.5	34954.9	7462	136705
Foci/lobe	MCF7	14	35904.3	32183.8	12518	119062
Left inferior	HCC1806	10	24916.6	23251.5	5051	84738
($p=0.368$)	Total	24	31326.1	28768.4	5051	119062

considered. Using the injection of tumor cells into the tail vein, it is not possible to perform a total correlation with the dissemination process, particularly considering the immune response and influence of stroma compounds. We aimed to

evaluate the differential metastasis of two breast cancer cell lines with distinct phenotypes. The inherent aspects of the metastatic model are present in both groups, which allows the investigation to be completed in a short period of time,

Table II. Number of metastatic liver foci.

Location	Injected cell line	n	Mean	Standard deviation	Minimum	Maximum
Total ($p=0.011$)	MCF7	17	1.2	2.04	0	6
	HCC1806	13	5.8	6.55	0	22
	Total	30	3.2	5.04	0	22
Left lateral lobe ($p=0.149$)	MCF7	4	1.8	0.96	1	3
	HCC1806	3	3.3	1.53	2	5
	Total	7	2.4	1.39	1	5
Left medial lobe ($p=0.214$)	MCF7	4	1.3	0.50	1	2
	HCC1806	7	4.0	4.00	1	11
	Total	11	3.0	3.41	1	11
Right medial lobe ($p=0.257$)	MCF7	3	2.3	1.53	1	4
	HCC1806	6	4.7	3.01	1	10
	Total	9	3.9	2.76	1	10
Right lateral lobe ($p=0.604$)	MCF7	1	1.0	–	1	1
	HCC1806	6	1.5	0.84	1	3
	Total	7	1.4	0.78	1	3

simultaneously showing reproducibility, which is confirmed by the metastasis that was observed in the lungs, developed by all animals from both groups, in accordance to previous reports (27-30). This model was developed to provide experimental data that allow evaluation of this type of metastasis. In fact, there are aspects that limit direct clinical implication, mainly the response of host immune system.

The necropsy revealed lung metastasis in all animals and liver metastasis in 47%. There was a case of bone metastasis and another with a pelvic mass in the HR-positive group. The distribution of circulating tumor cells does not seem be a random phenomenon (31). Tumor cells have dimensions that are superior to those of capillary vessels, with preferential interactions with microvasculature (32). In lungs and liver, the distribution of blood flow and cellular embolization are relatively uniform (33). This distribution of tumor cells in these organs is dependent on its vascularization. The clinical trials showed a prevalence of metastasis in lung of 70%, in bone of 70%, in the lymph nodes of 67%, in the liver of 62%, in the brain of 25% and in the ovary of 20% (28). The expression of HRs also influences the type of distant metastasis, bone metastasis being more frequent than visceral metastasis in HR-positive breast cancer (34). Our results emphasize significant metastasis to lungs in the HR-positive group. Hence there are factors that influence the metastatic growth and neovascularization to lungs that are targeted by HRs. In fact, the number of foci was similar between the HR-positive and TN groups, showing that the phenomena of extravasation and survival in an extravascular environment are due to the same molecular mechanisms. But the regulatory pathways for metastatic growth and neovascularization are particularly over-regulated in cells that express HRs. The expression and the function of adhesion molecules members

Table III. Immunohistochemistry of lung metastization considering estrogens receptors (ER), progesterone receptors (PR), human epidermal growth factor receptor 2 (ERBB2) and cytokeratin 5/6.

	MCF7 n (%)	HCC1806 n (%)	p-Value
ER			
Positive	4 (57.1%)	4 (80%)	0.546
Negative	3 (42.9%)	1 (20%)	
PR			
Positive	3 (42.9%)	2 (40%)	0.546
Negative	4 (57.1%)	3 (60%)	
ERBB2			
Positive		1 (20%)	0.125
Negative	7 (100%)	4 (80%)	
CK5/6			
Positive		1 (20%)	0.125
Negative	7 (100%)	4 (80%)	

of the integrin family, cadherins and matrix metalloproteinases may have an important role (35, 36). The C-X-C cytokine receptor type 4 (CXCR4) seems to have a specific function in lung metastasis from breast cancer and its specific ligand CXCL12 allows for activation of signalization pathways (37, 38). ER and PR also influence the expression of growth factors particularly Transforming growth factor alpha (TGF- α), Insulin-like growth factor-1 (IGF1) and -2 (39, 40). Tumor cells exposed to hypoxia activate genes associated with transcription of Hypoxia-inducible factor 1 (HIF1) and -2 (41). It is recognized that 40% of breast tumors present hypoxia areas, with $pO_2 < 2.5$ mmHg (42, 43). The mechanisms that decrease oxygen pressure in secondary tumor of the lungs may be particularly over-activated in ER-positive cells.

Regarding liver metastasis, the results showed that circulatory dissemination was more relevant in TN cells that are resistant in systemic circulation and metastasis occurs in a uniform way in the hepatic parenchyma. Hematogeneous dissemination has been described as preferential in basal-like cells that have a phenotype partially similar to TN cells (44, 45). Some pulmonary and hepatic studies demonstrated cellular viability several hours after placement of cells in circulation (27). This type of cell tolerates being in intravascular spaces in the lung and despite hemodynamic stress, is returned to the systemic circulation, leading to extra-pulmonary metastasis.

It is not established if the tumor phenotype in metastasis is a characteristic of natural history or a direct consequence of therapy. Considering ER, in lung metastatic foci, there was maintenance of the expression of the phenotype of the injected cells in 57.1%. In clinical trials, the expression of ER in locoregional recurrence and at distant metastasis can reach 97% (46). ER negativity in metastasis seems to be consistent (46, 47). But these conclusions refer to retrospective studies with divergent criteria and techniques of characterization of receptors. The results of our experimental study reflect a systemic evaluation of ER in metastasis as being predictive of hormone therapy response. The loss of expression of PR reached 57%, which correlates with inferior response to hormone therapy and promotion of metastasis. The metastatic process is a late event in the natural history of this type of tumor, usually with good prognosis. This points to possible systemic therapy in metastatic breast cancer under hormone therapy. Bone metastasis is another issue considering HR-positive breast cancer that was not described in our experimental data, that focused on secondary tumors of lung and liver.

Regarding ERBB2, we only found one case of overexpression in metastasis in the HCC1806 group, pointing to acquisition in the metastatic process, a phenomenon that elects patients to targeted-therapies. The overexpression of ERBB2 is not the same as the primary tumor (48-51). The alteration of its expression may be the result of the selection of an ERBB2-expressing clone or heterogeneous expression inside the tumor with higher potential for metastasis.

TN tumors are similar to basal-like ones, which are thought to derive from myoepithelial cells. CK5/6 expression is a method to identify basal-like tumors (12, 52). Our results emphasize a dichotomy in TN tumors. On the one hand, the basal-like phenotype in metastasis was present in only one part of the TN cells. On the other hand, applying this CK to evaluate the phenotype can be limiting. Some studies note a specificity of 80% of CK5/6 to the basal-like phenotype (53, 54). We found that the discordance of basal-like phenotype from the primary tumor can reach 30%. The expression of this and other markers with therapeutic implication are of great importance in studying tumor progression.

Conclusion

All animals developed lung metastases after injection of tumor cells into the tail vein. The area of metastases in animals injected with cells expressing HRs were significantly higher than those with TN tumors, emphasizing a possible influence of HRs in the expression of growth factors and neovascularization in the lung microenvironment. Liver metastases were superior in animals injected with TN cells, reflecting a higher resistance of this type of cell in the circulation. The predictive model applied showed an inferior risk of liver metastases with HR-positive cells and superior risk with TN cells. Lung metastases are not predictive of liver metastases.

The phenotype of secondary lesions was not significantly different in the two groups analyzed. The global maintenance of HRs reached 42.8% and in TN group, the markers changed in all cases. These experimental results highlight the complex behavior of metastatic breast cancer which clinically translates into the need for adequate targeted-therapies.

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References

- 1 Bezwoda WR, Esser JD, Dansey R, Kessel I and Lange M: The value of estrogen and progesterone receptor determinations in advanced breast cancer. Estrogen receptor level but not progesterone receptor level correlates with response to tamoxifen. *Cancer* 68: 867-872, 1991.
- 2 Elledge RM, Green S, Pugh R, Allred DC, Clark GM, Hill J, Ravdin P, Martino S and Osborne CK: Estrogen receptor (ER) and progesterone receptor (PgR), by ligand-binding assay compared with ER, PgR and pS2, by immunohistochemistry in predicting response to tamoxifen in metastatic breast cancer: a Southwest Oncology Group Study. *Int J Cancer* 89: 111-117, 2002.
- 3 Rastelli F and Crispino S: Factors predictive of response to hormone therapy in breast cancer. *Tumori* 94: 370-383, 2008.
- 4 Kuukasjärvi T, Kononen J, Helin H, Holli K and Isola J: Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. *J Clin Oncol* 14: 2584-2589, 1996.
- 5 Ravdin PM, Green S, Dorr TM, McGuire WL, Fabian C, Pugh RP, Carter RD, Rivkin SE, Borst JR and Belt RJ: Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: results of a prospective Southwest Oncology Group study. *J Clin Oncol* 10: 1284-1291, 1992.

- 6 Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer. *Cochrane Database Syst Rev* (1):CD000486, 2001.
- 7 Ross JS, Fletcher JA, Bloom KJ, Linette GP, Stec J, Clark E, Ayers M, Symmans WF, Pusztai L and Hortobagyi GN: HER-2/neu testing in breast cancer. *Am J Clin Pathol* 120: S53-S71, 2003.
- 8 Fernö M, Stål O, Baldetorp B, Hatschek T, Källström AC, Malmström P, Nordenskjöld B and Rydén S: Results of two or five years of adjuvant tamoxifen correlated to steroid receptor and S-phase levels. South Sweden Breast Cancer Group, and South-East Sweden Breast Cancer Group. *Breast Cancer Res Treat* 59: 69-76, 2001.
- 9 Gong Y, Booser DJ and Sneige N: Comparison of HER2 status determined by fluorescence *in situ* hybridization in primary and metastatic breast carcinoma. *Cancer* 103: 1763-1769, 2005.
- 10 Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, Ollila DW, Sartor CI, Graham ML and Perou CM: The triple-negative paradox: primary tumor chemosensitivity of breast cancer subtypes. *Clin Cancer Res* 13: 2329-2334, 2007.
- 11 Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P and Narod SA: Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res* 13: 4429-4434, 2007.
- 12 Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M and Perou CM: Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 10: 5367-5374, 2004.
- 13 van de Rijn M, Perou CM, Tibshirani M, Haas P, Kallioniemi O, Kononen J, Torhorst J, Sauter G, Zuber M, Köchli OR, Mross F, Dieterich H, Seitz R, Ross D, Botstein D and Brown P: Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. *Am J Pathol* 161: 1991-1996, 2002.
- 14 Tischkowitz M, Brunet JS, Bégin LR, Huntsman DG, Cheang MC, Akslen LA, Nielsen TO and Foulkes WD: Use of immunohistochemical markers can refine prognosis in triple-negative breast cancer. *BMC Cancer* 7: 134, 2007.
- 15 Bauer KR, Brown M, Cress RD, Parise CA and Caggiano V: Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer* 109: 1721-1728, 2007.
- 16 Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Borresen-Dale AL, Brown PO and Botstein D: Molecular portraits of human breast tumours. *Nature* 406: 747-752, 2000.
- 17 Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lønning PE and Borresen-Dale AL: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 98: 10869-10874, 2001.
- 18 Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lønning PE, Brown PO, Borresen-Dale AL and Botstein D: Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* 100: 8418-8423, 2003.
- 19 Tsuda H, Takarabe T, Hasegawa F, Fukutomi T and Hirohashi S: Large, central acellular zones indicating myoepithelial tumor differentiation in high-grade invasive ductal carcinomas as markers of predisposition to lung and brain metastases. *Am J Surg Pathol* 24: 197-202, 2000.
- 20 Bassa P, García Garzón JR, Piera C, Pavía A, Minoves A, Moragas M, Pavía J, Lomeña F and Setoain J: Procedure for red blood cell labelling with ^{99m}Tc-HMPAO. Methodology and quality control. *Nucl Med Biol* 21: 963-967, 1994.
- 21 Ogawa K and Saji H: Advances in drug design of radiometal-based imaging agents for bone disorders. *Int J Mol Imaging* 537687, 2011.
- 22 Wilkinson HA, Dahllund J, Liu H, Yudkovitz J, Cai SJ, Nilsson S, Schaeffer JM and Mitra SW: Identification and characterization of a functionally distinct form of human estrogen receptor beta. *Endocrinology* 143: 1558-1561, 2002.
- 23 Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics, 2007. *CA Cancer J Clin* 57: 43-66, 2007.
- 24 Vaidyan KS and Welch DR: Metastasis suppressors and their roles in breast carcinoma. *J Mammary Gland Biol Neoplasia* 12: 175-190, 2007.
- 25 Weiss L: Comments on hematogenous metastatic patterns in humans as revealed by autopsy. *Clin Exp Metastasis* 10: 191-199, 1992.
- 26 Fantozzi A and Christofori G: Mouse models of breast cancer metastasis. *Breast Cancer Res* 8: 212, 2006.
- 27 MacDonald IC, Groom AC and Chambers AF: Cancer spread and micrometastasis development: quantitative approaches for *in vivo* models. *Bioessays* 24: 885-893, 2002.
- 28 Tarin D: New insights into the pathogenesis of breast cancer metastasis. *Breast Dis* 26: 13-25, 2006.
- 29 Welch DR: Technical considerations for studying cancer metastasis *in vivo*. *Clin Exp Metastasis* 5: 272-306, 1997.
- 30 Morris VL, Schmidt EE, Koop S, MacDonald IC, Grattan M, Khokha R, McLane MA, Niewiarowski S, Chambers AF and Groom AC: Effects of the disintegrin eristatin on individual steps of hematogenous metastasis. *Exp Cell Res* 219: 571-578, 1995.
- 31 Carvalho MJ, Laranjo M, Abrantes M, Cabrita AS, Botelho MF and de Oliveira CF: Breast cancer circulating tumor cells. *Oncol rev* 2: 225-235, 2009.
- 32 Chambers AF, Naumov GN, Varghese HJ, Nadkarni KV, MacDonald IC and Groom AC: Critical steps in hematogenous metastasis: an overview. *Surg Oncol Clin N Am* 10: 243-255, 2001.
- 33 Cameron MD, Schmidt EE, Kerkvliet N, Nadkarni KV, Morris VL, Groom AC, Chambers AF and MacDonald IC: Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency. *Cancer Res* 60: 2541-2546, 2000.
- 34 Campbell FC, Blamey RW, Elston CW, Nicholson RI, Griffiths K and Haybittle JL: Oestrogen-receptor status and sites of metastasis in breast cancer. *Br J Cancer* 44: 456-459, 1981.
- 35 Cavallaro U and Christofori G: Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer* 4: 118-132, 2004.
- 36 Lopez JI, Camenisch TD, Stevens MV, Sands BJ, McDonald J and Schroeder JA: CD44 attenuates metastatic invasion during breast cancer progression. *Cancer Res* 65: 6755-6763, 2005.

- 37 Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verástegui E and Zlotnik A: Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410: 50-56, 2001.
- 38 Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ and Krek W: Chemokine receptor CXCR4 down-regulated by von Hippel-Lindau tumour suppressor pVHL. *Nature* 425: 307-311, 2003.
- 39 Lee AV, Cui X and Oesterreich S: Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. *Clin Cancer Res* 7: 4429s-4435s, 2001.
- 40 Cui X, Zhang P, Deng W, Oesterreich S, Lu Y, Mills GB and Lee AV: Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells *via* the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer. *Mol Endocrinol* 17: 575-588, 2003.
- 41 Semenza GL: Hypoxia, clonal selection, and the role of HIF1 in tumor progression. *Crit Rev Biochem Mol Biol* 35: 71-103, 2001.
- 42 Chaudary N and Hill RP: Hypoxia and metastasis in breast cancer. *Breast Dis* 26: 55-64, 2006.
- 43 Vaupel P, Mayer A, Briest S and Hockel M: Oxygenation gain factor: a novel parameter characterizing the association between hemoglobin level and the oxygenation status of breast cancers. *Cancer Res* 63: 7634-7637, 2003.
- 44 Fulford LG1, Reis-Filho JS, Ryder K, Jones C, Gillett CE, Hanby A, Easton D and Lakhani SR: Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival. *Breast Cancer Res* 9: R4, 2007.
- 45 Tsuda H, Takarabe T, Hasegawa F and Fukutomi T: Large Central a cellular zones indicating myoepithelial tumor differentiation in high-grade invasive ductal carcinomas as markers of predisposition to lung and brain metastases. *Am J Surg Pathol* 24: 197-202, 2000.
- 46 Gomez-Fernandez C, Daneshbod Y, Nassiri M, Milikowski C, Alvarez C and Nadji M: Immunohistochemically determined estrogen receptor phenotype remains stable in recurrent and metastatic breast cancer. *Am J Clin Pathol* 130: 879-882, 1996.
- 47 Kuukasjarvi T, Kononen J, Helin H, Holli K and Isola J: Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. *J Clin Oncol* 14: 2584-2589, 1996.
- 48 Vincent-Salomon A, Jouve M, Genin P, Fréneaux P, Sigal-Zafrani B, Caly M, Beuzeboc P, Pouillart P and Sastre-Garau X: HER2 status in patients with breast carcinoma is not modified selectively by preoperative chemotherapy and is stable during the metastatic process. *Cancer* 94: 2169-2173, 2002.
- 49 Cardoso F, Di Leo A, Larsimont D, Gancberg D, Rouas G, Dolci S, Ferreira F, Paesmans M and Piccart M: Evaluation of HER2, p53, bcl-2, topoisomerase II-alpha, heat-shock proteins 27 and 70 in primary breast cancer and metastatic ipsilateral axillary lymph nodes. *Ann Oncol* 12: 615-620, 2001.
- 50 Edgerton SM, Moore D, Merkel D and Thor AD: ErbB-2 (HER-2) and breast cancer progression. *Appl Immunohistochem Mol Morphol* 11: 214-221, 2003.
- 51 Gancberg D, Di Leo A, Cardoso F, Rouas G, Pedrocchi M, Paesmans M, Verhest A, Bernard-Marty C, Piccart MJ and Larsimont D: Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. *Ann Oncol* 13: 1036-1043, 2002.
- 52 Hicks DG, Short SM, Prescott NL, Tarr SM, Coleman KA, Yoder BJ, Crowe JP, Choueiri TK, Dawson AE, Budd GT, Tubbs RR, Casey G and Weil RJ: Breast cancers with brain metastases are more likely to be estrogen receptor negative, express the basal cytokeratin CK5/6, and overexpress HER2 or EGFR. *Am J Surg Pathol* 30: 1097-1104, 2006.
- 53 Lerma E, Barnadas A and Prat J: Triple-negative breast carcinomas: similarities and differences with basal like carcinomas. *Appl Immunohistochem Mol Morphol* 17: 483-494, 2009.
- 54 Pintens S, Neven P, Drijckoningen M, Van Belle V, Moerman P, Christiaens, A, Smeets MR, Wildiers H and Vanden Bempt I: Triple-negative breast cancer: a study from the point of view of basal CK5/6 and HER-1. *J Clin Pathol* 62: 624-628, 2009.

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