Title: Expression and clinical significance of metalloproteases and their inhibitors by endothelial cells from invasive breast carcinomas

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Corresponding Author's Institution:

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Abstract: Aims: Given that tumor blood vessels are important in tumor progression and metastasis, tumor endothelial cells are the main targets of anti-angiogenic therapy. The aim of the present work was to evaluate the phenotype of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) from endothelial cells (ECs) at the tumor center and its relationship with MMPs/TIMPs global expression, as its relationship with the occurrence of distant metastasis.
Methods: An inmunohistochemical study was performed using tissue arrays and specific antibodies against MMPs (2, 7, 9, 11, 13 and 14) and TIMPs (1, 2 and 3), at tumor center in 104 patients with primary ductal invasive breast tumors.
Results: Our results demonstrated that MMP-11 expression by ECs was related with a shortened relapse-free survival, whereas TIMP-3 expression was related with low occurrence of distant metastasis. In addition, MMP-11 and TIMP-2 expression by ECs was associated with a shortened overall survival, whereas TIMP-3 expression by ECs was associated with a higher overall survival. However, our findings indicate significant relationships between the expression of MMPs/TIMPs by ECs and the global expression of these factors at the tumor scene.
Conclusions: The present work shows a high MMPs/TIMPs expression by ECs from breast carcinomas, which may be consequence of the crosstalk between tumor cells and their surrounding microenvironment.

Response to Reviewers:

Dear Editor,

Please find enclosed the revised version of our manuscript (CBC MS# CBC-D-15-00331). We have revised the paper in response to the reviewer's comments, according to which it has been modified. We thank you and Reviewers for the valuable criticisms and suggestions, which will undoubtedly result in an improved version of our manuscript.

Our specific answers to the reviewer's comments are listed below.

1.   Is there any correlation between expression of any MMP and ER, her2Neu or PR status?

According to Referee's suggestion and as it was shown in Table 5 (previously named Table 4), we had conducted an statistical analysis regarding to the expression of metalloproteases (MMPs) and their inhibitors (TIMPs) by endothelial cells and clinico-pathological characteristics from 104 patients with breast cancer. We found a significant relation between ER and TIMP-3 expression. However, we did not find significant associations between PR or HER-2 and MMPs/TIMPs expressions. These results have been included in the Results section, page 11, paragraph 2: "Table 5 shows the relationships between MMPs/TIMPs expression by ECs and clinicopathological characteristics including age or menopausal status from patients, tumor size, nodal status, histological grade, estrogen or progestorone receptor status, and HER-2 status. Our results only demonstrated a significant and positive relation between MMP-7 expression by ECs and lymph node status, whereas TIMP-3 expression by tumor ECs was significant and negatively associated with lymph node status but significant and positively associated with ER-positive status."
For further clarification, we decided to highlight the significant correlations in Table 5 with bold letters and indicate the p-value above it.
We also included the following information in the Discussion section, page 14, paragraph 2: "In this sense and according to our results, it has also been described a positive correlation between ER and TIMP-3 expression, which is associated with a clinical benefit from endocrine treatment (Jozien Helleman, 2008)"

2.   The specific details, including batch number and catalog number of the specific antibodies and the specific conditions for their optimization in the IHC protocols for each are essential and must be included.

According to the Referee indication, we included the details regarding the catalog number of the antibodies. Also, we believed that we should clarified the specific conditions for inmuhistochemistry, therefore we replaced the following text (previously described in page 7, paragraph 2) by a new Table 2:

"Antibodies for MMPs and TIMPs were obtained from Neomarker (Lab Vision Corporation, Fremont, CA, USA). The dilution for each antibody was the following: 1:50 for MMP-2, -7, -13 and -14; 1:100 for MMP-9, TIMP-1, -2 and -3; and 1:400 for MMP-11. To enhance antigen retrieval, tissue sections were treated in a PT-Link (Dako) at 97°C for 20 min, in citrate buffer (pH 6.1) for MMP-1, -14, TIMP-1 and -3, or in ethylene diamine tetraacetic acid (EDTA) buffer (pH 9) for MMP-13 and TIMP-2. Antibodies against MMP-2, -7, -9 and -11 do not require antigen retrieval."

Regarding to the batch number, we are desolated to report that we can not supply said information. We are no longer in possession of those vials as we consumed them completely.

3.   How well was the generation of the TMAs controlled for tumor content in the tissue cylinder and what regions of the tumor were identified for "coring" and why? How long had the archival blocks been stored on average?

Following the Referee recommendation, we modified the paragraph corresponding to the TAs (Tissue Arrays) in the Materials and Methods section, page 7, paragraph 1, as follows :
"We used routinely fixed (overnight in 10% buffered formalin), paraffin embedded tumor samples stored in ideals conditions of temperature, humidity and light, in our pathology laboratory archives during 20 years on average. TAs blocks were obtained by punching a tissue cylinder (core) with a diameter of 1.5 mm through a histologically representative area of each 'donor' tumor block, which was then inserted into an empty 'recipient' tissue array paraffin block using a manual tissue arrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA) as described elsewhere. A total of 2 cores were employed for each case corresponding to tumor center area (defined as the area inner to the 2 mm surrounding the tumors). Collections of tissue cores was carried out under highly controlled conditions. Areas of non-necrotic cancerous tissue were selected for arraying by a experienced pathologist (LO González). This method, with two cores (double redundancy) has been shown to correlate well with conventional immunohistochemical staining (Vizoso, Br J Cancer, 2007)."

4.   Please explain the rationale for identifying endothelial cells that were selected for analysis.

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 "Stromal cell subsets were distinguished primarily by morphology: CAFs are spindle shaped cells, whereas MICs are round cells and endothelial cells were identify as tubular structures specially when the lumen can be identify". This sentence was included in Materials and Methods section, page 8, paragraph 2.

5.   What was the power of the study for the relapse or survival analysis? It would seem that the numbers would be low for such an analysis.

As recommended by the Referee, we determined the sample size requirements for the study, we calculate the number of events (recurrences or deaths, respectively) needed to obtain a power of 0.80 with an alpha of 0.05, as described by Collet, 1994.

In the table bellow, we include the numbers of events calculated for each analysis:

	Relapse-free survival		Overall survival	
	Number of recurrences required	Number of recurrences included	Number of deaths required	Number of deaths included
MMP-11	12	62	10	45
TIMP-2	60		20	
TIMP-3	60		45	

The number of events included in the study is higher than the number required for all statistically significant analysis. Therefore, we can conclude that the power of the analysis is over 80%.

We also included the following description in the Materials and Methods section, page 9, paragraph 3: "We determined that our sample size for the study achieve the requirements to obtain an 80% of statistical power with an alpha of 0.05".

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Dr. George W. Sledge, M.D.
Editor, Clinical Breast Cancer

January 17th, 2016

Dear Editor,

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Expression and clinical significance of metalloproteases and their inhibitors by endothelial cells from invasive breast carcinomas

Running title: MMPs/TIMPs expression by ECs in breast cancer

Sandra Cid[1], Noemi Eiro[1], Luis O. Gonzalez[1,2], Nana Beridze[1], Julio Vazquez[3] and Francisco J. Vizoso[1,4].

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

MICROABSTRACT

Given that tumor blood vessels are important in tumor progression and metastasis we evaluated the phenotype of matrix metalloproteinases and their tissue inhibitors from endothelial cells at tumor center in patients with breast tumors. We demonstrated a significant relationships between the expression of MMPs/TIMPs by ECs and the global expression of these factors at the tumor scene, which may be consequence of the crosstalk between tumor cells and their surrounding microenvironment.

ABSTRACT

Aims: Given that tumor blood vessels are important in tumor progression and metastasis, tumor endothelial cells are the main targets of anti-angiogenic therapy. The aim of the present work was to evaluate the phenotype of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) from endothelial cells (ECs) at the tumor center and its relationship with MMPs/TIMPs global expression, as its relationship with the occurrence of distant metastasis.

Methods: An inmunohistochemical study was performed using tissue arrays and specific antibodies against MMPs (2, 7, 9, 11, 13 and 14) and TIMPs (1, 2 and 3), at tumor center in 104 patients with primary ductal invasive breast tumors.

Results: Our results demonstrated that MMP-11 expression by ECs was related with a shortened relapse-free survival, whereas TIMP-3 expression was related with low occurrence of distant metastasis. In addition, MMP-11 and TIMP-2 expression by ECs was associated with a shortened

overall survival, whereas TIMP-3 expression by ECs was associated with a higher overall survival. However, our findings indicate significant relationships between the expression of MMPs/TIMPs by ECs and the global expression of these factors at the tumor scene.

Conclusions: The present work shows a high MMPs/TIMPs expression by ECs from breast carcinomas, which may be consequence of the crosstalk between tumor cells and their surrounding microenvironment.

KEY WORDS: Breast cancer, endothelial cells, MMPs, TIMPs, tumor stroma, prognosis.

1. INTRODUCTION

Breast cancer is the most common malignancy in women. Despite early diagnosis and current therapeutic approaches, including targeted therapies which have reduced cancer-specific mortality, there is still a considerable number of patients who experience recurrence with metastatic disease and death. In addition, in spite of an overwhelming amount of molecular data, breast cancer is currently treated on the basis of the status of only 3 clinical markers of cancer cells: estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). It proves to be necessary to find a new approach to elucidate the mechanisms intimately involved in breast cancer progression. It is known that tumors are composed not only of cancer cells but also of other cell types constituting the stroma. The "seed and soil" hypothesis postulates that an appropriate host microenvironment (the soil) is needed for the optimal growth of tumor cells (the seed) [1]. Nowadays, emerging evidences indicates that progression of tumors toward a malignant phenotype does not depend exclusively on the cell-autonomous properties of cancer cells themselves, but is also deeply influenced by tumor stroma reactivity [2-3]. These stromal cells include cancer associated fibroblasts (CAFs), immune cells, pericytes and endothelial cells (ECs).

ECs play a key role in the development and function of blood and lymph vessels. Given that tumors have abundant blood vessels to supply oxygen and nutrition, endothelial cells are ubiquitous within tumors. Angiogenesis is a multistep process that includes the activation of ECs by growth factors, the subsequent degradation of the extracellular matrix (ECM) by proteolytic enzymes such as matrix metalloproteinases (MMPs) followed by the invasion of the ECM, migration and proliferation of ECs, and finally the formation of new capillary tubes [4].

Although tumor blood vessels generally spout from pre-existing vessels and have thought to be genetically normal, they display a markedly abnormal phenotype, including morphological changes. In addition, there are phenotypic heterogeneity of tumor ECs. It has been reported that tumor ECs

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from highly metastatic tumors had more proangiogenic phenotypes than those from low metastatic tumors [5]. Therefore, it is relevant to know the different tumor EC phenotypes and identify their molecular signatures. MMPs and their tissue inhibitors (TIMPs) may be biomarkers of these phenotypes.

MMPs play an essential role in the degradation of the stromal connective tissue and basement membrane components, which are key elements in tumor invasion and metastasis. MMPs are also able to impact *in vivo* on tumor cell behavior as a consequence of their capacity to cleave growth factors, cell surface receptors, cell adhesion molecules, and chemokines/cytokines [6-7].

Furthermore, by cleaving proapoptotic factors, MMPs produce a more aggressive phenotype via generation of apoptotic resistant cells [6]. MMPs also regulate cancer-related angiogenesis, positively through their ability to mobilize or activate proangiogenic factors , and negatively via generation of angiogenesis inhibitors, such as angiostatin and endostatin, cleaved from large protein precursors . In addition, inflammatory cells from tumors stroma play a role in tumor angiogenesis by releasing other factors, such as VEGF, hepatocyte growth factor (HGF) or IL-8, which are able to promote ECs growth and migration. MMPs' activity is specifically inhibited TIMPs but it is now accepted that TIMPs are multifactorial proteins also involved in the induction of proliferation and the inhibition of apoptosis [8-9]. In previous studies, we reported the clinical value of MMP-2, -7, -9, -11, -13 and –14, and TIMP-1, -2 and -3 expression in different stromal cells [10-13], therefore we decided to study these MMPs' expression by ECs.

The aim of the present work was to evaluate the phenotype of MMPs/TIMPs expression by ECs at the tumor center and its relation with the occurrence of distant metastasis.

2. MATERIALS AND METHODS

2.1. Patient selection

This study comprised 104 women with a histologically confirmed diagnosis of early invasive ductal breast carcinoma treated between 1990 and 2001. Some of them were previously included in our preliminary studies on the expression of MMPs and TIMPs in breast cancer [10-13]. We selected women with the following inclusion criteria: invasive ductal carcinoma, T1 or T2 tumor size, at least 6 histopathologically-assessed axillary lymph nodes, and a minimum of 10 years of follow-up in those women without tumor recurrence. The exclusion criteria were the following: metastatic disease at presentation, prior history of any type of malignant tumor, bilateral breast cancer at presentation, having received any type of neoadjuvant therapy, development of loco-regional recurrence during the follow-up period, development of a second primary cancer, and absence of sufficient tissue in the paraffin blocks used for manufacturing the tissue arrays. We randomly selected a sample size of 104 patients, in accordance to 4 different groups of similar size and stratified with regard to nodal status and the development of metastatic disease, which were key variables in our study, to ensure the statistical power of the survival analysis. Patients' characteristics included in the main groups, with or without distant metastases (recurrence), are listed in Table 1. Patients underwent either modified radical mastectomy or wide resection with axillary lymphadenectomy. Data about the criteria for systemic adjuvant therapy or postoperative radiotherapy of the patients were described elsewhere.[10]. The median follow-up period in patients without metastasis was of 183 months, and 28.5 months in patients with metastatic disease. The study adhered to national regulations and was approved by our Institution's Ethics and Investigation Committee.

2.2. Tissue arrays (TAs)

We used Rroutinely fixed (overnight in 10% buffered formalin), paraffin embedded tumor samples stored in ideals conditions in of temperature, humidity and light, our pathology laboratory archives

during 20 years on average were used in this study. TAs blocks were obtained by punching a tissue cylinder (core) with a diameter of 1.5 mm through a histologically representative area of each 'donor' tumor block, which was then inserted into an empty 'recipient' tissue array paraffin block using a manual tissue arrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA) as described elsewhere [10]. A total of 2 cores were employed for each case corresponding to tumor center area (defined as the area inner to the 2 mm surrounding the tumors). Collections of tissue cores was carried out under highly controlled conditions. Areas of non-necrotic cancerous tissue were selected for arraying by a experienced pathologist (LO González). This method, with two cores (double redundancy) has been shown to correlate well with conventional immunohistochemical staining[10]. (ref PMID:17342087;.

2.3. Immunohistochemistry

Immunohistochemistry was carried out on TA sections 5 μm thick using a TechMate TM50 autostainer (Dako, Glostrup, Denmark). Antibodies use were listed in Table 2.for MMPs and TIMPs were obtained from Neomarker (Lab Vision Corporation, Fremont, CA, USA). The dilution for each antibody was the following: 1:50 for MMP-2, -7, -13 and -14; 1:100 for MMP-9, TIMP-1, -2 and -3; and 1:400 for MMP-11. To enhance antigen retrieval, tissue sections were treated in a PT-Link (Dako) at 97ºC for 20 min, in citrate buffer (pH 6.1) for MMP-1, -14, TIMP-1 and -3, or in ethylene diamine tetraacetic acid (EDTA) buffer (pH 9) for MMP-13 and TIMP-2. Antibodies against MMP-2, -7, -9 and -11 do not require antigen retrieval. The negative control was DakoCytomation mouse serum diluted at the same concentration as the primary antibody used. All dilutions were made in Antibody Diluent, (Dako, Glostrup, Denmark) and incubated for 30 min to 2 h at room temperature. Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 min. The EnVision Detection Kit (Dako) was used as the staining detection system.

Sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently coverslipped.

2.4. Immunostaining evaluation

For each antibody preparation studied, the location of immunoreactivity in each cell type was determined. In each case, immunoreactivity was classified into 2 categories depending upon the percentage of cells stained (negative: 0–10% positive cells; positive: >10% positive cells) in each cell type (cancer cells, CAFs and mononuclear inflammatory cells (MICs)). In the event that no tumor was present in a particular core, then the results of the other core analyzed was given. Two certified pathologists (LOG and NB) blinded to the clinical outcome of the patients performed the histological examination. We distinguished stromal cells from cancer cells on the basis of cell size (the latter cells are larger in size). Stromal cell subsets were distinguished primarily by morphology: (CAFs are spindle shaped cells, whereas MICs are round cells and endothelial cells were identify within tubular structures specially when the lumen ca be identify). Additionally, whereas cancer cells are arranged forming either acinar or trabecullar patterns, stromal cells are scattered throughout the tissue.

In the present study all the cases were also quantified for each protein-stained area. An image analysis system with the Olympus BX51 microscope and analysis software (analySISs, Soft imaging system, Münster, Alemania) was employed as follows: tumor sections were stained with antibodies according to the method explained above and counterstained with haematoxylin. There are different optical thresholds for both stains. Each core was scanned with a X400 power objective in two fields per core. Fields were selected searching for the protein-stained areas. The computer program selects and traces a line around antibody-stained areas (higher optical threshold), with the remaining, non-stained areas (haematoxylin-stained tissue with lower optical threshold) standing out as a blue background. Any field has an area ratio of stained vs non-stained areas. A final area ratio was

obtained after averaging two fields. To evaluate immunostaining intensity we used a numeric score ranging from 0 to 3, reflecting the intensity as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. Using an Excel spreadsheet, the mean score was obtained by multiplying the intensity score (I) by the percentage of stained cells (PC) (score: I X PC). This overall score (global expression) was then averaged with the number of cores that were carried out for each patient. If there was no tumor in a particular core, then no score was given. In addition, for each tumor, the mean score of two core biopsies was calculated.

Staining for ERs and PgRs was scored according to the method described by Allred et al [14] and HER-2 staining according to the criteria used for the Herceptest.

2.5. Data analysis and statistical methods

Differences in percentages were calculated with the chi-square test. Immunostaining score values for each protein were expressed as a median (range). Comparison of immunostaining values between groups was made with the Mann-Whitney or Kruskal-Wallis tests. For analysis of relapse-free survival and overall survival analysis, we employed Cox's univariate method. We determined that our sample size of the study achieve the requirements to obtain an 80% of statistical power with an alpha of 0.05.

Expression profiles were analyzed by unsupervised hierarchical clustering, a method that organizes the patterns of protein expression in a tree structure based on similarity. We used the Cluster 3.0 program (average linkage, Pearson's correlation). Results were displayed with the Treeview program. The PASW 18.0 software was used for all calculations.

3. RESULTS

In order to evaluate the MMPs/TIMPs expression by ECs at tumor center from invasive breast carcinomas, we performed over 3,700 determinations in arrays of cancer specimens from 104 patients with primary invasive ductal breast carcinoma.

Figure 1 shows representative examples of MMPs and TIMPs expression in tumors samples. Immunostaining for these proteins shows a cytoplasmic location so in positive cancer cells as in stromal cells (ECs, CAFs and MICs). With regard to MMP-14 expression, it is noteworthy the positive immunostaining at both cytoplasmic and membrane locations. In neoplasms positive for each one of these cell types expressing either MMPs or TIMPs, at least 70% of these cells showed a positive immunostaining of each evaluated field. It is also of note our observation that MMPs/TIMPs expression by ECs from adjacent non-neoplastic tissue were absent of very low compared with ECs from tumor (Figure 1-J).

Most of MMPs and TIMPs were mainly expressed so by ECs as by cancer cells in breast carcinomas (Table 23). However, as can be also seen in Table 23, these proteins were also expressed by CAFs and MICs in a significant percentage of tumors.

With regard to the global expression (score values) for each MMP or TIMP, there is a wide variability among tumors. Table 3 4 shows the relationships between the expression of each one of these factors by tumor ECs and their corresponding scores values. As it can be seen, there are significant and positive correlations.

To identify specific groups of tumors with distinct MMPs/TIMPs immunohistochemical expression profiles by ECs, the obtained data were subsequently evaluated by unsupervised hierarchical clustering analysis for each cell type. This algorithm places proteins on the horizontal axis and

samples on the vertical axis based on similarity of their expression profile, and if appropriate, generates dendrograms with well-defined clusters of cases for each cell type. However, this method did not establish a dendrogram with well-defined clusters of cases according to MMPs/TIMPs expression by ECs from tumors (Figure 2).

Table 4 5 shows the relationships between MMPs/TIMPs expression by ECs and clinicopathological characteristics including age or menopausal status from patients, tumor size, nodal status, histological grade, estrogen or progestorone receptor status, and HER-2 status. Our results only demonstrated a significant and positive relation between MMP-7 expression by ECs and lymph node status, whereas TIMP-3 expression by tumor ECs was significant and negatively associated with lymph node status but significant and positively associated with ER-positive status.

We also investigated the possible association between MMPs/TIMPs expression by ECs and relapse-free survival and overall survival from all patients included in the present study. Our results showed that MMP-11 expression by ECs was associated with a shortened relapse-free survival, whereas TIMP-3 expression is related with low occurrence of distant metastasis. In addition, MMP-11 and TIMP-2 expression by ECs was associated with a shortened overall survival, whereas TIMP-3 expression by ECs was associated with a higher overall survival (Figure 3). However, we did not find significant associations between the others MMPs/TIMPs studied and either relapse-free survival or overall survival.

4. DISCUSSION

This is, to the best of our knowledge, the first study analyzing the expression of wide range of MMPs and TIMPs by ECs from human breast cancer. Our results shown the relevance of MMPs/TIMPs expression by ECs in tumor microenvironment.

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ECs –the building blocks of tumor vasculature- are not merely responsible for provision of oxygen and nutrients. In tumor microenvironment, the crosstalk between ECs and tumor cells was initially shown to activate angiogenesis and vasculogenesis [15]. However, therapeutic agents targeting angiogenesis effectors molecules have merely shown transient patient survival resulting in tumor recurrence [16]. These findings indicate alternative roles for ECs in mediating tumor progression. These actions may be because ECs regulate tumor growth through the secretion of several paracrine factors which might directly regulate tumor growth in a perfusion-independent manner [17-19]. In addition, it has been demonstrated recently that ECs Jag1/notch mediated interaction with breast cancer cell increased their tumorigenicity, stemness and invasiveness, also through a perfusion-independent manner [20]. In this context, our results also support the concept of an active role of ECs in tumor progression. It is because MMPs and TIMPs, which are of key importance in tumor biology, are highly expressed by tumor ECs when compared with these ones from adjacent non-neoplastic tissues. Nevertheless, we found not different clusters of ECs when we consider their MMPs/TIMPs expression profile, which seems to reflect the existence of a wide phenotypic variability of ECs. However, it was remarkable our finding indicating a significant relationship between MMPs/TIMPs expression by ECs and global expression of these factors at the tumor scene. This suggest that ECs seem to adapt to the context they reside in by showing plasticity. In accordance with it, it has been recently demonstrated that in tumor microenvironment, ECs are activated by cell-to-cell contact with tumor cell leading to the up-regulation of mesenchymal phenotypes. Therefore, the tumor cells promote the acquisition of a transient contact-dependent mesenchymal phenotype in ECs contributing to the generation of a pro-tumoral niche [21].

In the present study, we find out some interesting differential aspects in the MMPs/TIMPs expression profile from ECs which may correspond to their phenotypic heterogeneity, but even so have prognostic relevance. Thus, we found that MMP-7 expression by ECs was associated with axillary lymphs involvement, whereas MMP-11 expression was related to distant metastasis development.

However, TIMP-3 expression was associated with a less incidence of both lymph node and hematogenous metastasis.

MMP-7 (matrilysin 1) is a stromelysin, that degrades type IV collagen, fibronectin and laminin. MMP-7 is aberrantly expressed in human breast tumors and its elimination is associated with low invasiveness and slow tumor growth [22].. Our results, indicating an association between MMP-7 expression by ECs and lymph node-positive status are in accordance with previous data of our group indicating that global expression (score values) of this MMPs correlate with lymphatic metastasis [10]. Nevertheless, the present study evaluates the expression of factors in tumor center where it was hypothesized that the putative absence of i lymphatics vessels in invasive breast carcinomas [23]. But, it is possible that MMP-7 expression by ECs might facilitate the lymphatic invasion at other tumor localizations.

MMP-11, also known as stromelysin-3, can only partially degrade some enzymes (e.g., serine proteases), but cannot directly hydrolyze extracellular matrix molecules [24]. Previous studies of our group indicate that MMP-11 expression by intratumoral MICs is a strong indicator of high risk of distant metastatic development [11, 25]. Also, tumors with MMP-11 positive MICs were associated with a high inflammatory molecular profile (higher mRNA levels of IL-1b, IL-5, IL-6, IL-17, IFNβ and NFκB) in breast carcinomas [26].

Recently, we confirmed MMP-11 expression by MICs and TIMP-2 expression by CAFs as the most potent independent prognostic factors [27]. Now, the results of the present work point to the possible value of MMPs/TIMPs expression by ECs as biological markers of distant metastasis. With regard to TIMPs expressions, if TIMPs inhibit MMPs *in vivo*, it should be expected that high levels of these inhibitors would prevent tumor progression and thus be related with low aggressiveness of tumors. However, TIMPs are multifunctional proteins that, in addition to its MMP-inhibitory effect, also

show distinct tumor stimulatory functions involved in the induction of proliferation and inhibition of apoptosis [8-9, 28].

As a reflect of the multifunctional role of TIMPs, it was of note our clinical finding of that TIMP-3 expression by ECs was associated with a low occurrence of distant metastasis. Accordingly, TIMP-3 has been classified as a potential tumor suppressor due to its negative correlation with an aggressive phenotype and shortened disease free survival in breast cancer [29-30]. In this sense and according to our results, it has also been described a positive correlation between ER and TIMP-3 expression, which is associated with a clinical benefit from endocrine treatment[29]. (Jozien Helleman, Clin Cancer Res 2008). In fact, epigenetic silencing of TIMP-3 occurs in a variety of solid tumors-. TIMP-3 is a naturally occurring inhibitor of angiogenesis that limits vessel density in the vascular bed of tumors and curtails tumor growth [31-32]. It has also been proposed, in colon cancer cells and melanoma, that TIMP-3 promotes apoptosis through stabilization of TNF-α receptors on the cell surface, leading to increased susceptibility to apoptosis [33-34]. In addition, it has been recently reported that TIMP-3 may induce apoptosis in ECs by triggering a caspase-independent cell death pathway and targeting a FAK-dependent survival pathway [35]. In this context our data suggest that TIMP-3 expression by ECs might reflect a less functionally pro-metastasis vessels.

In summary, the present work shows a high expression of MMPs and TIMPs by ECs from breast carcinomas, which may be consequence of the crosstalk between tumor cells and their surrounding microenvironment. In addition, we demonstrate that high expression of MMP-11 and TIMP-3 by ECs is associated with a shortened relapse-free survival. Therefore, our results contribute to the phenotype characterization from tumor ECs, which may add to improve a more precise prognostic evaluation in breast cancer.

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Clinical Practice Points

Although tumor blood vessels generally spout from pre-existing vessels and have thought to be genetically normal, they display a markedly abnormal phenotype, including morphological changes. It has been reported that tumor ECs from highly metastatic tumors had more proangiogenic phenotypes than those from low metastatic tumors. Therefore, it is relevant to know the different tumor EC phenotypes and identify their molecular signatures. The present work shows that MMP-11 expression by ECs was related with a shortened relapse-free survival, whereas TIMP-3 expression was related with low occurrence of distant metastasis. In addition, MMP-11 and TIMP-2 expression by ECs was associated with a shortened overall survival, whereas TIMP-3 expression by ECs was associated with a higher overall survival. Therefore MMPs and TIMPs may be biomarkers of these phenotypes.

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FIGURE LEGEND

Figure 1. Representative pictures of mammary cancer patient tissue array immunostaining for the different matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) analyzed in breast cancer patients (400X), (A) MMP-2, (B) MMP-7, (C) MMP-9, (D) MMP-11, (E) MMP-13, (F) MMP-14, (G) TIMP-1, (H) TIMP-2 and (I) TIMP-3. (J) adjacent non-neoplastic (100x). Arrows indicates the expression by different cell types, (1) ECs, (2) cancer cells, (3) CAFs and (4) MICs.

Figure 2. Graphical representation of two-dimensional unsupervised hierarchical clustering results based on immunohistochemistry expression profiles of 10 MMPs/TIMPs by endothelial cells from 104 breast cancer samples. *Rows:* samples; *columns*, proteins. Protein expression are depicted according to a color scale: *red*, positive staining; *green*, negative staining; *gray*, missing data. Dendogram of samples (*to the left of matrix*) and proteins (*above matrix)* represent overall similarities in expression profiles.

Figure 3. Prognostic significance of MMP/TIMPs expression by endothelial cells in patients with breast cancer. Kaplan–Meier survival curves for relapse-free survival and overall survival as a function of MMP-11 (A-B), TIMP-2 (C-D) and TIMP-3 (E-F) expression by endothelial cells.

Table 1
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Table 1. Basal demographics and clinical characteristics of 104 patients with invasive ductal carcinoma of breast included in our study.

Characteristics	Without recurrence No. (%)	With recurrence No. (%)
Total cases	42 (100)	62 (100)
Menopausal status		
Premenopausal	16 (38.1)	17 (27.4)
Postmenopausal	26 (61.9)	45 (72.6)
Tumor size		
T1	23 (54.8)	28 (45.2)
T2	19 (45.2)	34 (54.8)
Nodal status		
N (-)	23 (54.8)	26 (41.9)
N (+)	19 (45.2)	36 (58.1)
Histological grade		
Well Dif. (I)	17 (40.5)	14 (22.6)
Mod. Dif. (II)	21 (50.0)	26 (41.9)
Poorly Dif. (III)	4 (9.5)	22 (35.5)
Estrogen receptors		
Negative	15 (39.5)	33 (55.9)
Positive	23 (60.5)	26 (44.1)
Progesterone receptors		
Negative	17 (44.7)	38 (64.4)
Positive	21 (55.3)	21 (35.6)
Her-2 Status		
Negative	34 (82.9)	43 (81.1)
Positive	7 (17.1)	10 (18.9)
Adjuvant radiotherapy		
No	32 (76.2)	32 (51.6)
Yes	10 (23.8)	30 (48.4)
Adjuvant systemic therapy		
Chemotherapy	18 (42.9)	13 (21.7)
Tamoxifen	13 (31.0)	26 (43.3)
Chemotherpy + Tamoxifen	8 (19.0)	8 (13.3)
No treatment	3 (7.1)	13 (21.7)
Basal-like phenotype		
Non basal-like	22 (64.7)	22 (53.7)
Basal-like	12 (35.3)	19 (46.3)

Table 2
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Table 2: Antibodies used for immunocitochemistry.

Antibody	Antigen retrieval	Dilution	Incubation (min)	Source (Cat. No.)
MMP-2	-	1:50	120	Thermo (MA5-806-P0)
MMP-7	-	1:50	30	Thermo (MA5-14215)
MMP-9	-	1:100	60	Thermo (MA1-12894)
MMP-11	pH6	1:400	60	Thermo (MA1-26627)
MMP-13	pH9	1:50	120	Thermo (MA5-14238)
MMP-14	-	1:200	60	GeneTex (GTX61603)
TIMP-1	pH6	1:100	60	Thermo (MA5-13688)
TIMP-2	pH9	1:100	30	Thermo (MA5-12207)
TIMP-3	pH6	1:100	30	Santa Cruz (SC-9906)

Table 23. Expression of metalloproteases and their inhibitors in the different cellular types from 104 breast carcinomas.

Factors	Endothelial cells	Cancerous cells	CAFs	MICs
MMP-2	72 (62.6%)	37 (35.6%)	27 (26.0%)	1 (1.0%)
MMP-7	74 (71.2%)	89 (85.6%)	75 (72.1%)	52 (50.0%)
MMP-9	94 (90.4%)	82 (78.5%)	18 (17.3%)	12 (11.1%)
MMP-11	91 (87.5%)	92 (88.5%)	74 (71.2%)	36 (34.6%)
MMP-13	76 (73.1%)	80 (76.9%)	55 (52.9%)	36 (34.6%)
MMP-14	95 (91.3%)	96 (92.3%)	87 (83.7%)	59 (56.7%)
TIMP-1	92 (88.5%)	99 (95.2%)	50(48.1%)	29 (27.9%)
TIMP-2	78 (75.0%)	88 (84.6%)	46 (44.2%)	42 (40.4%)
TIMP-3	80 (76.9%)	92 (88.5%)	67 (64.4%)	59 (56.7%)

Data are represented as number of positive cases (percentage)

Abbreviations: CAFs: cancer associated fibroblast; MICs: mononuclear inflamatory cells.

Table 4
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Table ~~3~~4. Relationship between the expression of metalloproteases (MMPs) and their inhibitors (TIMPs) by endothelial cells and the global expression (score values) for each factor in 104 breast carcinomas.

Factors	Score values		
	Negative expression by endothelial cells	**Positive expression by endothelial cells**	**p-value***
MMP-2	0.0 (0-70.0)	0.0 (0-246.0)	**0.043**
MMP-7	25.0 (0-120.0)	145.0 (20.0-270.0)	**0.000**
MMP-9	20.5 (0-59.0)	76.0 (0-273.0)	**0.008**
MMP-11	55.1 (0-210.0)	164.0 (0-279.0)	**0.001**
MMP-13	49.4 (0-138.0)	65.3 (0-234.0)	**0.005**
MMP-14	0.0 (0-71.0)	87.9 (0-261.0)	**0.000**
TIMP-1	99.0 (62.0-136.0)	150.0 (0-285.0)	n.s.
TIMP-2	45.5 (0-180.0)	139.5 (0-243.0)	**0.000**
TIMP-3	62.7 (0-162.8)	138.9 (0-272.4)	**0.002**

The data are represented as median (range).

*Man-Whitney test; n.s.: not significant

Table 5
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Table 45. Relationship between the expression of metalloproteases (MMPs) and their inhibitors (TIMPs) by endothelial cells and clinico-pathological characteristics from 104 patients with breast cancer.

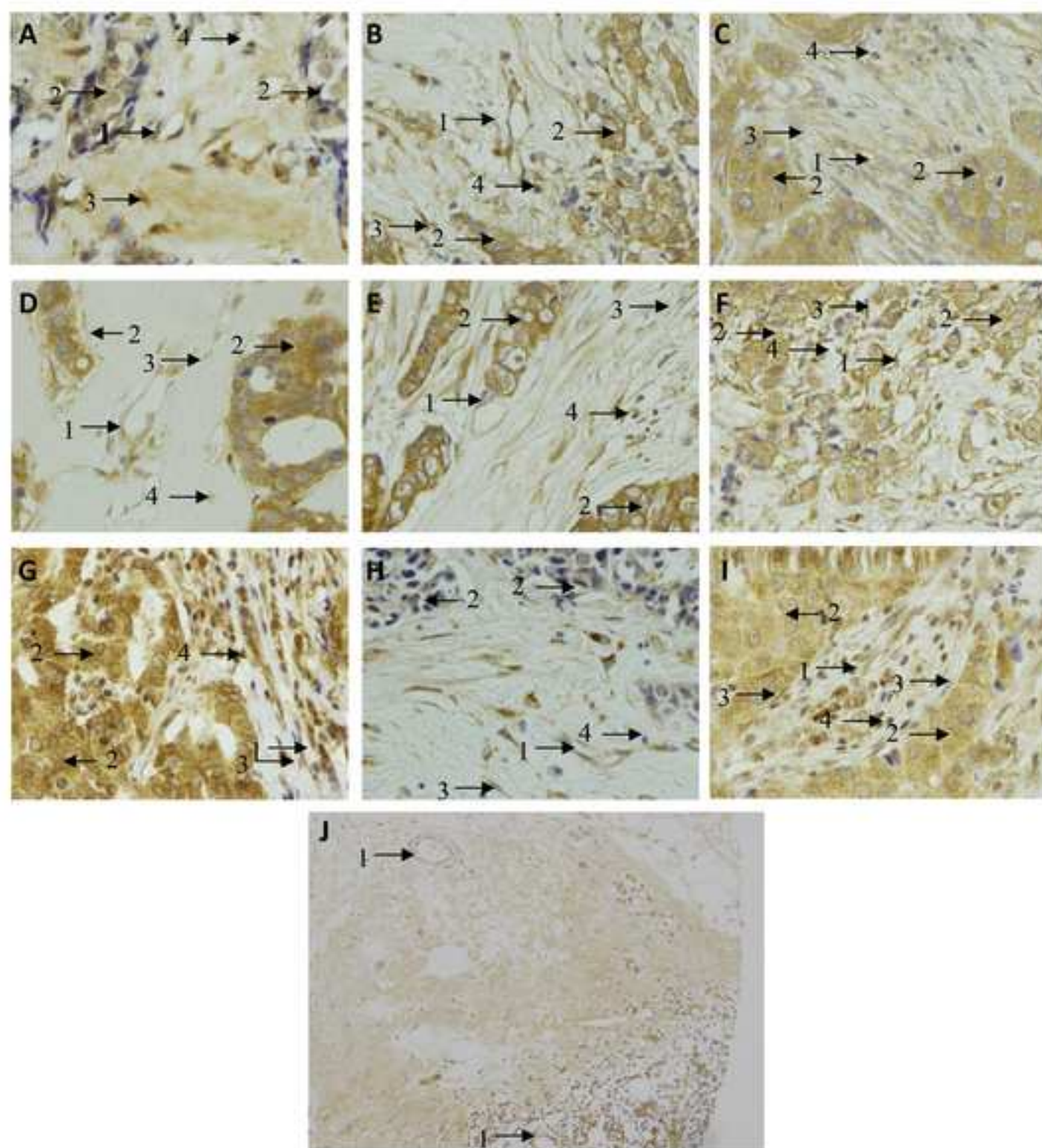| Characteristics	Nº	MMP-2	MMP-7	MMP-9	MMP-11	MMP-13	MMP-14	TIMP-1	TIMP-2	TIMP-3
						Endothelial cells				
Total cases	104	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Age										
≤ 56	55	38 (71.7)	40 (88.9)	51 (98.1)	49 (90.7)	43 (79.6)	49 (92.5)	50 (96.2)	40 (78.4)	42 (84.0)
> 56	49	34 (73.9)	34 (82.9)	43 (93.5)	42 (87.5)	33 (71.7)	46 (95.8)	42 (100)	38 (84.4)	38 (84.4)
Menopausal status										
Premenopausal	33	25 (80.6)	25 (92.6)	29 (96.7)	28 (87.5)	24 (75.0)	29 (90.6)	31 (96.9)	23 (74.2)	24 (80.0)
Postmenopausal	71	47 (69.1)	49 (83.1)	65 (95.6)	63 (90.0)	52 (76.5)	66 (95.7)	61 (98.4)	55 (84.6)	56 (86.2)
Tumor size										
T1	51	38 (77.6)	32 (80.0)	45 (93.8)	43 (87.8)	40 (78.4)	46 (92.0)	42 (95.5)	35 (77.8)	41 (89.1)
T2	53	34 (68.0)	42 (91.3)	49 (98.0)	48 (90.6)	36 (73.5)	49 (96.1)	50 (100)	43 (84.3)	39 (79.6)
Nodal status			p=0.001							p=0.042
N (-)	49	31 (66.0)	25 (67.6)*	41 (95.3)	41 (87.2)	36 (76.6)	45 (95.7)	39 (97.5)	35 (85.4)	42 (93.3)**
N (+)	55	41 (78.8)	49 (100)	53 (96.4)	50 (90.9)	40 (75.5)	50 (92.6)	53 (98.1)	43 (78.2)	38 (76.0)
Histological grade										
I	31	20(66.7)	15 (65.2)	26 (92.9)	24 (82.8)	23 (74.2)	29 (93.5)	23 (95.8)	19 (76.0)	26 (92.9)
II	47	33 (73.3)	34 (89.5)	43 (97.7)	43 (91.5)	22 (76.7)	43 (95.6)	44 (100)	40 (88.9)	97 (86.0)
III	26	19 (79.2)	25 (100)	25 (96.2)	24 (92.3)	20 (76.9)	23 (92.0)	25 (96.2)	19 (73.1)	17 (70.8)
Estrogen receptors										p=0.009
Negative	48	33 (73.3)	36 (92.3)	43 (97.7)	41 (87.2)	37 (78.7)	44 (95.7)	41 (97.6)	35 (81.4)	32 (72.7)***
Positive	49	34 (72.3)	34 (81.0)	44 (93.6)	44 (91.7)	32 (69.6)	45 (93.8)	45 (97.8)	38 (80.9)	42 (95.5)
Progesterone receptors										
Negative	55	37 (71.2)	40 (88.9)	48 (96.0)	49 (89.1)	41 (75.9)	50 (94.3)	48 (98.0)	43 (87.8)	40 (78.4)
Positive	42	30 (75.0)	30 (83.3)	39 (95.1)	36 (90.0)	28 (71.8)	39 (95.1)	38 (97.4)	30 (73.2)	34 (91.9)
Her-2										
Negative	77	56 (75.7)	57 (90.5)	70 (95.9)	68 (90.7)	58 (79.5)	69 (93.2)	67 (97.1)	56 (78.9)	59 (84.3)
Positive	17	9 (60.0)	11 (84.6)	14 (93.3)	14 (82.4)	12 (70.6)	17 (100)	16 (100)	13 (86.7)	12 (80.0)

* p= 0.0001, ** p= 0.042, *** p= 0.009

Data are represented as number of positive cases (percentage).

Figure 1
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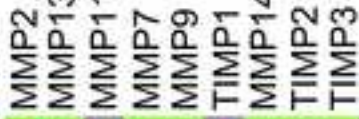
Figure 2
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Figure 3
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Figure 3