Risk of bone metastatization in breast cancer: role of matrix metalloproteinases and ADAMs tissue expression.

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INTRODUCTION

Breast cancer is the most common female neoplasm in western countries [1] and bone tissue is the preferred metastatic site [2]. Overall, 65-75% of the patients with advanced disease will develop bone metastasis[3], with several complications as pain, fractures and spinal cord compression. Adequate follow up and early diagnosis will reduce those complications. The etherogeneity of the biology of breast cancer has been a major obstacle for the definition of the biological mechanisms of malignant spreading and organ trophism [4]. Some series in the literature report correlation between extracellular matrix and bone metastasis.

The aim of our study is to evaluate the expression of molecules involved in the regulation or disregulation of extracellular matrix and their relationship with the risk of bone metastatization.
1. BREAST CANCER

Breast cancer is the most common female neoplasm in developed countries: it represents the first cause of death for cancer in women between 40-55 years old. The main risk factors for breast cancer are: age at menarche, age at the first pregnancy, the increase of the benign proliferative disease of the breast, obesity. In 10% of the cases the neoplasm has some hereditary tract. Primary prevention can reduce the incidence of breast cancer[5].

1.1 Epidemiology

Breast cancer is epidemiologically etherogeneous across different geographical areas. The higher incidence and prevalence are in North America and North Europe, where the female population has a relative risk 5-10 times of developing breast cancer compared with Asian and african population [1]. From the eighties, in the western world, the diagnosis of ductal carcinoma in situ and stage I invasive ductal carcinoma have increased of about 3.7 percentage points/year due to prevention programs as mammography screening. By the year 2000, the incidence and prevalence has progressively diminished by 2% yearly. This phenomenon can be due to the results of the Women’s Health Inititiative, an epidemiological study sowing an increase in incidence of breast cancer and myocardial infarction in people assuming estrogen-progestin hormonal therapy [6].

On the opposite, the low prevalence of breast cancer in African and asian population can be due to the lesser plasmatic concentration of estrogen-progestin hormones in the premenopausal period. However, the lifestyle modifications, such as higher caloric intake, especially lipids, menarche and pregnancy age has led to an increasing in the incidence in breast cancer even in these populations[7-9].
1.1.1 Mortality

The mortality rate for breast cancer has declined from 1975[1], due to mammographic screening and the new adjuvant treatments. The mortality has decreased significantly in people under 50 years old (3.8 % yearly) with regard to people with less than 50 years old (2.2 % yearly). This reduction was more significant for tumors expressing both estrogen and progesteron receptors[10].

1.1.2 The Italian scenario

In Italy there are 400000 women that are diagnosed as having breast cancer every year, the 20-25% of all female tumors, and 8000 die from this disease. The mortality rate for breast cancer has been increased until 27 every 100000 people and then has decreased of about 30% in the last three decades. There are screening programmes in Italy nowadays and the survival is one of the longest in Europe[11]. The 5-year survival for breast cancer has increased from 65% to 82% between 1978 and 1994 in Italy.

1.2 Pathogenesis

The malignant transformation is a multistep process that involves genetic and epigenetic mutations. The cancer cells became able to escape from antiproliferative and apoptotic signallings and acquire hypersensibility to proliferation stimuli. Genetic instability can play a role in the disregulation of cell growth[12, 13].

The morphological and biological characteristics of breast cancer are acquired at the in situ stage, as well as the relapses have the same characteristics of the early stage disease.

The interaction between epithelial and stomal cellss plays a role in the transition between in situ and invasive carcinoma. The loss of basal membrane, the increased epithelial proliferation, the loss of growth inhibition, angiogenesis and stromal
invasion are all acquired during tumorigenesis [14]. The loss of basal membrane and tissutual integrity has a further role in the malignant transformation.

1.3 Biomolecular classification of breast cancer

Breast cancer is a ethereogeneous disease, with different biological subtypes[15]. The pattern analysis of the genetic espression and activity can categorize breast cancer in different subtypes: the variants characterized by a major or minor estrogen and progestinic receptor espression respectively (Luminal A and B), the forms with hyperespression of HER-2/neu (human epidermal growth factor receptor 2), the basalioid type. The distinction between ER-positive and ER-negative forms is relevant, due to the distinct biological behaviour and origin [16].

The luminal carcinoma takes origin from omonymous cells, espress the luminal 8 and 18 cytokeratines and espress the estrogens and progestinic receptors. There is a further subclassification into a subtype A, more frequent, with a significative expression of genes correlated with estrogen receptors, low HER-2/neu espressione. The subtype B has a lower expression of ER-related genes, HER-2/neu expression.

HER-2/neu positive forms are the 10-15% of all breast cancers, have a low luminal genic clusters expression, and usually are ER and PR negative. The basalioid subtype espress the cytokeratines of mammarian precursors (p-cadherins) and of mioepithelial cells. These are poorly differentiated forms with a bad prognosis[17].

1.4 Clinical aspects

Lesions in early stage have generally a favorable evolution; rarely there are typical symptoms at the diagnosis. These include mastalgia, nodulations, morfological breast modifications or pathological nipple secretions[18]. The mastodinia is the most frequent mammarian symptom (10%). The neoplastic breast pain is usually modest, however 11% of patints refer severe, monolateral
The painful symptoms are typical of inflammatory carcinoma with skin modifications such as eczema. The presence of palpable nodules is the most frequent clinical sign. The breast mass is evident when it reaches 1-2 cm of diameter. The benign nodulations are frequent in premenopausal age. Only 10% of mammarian nodules in women under 40 years old are malign, while 60% of breast mass are tumors in post-menopausal women[21]. The clinical analysis has a positive predictive value under 73% and a negative predictive value of 87%[22]. The nipple secretion is a less frequent presenting clinical sign. The milky secretion is a presentation of a clinical disthyroidism and has been never associated with malignancies. An hemorrhagic secretion is typical of benign disease, however can be a presentation of malignant disease rarely. The most common cases of spontaneous nipple secretions are the solitary papilloma of the ducts, cysts and carcinomas. The latter has an incidence of 7% in women with less than 60 years and of 30% in older women [23].

Malign mammarian lesions can present with progressive nipple inversion or retraction. Axillary, supraclavicular, mediastinic and internal mammary lymphoadenopaties can be associated to this clinical picture.

1.5 Diagnosis and prevention

The early diagnosis, with the use of mammography screening, is considered the most important chance to cure and to improve survival [24]. The mortality rate is dependent of the initial stage. Annual screening reduces the probability of dying of about 25-30% [25].

Bilateral mammography is the gold standard for women with 50 years old or more; for women 40 to 49 years old mammography is indicate only for those with an increased risk of breast cancer [26].

Screening in Italy is performed every two years in women from 50 to 69 years old. From 2001 this procedure has been included into the “Livelli Essenziali di Assistenza” that every region should give to patients. Mammarian ultrasounds is
indicated in people with less than 40 years old due to the different density of mammarian tissue in this age population [27]. Microcalcifications can be seen in 60% of cancer lesions, however they can be a characteristic of benign lesions too[28, 29]. Magnetic resonance of the breast is indicated if there is a suspicion of multifocality, multicentricity or bilaterality. Special use can be considered in presence of mammarian prosthesis and in staging of locally advanced disease. Another indication is to search the primary tumor when there is presence of adenopathies with negative mammography [30-32]. The MRI is the most sensitive diagnostic exam for the mammarian prosthesis. The ductalgalattography is indicated in presence of ductal papillomatosis or in presence of nipple secretions. Sentinel node biopsy can be performed with the use of limphohoscintigraphy [33]. The negative predictive value of hystological exam is over 97%. This exam can avoid axillary dissection in patients with not palpable mass.

The cytological analysis can identify:
-C1: inadequate specimen.
-C2: negative specimen.
-C3: uncertain specimen.
-C4: suspected specimen; hystological confirmation is mandatory.
-C5: positive specimen for malignant tumor cells.

Hystopathological analysis:
The bioptic sampling can differentiate benign from malignant lesions. 15-30% of malignant lesions are in situ carcinoma, especially ductal. In 70-85% of cases there is a diagnosis of invasive carcinoma, most of them ductal, then lobular, tubular, mucinous and medullary[34].
## 1.6 Staging

TNM (tumor node metastasis) system is universally accepted for the anatomical extension of the disease. It is the principal prognostic factor and is necessary to define the therapeutic strategy.

**T:** anatomic extension of the primary lesion. This parameter is necessary to define a neoadjuvant strategy while the pathological staging after surgery is fundamental to define the optimal adjuvant therapy.

**N:** the nodal status is one of the most important prognostic parameter. The axillary nodes receive more than 85% of the lymphatic drainage from all mammalian quadrants, while part of the breast parenchyma drains to internal mammary chain nodes. The probability of node involvement depends from primary lesion dimension, his histological grade and his biological behavior.

**M:** the preferential metastatic sites are bone, lung and liver. Therefore the instrumental diagnostic is thorax x-rays, CT and bone scan (especially for stage III disease). MRI and PET/CT are used in selected case based on specific symptoms[35, 36].

<table>
<thead>
<tr>
<th>Stage 0</th>
<th>Tis</th>
<th>N0</th>
<th>M0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>T1 &lt;2 cm</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 2a</td>
<td>T1</td>
<td>N1(1-3ln)</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2 &gt;2&lt;5</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T0</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 2b</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3 &gt;5 cm</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 3a</td>
<td>T0-2</td>
<td>N2 (4-9 ln)</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1/N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 3b</td>
<td>T4 skin/chest wall</td>
<td>N0-2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 3c</td>
<td>any T</td>
<td>N3 (&gt;10 ln)</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 4</td>
<td>any T</td>
<td>any N</td>
<td>M1</td>
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1.7 Prognostic factors

Anatomic extension of the disease (coded by TNM staging system)
The presence of distant metastasis is associated to a median survival of two years. In
the presence of non-metastatic disease the prognosis is conditioned by the nodal
involvement. More important are nodal metastasis greater than 0,2 cm while
micrometsatsis are of uncertain prognostic value.
The 10-years disease free survival is 70-80%, it drops down to 20-30% with 1-3
positive nodes, 10-15% with more than 10 nodes positive. Invasion of the skin and
muscles is associated to a bad prognosis as well as inflammatory carcinoma[37].

Grade of differentiation
It is evaluated with Scarff-Bloom system, nuclear characteristics, tubuli formation
and proliferation index. The 10-year survival is 85% for well-differentiated forms
(G1), 60% for moderately differentiated form (G2), 15% if poorly differentiated
forms (G3) [38].

Hormonal receptors expression
The positivity for estro-progestinic receptors is associated with a more favorable
prognosis. It is predictive of response to endocrine therapy. 70% of post-menopausal
women have a ER-positive tumor.

Her2/neu expression
Hyper expression of Her2/neu protein, part of EGFR complex, is linked to the
amplification of the gene localized on chromosome 17 (17q21). The hyperexpression
of Her2/neu is present in about 25% of cancers and is associated with bad prognosis.
It is predictive of Trastuzumab response. The evaluation of the Her2/neu protein
hyper expression is done with himmunoistochemistry or gene amplification with
FISH[39, 40].

Vascular invasion
It is associated with bad prognosis.
**Proliferation index**
High DNA content in tumor cells, evaluated by cytometry, is an index of malignity. It is evaluated by the number of cells at S stage of cell cycle or by immunohistochemistry with monoclonal antibodies against Ki-67[41]. Other molecular markers have prognostic value: p53, histotype: E-caderine e tissue inhibitors of metalloproteinase (TIMPs) [39, 40].

1.8 Therapeutic approach
The therapy for breast cancer is multidisciplinary: surgery, radiotherapy, medical therapy, and supportive care.

1.8.1 Surgery and adjuvant therapy

**Early breast Cancer**
Surgery is the standard approach.
Conservative strategies such as nodulectomy, lumpectomy or quadrantectomy have the same outcomes of demolitive procedure such as mastectomy[42]. Complete nodal dissection including the first two stations, is considered to be the standard approach. Sentinel node dissection is indicated for patients with clinically negative axillary nodes [43].

**Adjuvant radiotherapy**
The radiation field should include the entire residual breast gland. Usually a fractioned scheme is adopted with 50 Gy in 25 sessions 5 times a week [44, 45]. Radiotherapy on thoracic wall is indicated after mastectomy if the primary tumor dimensions are over 5 cm or if more than 4 axillary nodes are interested.
Adjuvant medical treatment

The choice of adjuvant chemotherapy is based on the evaluation of predictive factors (hormonal status and Her2/neu expression) and of prognostic factors (anatomic extension, grading, vascular invasion, proliferation index).

Adjuvant hormonotherapy

The hormonal treatment is indicated in hormone responsive tumors with more than 1% of estrogen or progestins receptors. In premenopausal patients the 5 years treatment with Tamoxifen plus ovary ablation for 2-5 years is the standard approach. Overall, the reduction of the relapse risk and mortality rate is 39% and 31% respectively. In postmenopausal women the standard treatment is the administration of aromatase inhibitors for 5 years or the alternation with Tamoxifen [46].

Target therapy

In patients with Her2+ with T > 1 cm Trastuzumab treatment for one year is a standard approach. Trastuzumab is an monoclonal antibody directed against the extracellular domain of HER-2/neu receptor. Trastuzumab inhibits the tirosin-kinasic activity of Her2, favoring the apoptosis of the cell and the activate the cell mediated immunity (ADCC). Clinical trials have shown a reduction in relative risk of relapse of 50%.

1.8.2 Therapy of advanced disease

The objective is to prolong survival and to maintain a good quality of life. The systemic treatment is the treatment of choice. Again the treatment should take into account the presence of predictive and prognostic elements.
2. METASTASIS, MMPs, ADAMs and ADAMTSs

2.1 Metastatic phenotype

The proteolysis, the motility and the cellular adhesion are considered the most important elements for the cancer cell to metastasize. These functions are regulated by some proteins that send signals to the cellular and extracellular compartment of the host. Cytokines, motility factors, receptors, enzymes regulate the cross-talk between signals. The extracellular matrix (ECM) can be remodeled and favor the invasion.

I. Adhesion. The first step of the metastatic process is the separation from the primary tumor. The cadherin-E is a molecule that mediates the cell-cell adhesion in epithelial tumors. The loss or reduction of cadherin-E is associated with an advanced stage of disease. The extracellular part of this molecule is responsible of the homotypic interaction within cells.

The adhesion to surface glycoproteins of the extracellular matrix (ECM) is mediated by receptors. Among these, the integrines are a big family of heterodimeric receptors (about 24) composed of two subunits, α e β. The characteristic of these molecules is that the same ligand can bind more than one integrin and the same receptor can bound more than one ligand. The reduced expression of the integrines α1, α6, β1, β4 is involved in the loss of adhesion to the matrix, favoring the metastatization process of melanoma, colorectal cancer, breast and lung cancer. On the other side the hyper expression of the integrin α4β1 is a bad prognostic index for cutaneous melanoma.

FAK (focal adhesion kinase), whose phosphorylation is necessary for the migration signals, links the signal mediated by integrins with Ras-Raf e MAPK-ERK. It is described as bi-directional cross-talk and redundancy between signals with the increase in proliferation, survival and motility (outside-in signaling); these effects can influence, on the return, the expression of surface molecules (inside-out signaling).
Many other heterotypic cell-cell interaction are involved in the metastatic process. These include adhesion molecules (CAM), as ICAM (intracellular adhesion molecules), whose ligand is the integrin \( \beta_2 \) expressed on the circulant leukocytes; VCAM (vascular cell adhesion molecules) the ligand for cells expressing \( \alpha_4\beta_1 \), and NCAM (neural cell adhesion molecules). The selectins L, E, P link one carbohydrate and sialyl-Lewis-x, sialyl-Lewis-a present on carcinoma cells.

**II. Degradation and invasion.** The proteolytic modification of the extracellular matrix is an essential component for the tumoral invasion.

The main enzymes degradating the ECM in tumors are a) the matrix metalloproteinases (MMP); b) the lisin-ADAM; c) the serin-proteinases as the plasminogen activator and plasmin: d) the cistein-proteases with catepsine; e) the heparanase. The main substrates of the ECM are type I and III collagens localized in the stromal and type IV and V collagens localized on the basal membrane [47].

Every metastatization process can be summarized in the following events: interaction between tumoral and stromal cells; interaction with the ECM (process defined epithelial-to-mesenchymal transition); neovascularization and escape from apoptosis. These process requires the matrix metalloproteinase action[48]. In the bone progression the role of metalloproteinases is fundamental because the tumoral expression in bone tissue requires the destruction of a particularly abundant and resistant matrix.

**2.2 The bone “recess”**.

Bone metastasis are common in patients affected by advanced breast cancer. In patients affected by metastatic breast cancer (MBC), the bone represents the most common site of metastatization [49].
Some elements can explain the high frequency of bone metastasis: the blood flow is abundant in the bone marrow, the cancer cells produce adhesion molecules that link stromal cells and bone matrix, the bone tissue is a source of growth factors [50]. The bone recess provides homing signals to cancer cells; The physical (acid pH, high extracellular calcium concentration) and biochemicals properties (cytokines, growth factors) of the bone create a good micro environment for tumor growth [51]. The tumor cells express CXCR4 receptors for the chemokine that bound the stromal-cell derived factor 1 (SDF-1) in the bone environment [52]. The expression of RANKL in the bone seems to contribute to bone metastasis development binding his receptor to tumor cells surface [53].

### 2.2.1 Bone metastasis from breast cancer

Bone metastasis from breast cancer are usually osteolytic. Osteoclasts mediate the bone destruction. Cancer cells produce factors that induce the osteoclasts production. Bone matrix releases factors stimulating the tumor growth and bone destruction [54]. Cancer cells can secrete the PTHrP (parathyroid hormone-related peptide). When PTHrP bounds his receptor (PTHR1), on the osteoblasts, stimulates the expression of RANKL. RANKL binds his receptor on the osteoclasts precursors. The osteoclasts destroy bone tissue producing growth factors, proteins, IGF-1, TGF-beta.

### 2.3 Metalloproteinases

Every process of remodelling and repair of tissues require a controlled degradation of extracellular matrix (ECM). Cancer, arthritis and cardiovascular disease are characterized by a ECM remodelling generally in a pathological [55]. The main enzimatic group involved in the degradation of ECM is the superfamily of the
metalloproteinases Zinc-dependent, that includes the matrix metalloproteinases (MMPs), also known as matrixines, the ADAMs and the ADAMTSs. The MMPs are zinc-metalloproteasase multidomains, and the sequence homology with the catalytic domain of the collagenases of fibroblasts (Collagenase 1) or MMP-1 is a major criterion to belong to the family.

The metalloproteinases are able to degrade all ECM components, to release and activate/inactivate a great number of cellular functions. The MEROPS database classifies the MMPs as a subfamily of the metzincines (M10). The catalytic domain contains the bound site Zn2+ HEXXHXXGXXH and a methionine, to make a “met-turn” of eight remains, that sustains the pocket structure of the active site around the Zn2+ catalytic.

The MMPs are synthesized as pre-proenzimes, which have the “cistein switch” PRCGXPD motif, where the cysteine residue maintains the proMMPs inactive.

The MMPs are classified as: collagenase, gelatinase, stromelisine, matrilisine, membrane-type (MT)-MMPs.

Usually the MMPs are composed of a pro-peptide of about 80 amino acids, a catalytic domain of 170 amino acids, a link peptide (“hinge region”) and a hemopexinic domain of 200 amino acids. The only exceptions are the MMP-7 (matrilisine 1), MMP-26 (Matrilisine 2) and MMP-23 that lack of the link peptide and the “hemopexin-like” domain[56].

### 2.3.1 Collagenases

This group is composed of three proteins: collagenase 1 (MMP-1), collagenase 2 (MMP-8) e collagenase 3 (MMP-13). They have three domains: propetidic, catalytic and hemopexin-like. Their function is to degrade the fibrillar collagen type I, II, III in $\frac{3}{4}$ and $\frac{1}{4}$ fragments.
2.3.2 Gelatinases

Gelatinase A (MMP-2) and Gelatinase B (MMP-9) belong to this group. They can degrade the denaturated collagen, gelatins, native collagen type IV, V e XI, laminine. MMP-2 (in contrast to MMP-9) can degrade native collagen type I ,II, III as collagenases. The collagenolytic activity of MMP-2, in solution, is weaker than the MMP-1 one or other collagenases.

2.3.3 Stromelisines

MMP-3 (stromelisine 1), MMP-10 (stromelisine 2), MMP-11 (stromelisine 3) has teh same domain organization of the collagenases but they don’t clivate the interstitial collagen. MMP-3 and MMP-10, degrade great number of ECM proteins and participate to the activation of proMMPs. MMP-3 e MMP-10 are secreted by cells as inactive proMMPs, MMP-11 that is activated in the intracellular compartment.

2.3.4 Matrilisines

This group includes MMP-7 e MMP-26. They don’t have the hemopexin domain. MMP-7 is synthetized by epithelial cells and secreted from the apical part of the cell. It degrades ECM components but also cellular membrane molecules as Fas-ligand, proTNFα, syndecan1 and E-caderin to generate soluble forms. MMP-26 is expressed by normal cells as endometrial ones and by carcinomatous cells, it degrades ECM components.

2.3.5 Membrane-bound MMPs (MT-MMPs)

There are two types: transmembrane proteins type I (MMP-14, MMP-15, -16 e -24) and proteins bounded to glucosilphosfatidilinositol goups (MMP-17 and -25). They are activated in intracellular environment; the enzymes are expressed at the
membrane. All MT-MMPs, except MMP-17, can activate proMMP-2. MMP-14 can activate proMMP-13 on cellular surface. MMP-14 has an intrinsic collagen lytic activity, on collagen type I, II, III.

2.3.6 MMP-1

MMP-1 (Collagenase 1), member of the collagenases family (together with MMP-8 and MMP-13), is composed by 3 domains: pro-peptidic, catalytic and hemopexinic. It has the function to clivate fibrillar collagen type I, II, III. Hyperexpression of MMP-1 is found in many tumors [57, 58]and is correlated to a more advanced stage of disease. Several series show how the expression of MMP-1 is linked to the tumor progression ver for his proteolytic activity on protein G bounded to the receptor PAR1 (Boire et al. 2005). Murray et al. Have found an high expression of MMP-1 in bad prognosis colorectal cancer [59]. Weak expression of MMP-1 correlates with a better prognosis in patients affected by advanced colorectal cancer [60]. Xin Lu et al. Have shown that MMP-1 and ADAMTS-1 increase the invasivity through the ECM and endothelium, and favors the colonization of the bone microenvironment thorough pro osteolytic signals cascade that involve cancer cells, osteoblasts and osteoclasts. ADAMTS-1 and MMP-1 help the release of EGF-like ligands as AREG, HB-EGF, and TGF-alfa [61].

2.3.7 MMP-13

MMP-13 belongs, together with MMP-1 and MMP-8, to collagenase family. Constituted by three domains: pro peptidic, catalytic and hemopexin-like; it acts degrading mainly fibrillar collagen type I, II e III. The expression of MMP-13 has been associated to worse prognosis in colorectal cancer [61] and breast cancer [62] and is involved in the cell proliferation in melanoma[63]. In non small cell lung cancer, the cell clones that express MMP-13 have the potential to spread to teh bone marrow[64].
Cancer cells adherent to the type I collagen act through the signal cascade integrin-FAK-p38-MAPK to induce MMP-13 and increase the osteolytic activity[65]. The breast cancer cell line MDA-MB-232 can destroy bone tissue producing MMP-13, with the help of PTHrP[66]. In a murine-based experiment, the hyper expression of MMP-13 at the interface Tumor-Bone produce an increased osteolytic activity mediated by MMP-9 and TGF-beta activation[67].

2.4 Inhibitors of metalloproteinases

2.4.1 Endogen inhibitors of MMPs

They are the α-macroglobulin and the Tissue Inhibitors of MMPS (TIMPs). The human α-macroglobulin is a glycoprotein of 725 kDa composed of four subunits of 180 kDa. It acts as aspecific protease inhibitor and it is found in blood and interstitial liquids. Most of endopeptidases is inhibited by the macroglobulin. The TIMPs are four, TIMP-1, -2, -3, -4 of about 22-24 kDs. TIMP-1 and -3 are glycoproteins, TIMP-2 and -4 don’t contain carbohydrates. TIMP-1 is a weak inhibitor of MT1-MMP, MT3-MMP, MT5-MMP and MT-9-MMP. TIMPs are able to inhibit a large spectrum of metalloproteinases. TIMP-1 inhibits ADAM10, while TIMP-2 inhibits ADAM12. TIMP-3 has the broadest spectrum of action, including ADAM-10, -12, -17 and the ADAMTSs subgroup. The suppression of TIMP-3 in mice causes lung damage and apoptosis of the mammalian epithelial cells [68].

All the TIMPs have homology and are composed of 184-194 amino acids with 12 cysteinic remains. The inhibition of MMPs is mediated by N-terminal domain. The 4 N-terminal Cys1-Thr-Cys-Val4 remains bounded to Glu67-Ser-Val-Cys70 remains integrate in the active site of MMPs to chelate Zinc ion. TIMP-1 and TIMP-2 promotes the cell growth and protect cells from apoptosis; TIMP-3 causes apoptosis of cancer and smooth muscle cells TIMP-3 can bound to VEGFR2 and inhibit the angiogenesis.
2.4.2 Synthetic inhibitors of MMPs

The development of synthetic inhibitors of MMPs was based on the use of known peptidic, however they have a low selectivity. Most of clinical trials with MMPs inhibitors were conducted on oncological patients without any clinically relevant effect[69, 70]. New variants of inhibitors seem to be more specific and are under evaluation. Thyolic, hydrossipironic and barbituric inhibitors are under study. Innovative approaches include the synthesis of antibodies fragments specific for catalytic sites of MMPs, the inhibition of intracellular signals to down regulate MMPs (id the MAPK, NFkB, AP-1 pathways).

2.4.3 TIMP-2

The family of TIMPs is the principal regulator of the metalloproteinases. TIMP-2, discovered in 1989, has several pleiotropic effects. The TIMPs concentration usually exceeds the MMPs one in tissue and extracellular fluids, limiting the proteolytic activity[71, 72]. TIMP-2 block selectively the growth of human endothelial cells in vitro when stressed by proangiogenic factors as FGF-a and VEGF-a; it can inhibit the signals of tirosin kinase receptors independently from the metalloproteinases inhibition. TIMP-2 mediates the interaction between MMP-2 zimogen and MT1-MMP. At low concentrations TIMP-2 activates MMP-2; at higher concentrations, TIMP-2 creates stable complexes with MT1-MMP, inhibiting the activation of MMP-2[73, 74]. The TIMPs are down regulated or silenced in several cell lines. The hyper expression of TIMPs inhibits the development of metastasis from melanoma in experimental models. TIMPs have important anti tumoral activity [73]; TIMP-2 inhibits the growth of osteolytic bone metastasis from breast cancer cell lines MDA-231. In one series the hyper expression of TIMP-2 can protect cancer cells from apoptosis, through the activation of inflammatory signals mediated NF-kB[75].
2.4.4 TIMP-3

It has the broader spectrum of activity. It blocks the link between VEGF and VEGFR2, inhibiting the angiogenesis\[76\]. The hyper expression of TIMP-3 induces apoptosis in lung cancer cells A549 and AdCMVTIMP-3 (a viral vector), positively regulates the expression of p53, FAS-1, TNFR1 and 2. The use of adenovirus to transfect cancer cells A549 in nude mice with TIMP-3 induces the growth arrest [77].

2.5 MMPs and cancer

Many processes as neoplasms, cardiovascular disease, arthritis show a specific pattern of MMPs expression. Some animal models were developed to study the role of MMPs in the neoplastic progression. The loss of MMP-7 reduce the development of tumors in murine models[78, 79], the hyper expression of MMP-3 in the mammary gland brings to the spontaneous development of premalignant lesions. Some studies have shown the correlation between MMPs expression and disease outcome[80, 81]. MMPs promote cancer growth degradating ECM and secreting growth factors. MMP-9 makes VEGF available from ECM and clivates collagen type IV to generate Tumstatin, an angiogenesis inhibitor. In carcinomas, MMPs are associated to the stromal cells and this emphasized the relevance of the macroenvironment[80, 82].

The standard approach to study the tumorigenesis consists to delete some MMP and TIMP genes in murine models.

Mice knockout for MMP8 develop a significant number of cutaneous papilloma after treatment with carcinogens, mice knockout for MMP9 develop high-grade cutaneous tumors. High expression of MMP-12 on human squamocellular carcinoma is associated with aggressive disease[83].
2.6 ADAMs

ADAMs, or a disintegrin and metalloproteinase, are a family of multidomain transmembrane proteases $\text{Zn}^{2+}$-dependent, involved in mechanisms of proteolysis and cellular adhesion. They are correlated with other enzymes as ADAMTS (ADAM with trombospondin domains), the metalloproteinases of the matrix (MMPs) and the snake venom metalloproteinase (SVMP). 40 genes were identify in this family, 21 are considered functional in the humans[84].

2.6.1 Structure and function of ADAMs

The ADAMs are trans membrane proteins composed of 8 domains or regions, a prodomain, a metalloproteinases domain, a disintegrinic domain and integrins-ligands, a trans membrane sequence and an intracellular C-terminal. ADAMs functions include cellular adhesion, migration and signaling. Their principal substrates are trans membrane proteins as adhesion proteins and precursors of growth factors and cytokines. These proteases cut (shedding) and activate the precursors. ADAM10 and ADAM17, are able to activate different ligands for the epidermal growth factor receptor (EGR). These ligands include also the EGF, the TGF-$\alpha$, amphireguilina and the betacellulin that are involved in the genesis and progression of cancer. In some tumors the expression of ADAMs is correlated with the characteristic of the disease[84].

2.6.2 Shedding of HER

HER proteins (also called ErbB) belong to the superfamily of tyrosine kinase receptors. There are four type of HER: EGFR/ErbB1/HER1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4. Two members of this family (EGFR and HER2) mediate the cell growth, the survival and his migration. The impaired expression of
EGFR and HER2 is implicated in the genesis and progression of some tumor types. The HER tyrosine kinases are activated by some ligands synthetized as precursors. Specific ADAMs activates these precursors. The hyper expression of ADAM8, ADAM9, ADAM10, ADAM12, ADAM17 and ADAM19 can release EGF that will link to EGFR[84].

2.6.3 ADAM10

The hyper expression of ADAM10 promotes the growth of gastric and oral carcinoma, while his down regulation reduces the proliferation of cancer cells[85].

2.6.4 ADAM12

ADAM12 is expressed in two isoforms, one long, also called Long Form (ADAM12-L) and in short (ADAM12-S). The hyper expression of the two isoforms of ADAM12 is increased in the mammalian tumor tissue.

The hyperextension of the trans membrane isoform ie ADAM12-L, is significantly increased in the early stage of breast cancer while both isoforms are hyper expressed in advanced disease. An increase in the expression of the trans membrane isoform ADAM12-L, in stages I and II of breast cancer, could reflect his role on cancer growth through shedding way. ADAM12-S levels are higher in more advanced breast cancers. Data from Roy et al. suggest that the increased expression of ADAM12-S, favors local tumor invasion, vascular invasion and nodal metastatization. The down regulation of ADAM12 could be a potential therapeutic target in breast cancer.

ADAM12 has a low expression in most normal tissues while is highly expressed in cancer cells in carcinomas in situ (CIS) and invasive di carcinomas [86].

Three somatic mutations of ADAM12 were identified in breast cancer, one in the metalloproteinasic domain, one in the disintegrin domain and one in the intracellular domain. ADAM12 expressed in cells of breast cancer favors the progression of tumor inducing the apoptosis of the stromal cells[87]. Urinary levels
of ADAM12 correlate with disease stage in patients affected by breast cancer and those levels increase with disease progression[88-90]. ADAM12 degrades some molecules of the extracellular matrix including type IV collagen and fibronectin.

### 2.6.5 ADAM17

Also called TACE, ADAM17 is hyper expressed in breast, ovarian, renal, prostatic and colorectal carcinoma. Treating cell lines of breast cancer with antibodies anti-ADAM17 diminish cell proliferation [91]. High levels of ADAM17 are predictive of bad outcome inpatients affected by breast cancer and highest levels were found in high grade (G3) tumors[92].

### 2.7 ADAMs and cancer

ADAMs can promote the cancer growth. One of the mechanisms is the release of growth factors that stimulates the cell progression and growth. The most studied factor activated by ADAMs are ligands family of EGFR/HER. The activated form of these ligands binds to one or more receptors of the EGFR/HER family [93]. When activated, these receptors start a cascade of events that stimulate the proliferation, the motility and cell survival. There are correlations between ADAM-mediated release of growth factors, signalling EGFR mediated and proliferation or migration of malignant cancer cells. Treating murine embryonic fibroblasts with the platelet derived growth factor receptor beta increase the activation of ADAM17, the release of EGFR ligands, to the EGFR signaling [94]. In tumor lung tissue the hyper expression of ADAM28 seems to correlate with nodal metastasis[95]. ADAM9 is significantly increased in patients with breast cancer and nodal involvement [96]. ADAM17 levels can be an independent predictive factor of outcome [97].
2.8 ADAMTSs

ADAMTSs (a disintegrine and metalloproeinase with trombospondin motif) is a group of 20 metalloproteinasi correlated to ADAMs proteinases. ADAMTSs are secreted molecules[98]. Some ADAMTSs (ie ADAMTSs-1, 4 and 9) can bind to the ECM, with the mediation of central domain and the C-terminal[99].

2.8.1 ADAMTS1

ADAMTS1 promotes the development of lung metastasis in murine breast cancers through an increased proliferation, survival and tumor invasion. ADAMTS1 seems to favor cancer angiogenesis and is overexpressed in metastatic breast cancer [100].

Silencing ADAMTS1 and MMP1 dramatically reduces bone metastasis in animal. On the contrary, the hyper expression of ADAMTS1 and MMP1 increases the number of bone metastasis and osteolytic events[101].

ADAMTS-1 e ADAMTS-8 can inhibit angiogenesis VEGF induced[102].

3. OBJECTIVES OF THE STUDY

This mono institutional study evaluates the expression of a panel of biological and molecular markers in two cohorts of patients with breast cancers affected or not affected by bone metastasis.

The main end point is to verify if there is some marker significantly correlated with the risk of bine metastatization.
4. MATERIALS AND METHODS

4.1 Population

297 records of breast cancer patients (ductal, lobular, mucinous, papillary, tubular, apocrine) operated between 1985 and 2001 at San Paolo Hospital in Milan were analyzed; formalin fixed and paraffin included tissues were available. Only patients with at least 10 years of follow up were considered.

4.2 Tissue Microarray

All available slides were revised, stained with hematossilin and eosin, Tissue Microarray (TMA) was created with Tissue Arrayer Minicore instrument (Alphelys, Plaisir FR).

The TMA is a small block of paraffin composed by several tissue samples (cores) taken from a “donator” and put in a small block called “acceptor”.

This technique allows analyzing several tissue samples simultaneously.

1 mm “cores” were extract, n. 4 of the invasive neoplasia to represent his heterogeneity, n.1 of in situ neoplasia and n. 1 of nodal metastasis.

The “cores” were included in the small block “recipient” at 0,5 mm distance on from the other, disposed in 6 “cores” lines per patients (3 patients every line), with a overall number of 11 lines. In one “recipient” block were analyzed 33 patients contemporarily.
4.3 Immunohistochemistry

The block were cut by a microtome in 3-micron section and stained. The immunohistochemistry (IIC) is a technique that allow identifying cellular antigens on the normal and pathological tissues. It is based on the specificity between antigen and antibody.

In our series, we used antibodies directed against classical markers in breast cancer (estrogen receptors, clone 1D5; progesteron receptors, clone 636; ki-67, clone MIB-1; Her-2; Dako, Glostrup Denmark) and antibodies against:
- MMP1 (clone EP1247Y, diluizione 1:300; Epitomics, Inc., Burlingame CA, USA)
- MMP13 (clone M66, diluizione 1:50; Santa Cruz Biotechnology, Inc., Santa Cruz CA, USA)
- TIMP2 (clone 3A4, diluizione 1:100; Abcam, Cambridge, UK)
- TIMP3 (clone 136-13H4, diluizione 1:1000; Abcam, Cambridge, UK)
- ADAM10 (rabbit polyclonal ab 19997)
- ADAM12 (goat polyclonal ab 28747)
- ADAM17 (mouse polyclonal ab 57484)
- ADAMTS1 (rabbit polyclonal ab 28284)

When required, to ameliorate tissutal immunoreactivity, the sections, before incubation with primary antibody, were treated with antigenic unmasking using a solution of EDTA at pH 8 (MMP1) or citrate at pH 6 (TIMP3) in a thermostated small bath at 97,5°C for 35-40 minutes.

For the MMP1 antibody a blocking solution was used (Protein Block, Dako, Glostrup Denmark, 30 minutes at normal temperature) to make the coloration more precise.

All the immunohistochemistry labeling were done with the automatic i6000 BioGenex (Menarini Diagnostics, Firenze, It) and visualized with the revelation system NovoLink Polymer (Novocastra, Newcastle Upon Tyne, UK).
For all studied antibodies the presence or absence of immunoreactivity was evaluated, both in citoplasmatic and at membrane surface site. A score from 0 to 3 were used, as follows: 0= none immunoreactivity; 1= weak immunoreactivity; 2= moderate immunoreactivity; 3= intense immunoreactivity. A positive expression was defined as the presence of 1+, 2+ o 3+ score at immunohistochemistry [103]

The preparations were examined with a microscope LEICA DMLS (Leica Microsystems, CMS GmbH, Wetzler, Germany).

### 4.4 Statistical analysis

Descriptive statistics were computed to show the clinical and biological characteristics of the patients.

The antibody status, the receptors positivity and Her2/neu status were codified in dichotomic variables.

The variables were first tested with a univariate analysis, with a statistical significativity for p<0.10, to evaluate the independent impact of variables on bone metastatization. For those variables resulted significant at univariate analysis, a multivariable analysis were computed with a statistical significance for p<0.05.
5. RESULTS

A summary of the clinic-pathological features of the patients in the control group and those in the group with bone metastasis is shown in the table 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Bone metastases</th>
<th>No bone metastases</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of women – N (%)</td>
<td>207 (69.7)</td>
<td>90 (30.3)</td>
<td>297 (100)</td>
</tr>
<tr>
<td>Missing values</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Histology – N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDI</td>
<td>74 (30.0)</td>
<td>173 (70.0)</td>
<td>247 (83.2)</td>
</tr>
<tr>
<td>CDL</td>
<td>10 (28.6)</td>
<td>25 (71.4)</td>
<td>35 (11.8)</td>
</tr>
<tr>
<td>Mixed</td>
<td>6 (40.0)</td>
<td>9 (60.0)</td>
<td>15 (5.0)</td>
</tr>
<tr>
<td>Grade – N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11 (26.2)</td>
<td>31 (73.8)</td>
<td>42 (14.1)</td>
</tr>
<tr>
<td>2</td>
<td>41 (25.5)</td>
<td>120 (74.5)</td>
<td>161 (54.2)</td>
</tr>
<tr>
<td>3</td>
<td>38 (40.4)</td>
<td>56 (59.6)</td>
<td>94 (31.6)</td>
</tr>
<tr>
<td>ER – N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>17 (40.5)</td>
<td>25 (59.5)</td>
<td>42 (14.2)</td>
</tr>
<tr>
<td>Positive</td>
<td>73 (28.7)</td>
<td>181 (71.3)</td>
<td>254 (85.8)</td>
</tr>
<tr>
<td>Missing</td>
<td>.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PgR – N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>31 (36.5)</td>
<td>54 (65.5)</td>
<td>85 (28.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>59 (27.8)</td>
<td>153 (72.7)</td>
<td>212 (71.4)</td>
</tr>
<tr>
<td>C-erb – N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>30 (30.3)</td>
<td>69 (69.7)</td>
<td>99 (33.3)</td>
</tr>
<tr>
<td>1</td>
<td>21 (28.8)</td>
<td>53 (71.2)</td>
<td>74 (24.6)</td>
</tr>
<tr>
<td>2</td>
<td>15 (26.8)</td>
<td>41 (73.2)</td>
<td>56 (18.9)</td>
</tr>
<tr>
<td>3</td>
<td>24 (35.3)</td>
<td>44 (64.7)</td>
<td>68 (22.9)</td>
</tr>
<tr>
<td>Metastasis other than bone – N (%)</td>
<td>45 (17.9)</td>
<td>206 (82.1)</td>
<td>251 (84.5)</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>45 (97.8)</td>
<td>1 (2.2)</td>
<td>46 (15.5)</td>
</tr>
</tbody>
</table>

Mean age of the population is 61 years (standard deviation 11.7; range 27-89 years). The control group has 207 patients (69.7% of the total), which are, after a ten-year follow-up, free from disease or at least without bone metastasis. The group with bone metastasis consists of 90 patients (31.3% of the total). Of those, 90 patients, 45 (50%) have only bone localization of disease while 50% have both visceral and bone metastasis. In the control group, 173 patients out of 206 (83.6%) had ductal invasive carcinoma, 25 patients (12.1%) had lobular invasive carcinoma while 9 patients (4.3%) had other hystotype.
In the only bone metastasis group, 74 patients (82.2%) had ductal invasive carcinoma, 10 patients (11.1%) had lobular invasive carcinoma while 6 patients (6.7%) had other hystotype (in particular: apocrine, metaplastic, papillary carcinoma, mucinous carcinoma).

Grading is similar between groups and grade 2 was the more frequent (120/206 57.9%) in the control group and (41/90, 45.5%) in the bone metastasis group.

The hyper expression of Her2 was present in 182 /206 (87.9%) patients of the control group and in 73 /90 (81.1%) of the metastatic group.

The expression of PgR was positive in 153 /90 (73.9%) of patients in the control group, and in 59 / 90 (65.5%) of the group with bone metastasis.

In the control group 138 patients had positivity for Her2 protein (66.6%) and 60 (66.6%) were the patients positive for the protein with bone metastasis. Most of the patients received chemotherapy and hormonal treatment: 151/197 (50.8%) received adjuvant chemotherapy, 215/297 (72.4%) received endocrine therapy, 220/297 (74.1%) received radiotherapy.

At the moment the analysis was performed 89 metastatic patients (30%) were deceased, 21 with bone metastasis only and 37 with other type of metastatic site.

Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Died</th>
<th>Alive</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of women – N (%)</strong></td>
<td>89 (30.0)</td>
<td>208 (70.0)</td>
<td>297 (100)</td>
</tr>
<tr>
<td><strong>Bone metastases– N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>30 (30.3)</td>
<td>69 (69.7)</td>
<td>99 (33.3)</td>
</tr>
<tr>
<td>Yes</td>
<td>21 (28.8)</td>
<td>52 (71.2)</td>
<td>73 (24.6)</td>
</tr>
<tr>
<td><strong>Metastases other than bone– N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>52 (20.7)</td>
<td>199 (79.3)</td>
<td>251 (84.5)</td>
</tr>
<tr>
<td>Yes</td>
<td>37 (80.4)</td>
<td>9 (19.6)</td>
<td>46 (15.5)</td>
</tr>
</tbody>
</table>
Tab. 3 – Expression of the proteins studied in the tumoral tissue.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Bone metastases</th>
<th>No bone metastases</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of women – N (%)</strong></td>
<td>90 (30.3)</td>
<td>207 (69.7)</td>
<td>297 (100)</td>
</tr>
<tr>
<td><strong>Median Ki67 [min-max]</strong></td>
<td>15 [1-70]</td>
<td>10 [0-70]</td>
<td>10 [0-70]</td>
</tr>
<tr>
<td><strong>Q1-Q3</strong></td>
<td>10-23</td>
<td>5-20</td>
<td>7-20</td>
</tr>
<tr>
<td>Missing values</td>
<td>5</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td><strong>MMP1 – N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>35 (33.0)</td>
<td>71 (67.0)</td>
<td>106 (35.7)</td>
</tr>
<tr>
<td>Positive</td>
<td>55 (28.8)</td>
<td>136 (71.2)</td>
<td>191 (64.3)</td>
</tr>
<tr>
<td><strong>MMP13 – N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24 (27.6)</td>
<td>63 (72.4)</td>
<td>87 (29.3)</td>
</tr>
<tr>
<td>Positive</td>
<td>66 (31.4)</td>
<td>144 (68.6)</td>
<td>210 (70.7)</td>
</tr>
<tr>
<td><strong>TIMP2-N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>13 (14.4)</td>
<td>49 (24.7)</td>
<td>62 (21)</td>
</tr>
<tr>
<td>Positive</td>
<td>77 (85.6)</td>
<td>158 (76.3)</td>
<td>235 (79.1)</td>
</tr>
<tr>
<td><strong>TIMP3 – N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>9 (10)</td>
<td>23 (11.1)</td>
<td>61 (20.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>81 (32.8)</td>
<td>184 (67.2)</td>
<td>265 (79.4)</td>
</tr>
<tr>
<td>Missing value</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>ADAM17 – N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8 (19.0)</td>
<td>34 (81.0)</td>
<td>42 (14.1)</td>
</tr>
<tr>
<td>Positive</td>
<td>82 (32.2)</td>
<td>173 (67.8)</td>
<td>255 (85.9)</td>
</tr>
<tr>
<td><strong>ADAM12 – N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8 (19.0)</td>
<td>34 (81.0)</td>
<td>42 (14.1)</td>
</tr>
<tr>
<td>Positive</td>
<td>82 (32.2)</td>
<td>173 (67.8)</td>
<td>255 (85.9)</td>
</tr>
<tr>
<td><strong>ADAMTS1 – N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>7 (20.6)</td>
<td>27 (79.4)</td>
<td>34 (11.4)</td>
</tr>
<tr>
<td>Positive</td>
<td>83 (31.6)</td>
<td>180 (68.4)</td>
<td>263 (88.6)</td>
</tr>
<tr>
<td><strong>ADAM10 – N (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22 (29.7)</td>
<td>52 (70.3)</td>
<td>74 (25.1)</td>
</tr>
<tr>
<td>Positive</td>
<td>67 (30.3)</td>
<td>154 (69.7)</td>
<td>221 (74.9)</td>
</tr>
<tr>
<td>Missing value</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

The percentages in the column “Overall” are calculated having as denominator the total number of patients

In the control group: hyper expression of MMP-1 was present in 136 patients (65.7%); MMP-13 is present in 144 patients (69.6%); the positivity for TIMP-2 is present in 158 patients (76.3%); the positivity for TIMP-3 is present in 81 metastatic patients (90%) and in 184 patients (88.9%); positivity for ADAM17 173 patients (83.6%); the positivity for ADAM12 is present in 162 patients (78.3%); hyper expression of ADAMTS1 is present 180 patients (86.9%); positivity for ADAM10 is present in 154 patients (75.5%).

In table 4 all variables and their p value are shown.
Tab.4 Effect of different parameters on the risk of metastasizing- Univariate and Multivariate Logistic Regression Models

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>Age (increase of 10 years)</td>
<td>1.14 (0.92-1.41)</td>
<td>0.240</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CDI</td>
<td>0.64 (0.22-1.87)</td>
<td>0.415</td>
</tr>
<tr>
<td>CDL</td>
<td>0.60 (0.17-2.13)</td>
<td>0.429</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.96 (0.44-2.09)</td>
<td>0.924</td>
</tr>
<tr>
<td>3</td>
<td>1.91 (0.86-4.26)</td>
<td>0.924</td>
</tr>
<tr>
<td>Positive ER status</td>
<td>0.59 (0.30-1.16)</td>
<td>0.128</td>
</tr>
<tr>
<td>Positive PgR status</td>
<td>0.67 (0.39-1.15)</td>
<td>0.834</td>
</tr>
<tr>
<td>Positive MMP1</td>
<td>0.82 (0.49-1.37)</td>
<td>0.448</td>
</tr>
<tr>
<td>Positive MMP13</td>
<td>1.20 (0.69-2.09)</td>
<td>0.512</td>
</tr>
<tr>
<td>Positive TIMP3</td>
<td>1.12 (0.50-2.54)</td>
<td>0.777</td>
</tr>
<tr>
<td>Positive TIMP2</td>
<td>1.80 (0.92-3.52)</td>
<td>0.086</td>
</tr>
<tr>
<td>ADAM17</td>
<td>2.01 (0.89-4.54)</td>
<td>0.092</td>
</tr>
<tr>
<td>ADAM12</td>
<td>2.85 (1.28-6.32)</td>
<td>0.010</td>
</tr>
<tr>
<td>ADAMTS1</td>
<td>1.78 (0.74-4.25)</td>
<td>0.195</td>
</tr>
<tr>
<td>ADAM10</td>
<td>1.03 (0.58-1.83)</td>
<td>0.924</td>
</tr>
</tbody>
</table>

Non-statistically significant differences were found between the two groups regarding cancer hystotype (p value 0.77). Expression of ER, PgR and Her2 is similar between the two groups (p 0.13, p 0.14 and 0.83).

Tumoral grading, MMP1, ADAM 17 and ADAM 12 were the parameters selected by the univariate analysis.

ADAM12 expression was the only parameter significantly different between the two groups (78,26% vs 91,11% with p 0.036, OR=2.59, 95%IC 1.06-6.29).
6. DISCUSSION

Our study shows that ADAM 12 is the only hyper expressed protein in tumoral tissue that is significant related with bone metastatization.

Our results are discordant with those of Narita et al.[104], Where there was not a correlation between ADAM 12 and bone metastasis. In the studies previously mentioned the population analyzed had an inadequate sample (38 and 92 patients).

In a recent study of Roy et al. [105] ADAM12 induces estrogen-resistance in hormone sensitive tumors. Cancer cells hormone sensitive and expressing ADAM12, when put in a medium with low estrogen levels, became resistant to the hormonal treatment growing faster than cancer cells not hyper expressing ADAM12. It can be hypothetized that ADAM12 play a role as mediator of the resistance to the hormonal treatment, for example the EGFR way. Targeting ADAM12, together with hormonal treatment, could be a new approach to overcome anti estrogenic resistance.

Among metastatic patients, 73,3% had a ER-positive and ADAM12-positive disease. According to Roy et al. results, it can be hypothesized that some ormonosensitive patients, treated with hormones would develop bone metastatization due to hyperexpression of ADAM12. This subgroup of patients ADAM12 and ER-positive, an have developed ormonoresistant disease during hormonal treatment.

In conclusion, in our series, in the subgroup analysis of patients with ≥ 65 years no variable has been associated with bone metastatization. A possible explanation of that result could be a low ADAM12 hyperexpression in older population as found in other literature series (Narita et al., where ADAM12 is significantly hyperexpressed in women with less than 50 years than in older women[104]. In particular, the pre or post meopausal status could bring to different proteolytic cascades.

Our results are concordant with those of Sjoblom et al. [106] in the analysis of the breast cancer genome. They have highlighted ADAM12 as candidate gene cancer; in the 122 genes most frequently mutated in breast carcinoma, the only gene coding for one ADAMs, was the one coding for ADAM12.
Most of knowledge on MMPs and ADAMs comes from studies on animal models or cell cultures.

Syntetic inhibitors of MMPs and ADAMs tested in phase II and III studies have shown moderate activity[69, 70].

Even if our study showed the correlation between ADAM12 and bone metastasis, there is the need of further studies to establish the exact relationship between this protein and bone disease. ADAM12 can however be tested as a possible target of anticancer treatment.

7. BIBLIOGRAPHY


100. Liu YJ, Xu Y, Yu Q. Full-length ADAMTS-1 and the ADAMTS-1 fragments display pro- and antimetastatic activity, respectively. Oncogene 2006; 25: 2452-2467.