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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 comapared 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 sreening. By the year 2000, the incidence and prevalence has progressively diminished by 2% yearly. This phenomeon can be due to the results of the *Women's Health Iniziative*, an epidemiological study sowing an increase in inceidence of breast cancer and myocardial infarction in people assuming estro-progestinic 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 estro-progestinic hormones in the premenopausal period. However, the lifestyle modifications, such as higher caloric intake, especially lipids, menarce 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 mammografic 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 espressing 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 trasformation 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 ivasive carcinoma. The loss of basal membrane, the icreased epithelial proliferation, the loss of growth inhibition, angiogenesis and stromal

invasion are all acquired during tumorigenesis [14]. The loss of basal membrane and tissutal integrity has a further role in the malignant transformation.

1.3 Biomolecular classification of breast cancer

Breast cancer is a etherogeneous 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 hyperespession of HER-2/neu (human epidermal growth factor receptor 2), the basaliod 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 omonimous 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 espression of genes correlated with estrogen receptors, low HER-2/neu espressione. The subtype B has a lower espression of ER-related genes, HER-2/neu espression.

HER-2/neu positive forms are the 10-15% of all breast cancers, have a low luminal genic *clusters* espression, 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 Cinical 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

symtoms[19, 20]. 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 eviden when it reaches 1-2 cm of dimeter. 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 cuses 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, supraclavear, 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, multicentricy 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 mamarian 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 **limpohoscintigraphy** [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].

Stage	T	N	M
Stage 0	**Tis**	**N0**	**M0**
Stage 1	**T1 <2 cm**	**N0**	**M0**
Stage 2a	**T1**	**N1(1-3ln)**	**M0**
	T2 >2<5	**N0**	**M0**
	T0	**N1**	**M0**
Stage 2b	**T2**	**N1**	**M0**
	T3 >5 cm	**N0**	**M0**
Stage 3a	**T0-2**	**N2 (4-9 ln)**	**M0**
	T3	**N1/N2**	**M0**
Stage 3b	**T4 skin/chest wall**	**N0-2**	**M0**
Stage 3c	**any T**	**N3 (>10 ln)**	**M0**
Stage 4	**any T any N**		**M1**

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 immunihistochemstry with monoclonal antibodies against Ki-67[41].

Other molecular markers have prognostic value: p53, hystotype: 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, proferation index).

Adjuvant hormonoterapy

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 hetrodimeric 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 $\alpha 1$, $\alpha 6$, $\beta 1$, $\beta 4$ 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 $\alpha 4 \beta 1$ 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 β2 expressed on the circulant leukocytes; VCAM (vascular cell adhesion molecules) the ligand for cells expressing α4β1, 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 matastatization 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 than 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 ostheolytic.

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 bounds 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-metalloproteasese 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 ¾ and ¼ 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 thy 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, hyidrossipironic 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 he 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-l, 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 mocroenvironment[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 d*isintegrin *a*nd *m*etalloproteinase, are a family of multidomain trans membrane proteases 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-α, 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 disintegrinic 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 he 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 bounds 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 avaible 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 micrtome in 3-micron section and stained.

The immunohistochemstry (IIC) is a tecnique that allow identifying cellular antigens on the normal and pathological tissues. It is based on the specificity between antign 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 immunohistochemics 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 metastatzation. 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 1 - Demographic features and tumor characteristics of the sample

Variable	Bone metastases	No bone metastases	Overall
Number of women – N (%)	207 (69.7)	90 (30.3)	297 (100)
Median age [min-max]– years	62.5 [27-84]	62 [28-89]	61 [27-89]
Missing values	0	1	2
Histology – N (%)			
CDI	74 (30.0)	173 (70.0)	247 (83.2)
CDL	10 (28.6)	25 (71.4)	35 (11.8)
Mixed	6 (40.0)	9 (60.0)	15 (5.0)
Grade – N (%)			
1	11 (26.2)	31 (73.8)	42 (14.1)
2	41 (25.5)	120 (74.5)	161 (54.2)
3	38 (40.4)	56 (59.6)	94 (31.6)
ER – N (%)			
Negative	17 (40.5)	25 (59.5)	42 (14.2)
Positive	73 (28.7)	181 (71.3)	254 (85.8)
Missing	.	1	1
PgR – N (%)			
Negative	31 (36.5)	54 (65.5)	85 (28.6)
Positive	59 (27.8)	153 (72.7)	212 (71.4)
C-erb – N (%)			
0	30 (30.3)	69 (69.7)	99 (33.3)
1	21 (28.8)	53 (71.2)	74 (24.6)
2	15 (26.8)	41 (73.2)	56 (18.9)
3	24 (35.3)	44 (64.7)	68 (22.9)
Metastasis other than bone – N (%)			
No	45 (17.9)	206 (82.1)	251 (84.5)
Yes	45 (97.8)	1 (2.2)	46 (15.5)

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 thepatients 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

Variable	Died	Alive	Overall
Number of women – N (%)	89 (30.0)	208 (70.0)	297 (100)
Bone metastases– N (%)			
No	30 (30.3)	69 (69.7)	99 (33.3)
Yes	21 (28.8)	52 (71.2)	73 (24.6)
Metastases other than bone– N (%)			
No	52 (20.7)	199 (79.3)	251 (84.5)
Yes	37 (80.4)	9 (19.6)	46 (15.5)

Tab. 3 – Expression of the proteins studied in the tumoral tissue.

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

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

Variable	Univariate analysis OR (95%CI)	p-value	Multivariate analysis OR (95%CI)	p-value
Age (increase of 10 years)	1.14 (0.92-1.41)	0.240		
Histology Mixed	1			
CDI	0.64 (0.22-1.87)	0.415		
CDL	0.60 (0.17-2.13)	0.429		
Grade 1	1			
2	0.96 (0.44-2.09)	0.924		
3	1.91 (0.86-4.26)	0.113		
Positive ER status	0.59 (0.30-1.16)	0.128		
Positive PgR status	0.67 (0.39-1.15)	0.834		
Positive MMP1	0.82 (0.49-1.37)	0.448		
Positive MMP13	1.20 (0.69-2.09	0.512		
Positive TIMP3	1.12 (0.50-2.54)	0.777		
Positive TIMP2	1.80 (0.92-3.52)	0.086		
ADAM17	2.01 (0.89-4.54)	0.092	1.35 (0.54-3.37)	0.526
ADAM12	2.85 (1.28-6.32)	0.010	2.59 (1.06-6.29)	0.036
ADAMTS1	1.78 (0.74-4.25)	0.195		
ADAM10	1.03 (0.58-1.83)	0.924		

Non-statistically significant differencies 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 matastatization.

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 bonemetastatization. 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 frequntly 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 thisprotein and bone disease. ADAM12 can however be tested as a possible target of anticancer treatment.

7. BIBLIOGRAPHY

1.   Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012; 62: 10-29.
2.   Kominsky SL, Davidson NE. A "bone" fide predictor of metastasis? Predicting breast cancer metastasis to bone. J Clin Oncol 2006; 24: 2227-2229.
3.   Coleman RE, Rubens RD. The clinical course of bone metastases from breast cancer. Br J Cancer 1987; 55: 61-66.
4.   Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2002; 2: 584-593.
5.   Schnitt SJ. Benign breast disease and breast cancer risk: morphology and beyond. Am J Surg Pathol 2003; 27: 836-841.
6.   Marshall SF, Clarke CA, Deapen D et al. Recent breast cancer incidence trends according to hormone therapy use: the California Teachers Study cohort. Breast Cancer Res 2010; 12: R4.
7.   Danaei G, Vander Hoorn S, Lopez AD et al. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. Lancet 2005; 366: 1784-1793.
8.   Palmer JR, Wise LA, Horton NJ et al. Dual effect of parity on breast cancer risk in African-American women. J Natl Cancer Inst 2003; 95: 478-483.
9.   Bradley CJ, Given CW, Roberts C. Race, socioeconomic status, and breast cancer treatment and survival. J Natl Cancer Inst 2002; 94: 490-496.
10.   Jatoi I, Chen BE, Anderson WF, Rosenberg PS. Breast cancer mortality trends in the United States according to estrogen receptor status and age at diagnosis. J Clin Oncol 2007; 25: 1683-1690.
11.   Banca dati " I tumori in Italia". http://www.tumori.net.
12.   Miller K. Estrogen and DNA damage: the silent source of breast cancer? J Natl Cancer Inst 2003; 95: 100-102.
13.   Hahn WC, Weinberg RA. Rules for making human tumor cells. N Engl J Med 2002; 347: 1593-1603.
14.   Esteller M, Herman JG. Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J Pathol 2002; 196: 1-7.
15.   Perreard L, Fan C, Quackenbush JF et al. Classification and risk stratification of invasive breast carcinomas using a real-time quantitative RT-PCR assay. Breast Cancer Res 2006; 8: R23.

16.   Loi S, Haibe-Kains B, Desmedt C et al. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. J Clin Oncol 2007; 25: 1239-1246.

17.   Chia S, Norris B, Speers C et al. Human epidermal growth factor receptor 2 overexpression as a prognostic factor in a large tissue microarray series of node-negative breast cancers. J Clin Oncol 2008; 26: 5697-5704.

18.   Barton MB, Elmore JG, Fletcher SW. Breast symptoms among women enrolled in a health maintenance organization: frequency, evaluation, and outcome. Ann Intern Med 1999; 130: 651-657.

19.   Ader DN, Shriver CD. Cyclical mastalgia: prevalence and impact in an outpatient breast clinic sample. J Am Coll Surg 1997; 185: 466-470.

20.   Smith RL PS, Fitzpatrick LA. Evaluation and management of breast pain. Mayo Clin Proc 2004; 79:353.

21.   Donegan WL. Evaluation of a palpable breast mass. N Engl J Med 1992; 327: 937-942.

22.   Barton MB, Harris R, Fletcher SW. The rational clinical examination. Does this patient have breast cancer? The screening clinical breast examination: should it be done? How? JAMA 1999; 282: 1270-1280.

23.   Da Costa D TA, Cure ML, et al. Common and unusual diseases of the nipple-areolar complex. . Radiographics 2007; 27 suppl 1: 65.

24.   Baines CJ DRAtw. Factors likely to affect the efficacy of screening mammography. J NATL Cancer Inst 1999; 91: 833.

25.   Schonfeld SJ, Pee D, Greenlee RT et al. Effect of changing breast cancer incidence rates on the calibration of the Gail model. J Clin Oncol 2010; 28: 2411-2417.

26.   (CDC). CfDCaP. Vital signs: breast cancer screening amond women aged 50-74 years-United States, 2008. MMWR Morb Mortal Wkly Rep 2010; 59: 813.

27.   Berg WA BJ, Cormack JB,et al. Combined screening with ultrasound and mammography vs mammography alone in womwn at elevated risk of breast cancer. . JAMA 2008; 299:2151.

28.   Sanders MA, Roland L, Sahoo S. Clinical implications of subcategorizing BI-RADS 4 breast lesions associated with microcalcification: a radiology-pathology correlation study. Breast J 2010; 16: 28-31.

29.   Stomper PC, Geradts J, Edge SB, Levine EG. Mammographic predictors of the presence and size of invasive carcinomas associated with malignant microcalcification lesions without a mass. AJR Am J Roentgenol 2003; 181: 1679-1684.

30.   Bluemke DA, Gatsonis CA, Chen MH et al. Magnetic resonance imaging of the breast prior to biopsy. JAMA 2004; 292: 2735-2742.

31.   Peters NH, Borel Rinkes IH, Zuithoff NP et al. Meta-analysis of MR imaging in the diagnosis of breast lesions. Radiology 2008; 246: 116-124.

32.   Turnbull L, Brown S, Harvey I et al. Comparative effectiveness of MRI in breast cancer (COMICE) trial: a randomised controlled trial. Lancet 2010; 375: 563-571.

33.   Upponi S, Kalra S, Poultsidis A et al. The significance of intramammary nodes in primary breast cancer. Eur J Surg Oncol 2001; 27: 707-708.

34.   National Comprehensive Cancer Network (NCCN) guidelines. http://www.nccn.org.

35.   Edge SB BD, Compton CC, et al. AJCC (American Joint Committee on Cancer) Cancer Staging Manual 7th ed. New York 2010.

36.   Woodward WA, Strom EA, Tucker SL et al. Changes in the 2003 American Joint Committee on Cancer staging for breast cancer dramatically affect stage-specific survival. J Clin Oncol 2003; 21: 3244-3248.

37.   Vinh-Hung V, Cserni G, Burzykowski T et al. Effect of the number of uninvolved nodes on survival in early breast cancer. Oncol Rep 2003; 10: 363-368.

38.   Fitzgibbons PL, Page DL, Weaver D et al. Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. Arch Pathol Lab Med 2000; 124: 966-978.

39.   Lipton A, Ali SM, Leitzel K et al. Elevated plasma tissue inhibitor of metalloproteinase-1 level predicts decreased response and survival in metastatic breast cancer. Cancer 2007; 109: 1933-1939.

40.   Yoshida R, Kimura N, Harada Y, Ohuchi N. The loss of E-cadherin, alpha- and beta-catenin expression is associated with metastasis and poor prognosis in invasive breast cancer. Int J Oncol 2001; 18: 513-520.

41.   Yerushalmi R, Woods R, Ravdin PM et al. Ki67 in breast cancer: prognostic and predictive potential. Lancet Oncol 2010; 11: 174-183.

42.   Aebi S, Davidson T, Gruber G, Castiglione M. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2010; 21 Suppl 5: v9-14.

43.   Mansel RE, Fallowfield L, Kissin M et al. Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC Trial. J Natl Cancer Inst 2006; 98: 599-609.

44.   Clarke M, Collins R, Darby S et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 366: 2087-2106.

45.   Ragaz J, Olivotto IA, Spinelli JJ et al. Locoregional radiation therapy in patients with high-risk breast cancer receiving adjuvant chemotherapy: 20-year results of the British Columbia randomized trial. J Natl Cancer Inst 2005; 97: 116-126.

46.   Wong ZW, Ellis MJ. First-line endocrine treatment of breast cancer: aromatase inhibitor or antioestrogen? Br J Cancer 2004; 90: 20-25.

47.   Bani MR GR. Principles of Molecular Oncology. 2003.

48.   McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol Med Today 2000; 6: 149-156.

49.   Roodman GD. Mechanisms of bone metastasis. N Engl J Med 2004; 350: 1655-1664.

50.   Guise T. Examining the metastatic niche: targeting the microenvironment. Semin Oncol 2010; 37 Suppl 2: S2-14.

51.   Ibrahim T, Leong I, Sanchez-Sweatman O et al. Expression of bone sialoprotein and osteopontin in breast cancer bone metastases. Clin Exp Metastasis 2000; 18: 253-260.

52.   Yin JJ, Pollock CB, Kelly K. Mechanisms of cancer metastasis to the bone. Cell Res 2005; 15: 57-62.

53.   Jones DH NT, Sanchez OH, et al. Regulation of cancer cell migration and bone metastasis by RANKL. Nature 2006; 440: 692.

54.   Chirgwin JM, Guise TA. Molecular mechanisms of tumor-bone interactions in osteolytic metastases. Crit Rev Eukaryot Gene Expr 2000; 10: 159-178.

55.   Egeblad M, Werb, Z. New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer 2002; 2 (3): 161–174.

56.   G. M. Progress in matrix metalloproteinase research. Molecular Aspects of Medicine 2008; 29: 290-308.

57.   Brinckerhoff CE, Rutter JL, Benbow U. Interstitial collagenases as markers of tumor progression. Clin Cancer Res 2000; 6: 4823-4830.

58.   Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002; 2: 161-174.

59.   Sunami E, Tsuno N, Osada T et al. MMP-1 is a prognostic marker for hematogenous metastasis of colorectal cancer. Oncologist 2000; 5: 108-114.

60.   Bendardaf R, Lamlum H, Vihinen P et al. Low collagenase-1 (MMP-1) and MT1-MMP expression levels are favourable survival markers in advanced colorectal carcinoma. Oncology 2003; 65: 337-346.

61.   Leeman MF, McKay JA, Murray GI. Matrix metalloproteinase 13 activity is associated with poor prognosis in colorectal cancer. J Clin Pathol 2002; 55: 758-762.

62. Freije JM, Diez-Itza I, Balbin M et al. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. J Biol Chem 1994; 269: 16766-16773.

63. Meierjohann S, Hufnagel A, Wende E et al. MMP13 mediates cell cycle progression in melanocytes and melanoma cells: in vitro studies of migration and proliferation. Mol Cancer 2010; 9: 201.

64. Hsu CP, Shen GH, Ko JL. Matrix metalloproteinase-13 expression is associated with bone marrow microinvolvement and prognosis in non-small cell lung cancer. Lung Cancer 2006; 52: 349-357.

65. Ibaragi S, Shimo T, Iwamoto M et al. Parathyroid hormone-related peptide regulates matrix metalloproteinase-13 gene expression in bone metastatic breast cancer cells. Anticancer Res 2010; 30: 5029-5036.

66. Nannuru KC, Futakuchi M, Varney ML et al. Matrix metalloproteinase (MMP)-13 regulates mammary tumor-induced osteolysis by activating MMP9 and transforming growth factor-beta signaling at the tumor-bone interface. Cancer Res 2010; 70: 3494-3504.

67. Valente P, Fassina G, Melchiori A et al. TIMP-2 over-expression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis. Int J Cancer 1998; 75: 246-253.

68. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 2002; 295: 2387-2392.

69. Sparano JA, Bernardo P, Stephenson P et al. Randomized phase III trial of marimastat versus placebo in patients with metastatic breast cancer who have responding or stable disease after first-line chemotherapy: Eastern Cooperative Oncology Group trial E2196. J Clin Oncol 2004; 22: 4683-4690.

70. Witters L, Scherle P, Friedman S et al. Synergistic inhibition with a dual epidermal growth factor receptor/HER-2/neu tyrosine kinase inhibitor and a disintegrin and metalloprotease inhibitor. Cancer Res 2008; 68: 7083-7089.

71. Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Sci Signal 2008; 1: re6.

72. Stetler-Stevenson WG. The tumor microenvironment: regulation by MMP-independent effects of tissue inhibitor of metalloproteinases-2. Cancer Metastasis Rev 2008; 27: 57-66.

73. Montgomery AM, Mueller BM, Reisfeld RA et al. Effect of tissue inhibitor of the matrix metalloproteinases-2 expression on the growth and spontaneous metastasis of a human melanoma cell line. Cancer Res 1994; 54: 5467-5473.

74. Munshi HG, Wu YI, Mukhopadhyay S et al. Differential regulation of membrane type 1-matrix metalloproteinase activity by ERK 1/2- and p38 MAPK-modulated tissue inhibitor of metalloproteinases 2 expression controls transforming growth factor-beta1-induced pericellular collagenolysis. J Biol Chem 2004; 279: 39042-39050.

75. Sun J. Matrix metalloproteinases and tissue inhibitor of metalloproteinases are essential for the inflammatory response in cancer cells. J Signal Transduct 2010; 2010: 985132.

76. Qi JH, Ebrahem Q, Moore N et al. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. Nat Med 2003; 9: 407-415.

77. Finan KM, Hodge G, Reynolds AM et al. In vitro susceptibility to the pro-apoptotic effects of TIMP-3 gene delivery translates to greater in vivo efficacy versus gene delivery for TIMPs-1 or -2. Lung Cancer 2006; 53: 273-284.

78. Rudolph-Owen LA, Hulboy DL, Wilson CL et al. Coordinate expression of matrix metalloproteinase family members in the uterus of normal, matrilysin-deficient, and stromelysin-1-deficient mice. Endocrinology 1997; 138: 4902-4911.

79. Wilson CL, Heppner KJ, Labosky PA et al. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci U S A 1997; 94: 1402-1407.

80.   Bergers G, Brekken R, McMahon G et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000; 2: 737-744.

81.   Noel A, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor-host interface. Semin Cell Dev Biol 2008; 19: 52-60.

82.   Hamano Y, Zeisberg M, Sugimoto H et al. Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. Cancer Cell 2003; 3: 589-601.

83.   Balbin M, Fueyo A, Tester AM et al. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet 2003; 35: 252-257.

84.   Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. Mol Aspects Med 2008; 29: 258-289.

85.   Ko SY, Lin SC, Wong YK et al. Increase of disintergin metalloprotease 10 (ADAM10) expression in oral squamous cell carcinoma. Cancer Lett 2007; 245: 33-43.

86.   Frohlich C, Albrechtsen R, Dyrskjot L et al. Molecular profiling of ADAM12 in human bladder cancer. Clin Cancer Res 2006; 12: 7359-7368.

87.   Kveiborg M, Frohlich C, Albrechtsen R et al. A role for ADAM12 in breast tumor progression and stromal cell apoptosis. Cancer Res 2005; 65: 4754-4761.

88.   Pories SE, Zurakowski D, Roy R et al. Urinary metalloproteinases: noninvasive biomarkers for breast cancer risk assessment. Cancer Epidemiol Biomarkers Prev 2008; 17: 1034-1042.

89.   Roy R, Rodig S, Bielenberg D et al. ADAM12 transmembrane and secreted isoforms promote breast tumor growth: a distinct role for ADAM12-S protein in tumor metastasis. J Biol Chem 2011; 286: 20758-20768.

90.   Roy R, Wewer UM, Zurakowski D et al. ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem 2004; 279: 51323-51330.

91.   Lendeckel U, Kohl J, Arndt M et al. Increased expression of ADAM family members in human breast cancer and breast cancer cell lines. J Cancer Res Clin Oncol 2005; 131: 41-48.

92.   McGowan PM, McKiernan E, Bolster F et al. ADAM-17 predicts adverse outcome in patients with breast cancer. Ann Oncol 2008; 19: 1075-1081.

93.   Bublil EM, Yarden Y. The EGF receptor family: spearheading a merger of signaling and therapeutics. Curr Opin Cell Biol 2007; 19: 124-134.

94.   Mendelson K, Swendeman S, Saftig P, Blobel CP. Stimulation of platelet-derived growth factor receptor beta (PDGFRbeta) activates ADAM17 and promotes metalloproteinase-dependent cross-talk between the PDGFRbeta and epidermal growth factor receptor (EGFR) signaling pathways. J Biol Chem 2010; 285: 25024-25032.

95.   Ohtsuka T, Shiomi T, Shimoda M et al. ADAM28 is overexpressed in human non-small cell lung carcinomas and correlates with cell proliferation and lymph node metastasis. Int J Cancer 2006; 118: 263-273.

96.   O'Shea C, McKie N, Buggy Y et al. Expression of ADAM-9 mRNA and protein in human breast cancer. Int J Cancer 2003; 105: 754-761.

97.   McGowan PM, Ryan BM, Hill AD et al. ADAM-17 expression in breast cancer correlates with variables of tumor progression. Clin Cancer Res 2007; 13: 2335-2343.

98.   Tang BL. ADAMTS: a novel family of extracellular matrix proteases. Int J Biochem Cell Biol 2001; 33: 33-44.

99.   Kashiwagi M, Enghild JJ, Gendron C et al. Altered proteolytic activities of ADAMTS-4 expressed by C-terminal processing. J Biol Chem 2004; 279: 10109-10119.

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.

101.   Lu X, Wang Q, Hu G et al. ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. Genes Dev 2009; 23: 1882-1894.

102.   de Fraipont F, Nicholson AC, Feige JJ, Van Meir EG. Thrombospondins and tumor angiogenesis. Trends Mol Med 2001; 7: 401-407.

103.   Del Casar JM, Gonzalez-Reyes S, Gonzalez LO et al. Expression of metalloproteases and their inhibitors in different histological types of breast cancer. J Cancer Res Clin Oncol 2010; 136: 811-819.

104.   Narita D, Seclaman E, Ursoniu S, Anghel A. Increased expression of ADAM12 and ADAM17 genes in laser-capture microdissected breast cancers and correlations with clinical and pathological characteristics. Acta Histochem 2012; 114: 131-139.

105.   Roy R, Moses MA. ADAM12 induces estrogen-independence in breast cancer cells. Breast Cancer Res Treat 2012; 131: 731-741.

106.   Sjoblom T, Jones S, Wood LD et al. The consensus coding sequences of human breast and colorectal cancers. Science 2006; 314: 268-274.